# A preliminary survey on the presence of *Xylella fastidiosa* in olive, citrus and grapevine groves in Morocco

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**Abstract**

The bacterium *Xylella fastidiosa* is gram negative, xylem-inhabiting, devastating pathogen which causes various diseases on more than 300 plant hosts. Given the recent confirmed findings of *X. fastidiosa* in the European Union, this bacterium is becoming a serious threat to the Moroccan agricultural sector. A survey was conducted during May-September 2015 on the presence of *X. fastidiosa* in several commercial groves, covering olive, citrus and grapevine growing areas. In a few trees, severe symptoms which could be associated to the bacterium were observed. A total of 900 samples of different crops from different regions were randomly collected: 220 olive trees (cv. Picholine Marocaine) from two regions, 410 citrus trees belonging to 7 different cultivars collected in 4 regions and 270 grapevine plants belonging to 6 different cultivars from 3 regions; all these samples were tested for the presence of *X. fastidiosa* by using an ELISA commercial kit. The obtained results did not show any positive sample. These preliminary results are taken as an encouraging indication, considering that *X. fastidiosa* was not found in Morocco, at least in the surveyed crops. However, frequent extensive surveys in different regions are needed to prevent its entrance into the country.

**Keywords**: Morocco, *Xylella fastidiosa*, survey, olive, citrus, grapevine, ELISA.

## Enquête préliminaire sur la présence de *Xylella fastidiosa* sur l'olivier, les agrumes et la vigne au Maroc

**Résumé**

*Xylella fastidiosa*, bactérie gram-négative, confinée au xylème, très polyphage, provoque diverses maladies sur plus de 300 plantes hôtes. Compte tenu de la récente détection confirmée de *X. fastidiosa* dans l'Union Européenne, cette bactérie est en train de devenir une menace sérieuse pour le secteur agricole marocain. Une enquête a été menée durant Mai-Septembre 2015 sur la présence de *X. fastidiosa* dans plusieurs plantations de l'olivier, des agrumes et de la vigne à travers le pays. Des symptômes sévères qui pourraient être associés à la bactérie ont été relevés. Un total de 900 échantillons de différentes cultures à partir de différentes régions ont été collectés au hasard: 220 oliviers (cv. Picholine Marocaine) de deux régions, 410 arbres d'agrumes appartenant à 7 cultivars collectés dans 4 régions et 270 plants de vigne appartenant à 6 cultivars de 3 régions; tous ces échantillons ont été testés pour la présence de *X. fastidiosa* en utilisant un kit commercial ELISA. Les résultats obtenus ne montrent aucun échantillon positif. Ces résultats préliminaires sont considérés comme une bonne première indication, étant donné que *X. fastidiosa* n'a pas été détectée au Maroc. Cependant, des enquêtes continues à grande échelle sont nécessaires pour empêcher son entrée dans le pays.

**Mots-clés**: Maroc, *Xylella fastidiosa*, enquête, Olivier, agrumes, vigne, ELISA.

## INTRODUCTION

*Xylella fastidiosa*, a gram negative, devastating plant pathogenic bacterium and the causal agent of a number of severe diseases, among which olive quick decline syndrome, 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;Saponari *et al.,* 2013). Many wild plants may carry the pathogen with, but more often without showing symptoms, such as grasses, sedges and trees (Janse and Obradovic, 2010). Four subspecies of the bacterium have been discriminated so far: ssp. *fastidiosa*, ssp. *pauca*, ssp. *multiplex* and ssp. *Sandyi* (Schaad et *al*., 2004; Schuenzel et *al*., 2005). Randal et *al.,* (2009) proposed a fifth one (ssp. *tashke*), that can be differentiated by DNA: DNA hybridization (Schaad et *al.,* 2004) and multi-locus sequence typing (Scally et *al.,* 2005). *X. fastidiosa* causes various diseases, on more than 300 plant hosts, inducing various symptoms: marginal leaf scorching, wilting of foliage and withering of branches, dieback and stunting with eventual plant death in susceptible host plants (Djelouah et *al.,* 2014). *Xylela fastidiosa* is 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).

*X. fastidiosa* is a slow-growing (fastidious) bacterium that does not grow on many common culture media, but some good selective media are available (Schaad et *al*., 2001). In addition to symptom observation and isolation and culturing, detection and identification of the bacterium relies on several laboratory assays. Serological tests include enzyme linked immunosorbent assay (ELISA) (Sherald and Lei, 1991), dot immunobinding assay (DIBA), western blotting (Lee et *al*., 1992; Chang et *al.,* 1993), immuno-

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fluorescence (Carbajal et *al*., 2004) and direct tissue blot immunoassay (Djelouah et *al.,* 2014). Polymerase chain reaction (PCR)-based assays (Minsavage et *al*., 1994; Rodriguez *et al*., 2003; Huang, 2009), as well as real-time and loop-mediated isothermal amplification (LAMP) PCR (Oliveira *et al*., 2002; Francis et *al.,* 2006; 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. 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 (Loconsole et *al.,* 2014).

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» (Saponari et *al.,* 2013). In July 2015, the bacteria *X. fastidiosa* was detected in the island of Corsica (France) affecting ornamental plants of *Polygala myrtifolia* (EPPO, 2015a). Later, in October 2015, it was detected for the first time in Alpes-Maritimes department (Southern France) (EPPO, 2015b). In July 2016, the bacterium was reported for the first time in Germany on a single potted plant of *Nerium oleander* in a small nursery producing young vegetable and ornamental plants in Saxony (EPPO, 2016a). In November 2016, the presence of *X. fastidiosa* was confirmed in Islas Baleares. The bacterium was detected in sweet cherry (*Prunus avium*) in a garden centre in Porto Cristo, municipality of Manacor, on the island of Mallorca (EPPO, 2016b). More recently, in May 2017, *X. fastidiosa* was detected in Mallorca, Ibiza and Menorca on 12 plant species including citrus and grapevine (EPPO, 2017).

Following these confirmed findings of the bacterium in the European Union, it was decided to conduct a preliminary survey on the presence of the bacterium in olive, citrus and grapevine groves in Morocco.

## MATERIAL AND METHODS

Considering the potential risk presented by *X. fastidiosa* on several crops in Morocco, a preliminary survey was conducted across the country, covering olive, citrus and grapevine growing areas. Field surveys were conducted from May to September 2015 in six different regions. A total of 43 commercial groves were visited and their trees inspected for the presence of typical symptoms of *X. fastidiosa*. In a few trees, severe symptoms which could be associated to the bacterium were observed. A total of 900 samples of different crops from different regions were randomly collected: 220 olive trees (cv. Picholine Marocaine) from two regions (Azilal and Meknès), 410 citrus trees belonging to 7 different cultivars collected in 4 regions (Gharb, Haouz, Loukkos and Souss) and 270 grapevine plants belonging to 6 different cultivars (4 table and 2 wine grape cultivars) from 3 regions (Gharb, Haouz and Meknès) (Table 1). A detailed map was drawn up for each surveyed site, where information about position, cultivar, age, origin and location of each sampled tree were recorded. Any suspicious symptom and information potentially helpful for further sampling and investigation were recorded, and representative photos were also taken. Collected samples were stored in closed plastic bags in a cooling box during the delivery to the laboratory for analysis with ELISA and PCR diagnostic methods.

### Double antibody sandwich-ELISA

All the collected samples from olive, citrus and grapevine trees were tested by ELISA, using specific polyclonal antibodies to *X. fastidiosa* (Loewe Biochemica, Germany), according to the manufacturer's instructions. In the presence of PBS-buffer, extracts were obtained from leaf petioles and midveins, using mortars and pestles. Absorbance was measured after 30, 60, 120 and 180 minutes (min) with a microplate reader (ELX800, BioTek) at 405 nm.

**Table 1: Crops, location, number of visited and sampled groves, cultivars and number of collected samples during the survey (May-September, 2015).**

| Crops | Location | Number of visited and sampled groves | Number of sampled trees and (cultivars, cv.) |
|---|---|---|---|
| **Olive** | Azilal | 5 | 75 sampled trees (cv. Picholine Marocaine) |
| | Meknès | 10 | 145 (Picholine Marocaine) |
| **Citrus** | Gharb | 4 | 30 (Valencia Late Orange); 30 (Common Clementine); 40 (Thompson Navel) |
| | Haouz | 4 | 40 (Valencia Late Orange); 50 (Common Clementine) |
| | Loukkos | 2 | 50 (Common Clementine) |
| | Souss | 4 | 40 (Nour Clementine); 40 (Nules Clementine); 40 (Ortanique Mandarin); 50 (Salustiana Orange) |
| **Grapevine** | Gharb | 3 | 20 (Redglobe); 25 (Muscat d'Italie) |
| | Haouz | 5 | 30 (Muscat d'Italie); 30 (Abbou); 35 (Valency) |
| | Meknès | 6 | 30 (Redglobe); 20 (Muscat d'Italie); 40 (Carignan); 40 (Cinsault) |
| **Total** | | **43** | 900 samples from (1 olive, 7 citrus and 6 grapevine cultivars) |

### Polymerase chain reaction

A total of 10 samples per each tree crop (olive, citrus and grapevine) were randomly selected and tested by PCR for the detection of *X. fastidiosa*. Total nucleic acids were extracted from leaf petioles and midveins using a CTAB-based extraction buffer (Rodrigues et *al*., 2003; Djelouah *et al.,* 2014). 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 seconds (s), 50-55°C for 30-45 sec and 72°C for 30 sec, and a final extension of 5 min at 72°C (Djelouah *et al.,* 2014). PCR products were analyzed by electrophoresis in 1% Tris-Acetate-EDTA (TAE)-agarose gels and DNA bands visualized by staining the gel in ethidium bromide.

## RESULTS AND DISCUSSION

All the collected samples were tested for the presence of *X. fastidiosa* by using an ELISA commercial kit (Loewe Biochemica, GmbH). The obtained results did not show any positive sample. The ELISA test worked properly: the positive control of the kit reacted positively, whereas no color change was observed with the negative control of the kit. Loconsole et *al.* (2014) performed several laboratory tests, in which the reactivity of different commercially available ELISA kits was compared, and showed that a kit from Loewe (Biochemica GmbH, Germany) detected a higher number of known positive samples with reactions occurring within 2 hours, following manufacturer's instructions and using the controls supplied with the kit. Thus, this kit was used for this preliminary survey on the presence of *X. fastidiosa* in Morocco.

Moreover, no amplified DNA was obtained from any of the tested samples using PCR, confirming the absence of the bacterium in these samples. The used primers (RST31/33) 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 and Bazzi, 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).

These obtained preliminary results are taken as an encouraging indication, considering that *X. fastidiosa* was not found in Morocco, at least in the surveyed tree crops. However, frequent extensive surveys in different regions and on different potential host plants are needed to prevent its entrance into the country. It is noted that the presence of leaf scorch symptoms which were frequently observed during the survey may have multiple origins, abiotic or biotic (e.g. drought, salty winds, nutrient deficiency/toxicity, frost damage, fungal pathogens…). While the hazard presented by *X. fastidiosa* in other hosts (oak, plane, maple and others) still has to be evaluated, inspection services should be aware that these hosts also present a certain risk.

## CONCLUSION

*X. fastidiosa* is a regulated quarantine pest whose introduction and spread in Morocco is banned. Following the recent confirmed finding of *X. fastidiosa* in the European Union (Italy and France); this bacterium is becoming a serious threat to the Moroccan agricultural sector. In this context, the National Food Safety Office (ONSSA) adopted measures to prevent the introduction of *X. fastidiosa* into the national territory by deciding to suspend importation of host plant species to Pierce's disease from Italy. Results obtained in this study clearly indicated that *X. fastidiosa* was not found in Morocco. However, frequent extensive surveys in different regions are needed to prevent its entrance into the country.

For any effective control and containment strategy of *X. fastidiosa*, the correct identification of the pathogen by quick, sensitive and reliable laboratory tests is crucial. 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. Therefore, the use of serological and molecular diagnostic tools is of utmost importance for the detection of the bacterium in plant tissues and in potential insect vectors.

## REFERENCES

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.

Djelouah K., Frasheri D., Valentini F., D'Onghia A.M and Digiaro M.(2014).Direct tissue blot immunoassay for detection of *Xylella fastidiosa* in olive trees. *Phytopathologia Mediterranea* 53: 559-564.

European and Mediterranean Plant Protection Organization (EPPO) (2004). Diagnostic protocols for regulated pests. *Xylella fastidiosa. Bulletin OEPP/EPPO Bulletin* 34: 187-192.

EPPO.(2015a). European and Mediterranean Plant Protection Organization Reporting Service no. 08. Nr. 2015/144. https://gd.eppo.int/reporting/article-4942.

EPPO.(2015b). European and Mediterranean Plant Protection Organization Reporting Service no. 10. Nr. 2015/180. https://gd.eppo.int/reporting/article-5127.

EPPO. (2016a). European and Mediterranean Plant Protection Organization Reporting Service no. 07. Nr. 2016/133. ttps://gd.eppo.int/reporting/article-5878.

EPPO (2016b). European and Mediterranean Plant Protection Organization Reporting Service no. 11. Nr. 2016/213. https://gd.eppo.int/reporting/article-5958.

EPPO (2017). European and Mediterranean Plant Protection Organization Reporting Service no. 05. Nr. 2016/102.https://gd.eppo.int/reporting/article-6070.

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. and 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 Taq Man-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.

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. (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.

Krell R.K., Boyd E.A., Nay J.E., Park Y.L., Perring T.M.(2007). Mechanical and insect transmission of *Xylella fastidiosa* to *Vitis vinifera*. *American Journal of Enology and Viticulture* 58: 211-216.

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.

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

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.

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.

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.

Redak R.A., Purcell A.H., Lopes J.R.S., Blua M.J., Mizell III R.F., Andersen P.C. (2004). The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. *Annu. Rev Entomol.* 49: 243-270.

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., Jones J.B., Chun W.(2001).Laboratory Guide for Identification of Plant Pathogenic Bacteria. APS Press, St. Paul, MN, USA.

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.

Schuenzel E.L., Scally, M., Stouthamer R., Nunny L.(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.