Accumulation of Azelaic Acid in *Xylella fastidiosa*-Infected Olive Trees: A Mobile Metabolite for Health Screening

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ABSTRACT

Monitoring *Xylella fastidiosa* is critical for eradicating or at least containing this harmful pathogen. New low-cost and rapid methods for early detection capability are very much needed. Metabolomics may play a key role in diagnosis; in fact, mobile metabolites could avoid errors in sampling due to erratically distributed pathogens. Of the various different mobile signals, we studied dicarboxylic azelaic acid (AzA) which is a key molecule for biotic stress plant response but has not yet been associated with pathogens in olive trees. We found that infected *Olea europaea* L. plants of cultivars Cellina di Nardò (susceptible to *X. fastidiosa*) and Leccino (resistant to the pathogen) showed an increase in AzA accumulation in leaf petioles and in sprigs by approximately seven- and sixfold, respectively, compared with plants negative to *X. fastidiosa* or affected by other pathogens. No statistically significant variation was found between the *X. fastidiosa* population level and the amount of AzA in either of the plant tissues, suggesting that AzA accumulation was almost independent of the amount of pathogen in the sample. Furthermore, the association of AzA with *X. fastidiosa* seemed to be reliable for samples judged

as potentially false-negative by quantitative polymerase chain reaction (cycle threshold [C_t] > 33), considering both the absolute value of AzA concentration and the values normalized on negative samples, which diverged significantly from control plants. The accumulation of AzA in infected plants was partially supported by the differential expression of two genes (named *OeLTP1* and *OeLTP2*) encoding lipid transport proteins (LTPs), which shared a specific domain with the LTPs involved in AzA activity in systemic acquired resistance in other plant species. The expression level of *OeLTP1* and *OeLTP2* in petiole samples showed significant upregulation in samples positive to *X. fastidiosa* of both cultivars, with higher expression levels in positive samples of Cellina di Nardò compared with Leccino, whereas the two transcripts had a low expression level ($C_t > 40$) in negative samples of the susceptible cultivar. Although the results derived from the quantification of AzA cannot confirm the presence of the erratically distributed *X. fastidiosa*, which can be definitively assessed by traditional methods, we believe they represent a fast and cheap screening method for large-scale monitoring.

The spread of *Xylella fastidiosa* is reaching a worldwide scale, with a worrisome distribution in Europe (Almeida 2016; Stokstad 2015). In order to eradicate or at least contain this pathogen, monitoring is essential (Parnell et al. 2012) and involves finding novel methods for early detection (Zarco-Tejada et al. 2018), which are low cost and easy to execute (Chiriaco et al. 2018). In addition to the crucial role of traditional diagnostic tests for the verification of *X. fastidiosa* (i.e., molecular or immunoenzymatic tests), promising methods such as the analysis of volatile organic compounds are being developed to detect the disease before that it reaches the levels that can be detected by traditional methods. Such a new approach could be achieved by identifying the early plant–pathogen interaction mechanisms which can be highlighted by the analysis of biomarkers that are induced or altered in the host such as transcripts, proteins, and volatiles (Martinelli et al. 2015).

In addition to using biochemical markers as an early detection tool, such a strategy is low cost and rapidly applied. This is particularly useful for pathogens that require large-scale monitoring such as *X. fastidiosa* subsp. *pauca* strain CoDiRO (derived from the Italian name of the disease, “complesso del disseccamento rapido dell’olivo”), a bacterium associated with the olive quick decline syndrome (OQDS). The De Donno strain, derived from ST53 isolates retrieved from OQDS-affected plants, was recently sequenced (Giampetruzzi et al. 2017). This serious plant disease is destroying olive crops in Salento (Apulia, southern Italy) but also affects many

other species (Luvisi et al. 2017b; Martelli 2016; Saponari et al. 2013), as well as olive trees in Brazil (Coletta-Filho et al. 2016, 2017).

The identification of a resistant olive cultivar (i.e., Leccino) would provide a more useful resource than the susceptible ones commonly grown in Salento (i.e., Cellina di Nardò) (Martelli 2016; Giampetruzzi et al. 2016; Saponari et al. 2017). Transcriptome profiling indicates differences in gene expression according to cultivar and health status (Giampetruzzi et al. 2016), and metabolic profiling (Luvisi et al. 2017a; Sabella et al. 2018) suggests the potential role of biochemicals in host–pathogen interactions or their use as biomarkers.

New developments in metabolomics are promising to elucidate the complex mechanism of plant–biotic interactions (Tenenboim and Brotman 2016), which may play a role for diagnostic purposes; in particular, whether they are related to mobile metabolites that could overcome some limits of traditional tests (i.e., the sampling of infected material due to erratically distributed pathogens).

Because plants lack mobility and, thus, are not able to avoid exposure to environmental stresses, they have evolved both constitutive and inducible means to respond to many abiotic and biotic stresses. For example, the remodeling of membrane fluidity and some of its components, such as fatty acids (FAs), contributes to inducible stress resistance (Upchurch 2008) and could be used for the early detection of pathogens (Walley et al. 2013). In fact, the enzymatic and nonenzymatic cleavage of FAs within a plant produces a pool of chemicals that can provide specific information about the source of stress—which is generally biotic—that the plant is encountering (Zoeller et al. 2012). One of these chemicals is azelaic acid (AzA), a dicarboxylic acid, generated by the hydrolysis of C18 FAs oleic acid (18:1) or its unsaturated derivatives, linoleic acid (18:2) and linolenic acid (18:3), which carry a double bond at carbon 9 whose biosynthesis pathway is largely unknown (Shine et al. in press; Yu et al. 2013). AzA is a mobile metabolite of the

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

plant that confers local and systemic acquired resistance (SAR). It is also a signal against infection due to the pathogenic attack (Jung et al. 2009). Like many of the chemical SAR inducers, AzA requires a nonspecific lipid transfer protein (LTP) for its activity (Shine et al. in press). Because these FAs are essential for plant survival and they function redundantly in AzA biosynthesis, it is difficult to understand which plants do not contain AzA; however, its amount increases enormously as a result of the attack by pathogens (Ádám et al. 2018; Camañes et al. 2015; Nagy et al. 2017).

Because curative methods for the control of *X. fastidiosa* are not yet available, being able to identify the secondary metabolites involved in plant disease resistance and tolerance (Maddox et al. 2010) along with the mobile biochemical markers would be extremely useful as possible strategies for disease monitoring. However, to our knowledge, although AzA has been described in olive oil (Elkacmi et al. 2016; Godard et al. 2013), it has not been associated with the health status of *Olea europaea* L., regardless of the presence of other pathogens. Observations of the phenolic fraction of extra virgin olive oil produced from olive groves infected by *X. fastidiosa* indicated the presence of a significant amount of the metabolite AzA (data unpublished), suggesting further investigations in plant tissues infected by the pathogen. Thus, to better understand the interactions between the erratically distributed *X. fastidiosa* and olive trees and how they can affect a mobile metabolite such as AzA in naturally infected conditions, we investigated the presence of this chemical in petioles or sprigs of two cultivars previously defined as resistant or susceptible to *X. fastidiosa* (Leccino and Cellina di Nardò, respectively) (Saponari et al. 2017). Furthermore, two putative LTP genes that have been described for *O. europaea* var. *sylvestris* (GenBank accession numbers XM_023037740.1 and XM_023009062.1), likely related to the AzA activity, were isolated and their expression was evaluated.

MATERIALS AND METHODS

Plant material and experimental design. In order to limit the potential effects of plant growth or nutrient or water stress in the host–pathogen interaction, sampling was carried out during winter in orchards located in Lecce (Apulia, Southern Italy), on olive trees of Cellina di Nardò and Leccino. Selected plants (25 to 35 years old) had received the same agronomic practices (including insect control) over the previous 3 years, and phytosanitary treatments had been carried out by the farmers according to EU Decision 2015/789.

In order to avoid possible effects of different levels of water availability on the phenolic content among trees (Alagna et al. 2012; Machado et al. 2013), tree water status was assessed by Ψ_w measured 2 weeks before sampling, using a pressure chamber (Servili et al. 2007). No additional irrigation was considered necessary.

We evaluated the presence and the accumulation of AzA in petioles and sprigs (1 year old) in relation to (i) cultivar and health status of the plants (in terms of being positive or negative to *X. fastidiosa* or other pathogens) and (ii) level of infection of *X. fastidiosa*. A further trial was carried out to estimate the reliability of AzA as a biochemical marker. To evaluate AzA in relation to the cultivar and the health status, samples were collected from *X. fastidiosa*-positive trees (Xf-p, 10 naturally infected plants/cultivar); *X. fastidiosa*-negative trees, (Xf-n, 10 plants/cultivar); and *X. fastidiosa*-negative and pathogen-controlled trees (Xf-pc, 10 plants/cultivar), in which no symptoms due to other pathogens were observed (asymptomatic plants) and plants were negative to specific tests. The presence of *X. fastidiosa* (Xf-p) was assessed by real-time polymerase chain reaction (PCR) (Harper et al. 2010).

In both Xf-p and Xf-n samples, plants were monitored and tested for some of the most common pathogens in addition to *X. fastidiosa*, checking for symptoms caused by natural infection of *Colletotrichum gloeosporioides*, *Mycocentrospora cladosporioides*, *Spillocaea oleagina*, and *Pseudomonas savastanoi* pv. *savastanoi*. Symptoms of each pathogen were observed for each Xf-p and Xf-n

tree during the 12 months before sampling. The presence of symptoms was scored using a severity scale of 1 to 3, where 0 = symptomless, 1 = symptoms on a few branches (≤ 5), 2 = symptoms on several branches (> 5), and 3 = symptoms uniformly distributed throughout the canopy. Plants were selected according to higher severity (3) for at least two pathogens (most frequently, *S. oleagina* and *P. savastanoi* pv. *savastanoi*).

Diagnostic tests (real-time PCR) were carried out on leaves or woody chips, according to protocols reported in the literature, for *Verticillium dahliae* (Bilodeau et al. 2012); *Colletotrichum* spp., *C. acutatum*, and *C. gloeosporioides* (Garrido et al. 2009); *Phaeoconiella chlamydospora* (Martín et al. 2012); *Phaeoacremonium aleophilum* and *P. parasiticum* (Aroca et al. 2008; Carlucci et al. 2015); *Botryosphaeria dothidea* (Romanazzi et al. 2009); *Diplodia seriata* (Martín et al. 2014); and *Phytophthora* spp. (Drenth et al. 2006) but Xf-p and Xf-n trees were negative to every test, apart from *C. gloeosporioides*. Thus, we concluded that Xf-p and Xf-n trees mainly differ in terms of the presence or absence *X. fastidiosa*. Conversely, Xf-pc trees, in addition to not showing symptoms related to pathogens, were negative to all real-time PCR tests.

To evaluate the accumulation of AzA in relation to the infection levels, Xf-p samples were assayed by real-time PCR and the relative cycle threshold (C_t) was registered. The samples of both tissues were divided into five infection levels (five C_t ranges: 22 to 24, 25 to 27, 28 to 30, 31 to 33, and > 33 ; five trees per each infection level). The relative amount of AzA was calculated for samples belonging to each level.

To estimate the reliability of AzA as a biochemical marker of infection, two further tests were carried out. The first test aimed to estimate the sensitivity of AzA in samples collected from trees with a low level of infection which can lead to false-negative results ($C_t > 33$). Xf-p and Xf-n samples (leaves) were collected in spring from three different plants of Leccino (Xf-p 1, 2, and 3 and Xf-n 1, 2, and 3), a resistant cultivar that may have a negative impact on diagnostic sensitivity due to the erratic distribution of *X. fastidiosa*. Each Xf-p sample was divided in two subsamples for AzA quantification and real-time PCR assay. Three biological replications were carried out (tests 1, 2, and 3). For each test and Xf-p sample, the ratio (R) of AzA accumulation was calculated as the concentration of AzA in Xf-p_(n) (expressed as micrograms per gram dry weight) over the concentration of AzA in Xf-n (mean data obtained from three Xf-n samples). A second test estimated the robustness of AzA analysis, calculating R in Xf-p samples (leaves) collected from plants grown with different water management (summer sampling on the Adriatic coast within irrigated or nonirrigated orchards), in geographic areas (summer sampling in nonirrigated orchards on the Adriatic coast or Ionic coast), or sampled in different seasons (winter or summer sampling in nonirrigated orchards on the Adriatic coast). For each factor of variability, AzA accumulation was quantified in five Xf-p plants (chosen at random within the same orchard), calculating R regarding AzA concentration in irrigated Xf-n (mean data obtained from three Xf-n samples) (R_W) or nonirrigated Xf-n (R_{NW}).

DNA extraction and real-time PCR. In total, 25 leaves were collected from six branches per plant in order to verify the presence of *X. fastidiosa* in olive tree samples. Petioles and basal portions of leaf blades from each sample were cut with a sterile scalpel. Plant tissue from each sample (approximately 1.0 g of leaf petioles) was transferred into an extraction bag (BIOREBA) and 4 ml of extraction buffer (0.2 M Tris-HCl [pH 9], 0.4 M LiCl, and 25 mM EDTA) was added. Samples were homogenized using a semiautomatic homogenizer (Homex 6; BIOREBA) at 50% maximum speed. DNA was extracted according to Edwards et al. (1991), with some modifications, where the DNA solution was first extracted with a phenol-chloroform-isoamyl alcohol (25:24:1) mixture to remove protein contaminants, then precipitated with 100% isopropanol.

The isolated DNA was used as a template for *X. fastidiosa* detection by the TaqMan real-time PCR protocol with XF-F/R primers and an XF-P probe (Harper et al. 2010). Reactions were performed in a real-time thermal cycler (ABI PRISM 7900HT, Applied Biosystems).

Each reaction consisted of 5 μl from a 20-ng μl^{-1} dilution of DNA extracted from leaf petioles, 12.5 μl of Master Mix (Applied Biosystems), 400 nM forward and reverse primers, 200 nM TaqMan probe, and ultrapure DNase/RNase-free water (Carlo Erba Reagents) in a total volume of 25 μl . The following cycling conditions were implemented: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The final dissociation was at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s.

AzA extraction and quantification. Approximately 0.30 g of leaf petioles and relative sprigs was transferred into an extraction bag (BIOREBA) and 5 ml of extraction buffer, pH 2.8 (methanol-water-formic acid, 60:39.9:0.1) was added. A semiautomatic homogenizer (Homex 6) was used at 50% maximum speed. The extracts were centrifuged at $5,000 \times g$ for 3 min, filtered through a 0.20- μm syringe filter, and then analyzed. AzA was characterized using an Agilent 1200 liquid chromatography system (Agilent Technologies) equipped with a standard autosampler (Sabella et al. 2018). The high-performance liquid chromatography (HPLC) column was an Agilent Extend-C18 (1.8 μm , 2.1 by 50 mm). Separation was carried out at 40°C with a gradient elution program at a flow rate of 0.5 ml/min. The mobile phases used were water plus 0.1% formic acid (A) and acetonitrile (B). We used a multistep linear gradient: 0 min, 1% B; 13 min, 25% B; 19 min, 40% B; and 21 min, 90% B. The initial conditions were maintained for 5 min. The HPLC system was coupled to an Agilent 6320 time-of-flight (TOF) mass spectrometer, equipped with a dual electrospray ionization (ESI) interface (Agilent Technologies) operating in negative ion mode using a capillary voltage of +3.5 kV. The other optimum values of the ESI-TOF-mass spectrometry (MS) parameters were drying gas temperature, (N_2), 300°C; drying gas flow, 12 liters/min; and nebulizing gas pressure, 40 psig. A mass range of 50 to 1,700 m/z was used for detection. The total ion chromatograms of each peak were measured with an ISO Pump (Agilent G1310B) using a dual nebulizer ESI source that introduces a low flow (20 $\mu\text{l} \cdot \text{min}^{-1}$) of a calibration solution. This solution contained the internal reference masses at m/z 112.9856, 301.9981, 601.9790, and 1,033.9881, in negative-ion mode. Mass Hunter (Agilent Technologies) was used to process the mass data of the molecular ions.

A standard calibration graph for the quantification of the AzA of olive leaves and sprigs was prepared using a commercial standard (AzA, 98%; ACROS). The standard was dissolved in a mixture of methanol and water (60:40, vol/vol) and diluted in water plus 0.1% formic acid. The recovery was used to evaluate the accuracy of the method. Known amounts of standard were added to 0.30 g of the samples. The method described above was then used again for extracting and analyzing the mixture.

The tests were carried out in three replicates. Recovery (Rec) was calculated as $\text{Rec} = 100 (C_{\text{found}} - C_{\text{initial}}) / C_{\text{added}}$ for each compound, where C_{found} = measured concentration in the fortified sample, C_{initial} = initial concentration in the sample, and C_{added} = concentration in the standard solution used. An amount of standard that was five times higher than the endogenous concentration was added to the milled fresh leaves and sprig methanol solution in order to assess the analytical recoveries. Data were presented as analytical recovery (percent).

To reveal any variability in the extraction method, the procedure was repeated four times on the same pool of leaves and sprigs, with no statistically relevant difference being found among replicates.

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 the signal-to-noise ratio of 3:1. Intraday and interday precision was performed to assess the repeatability of the method, expressed by the relative standard deviation (RSD). A sprig extract and olive leaf were injected ($n = 5$) on the same day (intraday precision) for three consecutive days (interday precision, $n = 15$). The amount of AZA in leaves or sprig was expressed as micrograms per gram dry weight.

RNA extraction, cDNA synthesis, and isolation of *LTP* genes from *O. europaea*. Petioles from the samples Xf-p and Xf-n, 10 for each cultivar, were collected and frozen in liquid nitrogen. Total RNA was isolated from approximately 0.1 g of frozen petioles (per sample), and was performed with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was used to synthesize cDNA using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems). To obtain full-length cDNAs coding putative LTPs in Cellina di Nardò and Leccino trees, primers were designed based on the sequences of *LTP* genes from *O. europaea* var. *sylvestris* (GenBank accession numbers XM_023037740.1 and XM_023009062.1) and were used to amplify the cDNA fragment using PCR (Supplementary Table S1). All products were cloned into the pCR4TOPO vector (Life Technologies) according to the manufacturer's instructions, and both strands were sequenced. Full-length cDNAs were obtained which shared a high homology to *O. europaea* var. *sylvestris* genes, nonspecific LTP 1-like (XM_023037740.1) and nonspecific LTP 3-like isoform X2 (XM_023009062.1), respectively.

Bioinformatic and phylogenetic analysis. The coding sequence and relative amino acid sequences of *Arabidopsis AtDIR1* and *AtLTP1* were retrieved from the *Arabidopsis* Information Resource website (<https://www.arabidopsis.org/>). The sequences of *AtDIR1* and *AtLTP1* genes were used as queries to search for homologs in the *O. europaea* in the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>), using BLAST. The predicted protein information was conducted using EXPASY (<https://www.expasy.org/resources>). Signal peptides were revealed using the SignalP 3.0 prediction server at www.cbs.dtu.dk/services/SignalP. Conserved domains were identified using Conserved Domain Database (CDD; <https://www.ncbi.nlm.nih.gov/cdd/>).

To study the phylogenetic relationship of olive tree LTPs with other plant orthologs, full-length amino acid sequences were aligned with ClustalW (<https://www.ebi.ac.uk/Tools/msa/clustalw2/>). A phylogenetic tree based on LTP family members was constructed through Molecular Evolutionary Genetics Analysis, version 7.0 (Kumar et al. 2016) using the neighbor-joining method. The analysis involved 13 amino acid sequences. All positions containing gaps and missing data were eliminated. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the JTT matrix-based method and are in units of the number of amino acid substitutions per site.

Analysis of genes expression. Real-time quantitative, reverse-transcription (RT)-PCR was performed with a Power SYBR Green RT-PCR Master mix (Applied Biosystems). Real-time PCR conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The list of primers is provided in Table 1. All PCRs were performed in triplicate. For each sample, a C_T

TABLE 1. Primers used for real-time polymerase chain reaction to evaluate the *OeLTP1* and *OeLTP2* expression in *Olea europaea* 'Cellina di Nardò' and 'Leccino'

Gene symbol	Function	Primer sequence (5' to 3')
<i>UBQ</i>	Polyubiquitin OUB2 mRNA	GGTGGCCTCTAAATGTTCTTCTACTG CACACAGACTTCATTAGAAAGACAATCA
<i>OeLTP1</i>	Nonspecific lipid transfer protein like 1	GTCCATCTGCTACTGCCTGAAA CTTGCTGTAGTTGATGCCCTTAAA
<i>OeLTP2</i>	Plant lipid transfer protein (LTP_2)	TGCTGCGCTCAGGTGAAG GAGAGCATAACAGCGCAAAGG

value was calculated from the amplification curves. The fold change in relative gene expression was determined as $FC = 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_t \text{ target gene} - C_t \text{ reference gene}) \text{ infected sample} - (C_t \text{ target gene} - C_t \text{ reference gene}) \text{ healthy sample}$.

The C_t data are expressed as an average of the 10 samples. Amplifications that do not show any amplification curve within 40 cycles were considered out of detection range and marked as not detectable. For each gene, the relative amount of calculated mRNA was normalized to the level of the reference gene polyubiquitin (*UBQ*; AF429430). It was selected as a suitable reference gene because it was the most stable transcript in healthy and infected olive tree cultivars (Giampetruzzi et al. 2016).

Statistical analysis. The open source software R version 3.5.0 (R Foundation for Statistical Analysis) was used for all data analyses. After evaluating the normal distribution of variance among data (Shapiro's test), one-way or two-way analysis of variance (ANOVA) was performed. Pairwise comparisons (*t* test), were applied (*P* values < 0.05 were considered to be significant) to test the statistical significance of the AzA contents found among samples. Statistical significances were recorded (where *, **, and *** indicate *P* < 0.05, 0.01, and 0.001, respectively).

RESULTS

AzA identification. The AzA compound characterized by HPLC ESI-TOF is reported in Table 2, along with its retention time, *m/z* experimental and calculated, difference (ppm) between the

TABLE 2. Characterization of azelaic acid detected in olive leaf and sprig extract by HPLC ESI-TOF (ion M-H)^{-a}

Compound	RT (min)	(M-H) ⁻ Exp	<i>m/z</i> Exp	<i>m/z</i> Clc	Diff. (ppm) ^b	Score ^c
Azelaic acid	7.0734	C ₉ H ₁₅ O ₄	187.0976	187.0973	1.76	99.44

^a RT = retention time, Exp = experimental, and Clc = calculated.

^b Relative difference: the difference between the observed mass and the theoretical mass of the compound (ppm).

^c Isotopic abundance distribution match: a measure of the probability that the distribution of isotope abundance ratios calculated for the formula matches the measured data.

observed mass and the theoretical mass, isotopic abundance distribution match, and molecular formula generated by the software for the detected deprotonated molecule. Peaks were identified by comparing both migration time and MS spectral data obtained from samples with the authentic analytical standard. The calibration plot indicated a good correlation between peak area and analyte concentration and the regression coefficient was higher than 0.999. LOD had a value of 0.008 µg·ml⁻¹, while LOQ was 0.08 µg·ml⁻¹. The intraday repeatability of the peak area, expressed by RSD, was 0.85%, whereas the interday repeatability was 1.1%. The parameters of the calibration curve are summarized in Table 3.

Evaluation of accumulation of AzA according to cultivar, health status and level of infection. Amounts of AzA in *Xf-p*, *Xf-n*, and *Xf-pc* trees of Cellina di Nardò and Leccino are reported in Table 4. Two-way ANOVA showed that the AzA accumulation was influenced by the health status of the sample. Indeed, a high level of significance for “health status” was observed in both the petioles (*P* < 0.001) and sprigs (*P* < 0.001). Genotype was a factor that did not determine significant differences in the amount of AzA in either of the tissues (petioles or sprigs). Furthermore, the analysis of the health status–genotype variance was not significant to explain variations in the AzA accumulation in both tissues. Additionally, no statistically significant variation in leaf petioles or sprigs in the AzA amount was observed between either kind of negative sample (*Xf-n* and *Xf-pc*) of the two cultivars. Therefore, *X. fastidiosa* infection was associated with a statistically significant increase in AzA in both genotypes. In fact, *X. fastidiosa*-positive samples belonging to Cellina di Nardò and Leccino showed an increase of AzA in leaf petioles (approximately sevenfold) and in sprigs (approximately sixfold), regardless of the presence of other pathogens.

Subsequently, because the accumulation of AzA showed a significant variation only in relation to the PCR-confirmed health status (*Xf-p* or *Xf-n/Xf-pc*) and not to the genotype, the samples were analyzed in relation to the *X. fastidiosa* population level, evaluated by real-time PCR and relative C_t (Table 5). No statistically significant variation was found between the *X. fastidiosa* population level (expressed as C_t) and the amount of AzA in either of the plant tissues. In particular, we found a high amount of AzA even in samples collected from *Xf-p* trees that showed a C_t > 33.

TABLE 3. Parameters of calibration curve, limit of detection (LOD), limit of quantification (LOQ), relative standard deviation (RSD), and recovery for the high-performance liquid chromatography method validation of azelaic acid assay

	Slope	Intercept	<i>r</i> ²	LOD (µg ml ⁻¹)	LOQ (µg ml ⁻¹)	RSD (%)	Recovery (mean %)
Standard azelaic acid	6.46E+04	2.54E+03	0.999	0.008	0.08	0.85	85

TABLE 4. Amount of azelaic acid retrieved in leaves or sprigs of *Xylella fastidiosa*-positive or -negative samples^a

Cultivar, health status ^b	C_t	Leaf (µg g ⁻¹ dry weight)	Sprig (µg g ⁻¹ dry weight)
Cellina di Nardò			
<i>Xf-p</i>	22.8 – 29.9	13.13 ± 2.70	8.57 ± 2.65
<i>Xf-n</i>	...	1.15 ± 0.75	0.61 ± 0.59
<i>Xf-pc</i>	...	2.15 ± 0.54	2.00 ± 1.01
Leccino			
<i>Xf-p</i>	26.5 – 31.7	14.92 ± 2.93	10.52 ± 3.37
<i>Xf-n</i>	...	2.28 ± 1.78	0.92 ± 1.05
<i>Xf-pc</i>	...	1.97 ± 0.89	2.49 ± 0.60
Significance	...		
Health status	...	***	***
Genotype	...	ns	ns
Health status × genotype	...	ns	ns

^a Samples are classified according to the cultivar (Cellina di Nardò or Leccino). Mean real-time polymerase chain reaction cycle thresholds (C_t) are reported for positive samples; negative samples are classified according to the presence or absence of other pathogens. Two-way analysis of variance was carried out to evaluate statistical differences and interaction among factors (genotype and health status); *** indicates *P* < 0.001 and ns = not significant.

^b *Xf-p* = *X. fastidiosa*-positive sample, with other pathogens occurring; *Xf-n* = *X. fastidiosa*-negative sample, with other pathogens occurring; and *Xf-pc* = *X. fastidiosa*-negative sample, without other pathogens occurring.

Evaluation of AzA as a biochemical marker of infection.

In addition to the remarkable difference observed in the AzA accumulation between positive and negative *X. fastidiosa* samples, absolute values may not have taken into account that biochemical parameters can be influenced by the period of sampling and reproducibility of HPLC analysis. Thus, the evaluation of AzA accumulation was normalized, considering negative control plants as the baseline (mean data from samples collected from three *Xf*-n trees) and calculating the R of AzA to compare with standard real-time PCR values (Table 6). All computed ratios showed values >6.6, indicating that the amount of AzA in plants infected by *X. fastidiosa* was significantly higher than the concentration observed in negative plants. High ratios were also observed for two samples (test 1, *Xf*-p 1 and *Xf*-p 3), in which samples judged as being potentially false negative ($C_t > 33$) showed a ratio of 9.7 and 17.9.

With regard to tests in different growth conditions, all computed ratios of *Xf*-p showed values >4.9, regardless of the *Xf*-n chosen (irrigated or nonirrigated) (Table 7). Thus, the amount of AzA in positive samples to real-time PCR seems to be very high compared with those found in *X. fastidiosa*-negative plants, regardless of the sampling season, geographic area, or water management. Among all samples tested, the amount of AzA detected in *Xf*-n samples did not exceed 4 $\mu\text{g g}^{-1}$ dry weight, whereas the amount of AzA detected

in *Xf*-p samples was not lower than approximately 12 $\mu\text{g g}^{-1}$ dry weight in leaves. These results suggest that $R = 3$ could be considered as a threshold value to consider samples as positive to *X. fastidiosa*. However, a remarkable variation in absolute values of AzA was observed, indicating that a pluriannual test is required to assess the role of factors involved in accumulation of AzA and to define a reliable threshold value.

Identification of LTP genes in *O. europaea*. To identify *O. europaea* gene AZI from the *Arabidopsis* AzA-induced 1 (AZI1) gene sequence, a search in NCBI databases was carried out, with no result. Subsequently, degenerate primers were designed on the conservative sequences of known plant AZI genes for RT-PCR analysis but no cDNA for the *Olea* AZI gene was found. Therefore, the expression of *Olea* LTP genes belonging to the same superfamily of AZI1 and defective in induced resistance (*DIR1*) was analyzed for their involvement in the transport in a mobile signal such as AzA (Safi et al. 2015).

Two genes coding LTP were isolated in leaf petioles of Leccino and Cellina di Nardò. The genes were designated as *OeLTP1* and *OeLTP2*. They had significant homology with *LTP1* and *LTP2* genes retrieved in other plant species (*Arabidopsis thaliana*, *Capsicum annuum*, *Hordeum vulgare*, *Nicotiana tabacum*, and *Oryza sativa*). The *OeLTP1* nucleotide sequence (GenBank accession number MH594116) is composed by 489 nucleotides (nt), whereas the *OeLTP2* nucleotide sequence (GenBank accession number MH594117) is 383 nt in length.

Gene expression analysis. The expression level of *OeLTP1* and *OeLTP2* was evaluated in petiole samples that were positive and negative to *X. fastidiosa* in both cultivars (Table 8). In Leccino, a downregulation of both genes was observed in the *X. fastidiosa*-negative samples. In particular, *OeLTP1* in *Xf*-p samples showed a higher level of expression ($C_t = 26.3$) compared with *Xf*-n ($C_t = 27.8$), corresponding to a fold change of 6.52 ± 0.01 . Also, the level of expression of *OeLTP2* was higher in positive ($C_t = 31.8$) than negative samples ($C_t = 32.4$), with an estimated fold change of 2.82 ± 0.64 . For both genes, no expression was observed in up to 40 amplification cycles in *Xf*-n of Cellina di Nardò (Table 8). The *OeLTP1* and *OeLTP2* expression level in the positive samples was higher than in infected Leccino ($C_t = 22.5$ and $C_t = 28.7$, respectively). Thus, both transcripts in the two cultivars analyzed were differentially expressed in relation to sample health status, and a higher-level expression was associated with samples positive to *X. fastidiosa*, regardless to the presence of the most common olive tree pathogens.

Structure and biochemistry of *OeLTP1* and *OeLTP2* protein. Bioinformatic analysis showed that the *OeLTP1* and *OeLTP2* proteins are synthesized as precursors containing 26- and 27-amino-acid N-terminal signal peptides, respectively, whereas the mature proteins contained 90 residues. Conserved domains were predicted using NCBI CDD: *OeLTP1* displays a hydrophobic cavity and both proteins containing eight conservative cysteine residues forming four disulfide bonds, with the general pattern

TABLE 5. Amount of azelaic acid in leaves or sprig positive to *Xylella fastidiosa*^a

Health status	Leaf ($\mu\text{g g}^{-1}$ dry weight)	Sprig ($\mu\text{g g}^{-1}$ dry weight)
<i>Xf</i> -p, $C_t = 22-24$	12.06 $\pm$ 1.83	7.31 $\pm$ 0.60
<i>Xf</i> -p, $C_t = 25-27$	13.28 $\pm$ 2.17	7.87 $\pm$ 2.25
<i>Xf</i> -p, $C_t = 28-30$	12.77 $\pm$ 2.34	8.94 $\pm$ 2.78
<i>Xf</i> -p, $C_t = 31-33$	15.86 $\pm$ 3.00	9.50 $\pm$ 1.12
<i>Xf</i> -p, $C_t > 33$	16.66 $\pm$ 4.60	10.63 $\pm$ 1.06
Significance	ns	ns

^a Positive samples are classified according to the real-time polymerase chain reaction cycle threshold (C_t). *Xf*-p = *X. fastidiosa*-positive sample and ns = not significant.

TABLE 6. Ratio (R) of the azelaic acid (AzA) amount in the positive sample (*Xf*-p) and negative sample to *Xylella fastidiosa* (mean data obtained from three negative plants)^a

Test	<i>Xf</i> -p 1			<i>Xf</i> -p 2			<i>Xf</i> -p 3		
	AzA	R	C_t	AzA	R	C_t	AzA	R	C_t
Test 1	7.6	9.7	34.4	20.2	25.7	33.3	14.1	17.9	34.3
Test 2	4.7	6.6	32.8	24.8	35.1	26.2	18.8	26.5	26.5
Test 3	12.1	13.3	32.3	11.7	12.9	28.4	26.2	28.8	28.8

^a *X. fastidiosa*-positive (*Xf*-p) samples (leaves) were collected from three different plants (*Xf*-p 1, 2, and 3). Three tests are reported: AzA absolute value ($\mu\text{g g}^{-1}$ dry weight), R, and real-time polymerase chain reaction cycle threshold (C_t) calculated for each *Xf*-p sampled.

TABLE 7. Ratio (R) of the azelaic acid (AzA) amount in the positive sample (*Xf*-p) and negative sample (*Xf*-n) to *Xylella fastidiosa*^a

Variables	R _W	R _{NW}	AzA ($\mu\text{g g}^{-1}$ dry weight)	C_t (<i>Xf</i> -p)
Sampling season				
Winter	7.0	8.6	12.3	27.0
Summer	4.9	13.9	6.3	24.8
Geographic area				
Adriatic coast	5.5	15.6	7.0	25.8
Ionic coast	4.9	13.9	6.3	25.2
Water management				
Irrigated orchard	11.6	32.9	14.9	25.6
Nonirrigated orchard	5.1	14.5	6.6	26.0

^a Mean data were obtained from three negative plants. *Xf*-n samples belong to irrigated (R_W) or nonirrigated (R_{NW}) orchards. C_t = cycle threshold.

TABLE 8. Gene level expression of *OeLTP1* and *OeLTP2* in petiole samples of Leccino and Cellina di Nardò cultivars expressed as cycle threshold (C_t)^a

Gene	Genes level expression (C_t)			
	Leccino		Cellina di Nardò	
	<i>Xf</i> -p	<i>Xf</i> -n	<i>Xf</i> -p	<i>Xf</i> -n
<i>OeLTP1</i>	26.3 $\pm$ 0.6	27.8 $\pm$ 0.4	22.5 $\pm$ 2.7	n.d. (>40)
<i>OeLTP2</i>	31.8 $\pm$ 0.6	32.4 $\pm$ 0.3	28.7 $\pm$ 0.8	n.d. (>40)

^a *Xf*-p = *X. fastidiosa*-positive sample, with other pathogens occurring and *Xf*-n = *X. fastidiosa*-negative sample, with other pathogens occurring. Resulting values are given and show a distinct expression profile for *Xf*-p and *Xf*-n cDNA, respectively. Amplifications that do not show any amplification curve within 40 cycles were considered out of detection range and marked as n.d. (not detectable).

CXnCXnCCXnCxXnCXnC (Fig. 1). Protein–protein BlastP and multiple-alignment analysis showed that the deduced amino acid sequences had a high similarity to LTP sequences from other plant species, suggesting that *OeLTP1* and *OeLTP2* belong to the LTP family.

In order to determine the phylogenetic position, the two amino acid sequences, lacking the divergent signal sequence, were compared with pepper, tobacco, cotton, barley, rice, tomato, and *Arabidopsis* LTP sequences. This analysis showed two main clades: *OeLTP1* was grouped with *AtLTP1* and *GhLTP*, whereas *OeLTP2* had a common origin with *NtDIR1*, *AtDIR1*, and other LTP2 proteins (Fig. 2).

DISCUSSION

Understanding plant–pathogen interactions is critical to improving integrated management strategies of diseases and for developing diagnostic tools based on specific biochemical markers. A key role is played by lipids and their derivatives and knowledge of their functions in plant–microbe interactions has increased through advances in highly sensitive analytical technologies (Siebers et al. 2016). Several studies about *X. fastidiosa*–host plant interaction have reported a significant variation in the amount of different phenolic compounds such as hydroxytyrosol glucoside, quinic acid, caftaric acid, and lignin in xylem tissues (Luvisi et al. 2017a; Sabella et al. 2018; Wallis and Chen 2012), and some of these compounds have been correlated with resistance to infection or showed in vitro antibacterial properties (Maddox et al. 2010). Conversely, few data are available about the potential role of lipid-derivates in *X. fastidiosa*–host plant interaction, and our aim was to analyze in olive trees the correlation between the amount of one of these lipid derivatives, AzA, and a systemic pathogen such as *X. fastidiosa*. Previous studies in *Arabidopsis* have shown that AzA is a metabolite produced as a mobile molecule that signals infection due to the pathogenic attack and can also trigger systemic defenses (Kachroo and Robin 2013; Shah 2009; Yu et al. 2013).

We found a significant increase in AzA accumulation in olive trees of Cellina di Nardò and Leccino naturally infected by *X. fastidiosa* compared with *Xf*-n plants (approximately six- to sevenfold), regardless of the tested tissues (leaf and sprig) or the presence of other pathogens. Furthermore, we found no significant difference with respect to susceptible cultivar or resistant cultivar in AzA accumulation. As reported by Jung et al. (2009) and Shah (2009), AzA may prime the resistance to infection and antibacterial activity against pathogens and significant AzA increase was observed in various plant–pathogen interactions such as *Arabidopsis* infected by *Pseudomonas syringae* (approximately sixfold increase) and *Solanum lycopersicum* infected by *Botrytis cinerea* or *P. syringae* (approximately fivefold increase) (Camañes et al. 2015; Jung et al. 2009). Because *X. fastidiosa* is an aggressive pathogen with systemic distribution in olive trees, the significant increase of AzA observed in both plant tissues and its recovery even in samples

with low bacteria concentration could be explained by its role as a specific mobile signal produced after the activation of constitutive defenses during the systemic pathogen infection. To confirm this, we did not observe any correlation between the amount of AzA and level of infection, and a high amount of AzA was detected even in the samples potentially reported as being possible false negatives by real-time PCR, an event not uncommon for an erratically distributed pathogen such as *X. fastidiosa*. Furthermore, the low accumulation of AzA in *Xf*-n trees infected by other pathogens underlines the putative specific response of AzA. We speculate that the monitored pathogens (*Colletotrichum gloeosporioides*, *M. cladosporioides*, *Spilocaea oleagina*, and *P. savastanoi* pv. *savastanoi*), even if uniformly distributed throughout the canopy, did not significantly trigger the production of AzA, probably because they are not systemically distributed in the whole plant and do not cause a severe stress condition such as *X. fastidiosa* infection.

Tests carried out in different seasons or geographical areas or under different water management conditions suggested how AzA concentration may vary, although its amount was remarkably higher in infected plants compared with healthy ones. Thus, the AzA accumulation, expressed as an absolute value or as a ratio, seems to be almost independent of the amount of pathogen in the sample,

Fig. 2. Phylogenetic tree of *OeLTP1* and *OeLTP2* with other plants' characterized lipid transport protein (LTP). Sequence alignment was performed with ClustalW. The tree was constructed using the neighbor-joining method. Branches corresponding to partitions reproduced in <50% of bootstrap replicates are collapsed. Percentages of replicates trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Evolutionary distances were computed using the JTT matrix-based method. All positions containing gaps and missing data were eliminated. Protein accession numbers are *AtLTP1* (AEC09547.1), *AtDIR1* (OAO90722.1), *CaLTPIII* (AF208834_1), *HvLTP* (CAA42832), *NtDIR1* (XP_016480559.1), *NtLTP*(XP_009777694.1), *GhLTP* (Q43129.1), *OsLTP1* (AAP92127.1), *OsLTP2* (AAC50030.1), *SlLTP1* (NP001234074.1), and *SlLTP2* (NP001275625.1).

Fig. 1. Analysis of the deduced amino acid sequence of A, *OeLTP1* and B, *OeLTP2*; the 8-Cys patterns are in bold. The putative extracellular secretory signals (underlined) are displayed. The hydrophobic cavity of *OeLTP1* is highlighted by the light gray box.

suggesting a good reliability of this biochemical for the association with *X. fastidiosa* infection in olive plant tissues in both leaf petioles and sprigs (Tables 5, 6, and 7). However, to estimate the effective variation of AzA concentration throughout the whole growth season of the tree or to conclude that the accumulation of AzA occurs only in presence of *X. fastidiosa*, further pluriannual tests involving more biotic and abiotic factors are needed.

We speculate that, in both cultivars, AzA could be a mobile signal of *X. fastidiosa* infection which requires an LTP for its activity, as reported for other plant species (Cecchini et al. 2015; Shine et al. in press). The two identified genes, named *OeLTP1* and *OeLTP2*, and their protein sequence analysis showed that both proteins have specific domains commonly associated with the LTP family, to which the previously described AZI1 and DIR1 belong, and which are two key components of AzA activity in the SAR pathway (Cameron et al. 2016; Cecchini et al. 2015; Champigny et al. 2013; Yu et al. 2013). SAR provides long-lasting and broad-spectrum protection at the whole-plant level against virulent pathogens (Fu and Dong 2013) and it has been reported to confer fitness under conditions of high disease pressure (Traw et al. 2007). The identifying genes code for two proteins that are both characterized by a consensus motif of eight cysteine residues commonly associated with LTPs, which can be divided into multigenic families LTP1 and LTP2. In addition, the *OeLTP1* protein presents a central hydrophobic cavity, determined by training four disulphide bridges, which can bind long fatty acid chains (Cecchini et al. 2015; Lascombe et al. 2008; Yeats and Rose 2008) (Figs. 1 and 2). *OeLTP1* is clearly classified as an *nsLTP1* type, with a high similarity to the previously described LTP type 1 sequences in pepper, tomato, cotton, and *Arabidopsis*, as confirmed by the phylogenetic analysis. On the other hand, *OeLTP2* is part of the LTP2 family and is grouped with *NtDIR1* and *AtDIR1*, a protein required for the production or transport of SAR long-distance signals in *Arabidopsis* (Cameron et al. 2016).

In our study, the expression level of *OeLTP1* and *OeLTP2* in petiole samples showed a significant upregulation in samples positive to *X. fastidiosa*, with some differences among cultivars. In particular, in the susceptible Cellina di Nardò, the two transcripts were not detectable by real-time PCR up to 40 C_t, suggesting a very low expression level, whereas C_t values were much lower in positive samples of this cultivar than those belonging to Leccino. Although there are many reports about the role of LTPs in many physiological functions, we believe that our study is the first report on the effects caused by *X. fastidiosa* (or other pathogens) to *O. europaea* LTP gene expression. Previous studies in wheat showed the regulation of LTP transcripts in response to a pathogen, suggesting a key role in the early stages of pathogenesis of LTP in relation to the transport of signaling molecules (Safi et al. 2015; Wu et al. 2014). Similarly, in *Capsicum annum*, three genes coding LTP were up regulated by pathogen infection (Jung et al. 2003).

Conclusions. There are few metabolomic studies of plant-pathogen interactions and relative AzA production. Much of what is known about the metabolic intermediates involved in plant responses to pathogens in vegetative tissues comes from studies in *Arabidopsis*. Although further trials are needed to prove the robustness of our data (i.e., artificial infections, different sampling seasons, and evaluations of other cultivars), we propose a correlation between the accumulation of AzA and *X. fastidiosa* infection in olive trees, underlined by the six- to sevenfold increase of the compound in positive plants compared with the control. The upregulation of two genes encoding LTPs also suggest this correlation. Furthermore, analyzing the microbiome of the tissues of *Xf-p* and *Xf-n* samples (for example, throughout next-generation sequencing analysis) would be useful to demonstrate that the amount of AzA in the tested samples varies exclusively in relation to the presence or absence of *X. fastidiosa*, excluding differences due to other biotic factors.

However, as with other biochemical based tests, the quantification of AzA as a diagnostic method involves an indirect

determination of the pathogen. Thus, the results derived from the quantification of AzA cannot be used to confirm the presence of *X. fastidiosa* which, on the other hand, can be definitively assessed by traditional methods. Despite this limitation, our approach represents a rapid and low-cost screening method for the large-scale monitoring of an erratically distributed pathogen.

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