SHORT NOTE

Direct tissue blot immunoassay for detection of *Xylella fastidiosa* in olive trees

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Summary. A direct tissue blot immunoassay (DTBIA) technique has been compared with ELISA and PCR for detection of *Xylella fastidiosa* in olive trees from Apulia (southern Italy). Fresh cross-sections of young twigs and leaf petioles were printed onto nitrocellulose membranes and analyzed in the laboratory. Analyses of a first group of 61 samples gave similar efficiency for the three diagnostic techniques for detection the bacterium (24 positive and 36 negative samples), except for a single sample which was positive only with DTBIA and PCR. Similar results were obtained by separately analyzing suckers and twigs collected from different sectors of tree canopies of a second group of 20 olive trees (ten symptomatic and ten symptomless). In this second test the three diagnostic techniques confirmed the irregular distribution of the bacterium in the tree canopies and erratic detectability of the pathogen in the young suckers. It is therefore necessary to analyse composite samples per tree which should be prepared with twigs collected from different sides of the canopy. The efficiency comparable to ELISA and PCR, combined with the advantages of easier handling, speed and cost, make DTBIA a valid alternative to ELISA in large-scale surveys for occurrence of *X. fastidiosa*. Moreover, the printing of membranes directly in the field prevents infections spreading to *Xylella*-free areas, through movement of plant material with pathogen vectors for laboratory testing.

Key words: DTBIA, *Olea europea,* Olive quick decline syndrome, PCR, survey.

Introduction

Xylella fastidiosa, a gamma proteobacterium in the family *Xanthomonadaceae*, is a regulated quarantine pest, whose introduction and spread into EU Member States is banned. Four subspecies of the bacterium have been described: ssp. *fastidiosa*, ssp. *pauca*, ssp. *multiplex* and ssp. *sandyi* (Schaad *et al*., 2004; Schuenzel *et al*., 2005). These organisms induce various symptoms (marginal leaf scorching, wilting of foliage and withering of branches, dieback and stunting with eventual plant death) in susceptible host plants. *Xytella fastidiosa* is transmitted by xylem fluid feeding insect vectors (e.g. *Auchenorrhyncha*, mainly sharpshooter leafhoppers and froghoppers or spittlebugs) and is associated with a number of important diseases in a wide range of plant species. However, many host plants may remain latently infected, not showing any symptoms and serving as sources of inoculum for vectors (Hopkins and Purcell, 2002).

The first report of *X. fastidiosa* under field conditions in the European Union was in southern Apulia, Italy, associated with severe cases of an "olive quick decline syndrome" (Saponari *et al*., 2013), and the pathogen was characterized as a strain of the subspecies Pauca (Cariddi *et al.,* 2014). A monitoring campaign was immediately organized by the Regional Plant Protection Service to establish the exact distribution of the pathogen and to limit further spread.

Several samples of different plant species, primarily olive trees, were efficiently tested using ELISA for routine pathogen detection, and using PCR assays for confirmation of ELISA-positive and/or doubtful samples (Loconsole *et al.*, 2014).

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Timely and safe delivery of fresh tissue samples in the laboratory and their handling are critical issues for avoiding dissemination of this pathogen to "pathogen-free" areas. However, despite all precautions that can be taken, the risks associated with the handling and movement of such large quantities of plant material, which can often carry infected vectors, remain very high. To face such threats, Direct Tissue Blot Immunoassay (DTBIA) was investigated as an alternative diagnostic tool to ELISA, in order to directly perform mass analyses of plant samples directly in the field, thus avoiding the potential pathogen spread through sample delivery. Several lateral flow devices have been developed to be used in the field for the fast detection of plant pathogens in symptomatic material (Lopez *et al.*, 2003), including *X. fastidiosa*. During the present study these were not considered, due to their high costs for large scale surveys. DTBIA was already successfully applied in the mass detection of citrus disease agents, mainly *Citrus tristeza virus* and *Citrus psorosis virus* (Garnsey *et al.*, 1993; Cambra *et al.*, 2000; D'Onghia *et al.*, 2001). Some attempts to use DTBIA applications were also reported for the detection of *X. fastidiosa* from citrus affected by variegated chlorosis (Garnier *et al.*, 1993) and for other infectious agents (Lin *et al.*, 1990). High sensitivity, short assay time, and low costs are the main advantages of DTBIA, which can be performed directly in the field. Moreover, blotted membranes can be easily and safely shipped from one place to another, avoiding the risk of pathogen and vector dissemination (Cambra *et al.*, 2000; Djelouah and D'Onghia, 2001).

Here we report results from DTBIA for the detection of *X. fastidiosa* in olive plant material, and compare this method with ELISA and PCR assays for detection of the pathogen.

Materials and methods

The experimental work was conducted from November 2013 to March 2014, and subdivided in three different steps. In a preliminary step, a total of ten olive trees, five *Xf*-infected and five *Xf*-negative as previously identified by ELISA and PCR assays (Loconsole *et al.*, 2014), were used respectively as positive and negative controls for the assessing the DTBIA technique for detection of *X. fastidiosa* in olive.

In the second step, DTBIA performance was evaluated in comparison with ELISA and PCR us-

Figure 1. Decline of the canopy of an olive tree infected by *Xylella fastidiosa*.

ing 61 samples from 20 symptomatic olive trees (showing quick decline and leaf scorch: Figure 1) and 41 symptomless trees. Sampled trees were from four commercial orchards located in Gallipoli (2), Parabita and Taviano municipalities (Salento peninsula), largely infected by *X. fastidiosa*. In symptomatic trees, samples were preferentially collected from symptomatic branches.

In the third step, to investigate distribution of the pathogen in tree canopies, 20 out of the previous 61 tested olive trees were selected as follows: ten symptomatic (all positive) and ten symptomless trees (five positive and five negative). From each tree, five samples, one from basal suckers and one from each of the four branches at different positions in the canopy, were separately collected. A total of 100 samples were then analyzed.

During field operations, samples were stored in closed plastic bags in a cooling box during the delivery to the laboratory. Each sample was divided into three subsamples for comparative assays with ELISA and PCR diagnostic methods.

Dot blot immunoassay (DTBIA)

Tissue blots were prepared as described by Lin *et al.* (1990). Because *X. fastidiosa* is localized in host
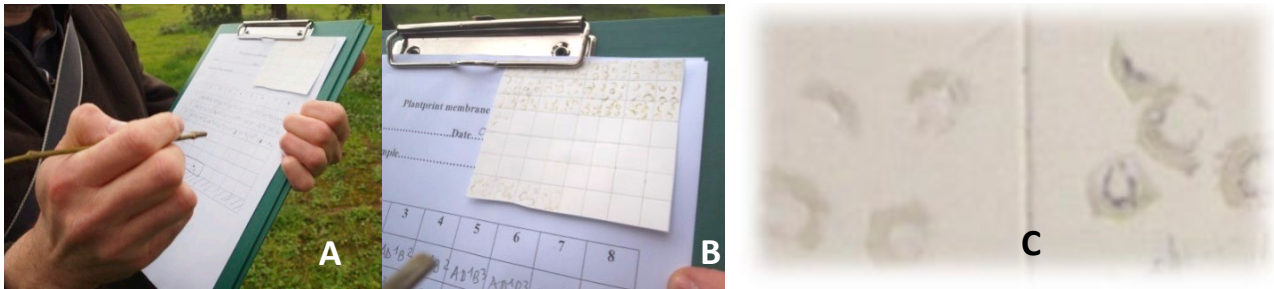
Figure 2. A) Printing of olive twigs in the field; B) detail of the nitrocellulose membrane: each sample is printed in two different grids and in each grid four prints representative of different branches of a tree canopy are blotted; C) purple coloration observed on stem sections of *Xf* infected olive samples (right), negative control (left).

xylem tissues, blots were made from cross sections of mature leaf petioles and twigs (2–5 mm in diameter), apical shoots excluded. Smooth fresh cuts were made with pruning shears, previously disinfected in a 10% solution of chlorine commercial bleach, and cut sections were gently pressed to the membrane (Figure 2). Each type of sample was printed twice. Gloves were used when handling the membranes and in the blotting process.

Two types of Protran (Sigma-Aldrich) nitrocellulose membranes were used, of 0.20 and 0.45 µm pore size, with high affinity for binding proteins and compatibility with a variety of detection methods. The selected membranes were cut to an appropriate size for the number of samples to be blotted, and pre-marked with a grid of suitable size so as to record the positions of individual samples. The same samples were blotted on both membranes that were processed at two intervals of time, within a few hours of collection and after 1 week.

Printed membranes were left to dry for 20–30 min at room temperature for fresh use or were stored in the dark always at room temperature. They were placed in the blocking solution using different incubation times and concentrations of Bovine Serum Albumin (BSA) or Fat milk solutions. After saturation of protein-binding sites with the selected solution on a shaker and washing with PBS containing 0.05% Tween 20, blotted membranes were exposed for 2 h to alkaline phosphatase-conjugated polyclonal antibodies to *X. fastidiosa* (Loewe Biochemica GmbH), at different dilutions of conjugate buffer (from 1:50 to 1:200).

Membranes were then stained by immersion in a solution obtained by dissolving one tablet of Sig-

maFast™ BCIP-NBT, in 10 mL of distilled water, and incubation at room temperature until a purple-violet colour appeared in the positive controls. The reaction was stopped by washing with tap water. After drying at room temperature, the membranes were observed under a low power magnification lens (×10 or ×20).

Comparison with other detection techniques

Olive leaves from twigs tested by DTBIA were also analysed by DAS-ELISA and PCR (Loconsole *et al.*, 2014).

DAS-ELISA. Samples were tested using specific antibodies to *X. fastidiosa* (Loewe Biochemica GmbH), according to the manufacturer's instructions. Extracts were obtained from leaf petioles and midveins macerated in plastic bags in the presence of PBS-buffer using an automated extractor. Absorbance was recorded at 405 nm using a microplate reader (Multiskan Ascent, Labsystems). Samples with absorbance readings exceeding three times that of the healthy control were considered as positive.

PCR. Total nucleic acids were extracted from olive samples using a CTAB-based extraction buffer (Rodrigues *et al.*, 2003). Petioles and vein tissues (600–800 mg), excised from surfaces of sterilized leaves, were homogenized in 1.5 mL of extraction buffer (20 mM EDTA, 350 mM sorbitol in 100 mM Tris-HCl, pH 7.5 plus 2.5% w/v PVP and 0.2% of β-mercaptoethanol) using a semi-automated homogenizer (Omex, Bioreba). Tubes were centrifuged at 16,000 g for 20 min and the pellets were re-suspended in 300 µL of buffer containing 20 mM EDTA, 350 mM sorbitol in 100 mM Tris–HCl, pH 7.0, and 300 µL of DNA lysis buf-

fer [50 mM EDTA, 2 M NaCl, 2% (w/v) CTAB in 200 mM Tris–HCl, pH 7.5, and 200 μL of 5% sarcosyl], mixed well and incubated at 65°C for 45 min. Extracted DNA was purified using chloroform-isoamyl alcohol (24:1) and precipitated with isopropanol after a 30 min incubation at -20°C. The total DNA preparation from each sample was re-suspended in 200 μL of 0.5× TE. For PCR, the RST31/RST33 set of primers targeting the 16S rDNA gene was used (Minsavage *et al.*, 1994). Reactions were conducted in a final volume of 25 μL, using 5 μL of 5× GoTaq polymerase (Promega), 250 nM each of forward and reverse primer, and 1 μL of total DNA template (50 ng μL^{-1}). Thermocycling conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 50–55°C for 30–45 sec and 72°C for 30 sec, and a final extension of 5 min at 72°C. Successful amplification was confirmed by an electrophoresis run in agarose gel of a 10 μL aliquot of reaction product.

Results and discussion

Preliminary tests carried out on previously tested negative and positive olive samples (Loconsole *et al.*, 2014) showed that DTBIA detected *X. fastidiosa.* Some differences were observed in the two types of nitrocellulose membranes, with the 0.45 μm membrane performing better in terms of blot colour reaction. Similarly, concerning the protein-binding sites, the use of 1% fat milk solution, coupled with a gentle stirring on a shaker for 1 h, gave better results than BSA, which has been commonly used in previous research (Garnsey *et al.*, 1993; Garnier *et al.*,1993; D'Onghia *et al.*, 2001). Using 1:100 dilution of AP-conjugated antibodies, the purple-stained area in the *X. fastidiosa*-infected imprints on blotted membranes was more intense and usually appeared within 2–5 min after the addition of substrate. Of the different plant tissues used for testing, the best reactions were generally obtained from twigs, which showed very distinct and homogeneous stained areas, whereas reactions from petioles were less clear-cut, and sometimes totally absent. Blots from negative controls remained virtually unstained (Figure 2). No apparent difference was observed between fresh and 1-week-stored blotted membranes, as well as between membranes printed directly in the field and those printed in the laboratory.

The protocol of DTBIA previously developed was adopted in the trials that followed.

In comparative tests with DAS-ELISA and PCR, results of the field application of DTBIA showed no relevant differences when 61 olive trees exposed to potential inoculum of *X. fastidiosa* in Salento area were analyzed. Twenty-four out of 61 tested trees were positive for *X. fastidiosa* with each of the three diagnostic techniques. Only in one symptomless tree was the bacterium detected by PCR and DTBIA, but not by ELISA (Table 1). All 20 olive trees showing quick decline and leaf scorch symptoms gave posi-

Table 1. Comparative results in the detection of *Xylella fastidiosa* (*Xf*) in symptomatic (S) and asymptomatic (A) olive trees using DTBIA, ELISA and PCR diagnostic techniques.

Location	Trees (S)/(A)	No. of samples	No. *Xf*-infected samples (%)		
			ELISA	PCR	DTBIA
Gallipoli	S	18	18 (100%)	18 (100%)	18 (100%)
	A	29	4 (13.8%)	5 (17.2%)	5 (17.2%)
Parabita	S	0	-	-	--
	A	10	0	0	0
Taviano	S	2	2 (100%)	2 (100%)	2 (100%)
	A	2	0	0	0
Total	S	20	20 (100%)	20 (100%)	20 (100%)
	A	41	4 (9.8%)	5 (12.2%)	5 (12.2%)

tive reactions. Of the numerous apparently symptomless olive trees (41), only five were positive in the laboratory tests (Table 1).

In the third trial, which evaluated pathogen distribution in the canopies and in suckers of 20 olive trees, the three diagnostic techniques (ELISA, DTBIA and PCR) each confirmed the presence of *X. fastidiosa* as reported in the second trial (Table 2). Conversely, *X. fastidiosa* was detected on six (instead of five) out of ten symptomless trees and in all the symptomatic trees in at least one of the five samples collected per tree.

On average, three out of four branches of symptomatic infected-trees (75%) and one out of four branches of asymptomatic infected trees (25%) were positive to *X. fastidiosa*. Only one symptomatic basal sucker sample was infected while the remaining were all negative.

Also in this trial, no relevant differences were observed in the results obtained using the three different diagnostic techniques. Discrepancies were observed only in three of 100 cases: in two cases the tests were positive by PCR and DTBIA, but not by ELISA, while one sample was positive by PCR and ELISA, but not by DTBIA.

Results obtained in this study clearly indicate that DTBIA is a reliable technique for detecting *X. fastidiosa* in olive trees, showing characteristics of sensitivity and reliability similar to ELISA. DTBIA also detected the presence of infections in asymptomatic branches of trees showing spotted symptoms in their canopies, or in totally symptomless trees (12.2%), where lower bacterial concentration occurs, presumably due to recent infections. The detection of *X. fastidiosa* in all symptomatic olive trees confirms the decisive role of this bacterium in the olive rapid decline syndrome, the aetiology of which possibly also involves some fungal and insect agents (Guario *et al.*, 2013).

Results obtained using the DTBIA also highlighted the importance of adopting appropriate sampling for effective pathogen detection in large scale monitoring. Especially in the early stages of infection, the bacterium can be localized in sectors of tree canopies, often symptomless, as was clearly shown by the third test conducted in this study. Six out of ten apparently asymptomatic trees were positive for the bacterium only in one of the four tested branches/canopy; conversely, on average, at least one out of the four branches was negative in ten symptomatic trees. Particularly in the case of asymptomatic trees, it is essential to use a composite sample representative of the whole of each olive tree, by collecting twigs from different sectors of the canopy. Samples of grouped trees for ELISA testing in large scale monitoring are not recommended. Young suckers should be avoided for sampling since they are probably infected at a later stage, as a result of xylem translocation of the bacterium. Because the bacterium is unevenly distributed within the canopy, and because four twigs can be printed in each quadrant of the grid drawn on a membrane, two quadrants can be allocated to each tree to be tested, so that a number of representative twigs is therefore analyzed.

The use of DTBIA for the analysis of a greater numbers of plants in olive nurseries is even more advantageous. Given the small size of these plants, the number of prints/plant can be reduced to two for these assays.

In some cases, lateral flow devices could be used; the recently developed kit (*Xylella fastidiosa* Loewe ®FAST Kit, Loewe BiochemicaGmBH) was set up for the rapid identification of the pathogen in sus-

Table 2. Comparative results in the detection of *Xylella fastidiosa* (*Xf*) in olive branches and suckers by DTBIA, ELISA and PCR.

Olive tree	Sample	No. of samples	No. Xf-infected samples (%)		
			ELISA	PCR	DTBIA
Symptomatic/ infected (ten trees)	Branches	40	28 (70%)	30 (75%)	29 (72.5%)
	Suckers	10	1 (10%)	1 (10%)	1 (10%)
Asymptomatic/ infected (five) and not-infected (five) trees	Branches	40	6 (15%)	6 (15%)	6 (15%)
	Suckers	10	0	0	0

pected cultures from agar plates. The sensitivity and specificity of this kit was not assayed and validated for detecting *Xylella fastidiosa* in olive plant tissues. Therefore, apart from the high costs to use these kits for extensive field surveys, their unconfirmed robustness led to their exclusion from our trials.

The DTBIA technique, which is proposed as a reliable alternative to ELISA in large-scale surveys, has several advantages that can be summarized as follows: i) the technique is easy to handle and sophisticated equipment or highly skilled operators are not required; ii) the method requires short times for execution (*ca* 4 h); iii) cost is about 50% less than for ELISA; iv) the number of daily analyzed samples per working unit is at least twice that for ELISA; v) the possibility to mail printed membranes or store them at room temperature for several days before testing is very adventageous; and vi) the printing of samples directly in the field avoids the movement of infected plant material, reducing the risk of spreading the bacterium to new uninfested areas.

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