LETTER TO THE EDITOR

DETECTION OF *XYLELLA FASTIDIOSA* IN OLIVE TREES BY MOLECULAR AND SEROLOGICAL METHODS

G. Loconsole[1], O. Potere[2], D. Boscia[1], G. Altamura[3], K. Djelouah[4], T. Elbeaino[4], D. Frasheri[4], D. Lorusso[4], F. Palmisano[3], P. Pollastro[3], M.R. Silletti[3], N. Trisciuzzi[3], F. Valentini[4], V. Savino[2] and M. Saponari[1]

[1]Istituto di Virologia Vegetale del CNR, UOS di Bari, Via Amendola 165/A, 70126 Bari, Italy
[2]Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro, Via Amendola 165/A, 70126 Bari, Italy
[3]Centro di Ricerca, Sperimentazione e Formazione in Agricoltura, Via Cisternino 281, 70100 Locorotondo (BA), Italy
[4]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70100 Valenzano (BA), Italy

SUMMARY

Xylella fastidiosa has recently been identified in the Apulian province of Lecce (south-eastern Italy) in olive trees affected by a devastating disease denoted Olive Quick Decline Syndrome (OQDS), that appeared suddenly in 2010. Symptoms of OQDS consist of withering and desiccation of scattered terminal shoots, which rapidly expands to the rest of the canopy, and results in the collapse and death of the tree. The identification of *X. fastidiosa* in OQDS-affected trees represents the first confirmed detection of this bacterium in the European Union (EU), but its exact role in the aetiology of this disease is yet to be determined. Since *X. fastidiosa* is a regulated quarantine pathogen in the EU, upon request of the Apulian Plant Protection Service, surveys were initiated in order to delineate the contaminated area. To this effect, diagnostic protocols based on ELISA and conventional PCR for *X. fastidiosa* detection in olive samples were compared and validated via an interlaboratory ring-test in which three accredited laboratories, all located in Italy, participated. Both procedures proved to be equally effective but, due to the simplicity of sample preparation, ELISA was chosen for the large-scale *X. fastidiosa* monitoring programme now in progress.

Key words: *Xylella fastidiosa*, olive, olive quick decline syndrome, PCR, ELISA

Corresponding author: M. Saponari
Fax: +39.080.5443608
Email m.saponari@ba.ivv.cnr.it

Xylella fastidiosa is a xylem-limited Gram-negative bacterium and the causal agent of a number of severe diseases, among which Pierce's disease of grapevine, leaf scorch of almond, oleander and coffee, citrus variegated chlorosis, and other disorders of perennial crops and landscape plants (Hopkins and Purcell, 2002; Janse and Obradovic, 2010; Purcell, 2013). Four subspecies of *X. fastidiosa* have been discriminated so far, i.e. subspp. *fastidiosa*, *multiplex*, *pauca* and *sandyi*, and a proposed fifth one (Randal *et al.,* 2009), that can be differentiated by DNA:DNA hybridization (Schaad *et al.,* 2004) and multi-locus sequence typing (Scally *et al.,* 2005). *X. fastidiosa* subsp. *fastidiosa* contains strains of low genetic diversity that cause Pierce's disease and almond leaf scorch. *X. fastidiosa* subsp. *multiplex* is endemic to North America only and infects numerous hosts, but generally not grapevine (Davis *et al.,* 1978; Hopkins and Mollenhauer, 1973). *X. fastidiosa* subsp. *pauca* contains South American strains causing citrus variegated chlorosis and coffee leaf scorch (Hopkins and Purcell, 2002). *X. fastidiosa* subsp. *sandyi* comprises closely related strains isolated from oleander in California and Texas (USA). As to olive (*Olea europaea*), there are three reports from California, where the bacterium was detected and/or isolated from trees with leaf scorching (Wong *et al*., 2004), unspecified symptomatology, if any (Hernandez-Martinez *et al*., 2007), or showing dieback and leaf scorch (Krugner *et al*., 2010). A bacterial isolate from Riverside proved to be phylogenetically related to subsp. *multiplex* (Hernandez-Martinez *et al*., 2007), whereas isolates from the San Joaquin Valley were classified as "Genotype A" (Chen *et al.,* 2005; Krugner *et al*., 2010). These isolates seem to differ from the one detected in southern Italian olives which, based on preliminary multilocus sequence typing assays, appears to be close to the subspecies *pauca* (M. Saponari, unpublished information). Californian pathogenicity tests were inconclusive because inoculations into olive plants did not reproduce the symptoms observed in the field (Krugner *et al.,* 2010).

In October 2013, an outbreak of *X. fastidiosa* was found in Apulia (south-eastern Italy) in olive trees affected by a disease denoted "Olive Quick Decline Syndrome" (OQDS), which appeared suddenly in 2010 (Saponari *et*

Fig. 1. Olive trees affected by the olive quick decline syndrome. A. Olive trees at the early stage of the disease, showing desiccation of terminal shoots; B. Advanced stage of the disease with the canopy of the trees severely compromised.

al., 2013) (Fig. 1). The confirmed presence in the EU of a regulated quarantine pathogen listed in Annex I, Part A, Section I to Council Directive 2000/29/EC called for the implementation of immediate actions among which, first and foremost, the carrying out of extensive surveys on olive and other susceptible hosts to identify the contaminated area and determine the most appropriate management strategies.

Detection and identification of *X. fastidiosa* relies on conventional field and laboratory approaches, as well as on serological and molecular assays. Symptom observation and isolation and culturing of the bacterium on agar media (Almeida *et al.,* 2004) are essential in confirmation of first occurrence of this important quarantine pathogen (EPPO, 2004). However, in routine monitoring and surveying, culturing *X. fastidiosa* is time consuming (with some subspecies it takes up to three weeks for the colonies to grow) and is labor-intensive, particularly when a large number of samples are processed. Although culturing remains the fundamental procedure to unequivocally confirm the presence of the bacterium, serological and molecular techniques are more suitable methods for screening a large number of samples.

Serological tests that were developed over the years include enzyme-linked immunosorbent assay (ELISA) (Sherald and Lei, 1991), dot immunobinding assay (DIBA), western blotting (Lee *et al*., 1992; Chang *et al.,* 1993) and immunofluorescence (Carbajal *et al*., 2004). More recently, PCR-based assays (Minsavage *et al*., 1994; Rodriguez *et al*., 2003; Huang *et al*., 2006; Huang, 2009) and PCR derivatives, including RFLP (restriction fragment length polymorphism) and RAPD (random-amplified polymorphic DNA) analysis (Pooler and Hartung, 1995; Mehta *et al*., 2001), as well real-time and LAMP PCR (Oliveira *et al*., 2002; Schaad *et al.,* 2002; Francis *et al.,* 2006; Blexine and Child, 2007; Harper *et al*., 2010; Li *et al*., 2013; Guan *et al*., 2013), have been used to detect the bacterium in grapevine, citrus, almond and other hosts. Extraction of *X. fastidiosa* DNA from host tissues for PCR and related molecular analyses has been achieved using standard commercial kits (Blexine and Child, 2007; Huang, 2009) and basic CTAB buffer (Hendson *et al.,* 2001; de Souza *et al.,* 2003; Rodrigues *et al.,* 2003).

When *X. fastidiosa* was first found in Apulia, no information was available on the strain or genotype causing infections in olive, nor procedures for the identification of the bacterium in locally grown olive cultivar tissues were known. Thus, validation of the molecular and serological protocols, reported for other perennial crops, was necessary prior to conducting a large scale monitoring program. To this aim, a ring-test was conducted amongst the three accredited laboratories operating in the area (listed further ahead), in which a set of 18 blind olive samples of known infection status was distributed and subjected to ELISA and PCR using a common set of serological and molecular reagents.

METHODS

Plant material and tissue preparation. Olive tissues used for bacterial detection consisted of leaf petioles and midveins excised from mature leaves. Samples were four hardwood cuttings per tree, 5-6 internode long, from which 2-3 basal leaves were excised and pooled to recover a total of 8-10 midveins, weighing approximately 0.4-0.6 g. Experiments were conducted either with or without prior surface sterilization of the leaves with 5% sodium hypochloride for 10 min. Petioles and midveins were then transferred to extraction bags with a plastic intermediate layer and homogenized using the semi-automated Homex 6 apparatus (Bioreba, Switzerland) or pulverized using liquid nitrogen.

ELISA. Tissue extracts obtained after homogenization of leaf tissues in extraction buffer (1:10) were transferred into microcentrifuge tubes prior to loading ELISA plates. Preliminary tests, in which the reactivity of different commercially available ELISA kits was compared, showed that

Table 1. Primers used for detection of the target genes RNA polymerase sigma factor (RST) and ribosomal RNA (16S rDNA).

Target gene	Primers	Amplicon size (bp)	Sequence (5' - 3')	References
RNA polymerase sigma factor	RST-31 RST-33	733	GCGTTAATTTTCGAAGTGATTCGATTGC CACCATTCGTATCCCGGTG	Minsavage *et al* ., 1994
16S rDNA	XF-1 XF-6	404	CAGCACATTGGTAGTAATAC ACTAGGTATTAACCAATTGC	Firrao and Bazzi, 1994
16S rDNA	S-S-X.fas-0838-a-S-21 S-S-X.fas-1439-a-A-19	603	GCAAATTGGCACTCAGTATCG CTCCTCGCGGTTAAGCTAC	Rodrigues *et al*., 2003

a kit from Loewe (Biochemica GmbH, Germany) detected a higher number of known positive samples with reactions occurring within 2 h, following manufacturer's instructions and using the controls supplied with the kit. Thus, this kit was used throughout, following a procedure in which plates were coated with 200 µl of anti *X. fastidiosa* IgG diluted 1:200 in coating buffer and incubated at 37°C for 4 h. Test samples were loaded in microplates and kept overnight at 4°C before the addition of alkaline-phosphatase-conjugated anti *X. fastidiosa* IgG diluted 1:200. Plates were then incubated at 37°C for 4 h prior to the addition of the substrate (1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8). Absorbance was measured after 30, 60, 120 and 180 min with a microplate reader at 405 nm. A reaction was determined to be positive if, after 120 min, the absorbance was three times greater than the mean absorbance of healthy control samples.

Isolation of total nucleic acid for PCR assay. Four different methods were compared to recover total nucleic acids (TNA) from olive tissues. Extracts from four infected and four healthy samples were used as template for *X. fastidiosa* detection by PCR.

Procedure A. 100 mg of leaf petioles and midveins were homogenized in liquid nitrogen and the powered tissue processed, using the DNeasy plant mini kit (Qiagen, The Netherlands) according to the manufacturer's instructions.

Procedure B. Small pieces of midveins and petioles (0.4-0.6 g) were crushed with a hammer in extraction bags and homogenized using the semi-automated homogenizer Homex 6 (Bioreba, Switzerland) with 5 ml of CTAB buffer (2% Hexadecyl trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8, 20 mM EDTA and 1.4 M NaCl). Extract aliquots (1 ml) were transferred into a 2 ml microcentrifuge tube, incubated at 65°C and chloroform-treated (1 ml). TNA were then isolated by precipitating the supernatant with 0.7 volume of cold 2-Propanol (Murray and Thompson, 1980).

Procedure C. 100 mg of leaf petioles and midveins were pulverized in liquid nitrogen and extracted in 1.5 ml of a buffer (20 mM EDTA, 350 mM sorbitol in 100 mM Tris-HCl, pH 7.5 plus 2.5% polyvinylpyrrolidone and 2% metabisulfite) as described by Lin and Walker (1997).

Procedure D. 100 mg of leaf petioles and midveins were pulverized in liquid nitrogen, instead of being lyophilized as in the original protocol (Chen *et al*., 2008). Then, 500 µl of sterile elution buffer [(AE buffer, DNeasy Plant mini kit (Qiagen, The Netherlands)] were added to the powdered tissues, which were allowed to soak at room temperature for 15 min, then vortexed for 10 sec and centrifuged for 1 min to recover the supernatant containing the bacterial DNA (EB-bacterial release).

TNA concentration and quality of the extracts recovered using methods A, B and C were evaluated by measuring the absorbance at 260 nm and the absorbance ratio 260/280 with a Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). TNA concentration was then adjusted to 80-100 ng/µl, and 2 µl were used for PCR reactions. As to method D, 5 µl of the EB-bacterial release were directly used for PCR along with ten-fold serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) prepared in sterile water.

PCR primers and cycling conditions. The primer sets tested in this work have previously been used for generic detection of *X. fastidiosa* by targeting conserved genomic regions (Table 1). The list includes primers RST31/33, which are widely accepted for the detection of the bacterium in quarantine programs (EPPO, 2004), as well as primers targeting the 16S rDNA genomic region (Firrao *et al*., 1994; Rodriguez *et al*., 2003), which are more suitable for accurate detection of a wider number of genetically diverse strains of *X. fastidiosa* (Harper *et al.,* 2010). PCR reactions were performed in 1X GoTaq buffer (Promega, USA) in a final volume of 25 µl containing 2 µl TNA, 160 µM dNTPs, 0.2 µM of each primer and 1.25 U of GoTaq DNA polymerase (Promega, USA). PCR conditions were different depending on the primer set used. They initially consisted of a denaturation step at 95°C for 5 min followed by 35 cycles of 30 sec at 94°C, 30 sec at 50°C for XF1-F/XF6-R or 55°C for RST31/RST33 and S-S-X.fas-0838-a-S-21/S-S-X.fas-1439-a-A-19, and 40 sec at 72°C. All reactions were finally extended at 72°C for 7 min and visualized on agarose gel. Samples were considered positive when the DNA band of the expected size was clearly visualized after electrophoresis.

Interlaboratory validation of PCR and ELISA assays. Validation of laboratory assays was carried out in November 2013 by the Institutions listed below, under the supervision of the reference laboratory CNR-UNIBA that first

Table 2. Olive samples used for the interlaboratory ring test validation.

ID blind samples	Information	Status for *Xylella fastidiosa*
XF 1	Symptomless field tree	Not infected
XF 2	Symptomless field tree	Not infected
XF 3	Certified virus-free plant (screenhouse-grown)	Not infected
XF 4	Certified virus-free plant (screenhouse-grown)	Not infected
XF 5	Tree showing OQDS	Infected
XF 6	Tree showing OQDS	Infected
XF 7	Certified virus-free plant (screenhouse-grown)	Not infected
XF 8	Tree showing OQDS	Infected
XF 9	Tree showing OQDS	Infected
XF 10	Symptomless field tree	Not infected
XF 11	Tree showing OQDS	Infected
XF 12	Certified virus-free plant (screenhouse-grown)	Non infected
XF 13	Tree showing OQDS	Infected
XF 14	Tree showing OQDS	Infected
XF 15	Tree showing OQDS	Infected
XF 16	Tree showing OQDS	Infected
XF 17	Tree showing OQDS	Infected
XF 18	Tree showing OQDS	Infected
XF PC	Sentinel OQDS tree	Positive control
XF NC	Certified virus-free plant (screenhouse-grown)	Negative control

identified and confirmed the presence of *X. fastidiosa* in olive trees affected by OQDS (Saponari *et al*., 2013).

– CNR-UNIBA: Istituto di Virologia Vegetale del CNR, UOS Bari (Italy), and Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi Aldo Moro, Bari (Italy) (UNIBA);
– CRSFA: Centro di Ricerca, Sperimentazione e Formazione in Agricoltura Basile Caramia, Locorotondo (BA), Italy;
– IAMB: Istituto Agronomico Mediterraneo, Valenzano (BA), Italy.

A set of 18 blind samples (Table 2) was tested by ELISA and PCR using the reagents and protocols previously described. Samples included 12 known positive samples, collected from OQDS-affected trees, grown in the contaminated area, and six healthy samples collected from known *X. fastidiosa*-free sources, that had been repeatedly checked by ELISA and PCR. Four of these samples were from certified screenhouse-grown olive plants obtained and maintained in compliance with the national phytosanitary certification procedure. Results obtained in each laboratory were summarized in a datasheet and the final report mailed to the reference laboratory CNR-UNIBA, where the data were collected and evaluated.

Composite leaf samples. Olive samples were collected either from a single tree (4 cuttings per tree) or, as a bulk, from four trees (1 cutting per tree). A panel of composite samples was prepared using different ratios between positive and negative leaves for each bulk (Table 3), and subjected to ELISA and PCR for defining the reliable detection threshold when composite samples were used. For each bulk, two independent samples (denoted a and b) were prepared and tested separately.

Table 3. Olive samples prepared for each bulk of 4 trees. Each composite sample was prepared using 8 leaves harvested from a total of 4 cuttings. For each sample, infected leaves were gradually substituted with leaves from healthy trees.

ID bulk samples	Cuttings and leaves infected	Cuttings and leaves healthy	Results	
			ELISA	PCR
1a	4, 8	0, 0	+	+
1b	4, 8	0, 0	+	+
2a	3, 6	1, 2	+	+
2b	3, 6	1, 2	+	+
3a	2, 4	2, 4	+	+
3b	2, 4	2, 4	+	+
4a	1, 2	3, 6	+	+
4b	1, 2	3, 6	+	+
5a	0, 0	4, 8	-	-
5b	0, 0	4, 8	-	-

RESULTS

Tissue preparation. Surface sterilization of olive leaves had no detectable effect on ELISA or PCR results. Likewise, testing of samples processed without surface sterilization was not influenced by the presence of possible epiphytic contaminants (data not shown). Indeed, sequence analyses of PCR products recovered from samples processed without surface sterilization proved that the amplified products were specific for *X. fastidiosa* (GenBank accession Nos. HG532023, HG532022, HG532020).

TNA recovery. TNA extracted with methods A, B and C varied from 15 ng/µl (DNeasy plant mini kit) to 1 µg/µl (CTAB). The quality of the extracted TNA was high, as shown by A260/280 ratios close to 1.8. Whereas extracts recovered using procedures A and B were correctly identified by PCR as positive or negative, extracts obtained with method C failed to detect *X. fastidiosa* in samples known to be infected (Table 4). Similarly, method D proved to be unsuitable for olive tissues, as detection failed in several samples known to be positive, regardless of whether undiluted or diluted EB-bacterial release was used.

In conclusion, although procedures A and B were equally effective for recovering suitable DNA templates from olive tissues for large scale tests, procedure B proved to be more user-friendly and cost-effective, as it does not require liquid nitrogen and the sample pre-processing

(homogenization) step is faster. This method was therefore chosen for validation tests in the three laboratories.

Interlaboratory validation of PCR and ELISA tests. *X. fastidiosa* was correctly identified by ELISA and PCR by all laboratories in the samples known to be infected (Table 5). None of the samples known to be *X. fastidiosa*-free gave false positive reactions. Only one infected sample was not correctly identified in one laboratory. However, this sample tested negative by both ELISA and PCR, indicating that the detection failure may have been due to the absence of the bacterium in the material examined. PCR reactions were consistent regardless of the primer sets used. However, signal intensity of specific amplicons in positive samples varied according to the primer pairs used. The primer set XF1/XF6 and S-S-X.fas-0838-a-S-21 S-S-X.fas-1439-a-A-19 gave comparable results, while primers RST31/RST33 amplified DNA bands showing a lighter signal (Fig. 2). ELISA results obtained by all three laboratories showed that infected samples produced clear and strong reactions similar to those of positive controls, with OD_{405} values ranging from 0.700 to 3.500 after 120 min.

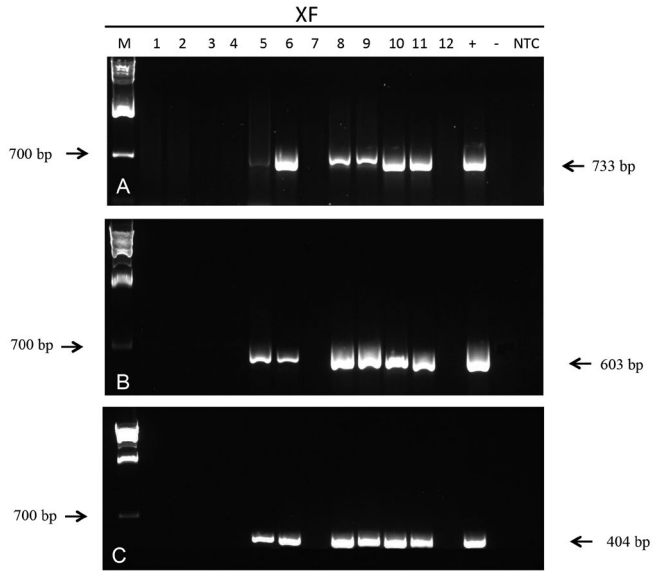

Fig. 2. Gel electrophoresis of PCR products recovered from the samples XF1-XF12 (Table 2). A. Primers sets RST31/RST33; B. Primers sets S-S-X.fas-0838-a-S-21 S-S-X.fas-1439-a-A-19; C. Primers sets XF1/XF6. M= DNA ladder; + = positive control; - = negative control; NTC = non-template control.

Comparison of molecular and serological tests. Ring test data showed the complete agreement of the outcome of PCR and ELISA tests carried out in the three laboratories. *X. fastidiosa* was detected with both types of assays in olive tissues, attesting the reliability and reproducibility of the two protocols tested (Table 5).

***X. fastidiosa* detection in composite leaf samples.** Positive ELISA and PCR reactions were obtained using composite leaf samples from as many as four trees, with at least one infected tree over the four sampled. This supports the feasibility of the use of composite samples in large scale monitoring, which increases the number of trees liable to be tested in each survey.

Given the recent identification of *X. fastidiosa* presence in southern Italy (Saponari *et al.*, 2013) there were many open questions that needed to be and are being addressed, such as: (i) determination of the size of the main outbreak area (currently estimated at *ca.* 8,000 ha); (ii) identification

Table 4. Comparison of different procedures used for total nucleic acid preparation for *Xylella fastidiosa* detection by PCR.

Procedure	No. of infected trees identified/ total infected trees	No of healthy trees identified/ total healthy trees	Total nucleic acids concentration (ng/µl) and quality (ratio A260/280)
A	4/4	4/4	15-24 (1.94)
B	4/4	4/4	300-1000 (1.78)
C	0/4	4/4	200-500 (1.85)
D	Undiluted extract 0/4 Dilution 10^{-1} 0/4 Dilution 10^{-2} 1/4 Dilution 10^{-3} 2/4	4/4	Not determined

Table 5. Results of laboratory performances using blind samples for *Xylella fastidiosa* detection by PCR and ELISA assays.

Laboratory	PCR		ELISA	
	Positive samples/ Positives provided	Negative samples/ Negatives provided	Positive samples / Positives provided	Negative samples/ Negatives provided
CNR-UNIBA	12/12	8/8	12/12	8/8
CRSFA	12/12	8/8	12/12	8/8
IAMB	11/12	8/8	11/12	8/8

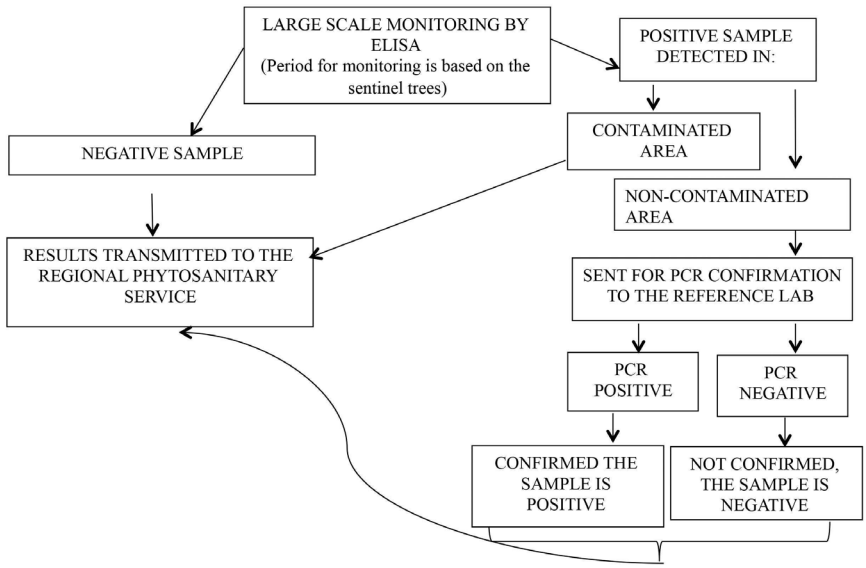

Fig. 3. Flow diagram of the diagnostic procedure used in Apulia for the *Xylella fastidiosa* monitoring programme.

Fig. 4. Map of Salento peninsula (southern Apulia). Boxed areas are the currently known locations of *Xylella fastidiosa* outbreaks.

of additional infection foci; (iii) identification of the insect vectors and their use in transmission trials; (iv) identification of the hosts serving as inoculum source for olive; (v) determination of secondary spreading in olive, if any; (iv) distribution and seasonal fluctuation of the bacterium in infected olive trees; (vii) isolation of the bacterium in axenic culture and its genotyping; (viii) pathogenicity to olive.

As it was considered that for any effective containment strategy of *X. fastidiosa* in an outbreak area the correct identification of the pathogen by quick, sensitive and reliable laboratory tests is crucial, the comparative detection trials described herein were given priority. This has allowed the finalization of the diagnostic tools to be used in a large-scale survey for *X. fastidiosa* detection, first in the province of Lecce, then in the rest of Apulia (Fig. 3). Of the 16,000 tests foreseen, more than 3,000 have already been done. Preliminary results have identified three active infection sites in the province of Lecce (Fig. 4), but none in the neighbouring provinces of Brindisi and Taranto. This is taken as an encouraging indication that the confinement of *X. fastidiosa* to the province of Lecce is feasible.

ACKNOWLEDGEMENTS

Grateful thanks are expressed to Prof. G.P. Martelli for encouragement, the useful suggestions and editing of the text, and to the Phytosanitary Services of Apulia for financial support.

REFERENCES

Almeida R.P.P., Mann R., Purcell A.H., 2004. *Xylella fastidiosa* cultivation on a minimal solid defined medium. *Current Microbiology* **48**: 368-372.

Blexine B., Child B., 2007. *Xylella fastidiosa* genotype differentiation by SYBR green-based qRT-PCR. *FEMS Microbiological Letters* **276**: 48-54.

Carbajal D., Morano K.A., Morano L.D., 2004. Indirect immunofluorescence microscopy for direct detection of *Xylella fastidiosa* in xylem sap. *Current Microbiology* **49**: 372-375.

Chang C.J., Garnier M., Zreik L., Rossetti V., Bové J.M., 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.

Chen J., Groves R., Civerolo E.L., Viveros M., Freeman M., Zheng Y., 2005. Two *Xylella fastidiosa* genotypes associated with almond leaf scorch disease on the same location in California. *Phytopathology* **95**: 708-714.

Chen J., Livingston S., Groves R., Civerolo E.L., 2008. High throughput PCR detection of *Xylella fastidiosa* directly from almond tissues. *Journal of Microbiological Methods* **73**: 57-61.

Davis M.J., Purcell A.H., Thompson S.V., 1978. Pierce's disease of grapevines: isolation of the causal bacterium. *Science* **199**: 75-77.

de Souza A.A., Takita M.A., Coletta-Filho H.D., Caldana C., Goldman G.H., Yanai G.M., Muto N.H., de Oliveira R.C., Nunes L.R., Machado M.A., 2003. Analysis of gene expression in two growth states of *Xylella fastidiosa* and its relationship with pathogenicity. *Molecular Plant-Microbe Interactions* **16**: 867-875.

EPPO, 2004. Diagnostic protocols for regulated pests. *Xylella fastidiosa. Bulletin OEPP/EPPO Bulletin* **34**: 187-192.

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

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

Guan W., Shao J., Singh R., Davis R.E., Zhao T., Huang Q., 2013. A TaqMan-based real time PCR assay for specific detection and quantification of *Xylella fastidiosa* strains causing bacterial leaf scorch in oleander. *Journal of Microbiological Methods* **92**: 108-112.

Harper S.J., Ward L.I., Clover G.R.G., 2010. Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. *Phytopathology* **100**: 1282-1288.

Hendson M., Purcell A.H., Chen D., Smart C., Guilhabert M., Kirkpatrick B., 2001. Genetic diversity of Pierce's disease strain and other pathotypes of *Xylella fastidiosa*. *Applied and Environmental Microbiology* **67**: 895-903.

Hernandez-Martinez R., de la Cerda K.A., Costa H.S., Cooksey D.A., Wong F.P., 2007. Phylogenetic relationships of *Xylella fastidiosa* strains isolated from landscape ornamentals in Southern California. *Phytopathology* **97**: 857-864.

Hopkins D.L., Mollenhauer H.H., 1973. Rickettia-like bacterium associated with Pierce's disease of grapes. *Science* **179**: 298-300.

Hopkins D.L., Purcell A.H., 2002. *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. *Plant Disease* **86**: 1056-1066.

Huang Q., Bentz J., Sherald J.L., 2006. Fast, easy and efficient DNA extraction and one-step polymerase chain reaction for the detection of *Xylella fastidiosa* in potential insect vectors. *Journal of Plant Pathology* **88**: 77-81.

Huang Q., 2009. Specific detection and identification of *Xylella fastidiosa* strains causing oleander leaf scorch using polymerase chain reaction. *Current Microbiology* **58**: 393-398.

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

Krugner R., Johnson M.W., Chen J., 2010. Evaluation of pathogenicity and insect transmission of *Xylella fastidiosa* strains to olive plants. California Olive Committee. *Final Report* 2010: http://calolive.org/wp-content/uploads/Research-Reports-2010.pdf

Lee R.F., Beretta M.J.G., Derrick K.S., Hooker M.E., 1992. Development of a serological assay for citrus variegated chlorosis: A new disease of citrus in Brazil. *Proceedings of the Florida State Horticultural Society* **105**: 32-35.

Li W., Teixeira D.C., Hartung J.S., Huang Q., Duan Y., Zhou L., Chen J., Lin H., Lopes S., Ayres A.J., Levy L., 2013. Development and systematic validation of qPCR assays for rapid and reliable differentiation of *Xylella fastidiosa* strains causing citrus variegated chlorosis. *Journal of Microbiological Methods* **92**: 79-89.

Lin H., Walker M.A., 1997. Extracting DNA from cambium tissue for analysis of grape rootstocks. *Horticulture Science* **32**: 1264-1266.

Mehta A., Leite R.P. Jr., Rosato Y.B., 2001. Assessment of the genetic diversity of *Xylella fastidiosa* isolated from citrus in Brazil by PCR-RFLP of the 16S rDNA and 16S-23S intergenic spacer and rep-PCR fingerprinting. *Antonie van Leeuwenhoek* **79**: 53-59.

Minsavage G.V., Thompson C.M., Hopkins D.L., Leite R.M.V.B.C., Stall R.E., 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. *Phytopathology* **84**: 446-461.

Murray M.G., Thompson W.F., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**: 4321-4325.

Oliveira A.C., Vallim M.A., Semighin, C.P., Araújo W.L., Goldman G.H., Machado M.A., 2002. Quantification of *Xylella fastidiosa* from citrus trees by real-time polymerase chain reaction assay. *Phytopathology* **92**: 1048-1054.

Pooler M.R., Hartung J.S., 1995. Genetic relationships among strains of *Xylella fastidiosa* from RAPD-PCR data. *Current Microbiology* **31**: 134-137.

Purcell A.H., 2013. Paradigms: Examples from the bacterium *Xylella fastidiosa*. *Annual Review of Phytopathology* **51**: 229-356.

Randal J.J., Golberg N.P., Kemp J.P., Radionenko M., French J.M., Olsen M.W., Hanson S.F., 2009. Genetic analysis of a novel *Xylella fastidiosa* subspecies found in the southwestern United States. *Applied and Environmental Microbiology* **75**: 5631-5638.

Rodrigues J.L.M., Silva-Stenico M.E., Gomes J.E., Lopes J.R.S., Tsai S.M., 2003. Detection and diversity assessment of *Xylella fastidiosa* in field-collected plant and insect samples by using 16S rRNA and gyrB sequences. *Applied and Environmental Microbiology* **69**: 4249-4255.

Saponari M., Boscia D., Nigro F., Martelli G.P., 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**: 668.

Scally M., Schuenzel E.L., Stouthamer R., Nunney L., 2005. Multilocus sequence type system for the plant pathogen *Xylella fastidiosa* and relative contributions of recombination and point mutation to clonal diversity. *Applied and Environmental Microbiology* **71**: 8491-8499.

Schaad N.W., Opgenorth D., Gaush P., 2002. Real-time polymerase chain reaction for one-hour on-site diagnosis of Pierce's disease of grape in early season asymptomatic vines. *Phytopathology* **92**: 721-728.

Schaad N.W., Postnikova E., Lacy G., Fatmi M., Chang C.J., 2004. *Xylella fastidiosa* subspecies: *X. fastidiosa* subsp *piercei*, subsp. nov., *X. fastidiosa* subsp *multiplex*, subsp. nov., *X. fastidiosa* subsp *pauca*, subsp. nov. *Systematic and Applied Microbiology* **27**: 290-300.

Sherald J.L., Lei J.D., 1991. Evaluation of a rapid ELISA test kit for detection of *Xylella fastidiosa* in landscape trees. *Plant Disease* **75**: 200-203.

Wong F., Cooksey D.A., Costa H.S., 2004. Documentation and characterization of *Xylella fastidiosa* strains in landscape hosts. *CDFA Pierce's Disease Control Program Progress Report. Proceedings Pierce's Disease Research Symposium San Diego, USA*: 121-123.