*Phytopathologia Mediterranea* (2017), 56, 2, 259–273
DOI: 10.14601/Phytopathol_Mediterr-20578

RESEARCH PAPERS

# *Xylella fastidiosa* subsp. *pauca* (CoDiRO strain) infection in four olive (*Olea europaea* L.) cultivars: profile of phenolic compounds in leaves and progression of leaf scorch symptoms

Andrea LUVISI, Alessio APRILE, Erika SABELLA, Marzia VERGINE, Francesca NICOLÌ, Eliana NUTRICATI, Antonio MICELI, Carmine NEGRO and Luigi De BELLIS

Department of Biological and Environmental Sciences and Technologies, University of Salento, via Prov.le Monteroni, I-73100 Lecce, Italy

**Summary.** *Xylella fastidiosa* subsp. *pauca (Xfp)*, strain CoDiRO, infects a broad range of olive (*Olea europaea* L.) cultivars. The profile of phenolic compounds, progression of leaf scorch symptoms and population density of *X. fastidiosa* were analyzed in response to *Xfp* infection, in four olive cultivars (Cellina di Nardò, Ogliarola di Lecce, Frantoio and Leccino). Differences in *X. fastidiosa* multiplication in xylem tissues were estimated using qPCR assays, showing that cvs. Cellina di Nardò and Ogliarola di Lecce were characterized by fewer threshold cycles than for cvs. Frantoio and Leccino. Periodical visual inspections of symptomatic plants estimated disease severity and progression using a disease rating scale; cvs. Frantoio and Leccino showed some disease resistance with up to 3-fold severity scores than those for cvs. Cellina di Nardò and Ogliarola. During vegetative growth, *Xfp*-positive leaf samples were analyzed using HPLC-ESI–TOF–MS. Among quantified phenolic compounds, *Xfp* infection modified hydroxytyrosol glucoside and quinic acid. Constitutive levels of hydroxytyrosol glucoside were greater in cvs. Frantoio and Leccino compared to Cellina di Nardò and Ogliarola di Lecce, while levels were strongly reduced in infected plants (95% reduction in Cellina di Nardò, 94% in Ogliarola di Lecce, 97% in Frantoio and 98% in, Leccino). Constitutive levels of quinic acid did not differ among cultivars, but strongly increased in infected Cellina di Nardò and Ogliarola di Lecce (5-fold increases), and to a lesser extent (4-fold increases) in infected Frantoio and Leccino. These results were consistent with the previously reported positive association of quinic acid with *X. fastidiosa* subsp. *fastidiosa* symptoms and titres in grapevine. Differences in the induced responses of these phenolic compounds among cultivars suggest that they play defensive roles in olive tree response to *X. fastidiosa* infection.

**Key words:** Frantoio, Leccino, hydroxytyrosol glucoside, quinic acid, selection marker.

## Introduction

*Xylella fastidiosa* is the bacterial causal agent of important diseases such as Pierce's disease (PD) of grapevine (caused by *X. fastidiosa* subsp. *fastidiosa*), citrus variegated chlorosis (CVC) or citrus X disease (subsp. *pauca*), and phony peach disease (subsp. *multiplex*) (Chatterjee *et al*., 2008; Janse and Obradovic, 2010). *Xylella fastidiosa* is no longer a plant pathogen limited to the Western Hemisphere, and its genetic diversity remains to be fully described (Janse and Obradovic, 2010; Nunney *et al*., 2014; Almeida and Nunney, 2015). Recently, *X. fastidiosa* was reported under field conditions in Italy (Apulia region), associated with severe cases of an "Olive Quick Decline Syndrome" (OQDS) (Saponari *et al*., 2013). The strain CoDiRO (Complesso del Disseccamento Rapido dell'Olivo, the Italian name of OQDS), found in infected Apulian olive trees (*Olea europaea* L.) and other plant species, was characterized as a strain clustered

Corresponding author: A. Luvisi
E-mail: andrea.luvisi@unisalento.it

in one clade with subsp. *pauca* strains (Cariddi *et al*., 2014), but was slightly distinct from subsp. *pauca* (Elbeaino *et al*., 2014). Olive leaf scorch caused by *X. fastidiosa* was observed in USA (caused by subsp. *multiplex*) (Wong *et al*., 2004; Krugner *et al*., 2010), Argentina (subsp. *pauca*) (Haelterman *et al*., 2015) and Brazil (subsp. *pauca*) (Coletta-Filho *et al*., 2016).

The pathogenesis and symptom formation from *X. fastidiosa* represent research challenges; in grapevine it is clear that the host response is mainly due to the extensive bacterial colonization of xylem tissues (Krivanek and Walker, 2005; Fritschi *et al*., 2007). Data about colonization behaviour of bacteria within olive xylem are not yet available, even if differences in population size in different olive cultivars were observed, suggesting correlation between bacterial multiplication ability and severity of symptoms (Giampetruzzi *et al*., 2016). While *X. fastidiosa* can infect more than 350 plant species, 22 species were found to be infected by the CoDiRO strain (EFSA, 2016). In the OQDS outbreak in Italy, *Philaenus spumarius* (Hemiptera, Aphrophoridae) was identified as the most important vector for transmission of the CoDiRO strain of *X. fastidiosa* subsp. *pauca* (*Xfp*) (Saponari *et al*., 2014; Cornara *et al*., 2016).

Methods for isolation of this pathogen, molecular testing and a pathogenicity test, were recently summarized by the European and Mediterranean Plant Protection Organization (EPPO, 2016). In olive trees, the pathogen is routinely detected using ELISA and PCR assays (Loconsole *et al*., 2014). Recently, new methods have been introduced for *X. fastidiosa* detection, including direct tissue blot immunoassay, Loop Mediated Isothermal Amplification (LAMP) and a new Polymerase Chain Reaction (PCR) assay (Djelouah *et al*., 2014; D'Onghia *et al*., 2014; Guan *et al*., 2015).

Because physical or chemical treatments for the control of *X. fastidiosa* are not (yet) available, particular attention has been given to identification of secondary metabolites that are essential for plant disease resistance and tolerance (Maddox *et al*., 2010) as possible strategies for disease management. Bacterial growth, aggregation and biofilm formation may be affected by xylem sap components (Cheng *et al*., 2009; Cruz *et al*., 2012; Shi *et al*., 2013). Phenolic acids such as gallic acid, caffeic acid, catechol, rutin and resveratrol have been shown to inhibit the *in vitro* growth of *X. fastidiosa* subsp. *fastidiosa*. Strong inhibitory activities against *X. fastidiosa* growth were observed with subgroups of flavonoid compounds, such as flavanone (naringenin), flavan-3-ol (catechin), and flavonol aglycone (quercetin) (Maddox *et al*., 2010). Other phenols such as the limonoid azadirachtin A and the flavanone hesperidin exhibited strong inhibitory effects against *Xfp* strain CVC (Ribeiro *et al*., 2008). In grapevine, *X. fastidiosa* subsp. *fastidiosa* infection may induce increased levels of catechin, digalloylquinic acid, and astringin in xylem sap, while multiple catechins, procyanidins, and stilbenoids were found at greater levels in xylem tissues. However, these effects were transient after pathogen infection (Wallis and Cheng, 2012). In grapevine, the potential induction of phenolics in response to *X. fastidiosa* infection did not affect phenolic levels in xylem sap and tissue as much as phenology or cultivar (Wallis *et al*., 2013a). It was also shown that the type of grapevine rootstock affected *X. fastidiosa* subsp. *fastidiosa* population levels and concentrations of caftaric acid, methyl salicylate (a procyanidin trimer), and quinic acid, which were all at greater levels in infected than non-infected grapevines (Wallis *et al*., 2013b). However, information on levels of phenolic compounds for olive cultivars infected by *Xfp* are not yet available. In olive tree, only the phenolic responses due to fungal infections have been investigated. Verbascoside is associated with resistance to *Verticillium dahliae* pathotypes (Markakis *et al*., 2010), whereas tyrosol and its derivatives were associated with constitutive resistance against *Spilocaea oleagina* infections; oleuropein and rutin were associated with induced resistance (Zine El Aabidine *et al*., 2010).

Under field conditions the most widespread South Apulia traditional olive cultivars Cellina di Nardò and Ogliarola di Lecce show clear symptoms of leaf scorching (Frisullo *et al*., 2014) when infected (Martelli, 2016), while the cultivar Leccino retains a green canopy with very little leaf scorching (Martelli, 2016). Furthermore, in the cvs. Leccino and Frantoio bacterial colonization and symptom development require longer incubation periods in comparison to cv. Cellina di Nardò (Saponari *et al*., 2016). It should be noted that in the infected area of South Apulia (Province of Lecce) Cellina di Nardò and Ogliarola di Lecce are the most planted cultivars (which together represent approximately 85% of plants). Leccino represents approximately 2% of planted olive trees, while other cultivars represent less than 1% of plants. Only recently it was proved that *X. fastidio-*

*sa* is able to systemically infect olive plants and to induce consistent symptoms of desiccation and leaf scorch 13–14 months post inoculation (Saponari *et al*., 2016) but a full replication of effects due to natural infection, as observed in mature trees, is not yet fully understood (Baccari and Lindow, 2011; Sun *et al*., 2011).

The objective of the research described in the present paper was to determine whether OQDS symptom progression, *Xfp* populations, or amounts of defense-associated phenolic compounds differ between cvs. of several naturally infected mature olive trees. Four olive cultivars, putative susceptible or resistant to the pathogen, were examined to observe if cultivar interferes with OQDS symptoms, and if plant responses to the pathogen are related to particular phenolic compounds.

## Materials and methods

### Experimental design, collection of samples and disease severity assessments

Trials were carried out in orchards located in the Lecce province of Apulia, Italy, in which OQDS symptomatic and symptomless trees were monitored. For each of four olive cultivars (Cellina di Nardò, Ogliarola di Lecce, Leccino and Frantoio), 12 plants (three groups of four plants per cultivar) were selected in *X. fastidiosa*-infected areas (in orchards where all plants showed OQDS since 2014, 1 year after pathogen detection), and 12 plants (three groups of four plants per cultivar) in orchards where the pathogen had not yet been detected. Olive trees within groups were selected to be of similar age (25-30 years), and to be growing with similar agronomic practices in the last 5 years and in similar pedoclimatic conditions.

Following management practices were the same for both symptomatic and asymptomatic plants. After winter pruning of trees, the canopy was not pruned during vegetative growth. Phytosanitary treatments were carried out in both plots according to EU Decision 2015/789, including controlling the insect vector (*Philaenus spumarius*) of strain CoDiRO and removal of wild plant hosts. Control of common insects such as the olive fly (*Bactrocera oleae*), was also carried out in order to minimize the effects of other pests. Orchards were monitored at weekly intervals to detect eventual insect or other pest outbreaks. No other treatments were carried out in orchards. According to Alagna *et al*. (2012), to avoid possible effects of different levels of water availability on the phenolic content among the trees, their water status was periodically monitored and, when necessary, irrigation was applied.

Evaluations of disease severity was carried out by visual inspections of the 12 plants every 2 months after pruning (from February 2015 to August 2016). In order to reduce the risk of overestimating disease due to the potential co-infection by other pathogens that can cause olive wilt, the canopies of the trees were each divided into upper and lower portions and subdivided in four sub-portions, according to cardinal directions. The presence of symptoms was recorded and scored using the following severity scale: 0 = symptomless; 1 = leaf scorching on few branches or few desiccated branches affecting the portion of the canopy; 2 = leaf scorching on several branches or desiccation affecting a large part of the portion of the canopy; and 3 = canopy with desiccated branches uniformly distributed.

Real-time PCR was used to determine *Xfp* population levels in the olive tree xylem tissues. Leaf samples were collected from 12 plants per cultivar belonging to infected areas and from 12 plants per cultivar belonging to non-infected areas. In April 2016, leaf samples (25 leaves collected from six branches per plant) were collected to assess *X. fastidiosa* presence by PCR with primers X.fas-0838-a-S-21-X.fas-1439-a-A-19 and XYgyr499-RXYgyr907 (Rodrigues *et al*., 2003). Samples from infected groups were always collected only from branches showing leaf scorching symptoms, to limit effects due to mixed infections of other pathogens with *Xfp*. Samples from not-infected groups did not show symptoms due to biotic or abiotic stress. The samples used for *Xfp* population level determinations were also used for analyses of phenolic compounds.

### Relative qPCR assay

To evaluate the presence and the relative population level of *Xfp* in olive trees, sampling (25 leaves collected from six branches per plant) was carried out 4 months after tree pruning (April 2016). For each sample, leaf petioles and basal portions of leaf blades were cut with a sterile scalpel. Plant tissue from each sample (approx. 1 g of leaf petioles) was transferred into an extraction bag (BIOREBA, Switzerland) and 4

mL of extraction buffer (0.2 M Tris – HCl pH 9, 0.4 M LiCl and 25 mM EDTA) was added. Sample homogenization was performed using a semi-automatic homogenizer (Homex 6, BIOREBA) at 50% maximum speed. DNA extraction was performed following the protocol of Edwards *et al*. (1991) which described the most commonly used method of purifying and concentrating DNA from samples. In this protocol, the DNA solution is first extracted with a phenol/chloroform/isoamyl alcohol mixture to remove protein contaminants and then precipitated with 100% ethanol.

The TaqMan quantitative PCR protocol with XF-F/R primers and XF-P probe (Harper *et al*., 2010) was used for analysis of populations of *Xfp* in samples. The primer pair XF-F/R (Harper *et al*., 2010) was also used with SYBR Green reagents to run qPCRs. TaqMan and SYBR Green qPCR reactions were performed in a real-time thermal cycler (ABI PRISM 7900HT, Applied Biosystems). Each reaction consisted of 5 µL from a 20 ng $\mu L^{-1}$ dilution of DNA extracted from 1 g of leaf petioles, 12.5 µL of SYBR Green PCR Master Mix (Applied Biosystems), 400 nM forward and reverse primers, ultrapure DNase/RNase-free water (Carlo Erba Reagents S.r.l.) in a total volume of 25 µL. The cycling conditions were: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, with the final dissociation at 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec. Taqman qPCR reactions were performed following the same protocol used for SYBR Green, adding a 200 nM TaqMan probe. The $2^{-\Delta Ct}$ method of relative quantification (semi-quantitative) was used to determine the fold change of the *Xfp* populations (Livak and Schmittgen, 2001; Cao and Shockey, 2012). The cycle threshold ($C_t$) of *O. europaea* chalcone synthase (*Chs*, GenBank accession no. KF935223. Forward primer: TCTCTC-CATTTTCTCTTTTGTACATCTATT. Reverse primer: CATCTCCAATGCAACAGTGTGA. Taqman Probe: CTCTTCTCCCCATTCCCATTTTGCTCGT 5′FAM – 3′ TAMRA) was used for calibration. Data analyses and $C_t$ calculations were carried out using SDS 1.2 software (Applied Biosystems). The relative amount of bacteria was determined by amplification of partial *rimM* open reading frame of *Xylella fastidiosa* (Harper *et al*., 2010) relative to the vegetal *Chs* gene, in the presence of SYBR Green.

qPCR reactions containing variable total DNA concentrations (0.8, 4, 20, 100 or 500 ng) from leaf petioles were used to estimate amplification efficiency based on the equation $E = (10^{\frac{1}{slope}} - 1) \times 100$ (Schneider *et al*., 2012).

### Analysis of phenolic compounds in plant tissues

Leaves used for analyses of phenolic composition were harvested as for the disease severity assessments described above, from plants grown under the described environmental and management conditions.

Leaf samples were collected from branches assayed by qPCR for *Verticillium dahliae* (Bilodeau *et al*., 2012), *Colletotrichum* spp., *C. acutatum* and *C. gloeosporioides* (Garrido *et al*., 2009), to ensure that differences found in phenolic composition were not influenced by mixed infections with other pathogens. The most common fungal species associated with olive wilt and decline in Southern Italy, *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *P. parasiticum* (Carlucci *et al*., 2015) were also assayed by qPCR, using wood chips obtained from branches (Aroca *et al*., 2008; Martín *et al*., 2012). Wood chips were also tested by PCR for *Botryosphaeria dothidea* (Romanazzi *et al*., 2009), *Diplodia seriata* (Martín *et al*., 2014) and *Phytopthora* spp. (Drenth *et al*., 2006). Only leaves positive to *Xfp* from branches without discolouration in the xylem tissues and negative to tests against these fungal pathogens were used for further assays. The widespread occurrence of the fungi *Stictis panizzei*, *Mycocentrospora cladosporioides*, *Spilocaea oleagina* and the bacterium *Pseudomonas savastanoi* pv. *savastanoi* did not permit limitation of tests to totally symptomless trees, but branches showing symptoms of these pathogens were also excluded for leaf sampling.

Immediately after harvesting, leaves were frozen in liquid nitrogen and stored at -80°C until further analysis. Extraction and analysis of phenolic compounds were carried out following the metods of Taamalli *et al*. (2012). Total phenolic compounds were assayed using a spectrophotometric methods with Fast Blue BB (4-benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi-[zinc chloride]) salt), and using a gallic acid standard dilution (Medina, 2011). Characterization of phenolics was carried out using an Agilent 1200 Liquid Chromatography system (Agilent Technologies) equipped with a standard autosampler. The HPLC column was an

Agilent Extended C18 (1.8 μm, 2.1 × 50 mm). Separation was carried out at 40°C with a gradient elution programme at a flow rate of 0.5 mL $min^{-1}$. The mobile phases consisted of water plus 0.1% formic acid (A) and acetonitrile (B). The following multistep linear gradient was applied: 0 min, 1% B; 13 min, 25% B; 19 min, 40% B; 21 min, 90% B. The initial conditions were maintained for 5 min. The injection volume in the HPLC system was 5 μL. The HPLC system was coupled to an Agilent 6320 TOF mass spectrometer equipped with a dual ESI interface (both from Agilent Technologies), operating in negative ion mode using a capillary voltage of +3.5 kV. The other optimum values of the ESI–TOF–MS parameters were; drying gas temperature, 300°C; drying gas flow, 12 L $min^{-1}$; and nebulising gas pressure, 40 PSIG. Detection was carried out within a mass range of 50–1700 m/z. The accurate mass data of the molecular ions were processed through Mass Hunter software (Agilent Technologies). Five standard calibration graphs for the quantification of the phenolic compounds from olive leaves were prepared using commercial standards (quinic acid, oleuropein, hydroxytyrosol, luteolin and luteolin 7-*O*-glucoside) (Sigma-Aldrich). The limit of quantification (LOQ) was determined as the signal-to-noise ratio of 10:1, and the limit of detection (LOD) was determined as signal-to-noise ratio of 3:1. Intra-day and inter-day precision was determined to assess repeatability of the methods, and were expressed by the relative standard deviations (RSD). An olive leaf extract was injected (n = 5) on one day (intraday precision) for three consecutive days (interday precision, n = 15).

### Statistical analyses

Software R version 3.3.1 (R Foundation for Statistical Analysis) was used for all data analyses. Due to disease severities being on a 0–3 scale, before variance analysis, the Shapiro-Wilk normality test and the Levene's test of homogeneity of variances were carried out to evaluate the applicability of analysis of variance (ANOVA). If data were not normally distributed, the non-parametric Kruskal–Wallis test was used to determine overall differences in disease severity. The Multiple Comparisons Dunn's Test (with Bonferroni correction of *P* value) was used to evaluate which groups were dissimilar from one another. A *P*-value ≤ 0.05 was considered to be statistically significant. Otherwise, if the data were normally distributed, the same software was used to perform one-way or two-way ANOVA and pairwise multiple comparisons were made using the Duncan's test. *P* value ≤0.05 was considered to be statistically significant.

## Results

### Disease severity assessments

Disease symptoms appeared 2 months after pruning in plants positive to qualitative PCR tests (Table 1). Disease severity data were not normally distributed (Shapiro-wilk test, $P \leq 0.05$), so the non-parametric Kruskal-Wallis test was used to determine overall differences in means. At early stages there were large differences (approx. 7-9 fold increased severity) in Cellina di Nardò and Ogliarola di Lecce compared to Frantoio and Leccino. In the later (April) assessments, severity was about 3-4 fold greater, in June about 2.5–3 and in August about 1.5 fold greater in in the PCR positive plants. However, canopies with desiccated branches were found on each cultivar from April.

### Relative qPCR assay

The quantification of *Xfp* in the different cultivars was shown with the TaqMan and SYBR Green qPCR assays, using the XF-F/R (Harper *et al*., 2010) set of primers employing the *O. europaea* chalcone synthase gene (*Chs*) as the internal calibrator.

To assess the differences among olive cultivars in relation to *Xfp* multiplication in xylem tissues, a qPCR assay was performed 4 months after pruning (April). For better evaluation, two different chemistries for fluorescent detection (TaqMan and SYBR) were used. The TaqMan qPCR with XF-F/R primers (Harper *et al*., 2010) generated an efficiency slope similar to that observed in SYBR Green qPCR tests (Figure 1). Both fluorescent methods gave a similar amplification efficiency (102.9 ± 2.6 for TaqMan PCR and 101.3 ± 2.4% for SYBR Green, n = 7), but a greater y-intercept was observed with TaqMan assay (31.6) compared with the SYBR Green assay (29.1) (Figure 1). As reported by Cao and Shockey (2012), the y-intercept corresponds to the theoretical limit of detection of the reaction, or the $C_t$ value expected at the lowest copy number of target sequences gives significant amplification. Since the difference between

**Table 1.** Disease progression in four olive cultivars, expressed as mean disease severity scores based on a visually assessed scale from 0 to 3. Observations were assessed 2, 4, 6 and 8 months after pruning. The data comprised an average of 12 *Xylella fastidiosa* subsp. *pauca* (*Xfp*) infected plants per cultivar.

| Cultivar | February | April | June | August |
|---|---|---|---|---|
| Cellina di Nardò | 0.73 ± 0.25 | 1.56 ± 0.19 | 2.00 ± 0.26 | 2.10 ± 0.33 |
| Ogliarola di Lecce | 0.73 ± 0.13 | 1.73 ± 0.23 | 2.06 ± 0.22 | 2.17 ± 0.37 |
| Frantoio | 0.10 ± 0.13 | 0.50 ± 0.11 | 0.77 ± 0.20 | 1.38 ± 0.33 |
| Leccino | 0.08 ± 0.12 | 0.46 ± 0.21 | 0.60 ± 0.31 | 1.33 ± 0.22 |

| Multiple comparisons | z-score | *P*-value | z-score | *P*-value | z-score | *P*-value | z-score | *P*-value |
|---|---|---|---|---|---|---|---|---|
| Cellina - Ogliarola | 0.0226 | 1.0000 | -0.9828 | 0.9771 | -0.4289 | 1.0000 | -0.3030 | 1.0000 |
| Cellina - Frantoio | 4.1835 | 0.0001 | 3.6333 | 0.0008 | 3.7118 | 0.0006 | 3.6430 | 0.0008 |
| Ogliarola - Frantoio | 4.3716 | 0.0001 | 4.6161 | 0.0000 | 4.1407 | 0.0001 | 3.9460 | 0.0002 |
| Cellina - Leccino | 4.3716 | 0.0000 | 3.9609 | 0.0002 | 4.3773 | 0.0000 | 4.0199 | 0.0002 |
| Ogliarola - Leccino | 4.3491 | 0.0000 | 4.9437 | 0.0000 | 4.8061 | 0.0000 | 4.3228 | 0.0000 |
| Frantoio - Leccino | 0.1881 | 1.0000 | 0.3276 | 1.0000 | 0.6655 | 1.0000 | 0.3769 | 1.0000 |

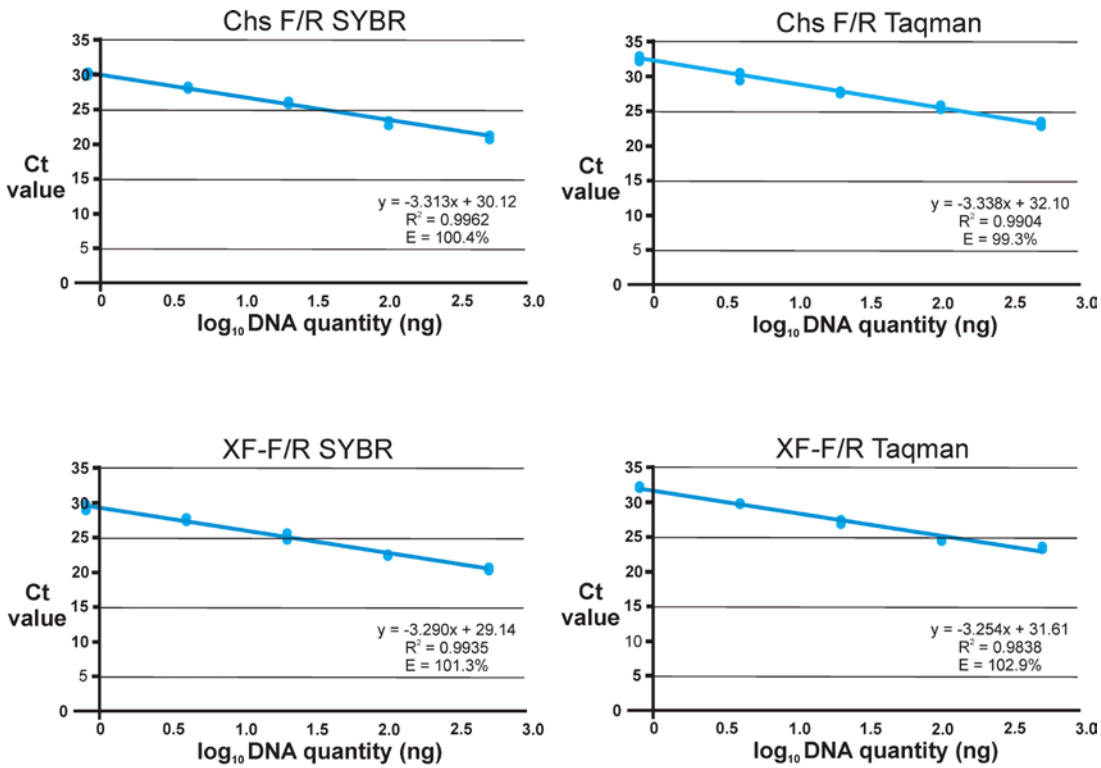


**Figure 1.** Real-time PCR standard curve graphically represented as a semi-log regression line plot of CI value (Y-axis) *vs.* log of input nucleic acid (X-axis). Five serial dilutions of DNA (extracted from 1 g of leaf petioles) between the range of 500 and 0.8 ng were used to design the standard curve.

y-intercepts was 2.5, this indicates that SYBR Green chemistry is 5.6-times more sensitive than TaqMan reagents. A similar (4-times) greater sensitivity of SYBR Green was observed also using primers for the calibrator *Chs* gene.

The copy number of the calibrator *Chs* was very stable among all the tested samples, and no differences were registered among cultivars (Table 2). The $C_t$ values from the SYBR Green assay among whole samples was 25.6 ± 1.0, while using TaqMan the $C_t$ was set at 23.9 ± 1.1.

For better interpretation of qPCR data, the level of infection of Frantoio cultivar was chosen as a baseline. The relative level of infection of the other cultivars was calculated as Fold Changes (FC) relative to Frantoio (Figure 2).

Using TaqMan chemistry, the relative level of infection of Leccino was very similar to that for Frantoio infection ($FC_{Lec}$ = 1.56). Conversely, Cellina and Ogliarola di Lecce showed greater levels of infection ($FC_{Cel}$ = 3.48, $FC_{Ogl}$ = 4.29).

As highlighted above from calculation of primer efficiency, SYBR Green chemistry was also more sensitive than TaqMan for detection of differences of infection among the cultivars. $FC_{Lec}$ obtained using SYBR Green chemistry was 3.25 ± 0.4, whereas the FC for Cellina di Nardò was 9.85 ± 1.0, and for Ogliarola di Lecce 21.11 ± 1.5. Moreover, statistical analyses of data from TaqMan assays resulted in two statistical groups, where Cellina di Nardò and Ogliarola di Lecce were characterized by twice greater relative levels of infection compared to Leccino and Frantoio (Figure 2). The output from SYBR Green assays resulted in four statistically different groups, one for each cultivar exhibiting a different relative level of infection, in increasing order of Frantoio, Leccino, Cellina di Nardò and Ogliarola di Lecce (Figure 2).

**Table 2.** Mean threshold cycles ($C_t$) of the calibrator chalcone synthase gene assayed by qPCR, using TaqMan or SYBR Green fluorescent chemistry.

| Cultivar | TaqMan | SYBR Green |
|---|---|---|
| Cellina di Nardò | 25.7 ± 0.1 a[a] | 24.0 ± 0.1 a |
| Ogliarola di Lecce | 25.4 ± 0.3 a | 23.9 ± 0.2 a |
| Frantoio | 25.8 ± 0.1 a | 23.6 ± 0.2 a |
| Leccino | 25.6 ± 0.1 a | 24.0 ± 0.1 a |

[a] Means in the same column followed by the same letter do not differ significantly ($P \leq 0.05$), according to Duncan's test.

## Analyses of phenolic compounds

Leaf samples from trees with OQDS symptoms were analyzed to evaluate the effect of *X. fastidiosa* infection on leaf phenolic composition. Leaves or wood chips were harvested from branches and were analyzed for the most common fungal species (according to previously reported lists of fungi) associated with olive wilt. This was to avoid detection of differences in phenolic compounds conditioned by the presence of other pathogens. *Phaeomoniella chlamydospora* (in 18.5% of analysed plants) *Botryosphaeriaceae* (12.5%) and *Colletotrichum* spp. (8.3%) were detected most abundantly. Presence of *Phaeoacremonium* spp. was sporadic (2.1% of analysed plants), while *Verticillium dahliae* was never found in the analysed olive tissues. Only leaves that tested negative against previously described fungi were used for the phenolic assays.

The calibration plots indicated good correlations between peak areas and analyte concentrations, and the regression coefficients were greater than 0.988 in all cases. LOD was within the range 0.002 and 0.005 mg $mL^{-1}$ while LOQ was within 0.005 and 0.009 mg $mL^{-1}$. The greatest intraday repeatability of the peak area among all peaks, expressed by RSD, was 0.82%, whereas the greatest interday repeatability among

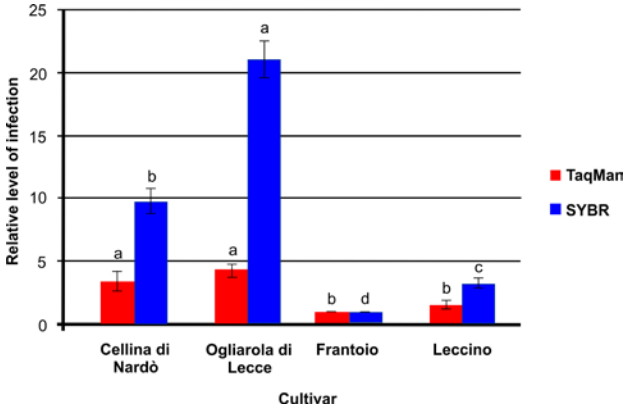


**Figure 2.** Relative levels of *Xylella fastidiosa* in four olive cultivars, expressed as fold changes ($2^{-\Delta Ct}$) calculated using the cultivar Frantoio as an arbitraryily chosen baseline and chalcone synthase as calibrator. Letters a, b, c and d indicate statistical groups that differ significantly ($P \leq 0.05$).

**Table 3.** Parameters of calibration curves, limit of detection (LOD), limit of quantification (LOQ) and relative standard deviation (RSD) for the HPLC method validation of phenolic assays.

| Standard phenolic compound | Slope | Intercept | $r^2$ | LOD (µg $mL^{-1}$) | LOQ (µg $mL^{-1}$) | RSD (%) |
|---|---|---|---|---|---|---|
| Quinic acid | 2.19E+05 | -7.17E+04 | 0.999 | 1.81 | 6.03 | 0.82 |
| Hydroxytyrosol | 1.95E+05 | -2.57E+05 | 0.999 | 2.60 | 8.66 | 0.78 |
| Oleuropein | 1.81E+06 | 5.01E+05 | 0.997 | 0.77 | 2.56 | 0.81 |
| Luteolin 7-*O*-glucoside | 1.37E+06 | 1.53E+06 | 0.988 | 1.25 | 4.15 | 0.77 |
| Luteolin | 1.61E+07 | 3.91E+06 | 0.989 | 0.13 | 0.45 | 0.74 |

**Table 4.** Characterization of olive leaf extract by HPLC-ESI-TOF (ion M-H)$^-$. Exp = experimental; Clc = calculated.

| Peak | Compound | RT (min) | $(M-H)^-$ Exp | m/z Exp | m/z Clc | Diff. (ppm)[a] | Score[b] | Reference[c] |
|---|---|---|---|---|---|---|---|---|
| 1 | *Quinic acid | 0.365 | $C_7H_{11}O_6$ | 191.0571 | 191.0561 | -5.89 | 90.44 | 1, 2, 3 |
| 2 | *Hydroxytyrosol glucoside | 2.965 | $C_{14}H_{19}O_8$ | 315.1095 | 315.1085 | -1.26 | 96.62 | 1, 2, 3 |
| 3 | Secologanoside is 1 | 3.960 | $C_{16}H_{21}O_{11}$ | 389.1095 | 389.1089 | -1.11 | 88.91 | 1, 2, 3 |
| 4 | Secologanoside is 2 | 6.116 | $C_{16}H_{21}O_{11}$ | 389.1101 | 389.1089 | -2.62 | 96.13 | 2 |
| 5 | Elenoic acid glucoside | 6.630 | $C_{17}H_{23}O_{11}$ | 403.1262 | 403.1246 | -3.68 | 80.9 | 2 |
| 6 | Oleuropein aglycon derivate | 7.194 | $C_{16}H_{25}O_{10}$ | 377.1459 | 377.1453 | -1.23 | 92.94 | 2 |
| 7 | Unknown | 7.825 | $C_{19}H_{27}O_{10}$ | 415.1621 | 415.1610 | -2.44 | 95.92 | 2 |
| 8 | Hydroxyolouropein | 9.036 | $C_{25}H_{31}O_{14}$ | 555.1732 | 556.1803 | -2.04 | 97.55 | 2, 4 |
| 9 | * Luteolin 7-*O*-glucoside is. 1 | 9.119 | $C_{21}H_{19}O_{11}$ | 447.0952 | 447.0933 | -3.93 | 77.64 | 3, 2 |
| 10 | Luteolin rutinoside | 9.517 | $C_{27}H_{29}O_{15}$ | 593.1517 | 593.1512 | -0.87 | 97.79 | 3 |
| 11 | * Luteolin 7-*O*-glucoside is. 2 | 9.998 | $C_{21}H_{19}O_{11}$ | 447.0948 | 447.0933 | -3.03 | 96.13 | 1, 2, 3 |
| 12 | Apigenin 7 glucoside | 10.010 | $C_{21}H_{19}O_{10}$ | 431.0988 | 431.0984 | -0.79 | 97.82 | 1, 2, 3 |
| 13 | Oleuropein diglucoside is. 1 | 10.545 | $C_{31}H_{41}O_8$ | 701.2307 | 701.2298 | -0.6 | 93.83 | 1, 2, 3 |
| 14 | Oleuropein diglucoside is. 2 | 10.728 | $C_{31}H_{41}O_8$ | 701.2306 | 701.2298 | -0.49 | 94.85 | 1, 2, 3 |
| 15 | Oleuropein diglucoside is. 3 | 10.893 | $C_{31}H_{41}O_8$ | 701.2291 | 701.2298 | 3.2 | 98.67 | 1, 2, 3 |
| 16 | 2-methoxy oleouropein is. 1 | 11.175 | $C_{26}H_{33}O_{14}$ | 569.1898 | 569.1876 | -3.76 | 85.77 | 3 |
| 17 | 2-methoxy oleouropein is. 2 | 11.258 | $C_{26}H_{33}O_{14}$ | 569.1899 | 569.1876 | -3.64 | 97.16 | 3 |
| 18 | Oleuropein | 11.406 | $C_{25}H_{32}O_{13}$ | 539.1772 | 539.1770 | 0.03 | 97.14 | 1,2,3,4 |
| 19 | *Luteolin | 11.939 | $C_{15}H_9O_6$ | 285.0419 | 285.0405 | -4.87 | 97.08 | 1,2,3,4 |
| 20 | Ligstroside | 12.611 | $C_{25}H_{31}O_{12}$ | 523.1823 | 523.1821 | -0.03 | 97.55 | 5 |
| 21 | *Apigenin | 13.382 | $C_{15}H_9O_5$ | 269.0461 | 269.0455 | -1.77 | 98.7 | 1 |
| 22 | Diosmetin | 13.929 | $C_{16}H_{11}O_6$ | 299.0566 | 299.0561 | -1.43 | 98.5 | 1 |

[a] Relative difference: the difference between the observed mass and the theoretical mass of the compound (ppm).
[b] Isotopic abundance distribution match: a measure of the probability that the distribution of isotope abundance ratios calculated for the formula matches the measured data.
[c] Reference list: 1 = Taamalli *et al*. (2013); 2 = Fu *et al*. (2010); 3 = Taamalli *et al*. (2012); 4 = Talhaoui *et al*. (2014); 5 = Quirantes-Piné *et al*. (2013).
* Confirmed by authentic chemical standard.

**Table 5.** Mean amounts of phenolic compounds (mg $g^{-1}$ fresh weight) in olive leaves infected by *Xylella fastidiosa* susp. *pauca* (I) compared to negative samples (H). Data are relative to 12 infected or healthy plants per cultivar. Two-way ANOVA was carried out to evaluate statistical differences and interactions among factors (cultivar (Cv) and health status (HS)). Letters indicate statistical groups that differ significantly (Duncan's test, $P \leq 0.05$).

| Phenolic compound | Cultivar (mg $g^{-1}$ FW) | | | | | | | | Statistic analyses | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cellina di Nardò | | Ogliarola di Lecce | | Frantoio | | Leccino | | | | |
| | H | I | H | I | H | I | H | I | Cv | HS | Cv x HS |
| 01. Quinic acid | 0.77 ±0.07$^{c}$ | 3.99 ±0.51$^{b}$ | 1.20 ±0.12$^{c}$ | 5.68 ±0.88$^{a}$ | 1.10 ±0.22$^{c}$ | 4.44 ±0.58$^{b}$ | 1.22 ±0.13$^{c}$ | 4.23 ±0.53$^{b}$ | *** | *** | *** |
| 02. Hydroxytyrosol glucoside | 4.09 ±0.40$^{c}$ | 0.20 ±0.07$^{d}$ | 4.12 ±0.52$^{c}$ | 0.24 ±0.05$^{d}$ | 5.33 ±0.40$^{b}$ | 0.13 ±0.05$^{d}$ | 6.15 ±0.82$^{a}$ | 0.11 ±0.05$^{d}$ | *** | *** | *** |
| 03. Secologanoside | 0.10 ±0.03 | 0.11 ±0.04 | 0.09 ±0.04 | 0.12 ±0.03 | 0.09 ±0.05 | 0.10 ±0.03 | 0.12 ±0.04 | 0.10 ±0.03 | NS | NS | NS |
| 05. Oleuropein aglycone | 0.08 ±0.02$^{bc}$ | 0.08 ±0.03$^{c}$ | 0.10 ±0.03$^{bc}$ | 0.08 0.02$^{bc}$ | 0.30 ±0.06$^{a}$ | 0.27 ±0.10$^{a}$ | 0.14 ±0.04$^{b}$ | 0.12 ±0.03$^{bc}$ | *** | NS | NS |
| 08. Luteolin 7-O-glucoside | 0.79 ±0.29 | 0.76 ±0.24 | 0.71 ±0.23 | 0.66 ±0.22 | 0.81 ±0.21 | 0.81 ±0.34 | 0.78 ±0.27 | 0.72 ±0.22 | NS | NS | NS |
| 10. Oleuoropein diglucoside | 0.68 ±0.31 | 0.88 ±0.59 | 0.73 ±0.55 | 0.69 ±0.47 | 0.71 ±0.51 | 0.73 ±0.44 | 0.75 ±0.47 | 0.63 ±0.51 | NS | NS | NS |
| 12. 2-methoxy oleouropein | 0.52 ±0.28 | 0.49 ±0.38 | 0.35 ±0.21 | 0.27 ±0.22 | 0.43 ±0.25 | 0.33 ±0.17 | 0.39 ±0.19 | 0.45 ±0.31 | NS | NS | NS |
| 14. Luteolin | 0.05 ±0.03 | 0.06 ±0.03 | 0.10 ±0.06 | 0.08 ±0.02 | 0.09 ±0.06 | 0.07 ±0.05 | 0.05 ±0.04 | 0.06 ±0.04 | NS | NS | NS |
| Total polyphenols | 20.01 ±2.34 | 21.14 ±3.04 | 20.45 ±3.45 | 21.11 ±2.77 | 18.42 ±2.47 | 19.23 ±1.39 | 19.92 ±1.49 | 19.99 ±1.12 | NS | NS | NS |

NS, Not significant; *, $P < 0.05$; **, $P < 0.01$;***, $P < 0.001$.

all peaks was 1.1%. The different parameters of the calibration curves are summarized in Table 3.

Characterization of olive leaf extracts using HPLC-ESI-TOF is reported in Table 4. At 4 months after pruning, differences in constitutive levels of phenols were observed for oleuropein aglycone and hydroxytyrosol glucoside (Table 5). The cultivars Frantoio and Leccino showed levels of oleuropein aglycone that were, respectively, 4-fold or 2-fold greater compared to the putative susceptible cultivars. Frantoio and Leccino showed levels of hydroxytyrosol glucoside that were, respectively, 12.8–12.9 or 14.8–14.9% greater compared to the putative susceptible cultivars. No differences in quinic acid, secologanoside, luteolin-7-*O*-glucoside, oleuropein diglucoside, 2-methoxy oleuropein or luteolin were observed among healthy plants of the four cultivars.

*Xfp*-positive samples showed significant differences in hydroxytyrosol glucoside and quinic acid levels. For both compounds, the interaction between health status and cultivar was observed. For hydroxytyrosol glucoside, strong reductions were observed in the putative susceptible infected plants of Cellina di Nardò (95.1% reduction) and Ogliarola di Lecce (94.2%), or in the putative resistant Frantoio (97.6% reduction) and Leccino (98.2%). Only quinic acid had greater amounts in *Xfp*-infected plants, with large increases (5-fold increase) in Cellina di Nardò and Ogliarola di Lecce, and lesser (4-fold increases) in Frantoio and Leccino (Table 5). A representative chromatogram of phenolic compounds with regard to health status is shown in Figure 3 (A and B).

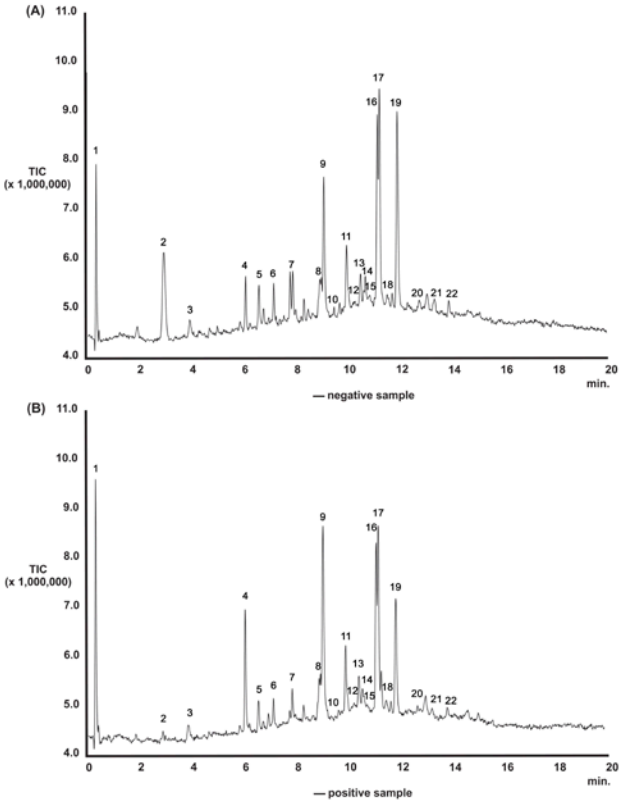


**Figure 3.** Representative chromatogram of phenolic compounds in *Xylella fastidiosa* subsp. *pauca* (*Xfp*)-positive or negative olive trees (cultivar Ogliarola di Lecce). Amounts of phenolic compounds in leaves of (A) *Xfp*-negative or (B) *Xfp*-positive plants. Characterization of peaks is detailed in Table 4.

## Discussion

In naturally *Xfp*-infected olive plants, disease severity was up to 3-fold greater in the cultivars Cellina di Nardò and Ogliarola di Lecce compared to Frantoio and Leccino, where mean disease severity was constantly less than for the other two cultivars (Saponari and Loconsole, personal communication; Giampetruzzi *et al*., 2016). Using level of infection of Frantoio as a baseline, the relative level of infection expressed as FC was significantly less in Leccino (1.6-fold in TaqMan assays) from those observed in putative susceptible cultivars such as Cellina di Nardò (3.5-fold in TaqMan) or Ogliarola di Lecce (4.3-fold in TaqMan), according to preliminary results in bacterial concentration observed in Leccino ($1.3 \times 10^4$ CFU $mL^{-1}$ tissue extract *vs* $2.1 \times 10^5$ CFU $mL^{-1}$ tissue extract in Ogliarola di Lecce) (Saponari and Loconsole, personal communication; Martelli, 2016). This was supported by evaluation of disease severity, which was less in Leccino and Frantoio during the sampling period. Disease severity of naturally infected plants could be influenced by fungal pathogens, because many fungal species can be found in olive plants with decline symptoms in Southern Italy, and old olive plants are frequently infected by *P. chlamydospora* (Nigro *et al*., 2013), *Phaeoacremonium* spp. or *Botryosphaeriaceae* (Carlucci *et al*., 2015). Our results confirmed the presence of such fungi in olive trees naturally infected by *Xfp*, and no cultivar was unaffected by at least one fungus related to decline. The most frequently detected fungi were *P. chlamydospora* and *Botryosphaeriaceae*, which were detected, respec-

tively, in 9 or 6 plants out of 48 tested), even when unaffected branches were used for further analysis.

*Xylella Fastidiosa* primers designed by Harper *et al*. (2010) responded positively to SYBR Green. Dissociation curve analysis was used to determine that the intercalating dye produced single, specific products. No qualitative differences with PCR or TaqMan methods were observed. Both the qPCR assays gave high correlation coefficients and similar primer amplification efficiency, although SYBR Green showed a better limit of detection (5.6-times less) than TaqMan, using *X. fastidiosa*-specific primer pairs (Figure 1). The differences between TaqMan and SYBR Green qPCR assay results derived from the different chemistry employed: in SYBR Green, the amount of signal is dependent on the mass of double stranded DNA produced in the reaction, while TaqMan chemistry requires the use of a fluorogenic probe and a single fluorophore is released for each amplified molecule synthetized. Multiple dyes binding a single amplified molecule increase sensitivity for detecting amplification products. The SYBR green qPCR method was as reliable as TaqMan for determining *X. fastidiosa* in petioles of olive leaves, independently from cultivars, and may represent an improved diagnostic method since no probe is required reducing costs for assay setup and running. The designed internal calibrator gene *Chs* did not fluctuate during tests (constant cycle threshold values); therefore, this could be efficiently employed as a calibrator to control each sample for the presence of PCR inhibitors, to determine a cut-off value of sensitivity for negative samples and to normalize positive samples for the efficient DNA recovery. That procedure was necessary to discern between infected and uninfected (healthy) olive plants and to accurately evaluate the levels of *Xfp* infection.

Phenolic compounds have been associated with host resistance to bacterial and other diseases (Hammerschmidt, 2004; Gutha *et al*., 2010; Markakis *et al*., 2010; Zine El Aabidine *et al*., 2010; Rusjan *et al*., 2012). For the two phenolic compounds mainly affected by the presence of *Xfp*, hydroxytyrosol has been shown to inhibit or delay the rate of growth of a range of bacteria and fungi that cause disease of animals (Bisignano *et al*., 1999; Sousa *et al*., 2006) or plants (Yangui *et al*., 2010a, 2010b; Medina *et al*., 2011).

The present study did not reveal significant effects of *Xfp* on the phenolics commonly present at greatest concentrations in olive leaves, such as secoiridoids. Phenolics associated to disease resistance in olive, such as oleuropein (Zine El Aabidine *et al*., 2010), were not affected by the presence of *Xfp*. Infection, however, modified concentrations of some minor phenolic compounds, including hydroxytyrosol glucoside and quinic acid. Furthermore, significant interactions were observed among health status and cultivars. The olive cultivars Ogliarola di Lecce and Cellina di Nardò showed lower constitutive levels of hydroxytyrosol glucoside compared to the cultivars Frantoio and Leccino, confirming different baselines of this compound (in healthy plants), as has been observed in other olive cultivars (Petridis *et al*., 2012a). Bacterial infection was associated with a statistically significant decrease of hydroxytyrosol glucoside in mature naturally-infected olive trees (Table 5), with lower amounts in Leccino (1.8- and 2.2-fold less, respectively, than in Cellina di Nardò and Ogliarola di Lecce) and in Frantoio (1.5- and 1.8-fold less respectively, than in Cellina di Nardò or Ogliarola di Lecce). The decrease in hydroxytyrosol glucoside concentration has also been observed in olive trees under high salinity stress (Petridis *et al*., 2012a), or after cold stress (Ortega-Garía and Peragón, 2009). In the case of grapevines with Pierce's diseases, symptomatic leaves developed lower water potentials than healthy plants around noon each day, which was related to a reduction in the leaf osmotic potential (Goodwin and Meredith, 1988). Moreover, hydroxytyrosol levels decreased with water stress in four Greek olive tree cultivars (Petridis *et al*., 2012b). Petridis *et al*. (2012b) suggested that since hydroxytyrosol is one of the main phenolic compounds in olive, and this compound has high antioxidant activity, increased concentrations under adverse conditions would be expected. Involvement of hydroxytyrosol in oleuropein synthesis could explain this behaviour (Petridis *et al*., 2012b), but conversely to water stress tests, the present study did not reveal increases in oleuropein levels in *X. fastidiosa*-infected plants.

Constitutive levels of quinic acid were similar in the four olive cultivars analyzed. However, statistically significant interactions between health status and cultivar was observed for this compound, because greater amounts of quinic acid were detected in putative susceptible cultivars (5-fold greater) compared to Leccino and Frantoio (4-fold increases) (Table 5).

Wallis and Chen (2012) indicated that in grapevine, even though plants may initially respond to *X. fastidio-*

*sa* infections with increased production of phenolic compounds, the bacteria ultimately cause plants to decline because they no longer have resources to support secondary metabolite production, including defense-associated phenolic compounds. These authors defined symptomatic plants as entering "survival mode", where most photosynthate is allocated to basic growth and maintenance (also see Bonello *et al*., 2006). Our results suggest similar behaviour in olive trees; the only phenolic compound for which the concentration was greater in *Xfp*-infected olive trees was quinic acid, which is associated with suppression of disease development caused by fungi in tomato (Liu, 2001; Bartz *et al*., 2013). Wallis *et al*. (2013b) demonstrated that quinic acid was the only compound positively associated with Pierce's Disease symptoms in grapevine, although other molecules were positively associated with bacterial concentration. Their results suggested a further linkage between behaviour of *Xfp*-infected olive trees and infected grapevines. Due to lack of effective protocols for inducing OQDS in healthy olive plants, the present study focused on 1-year-old branches in naturally-infected plants, aiming to evaluate relationships between phenolic levels with disease. As proposed for grapevine (Wallis *et al*., 2013b), future studies should consider monitoring earlier shifts in phenolics in olive that occur during *Xfp* infections, and how those relate to progression.

In future, we will develop additional data to support the possibility that changes in the levels of hydroxytyrosol glucoside and quinic acid could be used as markers of *Xfp* infection in olive trees, and to reinforce the hypothesis that constitutive amounts of hydroxytyrosol glucoside could be associated with olive cultivars that are tolerant to OQDS.

**Conflict of interest.** The authors declare that they have no conflicts of interest.

## Acknowledgments

This research was partially financed by the Apulia Region "Cluster Tecnologici Regionali" TAPASS Project. We thank farmers/growers for their support in the field observations.

## Literature cited

Alagna F., R. Mariotti, F. Panara, S. Caporali, S. Urbani, G. Veneziani, S. Esposto, A. Taticchi, A. Rosati, R. Rao, G. Perrotta, M. Servili and L. Baldoni, 2012. Olive phenolic compounds: metabolic and transcriptional profiling during fruit development. *BMC Plant Biology* 12, 162.

Almeida R.P.P. and L. Nunney, 2015. How do plant diseases caused by *Xylella fastidiosa* emerge? *Plant Disease* 99, 1457–1467.

Aroca A., R. Raposo and P. Lunello, 2008. A biomarker for the identification of four *Phaeoacremonium* species using the β-*tubulin* gene as the target sequence. *Applied Microbiology and Biotechnology* 80, 1131–1140.

Baccari C. and S.E. Lindow, 2011. Assessment of the process of movement of *Xylella fastidiosa* within susceptible and resistant grape cultivars. *Phytopathology* 101, 77–84.

Bartz F.E., N.J. Glassbrook, D.A. Danehower and M.A. Cubeta, 2013 Modulation of the phenylacetic acid metabolic complex by quinic acid alters the disease-causing activity of *Rhizoctonia solani* on tomato. *Phytochemistry* 89, 47–52.

Bilodeau G.J., S.T. Koike, P. Uribe and F.N. Martin, 2012. Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. *Phytopathology* 102, 331–343.

Bisignano G., A. Tomaino, R. Lo Cascio, G. Crifasi, N. Uccella and A. Saija, 1999. On the in-vitro activity of oleuropein and hydroxytyrosol. *Journal of Pharmacy and Pharmacology* 51, 971–974.

Bonello P., T.R. Gordon, D.A. Herms, D.L. Wood and N.A. Erbilgin, 2006. Nature and ecological implications of pathogen-induced systemic resistance in conifers: A novel hypothesis. *Physiological and Molecular Plant Pathology* 68, 95–104.

Cao H. and J.M. Shockey, 2012. Comparison of TaqMan and SYBR Green qPCR methods for quantitative gene expression in Tung tree tissues. *Journal of Agricultural and Food Chemistry* 60, 12296–12303.

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.

Carlucci A., F. Lops, F. Cibelli and M.L. Raimondo, 2015. *Phaeoacremonium* species associated with olive wilt and decline in Southern Italy. *European Journal of Plant Pathology* 141, 717–729.

Chatterjee S., R.P.P. Almeida and S. Lindow, 2008. Living in two Worlds: The Plant and Insect Lifestyles of *Xylella fastidiosa*. *Annual Review of Phytopathology* 46, 243–271.

Cheng D.W., H. Lin, M.A. Walker, D.C. Stenger and E.L. Civerolo, 2009. Effects of grape xylem sap and cell wall constituents on in vitro growth, biofilm formation, and cellular aggregation of *Xylella fastidiosa*. *European Journal of Plant Pathology* 125, 213–222.

Coletta-Filho H.D., C.S. Francisco, J.R.S. Lopes, A.F. De Oliveira and L.F.D.O. Da Silva, 2016. First report of olive leaf scorch in Brazil, associated with *Xylella fastidiosa* subsp. pauca. *Phytopathologia Mediterranea* 55, 130–135.

Cornara D., M. Saponari, A.R. Zeilinger, A. de Stradis, D. Boscia, G. Loconsole, D. Bosco, G.P. Martelli, R.P.P. Almeida and F. Porcelli, 2016. Spittlebugs as vectors of *Xylella fastidiosa* in olive orchards in Italy. *Journal of Pest Science* 90, 521–530.

Cruz L.F., P.A. Cobine and L. Da La Fuente, 2012 Calcium increases *Xylella fastidiosa* surface attachment, biofilm formation, and twitching motility. *Applied and Environmental Microbiology* 78, 1321–1331.

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

D'Onghia A.M., F. Santoro, T. Yassen, K. Djelouah, A. Guario, A. Percoco, T. Caroppo and F. Valentini, 2014. An innovative monitoring model of *Xylella fastidiosa* in Apulia. International Symposium on the European Outbreak of *Xylella fastidiosa* in Olive. *Journal of Plant Pathology* 96, 99.

Drenth A., G. Wagels, B. Smith, B. Sendall, C. O'Dwyer, G. Irvine and J.A.G. Irwin, 2006. Development of a DNA-based method for detection and identification of *Phytophthora* species. *Australasian Plant Pathology* 35, 147–159.

Edwards K., C. Johnstone and C. Thompson, 1991 A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* 19, 1349.

EFSA, 2016. Update of a database of host plants of *Xylella fastidiosa*: 20 November 2015. EFSA Journal 14, 4378.

EPPO, 2016. PM 7/24 (2) *Xylella fastidiosa*. *EPPO Bulletin* 46, 463–500.

Elbeaino R., F. Valentini, R. Abou Kubaa, P. Moubarak, T. Yaseen and M. Digiaro, 2014. Multilocus sequence typing of *Xylella fastidiosa* isolated from olive affected by "olive quick decline syndrome" in Italy. *Phytopathologia Mediterranea* 53, 533–542.

Frisullo S., I. Camele, G.E. Agosteo, D. Boscia and G.P. Martelli, 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. *Journal of Plant Pathology* 96, 441–449.

Fritschi F.B., H. Lin and M.A. Walker, 2007. *Xylella fastidiosa* population dynamics in grapevine genotypes differing in susceptibility to Pierce's disease. *American Journal of Enology and Viticulture* 58, 326–332.

Fu S., D. Arráez-Roman, A. Segura-Carretero, J.A. Menéndez, M.P. Menéndez-Gutiérrez, V. Micol, A. Fernández-Gutiérrez, 2010. Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells. *Analytical and Bioanalytical Chemistry* 397, 643–654.

Garrido C., M. Carbú, F.J. Fernández-Acero, N. Boonham, A. Colyer, J.M. Cantoral, G. Budge, 2009. Development of protocols for detection of *Colletotrichum acutatum* and monitoring of strawberry anthracnose using qPCR. *Plant Pathology* 58, 43–51.

Giampetruzzi A., M. Morelli, M. Saponari, G. Loconsole, M. Chiumenti, D. Boscia, V.N. Savino, G.P. Martelli and P. Saldarelli, 2016. Transcriptome profiling of two olive cultivars in response to infection by the CoDiRO strain of *Xylella fastidiosa* subsp. *pauca*. BMC Genomics 1, 475

Goodwin P.H. and C.P. Meredith, 1988. New clues in understanding Pierce's disease. *California Agriculture* 42, 6–7.

Guan W., J. Shao, T. Elbeaino, R.E. Davis, T. Zhao and Q. Huang, 2015. Infecting and Italian Olive-Associated Strains of *Xylella fastidiosa* by Polymerase Chain Reaction. *PLoS ONE* 10:e0129330.

Gutha L.R., L.F. Casassa, J.F. Harbertson and R.A. Naidu, 2010. Modulation of flavonoid biosynthetic pathway genes and anthocyanins due to virus infection in grapevine (*Vitis vinifera* L.) leaves. *BMC Plant Biology* 10, 187.

Haelterman R.M., P.A. Tolocka, M.E. Roca, F.A. Guzmán, F.D. Fernández and M.L. Otero, 2015. First presumptive diagnosis of *Xylella fastidiosa* causing olive scorch in Argentina. *Journal of Plant Pathology* 97, 393.

Hammerschmidt R., 2004. The metabolic fate of resveratrol: key to resistance in grape? *Physiological and Molecular Plant Pathology* 65, 269–270.

Harper S.J., L.I. Ward and G.R.G. Clover, 2010. Development of LAMP and QPCR Methods for the Rapid Detection of *Xylella fastidiosa* for Quarantine and Field Applications. *Phytopathology* 100, 1282–1288.

Janse J.D. and A. Obradovic, 2010. *Xylella fastidiosa*. Its biology, diagnosis, control and risks. *Journal of Plant Pathology* 92, 35–48.

Krivanek A.F. and M.A. Walker, 2005. *Vitis* resistance to Pierce's disease is characterized by differential *Xylella fastidiosa* populations in stems and leaves. *Phytopathology* 95, 44–52.

Krugner R., M.W. Johnson and J. Chen, 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

Liu C., 2001. Quinic Acid-Mediated Induction of Hypovirulence and a Hypovirulence-Associated Double-Stranded RNA in *Rhizoctonia solani*. Ph.D. Thesis, The University of Maine. http://digitalcommons.library.umaine.edu/cgi/viewcontent.cgi?article=1337&context=etd

Livak K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25, 402–408.

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.

Maddox C.E., L.M. Laur and L. Tian, 2010. Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa*. *Current Microbiology* 60, 53–58.

Markakis E.A., S.E. Tjamos, P.P. Antoniou, P.A. Roussos, E.J. Paplomatas and E.C. Tjamos, 2010. Phenolic responses of resistant and susceptible olive cultivars induced by defoliating and nondefoliating *Verticillium dahliae* pathotypes. *Plant Disease* 94, 1156–1162.

Martelli G.P., 2016. The current status of the quick decline syndrome of olive in Southern Italy. *Phytoparasitica* 44, 1–10.

Martín M.T., R. Cobos, L. Martín and L. López-Enríquez, 2012. QPCR Detection of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*. *Applied and Environmental Microbiology* 78, 3985–3991.

Martín M.T., M.J. Cuesta and L. Martín, 2014. Development of SCAR Primers for PCR Assay to Detect *Diplodia seriata*.

*International Scholarly Research Notices*, 824106

Medina M.B., 2011. Determination of the total phenolics in juices and superfruits by a novel chemical method. *Journal of Functional Foods* 3, 79–87.

Medina E., C. Romero, B. de Los Santos, A. de Castro, A. Garcia, F. Romero and M. Brenes, 2011. Antimicrobial activity of olive solutions from stored Alpeorujo against plant pathogenic microorganisms. *Journal of Agricultural and Food Chemistry* 59, 6927–6932.

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.

Nunney L.R., B. Ortiz, S.A. Russell, R.R. Sánchez and R. Stouthamer, 2014. The complex biogeography of the plant pathogen *Xylella fastidiosa*: genetic evidence of introductions and subspecific introgression in Central America. *PLoS ONE* 9:e112463.

Ortega-García F. and J. Peragón, 2009. The response of phenylalanine ammonia-lyase, polyphenol oxidase and phenols to cold stress in the olive tree (*Olea europaea* L. cv. Picual). *Journal of the Science of Food and Agriculture* 89, 1565–1573.

Petridis A., I. Therios, G. Samouris and C. Tananaki, 2012a. Salinity-induced changes in phenolic compounds in leaves and roots of four olive cultivars (*Olea europaea* L.) and their relationship to antioxidant activity. *Environmental and Experimental Botany* 79, 37–43.

Petridis A., I. Therios, G. Samouris, S. Koundouras and A. Giannakoula, 2012b. Effect of water deficit on leaf phenolic composition, gas exchange, oxidative damage and antioxidant activity of four Greek olive (*Olea europaea* L.) cultivars. *Plant Physiology and Biochemistry* 60, 1–11.

Quirantes-Piné R., J. Lozano-Sánchez, M. Herrero, E. Ibáñez, A. Segura-Carretero and A. Fernández-Gutiérrez, 2013. HPLC-ESI-QTOF-MS as a powerful analytical tool for characterising phenolic compounds in olive-leaf extracts. *Phytochemical Analysis* 24, 213–223.

Ribeiro A.B., P.V. Abdelnur, C.F. Garcia, A. Belini, V.G. Pasqualotto Severino, M.F.D.G.F. Da Silva, J.B. Fernandes, P.C. Vieira, S.A. De Carvalho, A.A. De Souza and M.A. Machado, 2008. Chemical characterization of *Citrus sinensis* grafted on *C. limonia* and the effect of some isolated compounds on the growth of *Xylella fastidiosa*. *Journal of Agricultural and Food Chemistry* 56, 7815–7822.

Rodrigues J.L.M., M.E. Silva-Stenico, J.E. Gomes, J.R.S. Lopes and S.M. Tsai, 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.

Romanazzi G., S. Murolo, L. Pizzichini and S. Nardi, 2009. Esca in young and mature vineyards, and molecular diagnosis of the associated fungi. *European Journal of Plant Pathology* 125, 277–290.

Rusjan D., R. Veberič and M. Mikulič-Petkovšek, 2012. The response of phenolic compounds in grapes of the variety 'Chardonnay' (*Vitis vinifera* L.) to the infection by phytoplasma Bois noir. *European Journal of Plant Pathology* 133, 965–974.

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, 668.

Saponari M., G. Loconsole, D. Cornara, R.K. Yokomi, A.D. Stradis, D. Boscia, D. Bosco, G.P. Martelli, R. Krugner and F. Porcelli, 2014. Infectivity and transmission of *Xylella fastidiosa* by *Philaenus spumarius* (Hemiptera: Aphrophoridae) in Apulia, Italy. *Journal of Economic Entomology* 107, 1316–1319.

Saponari M., D. Boscia, G. Altamura, G. D'Attoma, V. Cavalieri, S. Zicca, M. Morelli, D. Tavano, G. Loconsole, L. Susca, O. Potere, V. Savino, G.P. Martelli, F. Palmisano, C. Dongiovanni, A. Saponari, G. Fumarola and M. Di Carolo, 2016. Pilot project on *Xylella fastidiosa* to reduce risk assessment uncertainties. *EFSA Supporting Publications* 13, 3.

Schneider U.V., N.D. Mikkelsen, A. Lindqvist, L.M. Okkels, N. Johnk and G. Lisby, 2012. Improved efficiency and robustness in qPCR and multiplex end-point PCR by twisted intercalating nucleic acid modified primers. *PLoS ONE* 7:e38451.

Shi X.Y., J.L. Bi, J.G. Morse, N.C. Toscano and D.A. Cooksey, 2013. Effect of xylem fluid from susceptible and resistant grapevines on developmental biology of *Xylella fastidiosa*. *European Journal of Plant Pathology* 135, 127–135.

Sousa A., I.C.F.R. Ferreira, R. Calhelha, P.B. Andrade, P. Valentão, R. Seabra, L. Estevinho, A. Bentoa and J.A. Pereira, 2006. Phenolics and antimicrobial activity of traditional stoned table olives 'alcaparra'. *Bioorganic & Medicinal Chemistry* 14, 8533–8538.

Sun Q., L.C. Greve and J.M. Labavitch, 2011. Polysaccharide compositions of intervessel pit membranes contribute to Pierce's disease resistance of grapevines. *Plant Physiology* 155, 1976.

Taamalli A., D. Arráez-Román, E. Ibañez, M. Zarrouk, A. Segura-Carretero and A. Fernández-Gutiérrez, 2012. Optimization of microwave-assisted extraction for the characterization of olive leaf phenolic compounds by using HPLC-ESI-TOF-MS/IT-MS(2). *Journal of Agricultural and Food Chemistry* 60, 791–798.

Taamalli A., L. Abaza, D. Arráez Román, A. Segura Carretero, A. Fernández Gutiérrez, M. Zarrouk and B.Y. Nabil, 2013. Characterisation of phenolic compounds by HPLC-TOF/IT/MS in buds and open flowers of 'Chemlali' olive cultivar. *Phytochemical Analysis* 24, 504–512.

Talhaoui N., A.M. Gómez-Caravaca, L. León, R. de la Rosa, A. Segura-Carretero, A. Fernández-Gutiérrez, 2014. Determination of phenolic compounds of 'Sikitita' olive leaves by HPLC-DAD-TOF-MS. Comparison with its parents 'Arbequina' and 'Picual' olive leaves. *LWT Food Science and Technology* 58, 28–34.

Wallis C.M. and J. Chen, 2012. Grapevine phenolic compounds in xylem sap and tissues are significantly altered during infection by *Xylella fastidiosa*. *Phytopathology* 102, 816–826.

Wallis C.M., A.K. Wallingford and J. Chen, 2013a. Effects of cultivar, phenology, and *Xylella fastidiosa* infection on grapevine xylem sap and tissue phenolic content. *Physiological and Molecular Plant Pathology* 84, 28–35.

Wallis C.M., A.K. Wallingford and J. Chen, 2013b. Grapevine rootstock effects on scion sap phenolic levels, resistance

to *Xylella fastidiosa* infection, and progression of Pierce's disease. *Frontiers in Plant Science* 4, 502.

Wong F., D.A. Cooksey and H.S. Costa, 2004. Documentation and characterization of *Xylella fastidiosa* strains in landscape hosts. In: *California Department of Food and Agriculture Symposium Proceedings, Pierce's Disease Research*, December 7-10, 2004, Coronado, CA, USA, pp. 238–241.

Yangui T., S. Sayadi, A. Rhouma and A. Dhouib, 2010a. Potential use of hydroxytyrosol-rich extract from olive mill wastewater as a biological fungicide against *Botrytis cinerea* in tomato. *Journal of Pest Science* 83, 437–445.

Yangui T., S. Sayadi, A. Gargoubi and A. Dhouib, 2010b. Fungicidal effect of hydroxytyrosol-rich preparations from olive mill wastewater against *Verticillium dahliae*. Crop Protection 29, 1208–1213.

Zine El Aabidine A., Y. Baissac, A. Moukhli, C. Jay-Allemand, B. Khadari and C. El Modafar, 2010. Resistance of olive-tree to *Spilocaea oleagina* is mediated by the synthesis of phenolic compounds. *International Journal of Agriculture and Biology* 12, 61–67.

*Accepted for publication: 22 August 2017*