Journal of Multidisciplinary Engineering Science and Technology (JMEST)
ISSN: 2458-9403
Vol. 3 Issue 10, October - 2016

Xylella fastidiosa does not occur in Albania: Preliminary results

Magdalena Cara, Jordan Merkuri, Edmond Koka

Agricultural University of Tirana, Faculty of Agriculture and Environment, Tirana, Albania
magdacara@ubt.edu.al

Mendim Bacaj
Center for Agricultural Technologies Transfer Vlora, Albania

Orges Cara
University of Tirana, Faculty of Natural Science, Tirana, Albania

Abstract — *Xylella fastidiosa* has been reported as responsible for a devastating disease on olive trees in Apulia region (South–Eastern in Italy) characterized by a quick decline syndrome. The identification of *X. fastidiosa* in the olive quick decline syndrome affected trees represents the first confirmed detection of this bacterium in the European Union. Since, *X. fastidiosa* is a regulated quarantine pathogen and Albania is a neighbor country with Italy, the risk of introduction and spread of this bacterium is very high. Since 2013 technicians and farmers reported the presence of similar symptoms associated with leaf scorch and dieback of olive tree branches in Lushnja, Vlora and Saranda regions. To assess the occurrence and distribution of the pathogen in Albania, samples of twigs from olive cultivars imported from Italy or local varieties and oleander were analyzed by serological techniques (ELISA, DTBIA) using *X. fastidiosa* – specific antibodies and PCR using tree set of primers. Results demonstrated that all olive and oleander collected samples were free from the pathogen. Nevertheless, continuous monitoring and rigorous control measures of propagative materials are necessary to prevent the introduction of *Xylella fastidiosa* in Albania. Moreover from 21.08.2014 has banned by Order No. 409 of the Minister of Agriculture, Rural Development and Water Resources, the import of olive seedlings from Apulia region. Furthermore, different fungal colonies morphologically similar to *Phaemoniella* and *Phaeoacremonium* species were isolated in PDA media stems collected from symptomatic olive trees *Ogliarola* and *Frantoio* in Shamogjin. The identification is ongoing.

Keywords — Xylella fastidiosa, Olive, ELISA, DTBIA, PCR, Phaeoacremonium sp, Phaeomoniella sp.

I. INTRODUCTION

Xylella fastidiosa is a xylem-limited bacterium and the causal agent of a number of several diseases, among which Pierce's diseases of grapevine, leaf scorch of almond, oleander and coffee, citrus variegated chloroses, and other disorders of perennial crops and landscape plants [1]. Four subspecies of the bacterium have been described: (i) *X. fastidiosa* subsp. *Fastidiosa,*(ii) *X.f*. subsp. *pauca*, (iii) *X.f*, subsp. *multiplex* and (iv) *X.f*.subsp. *sandyi* [2] [3].

Xylella fastidiosa is transmitted by xylem fluid feeding insect vectors (e.g. *Auchenorrhyncha*, mainly sharpshooter leafhoppers and spittlebugs). However, many hosts' plants may remain latently infected, not showing any symptoms and serving as sources of inoculums for vectors [1].

The first report of *X. fastidiosa* under field condition in the European Union was in southern Apulia, Italy, associated with severe cases of an "Olive Quick Decline Syndrome" (OQDS) [4], and the pathogen was characterized as a strain of subspecies *Pauca* [5].

Affected plants showed leaf scorch symptoms, galleries in trunks and branches made by larval stages of *Zeuzera pyrina* (leopard moth), and extended discoloration of the wood from which different fungi were isolated [6]; [5].

Serological and molecular diagnosis methods and protocols were developed for the outbreak in Apulia [7] for screening a large number of samples during the monitoring campaign organized by Regional Plant Protection Service to establish the distribution of the pathogen and to limit further spread.

Since May 2013, symptoms of leaf scorching, withering of branches, dieback and plant death have been observed on olive in Lushnja region (Fig.1), and later in the other regions as Vlora and Saranda. Considering the risk of pathogen and the devastating disease it can cause in Albania, on both agriculture and landscaping plants, it was crucial to monitor the areas in which the symptoms were observed.

Moreover, rapid and accurate diagnostic tools are necessary to screen a large number of plants and to set up a phytosanitary control plan for both the imported and locally produced plant propagative materials. Therefore, to ascertain the presence of *X. fastidiosa*, the survey was conducted in Albanian territory, covering suspected olive growing areas, and other potential hosts. The preliminary results are reported in this paper.

II. MATERIALS AND METHODS

A. Sampling

Survey on the spread of *X. fastidiosa* in Albania was organized from May 2014 to early Spring 2016, starting from the olive areas in which symptoms similar to those described for OQDS [8]; [6]. (Fig.1,2,3) were observed. Samples were collected during three vegetative seasons: spring and fall 2014, 2015 and spring 2016.

A total of 100 different olive trees (Table 1), were sampled by collecting four twigs (20-30 cm long) for every tree, located at different height of the four branches of the canopy. In addition, tree twigs per plant were sampled from 10 Oleander plants expressing leaf scorch symptoms (Fig. 4) and located in the surrounding landscape areas. During field operations, samples were stored in closed plastic bags in a cooling box during the delivery to the laboratory in Department of Plant Protection (DPP) of Agricultural University of Tirana.

Fig.1 Defoliation and withering of branches in Dushk, Lushnje.

Fig.2 Leaf scorches symptoms on olive in Rade - Maminas, Durres.

Fig.3. Leaf scorches symptoms on olive in Shamogjin, Vlore

Fig.4. Leaf scorches symptoms on oleander in Golem – Durres.

B. Serological detection

Double Antibody Sandwich ELISA

Tissue extracts were obtained from leaf petioles and midveins excised from 6–8 mature leaves and macerated in plastic bags [7] in the presence of extraction buffer (polyvinyl pyrrolidone 20 g/l, Tween-20 - 0.05%, BSA 2g/l in 1 X PBS buffer, pH = 7.4) using a semi-automated Homex 6 apparatus (Bioreba) (Fig. 5).

Fig. 5 Tissue extracts for ELISA test in DPP laboratory.

Samples were tested using specific antibodies to *X. fastidiosa* (Loewe Biochemica GmdH, Germany), following manufacturer's instructions and using the controls supplied with the kit. Plates were coated with 100ml/weal 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 incubated at 37°C for 4 h before the addition of the substrate (1mg/ml p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8). Absorbance was recorded at 405 nm using a microplate reader (ELx800 Absorbance Reader). Samples were considered positive if their absorbance was three times higher than the mean absorbance of healthy control samples [9] (Fig. 6).

Direct Tissue Blot Immunoassay

Direct Tissue Blot Immunoassay (DTBIA) technique for the detection of *X. fastidiosa* was carried out as described by [10]. Because *X. Fastidiosa* is located in host xylem tissues, blots were made from cross sections of 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 section were gently pressed to a 0.45 μm Protran nitrocellulose membrane (Sigma-Aldrich) where a positive and a negative control were blotted previously. Gloves were

used when handling the membranes and during blotting process. Membranes were left to dry for 20–30 min at room temperature and were placed in 1% fat milk solution on a shaker for 1 h for saturation of protein-binding sites. After washing with 1 X PBS buffer pH = 7.4 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 1:100 dilution in conjugate buffer. Membranes were then stained by immersion in a solution obtained by dissolving one tablet of Sigma-Fast BCIP-NBT, in 10 ml distilled water, and gently stirred by hand at room temperature for 3 min until a purple-violet color appeared in the positive control. The reaction was stopped by washing with tap water. After drying at room temperature, the membrane was observed under a low power magnification lens (x10 or x20) (Fig. 7).

C. *Molecular detection*

Isolation of DNA and PCR assay

Total nucleic acids extracted from olive samples that were CTAB-based extraction buffer [11]. Petioles and midveins tissues (0.4–0.6 g) excited from surfaces of sterilized leaves, were placed in plastic bags, and homogenized in 1,5 ml of extraction buffer (20 mM EDTA, 350 mM sorbitol in 100 mM Tris-HCl 2% hexadecyl trimethyl ammonium bromide, 0.1 M Tris-HCl pH 7.5 plus 2.5%w/v PVP and 0.2% of ß-mercapto ethanol) using a semi automated homogenizer (Homex, 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 buffer [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 30 min incubation at -20°C. The total DNA preparation from each sample was re-suspended in 200 µl of 0.5x TE.

For PCR the RST31/RST33 set of primers targeting the 16S rDNA gene was used Minsavage et al., 1994 [12]. Reaction were conducted in a final volum of 25 µl, using 5µl of 5x GoTaq polymerase (Promega), 250 nM each of forward and reverse primers, and 1 µl of total DNA template (50ng μl^{-1}). Thermocycling conditions were as following: 94°C for 3 min, 35 cycles at 94°C for 30 sec, 50-55°C for 30-40 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 [13].

III. RESULTS AND DISCCUSION

All olive and oleander samples tested by ELISA and DTBIA were negative for *X. fastidiosa*. Moreover the four samples, including the ELISA–doubtful sample gave negative results for *X. fastidiosa* using PCR assay (Table1).

Both ELISA and PCR techniques are reliable for detection of *X. fastidiosa* in plant tissues [13]; however, in previous studies [14]; [15], it has been demonstrated that ELISA may show false-positive cases [16], as for olive sample from Shamogjin, Vlore.

In ELISA tests, positive controls produced clear and strong reactions with average OD_{405} value of 1.059 after 2 h, six times higher than the OD_{405} value of the tested samples, which ranged from 0.168 to 0.180. Moreover, in DTBIA assay, intense purple coloration appeared in positive control. PCR products of olive positive control, amplified with the RST31/RST33 set of primers, showing expect of bands on the agarose gel electrophoresis (Fig.8).

The leaf scorching symptoms observed during the survey may have multiple origins, either abiotic or biotic [17]. Any physical cause that affects water supply to the leaf margin cell (e.g. dry soil, rapid loss from the leaves, damaged roots) or, salt winds, nutrient deficiency/toxicity, can induce leaf scorch [18]. Conversely, weak foliar pathogens or other xylem-invading pathogens can be biotic factors causing leaf scorch [19].

Nevertheless, continuous monitoring and rigorous control measures of propagative materials are necessary to prevent the introduction of *Xylella fastidiosa* in Albania.

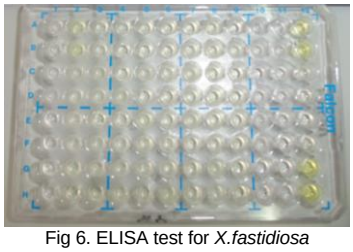

Fig 6. ELISA test for *X.fastidiosa*

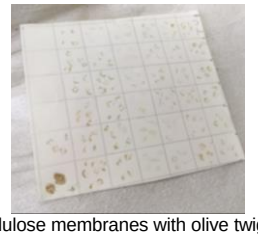

Fig. 7 Blotted nitrocellulose membranes with olive twigs for DTBIA test

M 1 2 3 4 5 6 7

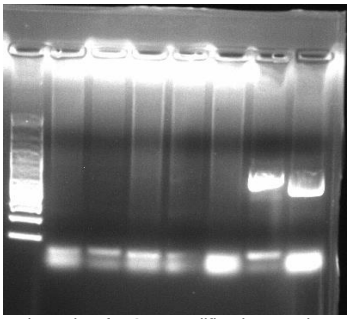

Fig.8. Gel electrophoresis of PCR amplification products recovered from samples of olive [ELISA–doubtful samples (1), ELISA –negative samples (2-4), negative control (5), positive control (6-7)]

Table 1. Results of laboratory analyses performed on samples collected for the survey of *X.fastidiosa* in Albania during 2014-2016

Host plant	Area of sampling	Sampled plants (No.)	Infected plants		
Commercial fields Olive (*Olea europaea)*			DAS ELISA	DTBIA	PCR
Cazzinicchia	Vlorë – Shamogjin	5	-	-	-
Ogliarola	Vlorë – Shamogjin	5	-	-	-
Karolea	Vlorë – Shamogjin	5	-	-	-
Nocellara Messinese	Vlorë – Shamogjin	5	-	-	-
Coratina	Vlorë – Shamogjin	5	-	-	-
Cazzinicchia	Vlorë – Shamogjin	5	-	-	-
Itrana	Vlorë – Shamogjin	5	-	-	-
Picholin	Vlorë – Shamogjin	5	-	-	-
Frantoio	Vlorë – Shamogjin	5	-	-	-
Frantoio	Vlorë – Shamogjin	5	-	-	-
Frantoio	Vlorë – Shamogjin	5	-	-	-
Frantoio	Vlorë – Shamogjin	5	-	-	-
Frantoio	Vlorë – Shamogjin	5	-	-	-
Frantoio	Vlorë – Shamogjin	5	-	-	-
Fragivento	Vlorë – Shamogjin	4	-	-	-
Fragivento	Vlorë – Shamogjin	4	-	-	-
Kallamon	Vlorë – Shamogjin	4	-	-	-
Frantoio	Vlorë - Skrofotinë	3	-	-	-
Ulli i Bardhe	Sarande - Xarre	3	-	-	-
Kripës	Maminas - Rade	3	-	-	-
Ullashk	Maminas - Rade	3	-	-	-
Ulli i Bardhe	Lushnje - Dushk	3	-	-	-
Haragjel	Maminas - Rade	3	-	-	-
Nerium oleander	Golem – Durrës	4	-	-	-
Nerium oleander	Vlorë	4	-	-	-
Nerium oleander	Durrës	2	-	-	-

D. Fungal isolation

Samples of parts of stems were collected from symptomatic olive trees (cv. *Ogliarola and Frantoio*) 14 and 40 years old sites in Shamogjin (Vlora reagion). The samples were transported to the lab for analysis. The samples were washed with distilled sterile water and surface – sterilized by immersion in sodium hypochlorite solution 0.5% (v/v) for 2 min [20], the bark was removed and small pieces (1-3 mm^2) were taken from sub-cortical tissues with a scalpet. These samples were placed on potato dextrose agar (PDA, Oxoid Ltd.) and incubated at 25°C in the dark.

The fungal colonies morphologically similar to *Phaemoniella and Phaeoacremonium species* were grown until they sporulated and the conidial suspension was spread on agar plate (Fig.9-10). After 24-26 h of incubation single germinated conidia were transferred to fresh plates of potato dextrose agar. The identification is ongoing.

Fig. 9. *Phaemoniella spp.* (Photo J. Merkuri 2014)

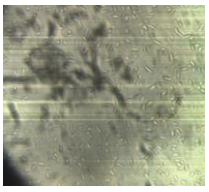

Fig. 10. *Phaeoacremonium spp* (Photo J.Merkuri 2014)

ACKNOWLEDGMENT

Authors would like to thank the Mediterranean Agronomic Institute of Bari (CIHEAM-MAIB). Justus-Liebig-University Giessen, Germany: Quality Network Biodiversity and Agriculture on the Balkan Peninsula (BioAgBal) in the frame of the DAAD Programme.

REFERENCES

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

[2] Schaad N.W., E. Postnikova, G. Lacy, M. Fatmi and C.J. Chang, 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.

[3] Schuenzel E.L., M. Scally, R. Stouthamer and L. Nunny, 2005. A multigene phylogenetic study of clonal diversity and divergence in North American strains of the plant pathogen Xylella fastidiosa. Applied Environmental Microbiology 71, 3832–3839.

[4] 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.

[5] Cariddi C., M. Saponari, D. Boscia, A. De Stradis, G. Loconsole, F. Nigro, F. Porcelli, O. Potere and G. P. Martelli, 2014. Isolation of a Xylella fastidiosa strain infecting olive and oleander in Apulia, Italy. Journal of Plant Pathology 96, 1–5.

[6] Nigro F., D. Boscia, I. Antelmi and A. Ippolito, 2013. Fungal species associated with a severe decline of olive in southern Italy. Journal of Plant Pathology 95, 668.

[7] Loconsole G., O. Potere, D. Boscia, G. Altamura, K. Djelouah, T. Elbeaino, D. Frasheri, D. Lorusso, F. Palmisano, P. Pollastro, M.R. Silletti, N. Trisciuzzi, F. Valentini, V. Savino and M. Saponari, 2014. Detection of Xylella fastidiosa in olive trees by molecular and serological methods. Journal of Plant Pathology 96, 7–14.

[8] Martelli GP. (2013) Disseccamento rapido dell'olivo. Georgofili INFO, 30 Ottobre 2013. (www.georgofili.info) (verified Oct 8, 2015).

[9] Sutula CL, Gillett JM, Morrissey SM, Ramsdell DC. (1986) Interpreting ELISA data and establishing the positive negative threshold. Plant Disease 70:722–726.

[10] Djelouah K, Frasheri D, Valentini F, D'Onghia AM, Digiaro M. (2014) Direct tissue blot immunoassay for detection of Xylella fastidiosa in olive trees. Phytopathology Mediterranean 53:559–564.

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

[12] Minsavage G.V., C.M. Thompson, D.L. Hopkins, R.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, 446–461.

[13] Martelli G.P., D. Boscia, F. Porcelli and M. Saponari, 2016. The olive quick decline syndrome in south-east Italy: a threatening phytosanitary emergency. European Journal of Plant Pathology 144, 235–243.

[14] Almeida RPP, BluaMJ, Lopes JRS, Purcell AH. (2005) Vector transmission of Xylella fastidiosa: applying fundamental knowledge to generate disease management strategies. Ann Entomol Soc Am 98:775–786.

[15] Carbajal D, Morano KA, Morano LD. (2004) Indirect immunofluorescence microscopy for direct detection of Xylella fastidiosa in xylem sap. Curr Microbiol 49:372–375.

[16] Costa HS, Raetz E, Pinckard TR, Gispert C, Hernandez-Martinez R, Dumenyo CK, Cooksey DA. (2004) Plant hosts of Xylella fastidiosa in and near southern California vineyards. Plant Dis 88:1255–1261.

[17] Sherald JL. (2001) Xylella fastidiosa, a bacterial pathogen of landscape trees. In: Ash CL. (ed) Shade Tree Wilt Diseases. St Paul, MN, American Phytopathological Society, pp 191–202.

[18] Sanzani S, Schena L, Nigro F, Sergeeva V, Ippolito A, Salerno MG. (2012) Abiotic diseases of olive. J Plant Pathol 94:469–491.

[19] Frisullo S, Camele I, Agosteo GE, Boscia D, Martelli GP. (2014) Brief historical account of olive leaf scorch ("brusca") in the Salento peninsula of Italy and state-of the- art of the olive quick decline syndrome. J Plant. Pathol 96:441–449.

[20] Fisher P.J., O. Petrini, L.E., Petrini and E. Descals, 1992. A preliminary study of fungi inhabiting xylem and whole stems of Olea europaea. Sydowia 44, 117–121.