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A survey of potential insect vectors of the plant pathogenic bacterium *Xylella fastidiosa* in three regions of Spain

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Abstract

The emergence of a rapid-spreading olive disease associated with *Xylella fastidiosa* in southern Italy represents a high risk to susceptible crops in other countries of the Mediterranean basin, if insect vectors occur in the region. The goal of this study was to identify xylem-feeding Auchenorrhyncha that could potentially act as vectors of *X. fastidiosa* in three regions of Spain (Andalucía, Murcia and Madrid). Samplings with sweep net and stem tap were carried out in October/2004 on grapevines and adjacent crops (olives, nectarine, citrus, *Prunus* spp.), ornamental trees and herbaceous weeds. Yellow sticky cards were placed in ten vineyards located across 100 km in Andalucía and in three vineyards distant 10-15 km apart in Murcia. Specimens of frequently-trapped species were tested by nested- or multiplex-PCR for the presence of *X. fastidiosa*. The Typhlocybinae leafhopper, *Austroasca (Jacobiasca) lybica* (Hemiptera: Cicadellidae) was the most abundant species in vineyards and citrus orchards. Planthoppers (Hemiptera: Fulgoroidea) and psyllids (Hemiptera: Psylloidea) were prevalent on olives. Cicadellinae leafhoppers (known as sharpshooters), which are major vectors of *X. fastidiosa* in the Americas, were not found in the samples. The only potential vectors were spittlebugs (Hemiptera: Cercopoidea) collected on *Populus* sp., herbaceous and on conifer trees (*Pinus halepense*); the spittlebug *Neophileanus* sp. was common on conifer trees adjacent to a vineyard in Jumilla. None of the insect samples tested positive for *X. fastidiosa* by PCR assays. However, spittlebugs already associated with susceptible crops in Spain may allow fast spread of *X. fastidiosa* in case this pathogen is introduced.

Additional key words: exotic disease; xylem-limited bacterium; spittlebug vectors; Cercopoidea; epidemiology.

Xylella fastidiosa is a xylem-inhabiting bacterium that is vector-transmitted, gram-negative, shows a very slow growth *in vitro* and was cultured and properly described for the first time in 1987 in the USA (Hopkins, 1989; Chang *et al*., 1993; Janse & Obradovic, 2010). This bacterium causes enormous yield losses as the etiological agent of Pierce's disease (PD) of grapevine, *Vitis vinifera*; phony peach disease in peach, *Prunus persica*; and citrus variegated chlorosis in *Citrus* spp. Moreover, it also causes a number of so-called leaf scorch diseases in *Prunus* spp. (including almond leaf scorch in *Prunus amygdalus/P. dulcis* and plum leaf scald in *Prunus domestica*), *Acer* spp., *Carya illinoinensis*, *Coffea arabica*, *Hedera helix*, *Morus rubra*, *Nerium oleander, Platanus occidentalis*, *Quercus* spp., and *Ulmus americana*. It is able to infect many herbaceous plants such as *Medicago sativa* and *Vinca major* and wild plants including grasses sedges and trees, which may carry the pathogen without showing symptoms (Hopkins, 1989; Janse & Obradovic, 2010).

Several pathogenic variants of the bacterium have been described, that are often host specific, and that have been given the category of subspecies (Schaad *et al*., 2004): (i) *Xylella fastidiosa* subsp. *fastidiosa* including strains from cultivated grape, alfalfa, almond, and maple; (ii) *X. fastidiosa* subsp. *multiplex*, including strains isolated from peach, elm, plum, pigeon grape, sycamore and almond; (iii) *X. fastidiosa* subsp. *pauca*, including strains isolated from citrus and coffee; and (iv) *X. fastidiosa* subsp. *sandyi*, including strains isolated from *Nerium oleander*.

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Since *X. fastidiosa* has more than 150 hosts and for many of them (including nursery productions, cut flowers, propagating material, fruit trees) planting material is imported in different countries, the risk of introduction (especially in latent form) is very high. This risk is especially high for grapes, citrus (sweet oranges, mandarins, and tangerines), almond, olive, plum and peach that are widely grown in southeast and southwest Europe, especially in the warmer Mediterranean basin, where a disease-favorable combination of warm nights, regular rainfall/high humidity and long growing season is present (Janse & Obradovic, 2010). Only in Spain the four first above mentioned crops represent more than 4 million ha (olive, $2.5 \cdot 10^6$ ha; grapes, $9.6 \cdot 10^5$ ha; almond, $5.4 \cdot 10^5$ ha; citrus $3.2 \cdot 10^5$ ha) (MAGRAMA, 2012) which gives an idea of the devastating situation for the Spanish agriculture that could occur if this pathogenic bacterium enters and establishes.

Due to its potential risks in the region, *X. fastidiosa* is on the A1 Quarantine list of EPPO (EPPO, 2011), as well as its most invasive vector, *Homalodisca vitripennis* (Germar) (recently included on the EPPO alert list). For Europe, until recently there were only a few unconfirmed records of *X. fastidiosa* on grapevine from Kosovo and France (Berisha *et al*., 1998; EPPO, 2004). However, very recently the bacterium has been detected by specific molecular tests to be associated to a rapidly spreading decline of aged olive trees in a large area of the Salento peninsula (Apulia, south of Italy) (Saponari *et al*., 2013), which has prompted to inform the EPPO authorities. More importantly, molecular tests extended to almond and oleander trees with leaf scorching symptoms, growing nearby to the diseased olive orchards, were also positive for the bacterium. Before this recent report in Italy, infection of olive by *X. fastidiosa* had only been reported in California, USA, where genotypes of the bacterium, isolated from olive were shown to be pathogenic to almond, but not to grapevine and olive (Krugner *et al*., 2014).

The establishment and spread of *X. fastidiosa* in a new area depends on the presence of appropriate environmental conditions, host plants and vectors (Rathé *et al*., 2012). In a computer simulation program (CLIMEX) study concerning climatic conditions and possibilities of spread of *X. fastidiosa*, Hoddle (2004) concluded: "CLIMEX predicted that cold stress accumulation would exclude Pierce's disease-causing strains of *X. fastidiosa* from France and northern and central grape producing areas of Spain and Italy". Southern Spain, on the other hand, has suitable climate (milder winter temperatures) and various plants known as hosts of this bacterium, but there is no information on the availability of vectors.

Xylela fastidiosa can be transmitted by several species of sharpshooter leafhoppers (Hemiptera: Cicadellidae: Cicadellinae) and spittlebugs or froghoppers (Hemiptera: Cercopoidea), which are xylem-fluid feeders (Redak *et al*., 2004). There is also evidence that cicadas (Hemiptera: Cicadoidea), another group of xylem-fluid feeders, transmit *X. fastidiosa* in grape (Krell *et al*., 2007). Although differences in vector efficiency exist (Lopes *et al*., 2009), there is little or no vector specificity for transmission of *X. fastidiosa* (Redak *et al*., 2004). Because distantly related groups of specialized xylem-fluid feeders within Auchenorrhyncha have been reported as vectors, the xylem feeding habit appears to be one of the few requirements for transmission of this pathogen (Purcell, 1989). Therefore, all Cicadellinae, Cercopoidea and Cicadoidea species, as well as any other group of xylem-fluid feeders should be considered as potential vectors if they are able to feed on plants where *X. fastidiosa* is present.

The low vector specificity of *X. fastidiosa* suggests that indigenous species of xylem-feeding Auchenorrhyncha could transmit this pathogen if introduced in a new region. Thus, the purpose of our work was to do a preliminary identification of the xylem-feeding Auchenorrhyncha present in three regions in Spain in addition to those previously reported (Battle *et al*., 2000; Sabaté *et al*., 2007) that could potentially act as vectors of *X. fastidiosa*. Knowledge of potential vectors already present in a country is essential to understand epidemiology of crop diseases caused by *X. fastidiosa* and to establish eradication control measures if needed.

To accomplish this objective, field surveys were conducted in the autumn (late September to mid-October) of 2004 in different regions of Spain including Andalucía (southwestern Spain), Madrid (central Spain), and Murcia (southeastern Spain) to determine the presence of hemipteran species of the suborder Auchenorrhyncha (especially sharpshooters and spittlebugs) that could be potential vectors of *X. fastidiosa*. Surveys were performed in some of the main crops (grapevines, citrus and olives) that are known hosts of *X. fastidiosa,* as well as in riparian vegetation and weeds nearby those crops. Grapevines were ran-

domly selected in each region and were sampled by yellow sticky cards (10 × 30 cm) in ten vineyards located across 100 km in the Montilla-Moriles Denomination of Origin (Córdoba, Andalucía) and three vineyards distant 10-15 km apart in the Jumilla Denomination of Origin (Murcia). A total of 10 cards were used per region during 2 weeks, placed at 0.8 m of height in the border of the vineyards. Samples with sweep net (20 sweeps per sample) and stem tap (by counting the number of insects falling into a white tray after tapping the tree stem three times) were carried out on a single date (second week of October/2004) on grapevines and adjacent crops (olives, nectarine, citrus, *Prunus* spp.), ornamental trees (*Nerium oleander*, *Pinus halepense*, *Retama* sp.) and herbaceous weeds. Sweep net samples were also collected on grasses (*Phragmites* sp.) and shrubs (*Populus* sp., *Juniperus* sp.) growing in a riparian area at La Poveda CSIC Experimental Farm (Arganda del Rey, Madrid). No symptoms similar to those induced by *X. fastidiosa* were observed in the sampled crops, ornamental trees or weeds. The auchenorrynchan specimens trapped in the various localities were identified at the family and subfamily level. Some abundant leafhopper and spittlebug species were identified at the genus or species level.

Leafhoppers (Hemiptera: Cicadellidae) of the subfamily Typhlocybinae were the most abundant specimens trapped in vineyards, citrus orchards, *Juniperus* sp. and *Populus* sp., and the only group directly observed on grapes (Table 1). They were very abundant in vineyards of the Andalucía region, reaching population levels ranging from ≈1,200 to 5,800 individuals per yellow sticky trap. In most vineyards of the Murcia region (Rubializas and Las Hermanas), Typhlocybinae leafhoppers were also predominant, but were collected in much lower numbers by sticky traps (0-23 specimens/trap) (sticky trap data not shown). The species collected by sweep net and stem tap were identified as *Austroasca (Jacobiasca) lybica* (Bergevin & Zanon) on grapevines and citrus, *Tamaricella tamaricis* (Puton) on *Juniperus* sp., and *Zyginidia scutellaris* (Herrich-Schäffer) on *Populus* sp. and on the grass *Phragmites* sp. Specimens of other subfamilies of

Table 1. Number of Auchenorrhyncha specimens sampled on different plants and localities of Spain

Region[a]	Locality	Plant	Sampling method	Auchenorrhyncha taxa[b,c]		
				Cicadellidae	Cercopoidea	Fulgoroidea
Andalucía	Los Arenales	*Vitis vinifera*	Sweep net	99 (Ty, **10**)		
	Moriles	*Olea europaea*	Sweep net	1 (Ty)		3 (Is)
	Puente Genil	*Olea europaea*	Sweep net	1(De), 2 (Ty)		1 (Is)
		Vitis vinifera	Sweep net	27 (Ty, **5**)		
	Rotiles	Cedrella sp.	Sweep net	9 (Ty)		
		Prunus sp.	Sweep net	3 (Ty)		
		Capparis spinosa	Sweep net	1 (De)		
	Las Puentes	*Amaranthus*	Sweep net	1 (De), 11 (Ag. **5**)		
		Digitaria	Sweep net	1 (De)		
	Fuente Nueva	*Vitis vinifera*	Sweep net	36 (Ty, **10**)		
	Carchena	*Vitis vinifera*	Sweep net	20 (Ty)		
	Carchena	*Citrus sinensis*	Sweep net	34 (Ty, 5)		
	Carchena	*Olea europaea*	Sweep net	4 (Ty)		15(?)
	Cristóbal Trenas	*Digitaria* sp.	Sweep net	10 (De)		
Murcia	Rubializas	*Vitis vinifera*	Stem tap	5(Ty,**1**)		
	Las Hermanas	*Pinus halepensis*	Stem tap	5 (De)	5 (Ap, 2)	
	Alhama de Murcia	*Citrus sinensis*	Stem tap	9 (Ty)		
Madrid	La Poveda	*Phragmites* sp.	Sweep net	1 (De), 7 (Ty)		2 (Dp)
		Juniperus sp.	Sweep net	350 (Ty, De)		
		Populus sp.	Sweep net	3 (Id), 13 (Ty)	1?	
		Leguminosae	Sweep net	129 (Ty, 5)	1?	
		Grasses	Sweep net	215 (Ty, 5), 4 (De, **4**)	1?	41 (Dp, **4**)

[a] Sampling dates: Madrid: Oct 6, 2004; Andalucía (Córdoba province, Montilla-Moriles Denomination of Origin): Oct 7-8, 2004; Murcia (Jumilla Denomination of Origin): Oct 14-15, 2004. [b] Taxa abbreviations - Ag: Agalliinae; Ap: Aphrophoridae; De: Deltocephalinae; Dp: Delphacidae; Id: Idiocerinae; Is: Issidae; Ty: Typhlocybinae; ?: unidentified. [c] Numbers of specimens tested by PCR for the presence of *X. fastidiosa* are shown in bold case within parenthesis.

leafhoppers (Agalliinae, Deltocephalinae and Xestocephalinae) were trapped in lower numbers, mainly on herbaceous weeds *e.g. Amaranthus* sp. and *Digitaria* sp. (Table 1). These subfamilies of leafhoppers are not potential vectors of *X. fastidiosa*, because they feed primarily in the phloem and/or mesophyll tissues. However, some species in these leafhopper subfamilies (especially Deltocephalinae) are important vectors of phytoplasmas and plant viruses in several crops. In sweep net samples on olive trees in the Andalucía region, only one specimen of leafhopper (Deltocephalinae) was collected (Table 1). Planthoppers (Hemiptera: Fulgoroidea) and psyllids (Hemiptera: Sternorrhyncha: Psylloidea) were trapped in higher numbers. Planthoppers and psyllids are known as phloem feeders and some species are important vectors of phytoplasmas; psyllids can also transmit liberibacters associated with diseases in citrus and vegetable crops.

In previous studies, several auchenorrhynchan species belonging to the families Cicadellidae and Delphacidae were sampled in vineyards from different regions of Northeastern Spain (Battle *et al*., 2000; Sabaté *et al*., 2007) and some were found to be infected by the "Bois noir" (BN) stolbur phytoplasma. Most abundant species carrying the phytoplasma included *Adarrus taurus* Ribaut*, Agallia laevis* Ribaut*, Aphrodes bicinctus* (Schrank)*, Euscelidius variegatus* (Kirschbaum), *Hyalesthes obsoletus* Signoret, *Laodelphax striatellus* (Fallén)*, Macrosteles quadripunctulatus* (Kirschbaum)*, M. sexnotatus* (Fallén)*, Neoaliturus fenestratus* (Herrich-Schäffer)*, Psammotettix striatus* (L.), and *Zyginidia scutellaris* (Herrich-Schäffer).

Sharpshooter leafhoppers (Hemiptera: Cicadellinae), which represent the major group of vectors of *X. fastidiosa* in the American continent, were not found in any of the samples in this whole survey. The only potential vectors found were spittlebugs (Hemiptera: Cercopoidea), which were not observed directly on the sampled crop plants (grapevines, olives and citrus), but were collected by sweep net on *Populus* sp. and herbaceous weeds in a riparian vegetation in La Poveda, Madrid, and by stem tap on conifer trees (*P. halepense*) adjacent to a vineyard in the region of Jumilla (Murcia) (Table 1). Interestingly, several specimens of the spittlebug *Neophilaenus* sp. (Aphrophoridae) and of the deltocephaline leafhopper *Grypotes staurus* Ivanoff were found on *P. halepense,* which is an evergreen tree and thus may be an important host or shelter plant for these hopper species during the winter or dry seasons, when other possible hosts are unsuitable. Because *P. halepense* is a well-adapted (native) and common tree in semi-arid regions of southern Spain, it may play a role as a source of potential vectors of *X. fastidiosa* and other pathogens.

The presence of *X. fastidiosa* in specimens of the most frequent leafhopper and spittlebug species collected by sweep net and stem tap in this survey was tested either using nested-PCR (Ciapina *et al*., 2004) or multiplex-PCR (Rodrigues *et al*., 2003) assays. For extraction of total DNA, insect tissues combined in samples from the same plot (Table 1) were placed directly in a 1.5-mL Fast DNA tube containing lysing matrix A, 500 µL of CLS-VI solution (Qbiogene), and 200 µL of Protein Precipitation Solution (PPS) (for insect material). Insect tissues were disrupted mechanically in a Fast Prep System Bio 101 (Qbiogene) by reciprocal shaking of the samples for 30 s at 5.5 speed level (twice), incubating the samples in ice for 2 min between successive homogenizations. Then, the supernatant was collected by centrifugation (10 min at 14,000 rpm) and processed with the Fast DNA kit (Qbiogene) according to the manufacturer's instructions. The DNA pellet was finally resuspended in ultrapure water, quantified fluorimetrically using the Quant-iT DNA Assay Kit Broad Range fluorometric assay (Molecular Probes Inc., Leiden, The Netherlands) with a Tecan Safire fluorospectrometer (Tecan Spain, Barcelona) according to manufacturer's instructions, diluted with ultrapure distilled water to 20 ng μL^{-1}, and used directly as template for PCR assays. Additionally, DNA from *X. fastidosa* strain 95aC and DNA extracted from Cicadellidae that were fed in citrus plants infected with *X. fastidiosa* was extracted as described above in Brasil and send to Spain to be used as positive controls for PCR assays.

For the nested-PCR assay reaction conditions the primer pairs 272-1/272-2 and CVC1/272 int (Pooler & Hartung, 1995) were used for the first and second round of PCR amplification, respectively using same amplification conditions described in Ciapina *et al.* (2004). For the multiplex-PCR assay two sets of primers were used in same reaction tube to amplify the *gyrB* gene (FXYgyr499/RXYgyr907 primers) and the 16S rRNA (S-S-X.fas-0067-a-S-19/S-S-X.fas-0838-a-A-21 primers) using same amplification conditions described in Rodrigues *et al*. (2003). The PCR products were separated by 1.5% ethidium bromide agarose gel electrophoresis at 100 V, in 1X TBE buffer for 1.5 h.

None of the insect samples from Spain fields gave a positive amplification signal for *X. fastidiosa* using

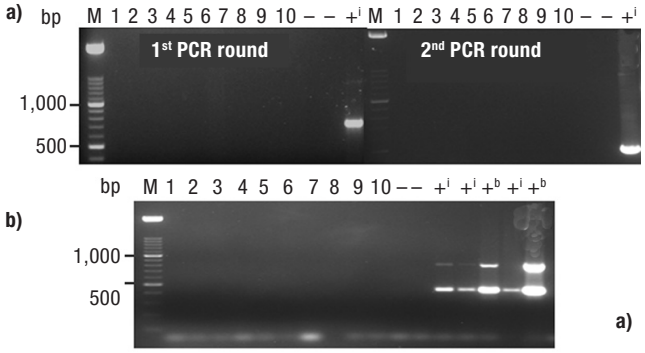

Figure 1. Ethidium bromide-stained gel of (a) first and second round amplification products of the nested PCR assay or (b) multiplex-PCR assays to determine the presence of *Xylella fastidiosa* in insect samples. M: molecular marker (100-bp ladder); lanes 1 to 10, DNA extracted from insect samples listed in Table 1; (–) = negative control, water; ($+^{i}$) = positive control, DNA extracted from insect fed with plants infected by *X. fastidiosa;* ($+^{b}$) = positive control, DNA extracted from *X. fastidiosa* strain 9a5c. The expected sizes are 500 bp for the nested PCR assay, and 745 and 429 bp for 16S rRNA and gyrB-specific genes of multiplex-PCR, respectively.

either the nested-PCR (Fig. 1a), or multiplex-PCR (Fig. 1b) assays. Conversely, a DNA sample of a sharpshooter vector [*Bucephalogonia xanthophis* (Berg)] fed on plants infected with *X. fastidiosa* and used as positive controls for PCR assays gave a positive amplification signal of the expected size as well as the DNA sample from *X. fastidiosa* strain 9a5c (Fig. 1). Uneven amplifications of 16S or *gyrB* genes were found in some cases for the positive control samples. A direct explanation for this result is the presence of only one *gyrB* gene in the *X. fastidiosa* genome, whereas two copies of the 16S rRNA are found (Rodrigues *et al*., 2003).

The emergence of a serious olive disease associated with *X. fastidiosa* in Italy (Saponari *et al*., 2013) and its potential to spread to neighboring countries in the Mediterranean basin, represents a serious threat not only to olive, *Vitis* and *Citrus,* but also to stone fruits (almond, peach and plum) and oleander. The climatic conditions of areas where *X. fastidiosa* causes severe diseases in California are similar to those prevailing in the Mediterranean basin, suggesting that *X. fastidiosa* will find a suitable environment for successful establishment in the latter region (Hoodle, 2004).

The present survey, although restricted to two weeks in the autumn of 2004, represents to our knowledge the first survey in Spain to test for the presence of *X. fastidiosa* in potential vector species belonging to the Hemiptera: Auchenorrhyncha suborder. The study shows that spittlebug or froghopper species already present in association with susceptible crops in Spain may allow *X. fastidiosa* spread if the pathogen is introduced in this country. A list of potential vectors of *X. fastidiosa in Europe*, extracted from the Fauna Europaea database (De Jong, 2013), includes several species of Cercopoidea and a few sharsphooters (Cicadellinae) already reported in Europe (EFSA, 2013). Compared with the American Continent, Europe shows a lower diversity of Cicadellinae species (Wilson *et al*., 2009), and it is likely that species composition and abundance also vary within Europe. Therefore, more extensive surveys in different regions, seasons and years of xylem-feeding Auchenorrhyncha are needed to characterize the range of possible vectors of *X. fastidiosa,* since new potential vector species could have been introduced since 2004, and assess risks of disease spread in different regions of Europe, especially in the Mediterranean basin.

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