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SHORT COMMUNICATION

ISOLATION OF A *XYLELLA FASTIDIOSA* STRAIN INFECTING OLIVE AND OLEANDER IN APULIA, ITALY

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SUMMARY

The isolation in pure culture of the *Xylella fastidiosa* strain associated with the quick decline syndrome of olive, recently observed in Apulia (Salento peninsula, southern Italy) was attempted from symptomatic, naturally infected olive and oleander plants, and a periwinkle seedling that had been exposed to, and was infected by *Xylella*-positive spittlebugs. Prior to isolation, the presence of *Xylella* was ascertained in all donor hosts by PCR, indirect immunofluorescence and electron microscopy. Isolations from olive failed because of the heavy contamination by bacteria other than *Xylella.* By contrast, pure bacterial cultures were obtained from oleander and periwinkle extracts plated in periwinkle wilt gelrite (PWG) and buffered cysteine-yeast extract (BCYE) media. In both media, colonies were slow-growing, small-sized (less than 1 mm 25 days from plating), non pigmented, opalescent and exhibited the same morphology, except for the margin that was entire in BCYE and somewhat irregular in PWG. Bacterial cells were rod-shaped with rounded ends, had a thick and rippled cell wall, an average width of 0.35 µm, and a maximum length of *ca.* 5 µm. They gave a positive reaction in immunofluorence assays and were clearly decorated by colloidal gold in immunogold labelling tests. Sequenced PCR products amplified from periwinkle and oleander colonies shared 97-99% sequence identity with known *X. fastidiosa* strains from database and were 100% identical to one another and to comparable sequences obtained from infected olive trees. These sequences grouped in a distinct cluster of a branch comprising *X. fastidiosa* isolates belonging to the subspecies *pauca.*

Key words: *Xylella*, olive decline, electron microscopy, immunofluorescence, PCR, genotyping, subspecies *pauca.*

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The quick decline syndrome of olive (OQDS) is a destructive disorder that appeared suddenly a few years ago in the groves of a restricted area of the Apulian province of Lecce, Salento peninsula, south-east Italy (Martelli, 2013). From there, the disease expanded to a wider area, currently estimated at some 8,000 ha. In some instances, especially in relatively young olive stands (50-60 years of age), symptoms may be limited to withering and desiccation of scattered shoots and small branches. By contrast, in the case of OQDS proper, the desiccation extends to the rest of the canopy and is followed by a collapse of the trees. Centenarian olives seem to be the most seriously affected by this severe syndrome. These big trees, when collapsed, are heavily pruned by the growers in the hope to incite sprouting of new vegetation. This rarely happens, although the trees are not dead, as shown by the abundant production of suckers from the crown (Fig. 1A).

The pathological condition of very aged and declining trees is complex, for three major factors seem to be involved in its induction: (i) infestations by the leopard moth (*Zeuzera pyrina*), a lepidopteran, whose galleries excavated in trunks and branches facilitate the entry of the second agent, (ii) a set of xylem-inhabiting fungi of various genera, with a prevalence of *Phaeoacremonium parasiticum* (Nigro *et al.*, 2013) which colonize and necrotize the sapwood and, (iii) the xylem-limited bacterium *Xylella fastidiosa* (Saponari *et al.*, 2013). The identification of the latter agent, a regulated quarantine pathogen in the European Union, was substantiated by an extensive survey of the olive groves in the province of Lecce which, in agreement with EPPO's (2004) specifications, was carried out with serological (ELISA) and molecular (PCR) methods (Loconsole *et al.*, 2014).

Because the specific, but still undetermined, role of *X. fastidiosa* in the aetiology of the OQDS needs to be ascertained by pathogenicity tests, the availability of pure bacterial cultures to be used as inoculum is required. Thus, the recovery in axenic culture of the olive-infecting isolate of *X. fastidiosa* (Salento strain) was attempted and successfully achieved, as described in the present paper.

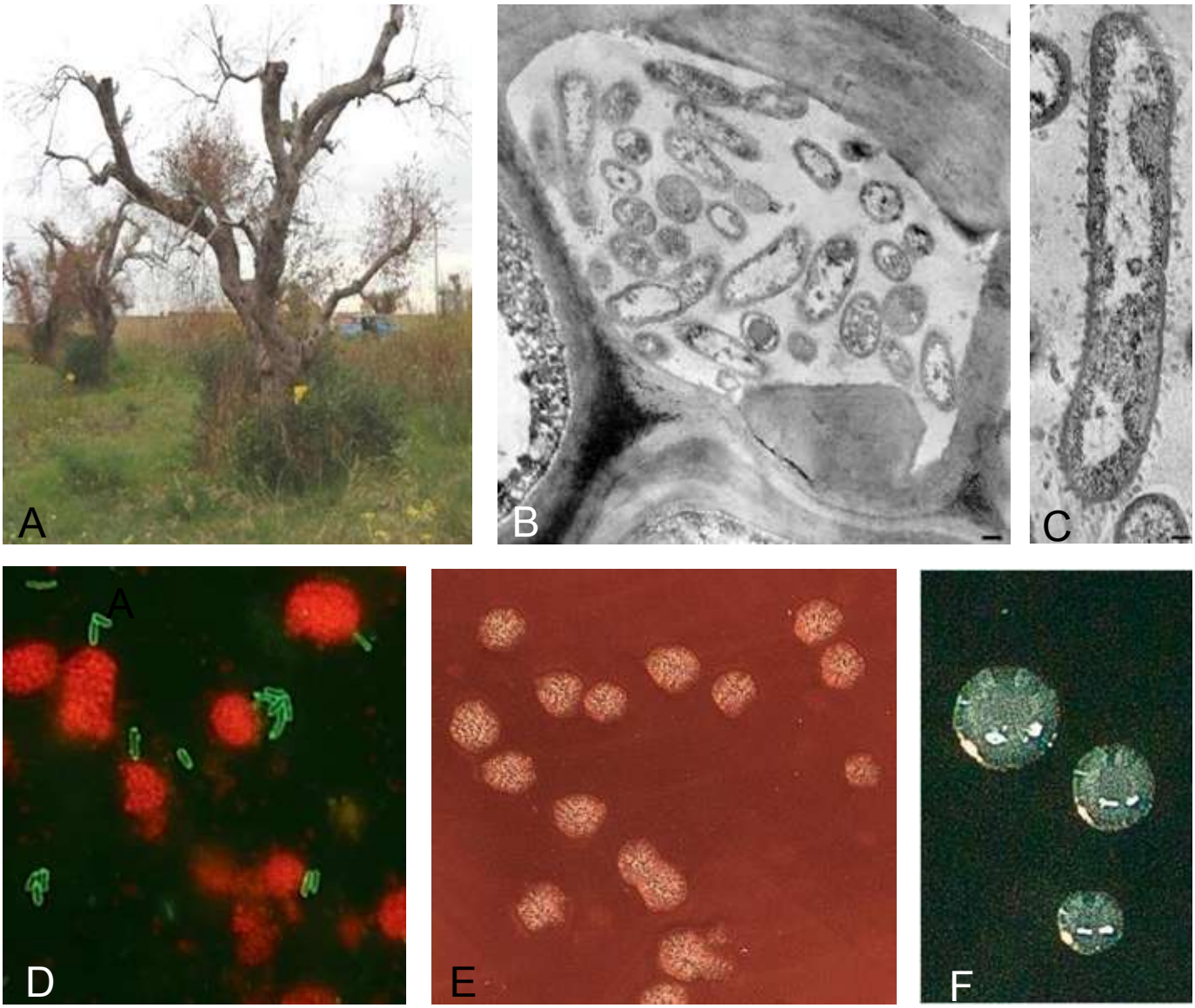

Fig. 1. A. Aged olive tree affected by quick decline. The tree is desiccated but suckers are sprouting from the crown. B. Electron micrograph of a cross-sectioned leaf petiole of a diseased olive showing an accumulation of bacterial cells in a tracheary element. Bar = 200 nm. C. Details of a *Xylella* cell in a tracheary element of a diseased olive tree. Bar = 100 nm. D. Immunofluorescence-positive green-fluorescing bacterial cells in a periwinkle extract used for isolation in culture. E, F. Colonies of the *Xylella fastidiosa* strain isolated from a spittlebug-infected periwinkle growing on PWG (E) and BCYE (F) medium, respectively.

Xylella-infected sources used for isolation were field-grown symptomatic olive trees and oleanders with leaf scorching, and a glasshouse-grown periwinkle (*Catharanthus roseus*) seedling that had been exposed to, and was infected by field-collected *Xylella*-positive spittlebugs (*Philaenus spumarius*) (Saponari *et al*., 2014).

Donor plants chosen for isolation were preliminarly tested for the presence of *Xylella* by ELISA and PCR according to Loconsole *et al.* (2014). Moreover, leaf extracts were exposed to indirect immunofluorescence (IF), and thin-sectioned tissues were observed under the electron microscope.

For the indirect IF test, 20 µl of a tissue extract like that used for bacterial isolations (see below) plus the negative (extract from healthy hosts) and positive (Loewe 07319PC, Germany) controls were pipetted at dilutions of 1:10 and 1:100 in PBS pH 7.2 into the wells of multiwell glass slides. These were allowed to dry under a laminar flow cabinet for 20 min on a heated support. Ten µl of the primary antibody (Loewe 07319/02) diluted 1 : 1000 in PBS were added to each well and the slides were incubated for 30 min in the dark in a humid chamber at room temperature, then rinsed three times with PBS supplemented with 0.1% Tween 20 and gently dried with filter paper. Subsequently, 10 µl of conjugated antiserum diluted 1:150 in PBS Goat-antirabbit IgGs [(H+L) FITC-Loewe 07201] were added to each well and the slides were incubated, rinsed and dried as above. Aliquots (10 µl) of phosphate glyceric buffer ($Na_2HPO_4 \cdot 12H_2O$ 3.2 g; $NaH_2PO_4 \cdot 2H_2O$ 0.15 g; glycerol 50 ml, distilled water 100 ml, pH 7.6) were pipetted

in each well, the slides were covered with coverslips and observed with an epifluorescence microscope (Zeiss Axioskop) equipped with a HBO 50 mercury incidence light source and filter system at 400× and 1000× magnification.

For electron microscopy small fragments of leaf petioles and midveins were processed according to standard procedures at 4°C throughout (Martelli and Russo, 1984), i.e. fixation in 4% glutarldehyde in 0.5 M potassium phosphate buffer for 2 h, post-fixation in 1% osmium tetroxide for 2 h, staining overnight in 2% aqueous uranyl acetate, dehydration in graded ethanol dilutions and embedding in TAAB low viscosity resin (Agar Scientific, UK). Thin section were stained with lead citrate prior to viewing under a Philips Morgagni electron microscope. Immunogold labelling was carried out as described by Louro and Lesemann (1984) with some modifications. Carbonated grids were floated for a few minutes on a drop of bacterial suspension from a colony, rinsed on 0.05 M phosphate-buffered saline (PBS) containing 0.01% Tween 20 and 0.01% Triton (PBSTT) for 10 min and incubated for 30 min on a drop of antiserum to *Xylella fastidiosa* (Loewe 07319/220601) diluted 1:300 with PBSTT. After floating on a drop of PBSTT for 10 min, the grids were exposed for 15 min to a preparation of colloidal gold 15 nm in diameter conjugated with anti-rabbit antibodies (Sigma, USA) diluted 1:50 with PBSTT. Grids were rinsed with PBS and glass-distilled water prior to viewing under the electron microscope.

ELISA and PCR assays (Loconsole *et al*., 2014) detected *Xylella* in the three hosts examined (olive, oleander and periwinkle), and tissue extracts exposed to indirect immunofluorescence showed the presence of green fluorescent bacterial cells singly or in groups (Fig. 1D). Electron microscopy observations disclosed in the tracheary elements of all hosts accumulations of bacterial cells with the thick and rippled cell wall (Fig. 1B, C) that characterizes *Xylella fastidiosa* (Wells *et al*., 1987).

For isolations, petioles and midribs excised from 10-15 leaf batches (*ca.* 0.5 to 0.6 g) from donor plants, were surface-disinfected for 2 min each in 70% ethanol and 2% sodium hypochlorite, rinsed for three times in sterile distilled water and cut to small pieces with a sterile scalpel on filter paper disks. Tissue fragments were placed in 55 mm Petri dishes containing 1-1.5 ml of succinate-citrate-phosphate buffer (SCP), chopped to mush with a sterile scalpel and left to soak for 10-15 min. The slurry was filtered and the filtrate used, as such and after dilution 1:10 and 1:100, for plating onto periwinkle wilt gelrite (PWG) (Davis and Schaad., 1981; Hill and Purcell, 1994) and buffered cysteine-yeast extract (BCYE) (Wells *et al*., 1981) media. Fifty µl of the initial suspension and 100 µl of each dilution were spread in PWG and BCYE plates and incubated at 28°C. After about 25 days, many very slow-growing bacterial colonies developed from oleander and periwinkle extracts in both media (Fig 1E, F). These colonies had the morphological properties summarized in Table 1 and the same general aspect in both culture media. A number of isolations from olive samples yielded colonies containing green fluorescent bacterial cells and gave positive reactions in PCR. However, these colonies were heavily contaminated with bacteria other than *Xylella fastidiosa* and could not be obtained in purity.

Bacterial isolates from oleander and periwinkle were Gram-negative, oxidase-negative and catalase-positive. Observations with a phase contrast microscope at 1000× magnification revealed the presence of rod-shaped cells with an average width of 0.35 µm and a maximum length of *ca.* 5 µm usually present in aggregates and in a tightly knit matrix of filamentous strands (Fig. 2A). Bacterial cells from colonies were positive in the IF test and fluoresced green (Fig. 2B). In the electron microscope, they appeared rod-shaped with rounded ends (Fig. 2C) and were recognized by colloidal gold-tagged *Xylella*-specific antibodies, which clearly decorated body and fimbriae (Fig. 2D).

For a preliminary molecular analysis, individual colonies from periwinkle and oleander were collected with a sterile toothpick and gently suspended in 20 µl of elution buffer [(AE buffer, DNeasy plant mini kit (Qiagen, The Netherlands)]. Aliquots (2 µl) of the suspension were used for PCR in a reaction mix containing 1× GoTaq master Mix (Promega, USA) and 0.2 µM of the primers FXYgyr499 and RXYgyr907 targeting the *gyrase* subunit B gene (Rodrigues *et al*., 2003). PCR conditions were as described by Rodrigues *et al.* (2003). Colonies of *Xanthomonas arboricola* pv. *pruni* (No. 416, National Collection of Plant Pathogenic Bacteria, Sand Hutton, UK) and DNA extracts from a *X. fastidiosa*-infected olive tree served as negative and positive controls, respectively. The PCR products amplified from periwinkle and oleander colonies and from the control olive tree were sequenced and used for genetic analysis.

Amplicons of the expected size (429 bp) were obtained from periwinkle (PW1) and oleander (OLDR) colonies and from the positive control (OL-G2), but not from the *X. arboricola* pv *pruni* colony. All these amplicons shared 97-99% sequence identity with known *X. fastidiosa* strains from GenBank and were 100% identical to one another and to comparable sequences obtained from infected

Table 1. Morphology 25-day-old colonies of the Apulian strain of *Xylella fastidiosa* in PWG and BCYE media

Colony morphology	PWG	BCYE
Elevation	Convex	Convex
Form	Nearly circular	Circular
Margin	Irregular	Entire
Size (mm)	Less than 1.0	Less than1.0
Surface appearance	Smooth	Smooth
Texture	Viscous	Viscous
Opacity	Opalescent	Opalescent
Pigmentation	None	None

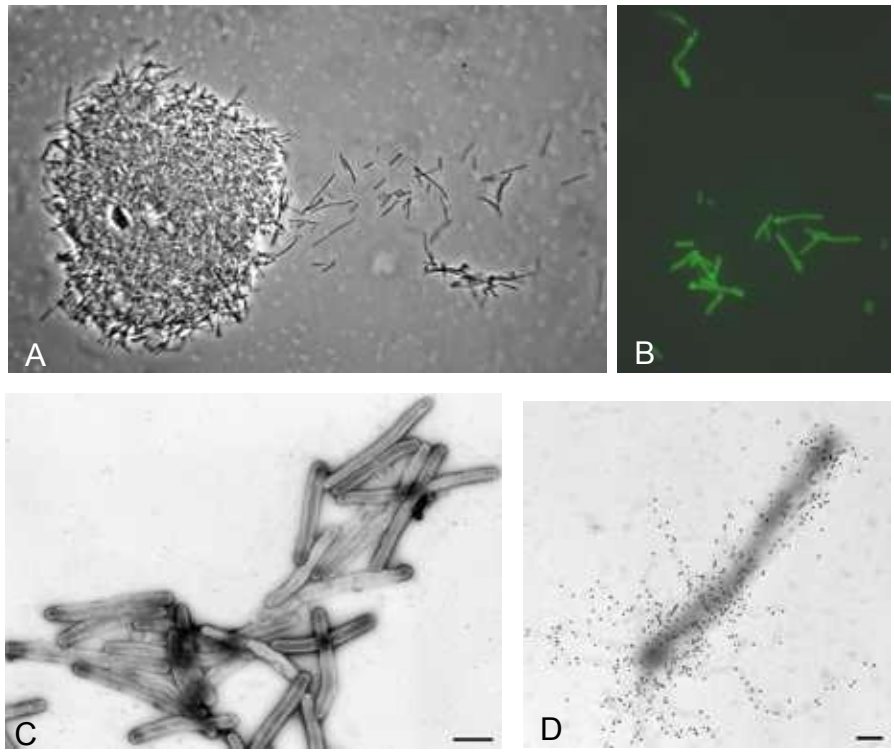

Fig. 2. A. A bacterial aggregate from a BCYE colony seen under a phase contrast microscope. B. Immunofluorescence test-positive green fluorescing bacterial cells from the same colony. C. Electron micrograph of bacterial cells from the same colony. Bar = 1 µm. D. Cell wall and fimbriae of a bacterial cell decorated by gold particles following immunogold labelling. Bar = 250 nm.

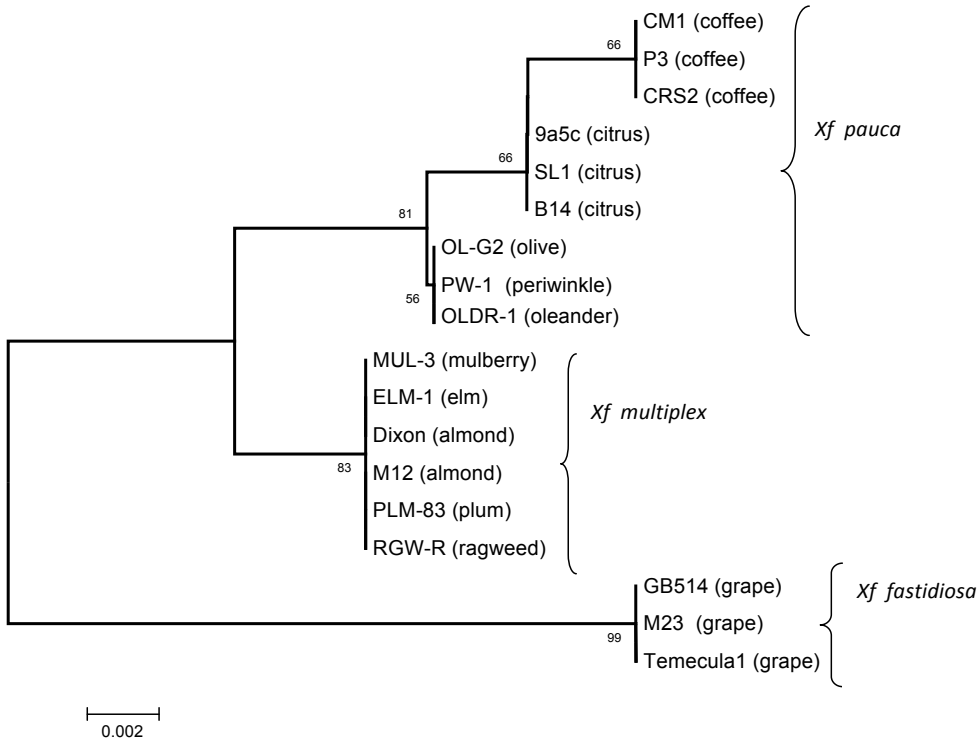

Fig. 3. Phylogenetic trees generated by the Neighbor-Joining method from the alignment of the partial nucleotide sequence of the DNA *gyrase* subunit B (gyrB) gene using MEGA (Version 5). Bootstrap values for 1000 replicates are shown at the main branches. Branch length is proportional to the number of nucleotide changes. Accession Nos. of GenBank sequences are: AF534974 (CM1); AF534972 (P3); AF534970 (CRS2); AE003849 (9a5c); AF534969 (SL1); AF534968 (B14); AF534965 (MUL-3); AF534966 (ELM-1); EU026153 (Dixon); CP000941 (M12); AF534962 (PLM-83); AF534963 (RGW-R); CP002165 (GB514); CP001011 (M23); AE009442 (Temecula). Sequences from olive, periwinkle and oleander were deposited under the following accession Nos.: KJ406212 (OL-G2); KJ406260 (PW-1); KJ406261 (OLDR-1).

olive trees in the course of field surveys. PW1, OLDR and OL-G2 sequences grouped in a distinct cluster of the branch comprising *X. fastidiosa* isolates that belong to the subspecies *pauca* (Fig. 3). Although this confirms the indications obtained with an earlier analysis (M. Saponari, unpublished information), further isolation attempt from olive are being made and multilocus sequence typing is under way for the ultimate taxonomic assignment of the Salento bacterial strain.

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