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Xylella fastidiosa subsp. *pauca* on olive in Salento (Southern Italy): infected trees have low *in planta* micronutrient content

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Abstract. *Xylella fastidiosa* subsp. *pauca* is associated with the olive quick decline syndrome in Salento (Apulia region, Southern Italy). The first outbreak of the disease was noticed in the Gallipoli district, from where it subsequently reached nearby areas. To date, no specific study has verified if abnormal soil or leaf micronutrients is associated with the disease. Soil and leaf samples were taken from 23 olive farms showing symptoms of the syndrome located in Gallipoli and nearby areas. Each sample was analyzed for magnesium and micronutrients content using inductively coupled plasma atomic emission spectroscopy. Real-time PCR indicated that *X. f.* subsp. *pauca* was present in each sampled tree. There is a general lack of molybdenum in soil and low bioavailability of copper and molybdenum in tree leaves. Low content of manganese in soil was also found in some farms located in Gallipoli, Galatone and Trepuzzi. Olive trees grown in Gallipoli area also had low content of boron. Principal component analysis showed that soil and leaf samples from this area had lower micronutrient contents compared with other areas. General copper depletion in leaves was uncommon and has not been previously recorded in Italy in a large area. This could indicate that *X. f.* subsp. *pauca* infection causes a depletion of copper within olive leaves. Reduced copper content has been previously recorded in leaves infected with *X. f.* subsp. *multiplex* and for other bacterial pathogens. The role of copper in relation to *X. fastidiosa* infection is discussed.

Keywords. Olive Quick Decline Syndrome, quarantine bacteria, micronutrients, Inductively Coupled Plasma Atomic Emission Spectroscopy Analysis.

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INTRODUCTION

First evidence of a severe and spreading decline of olive trees (shoot, twig and branch dieback, and tree death) growing in Gallipoli area of the Salento peninsula, Apulia, Southern Italy) was noticed by farmers dur-

ing 2008 (Martelli *et al*., 2016). Some fungi, such as *Phoeoacremonium parasiticum*, *P. rubrigenum*, *P. aleophilum* and *P. alvesi* as well as *Phaemoniella* spp. were reported to be associated with the diseased olive trees (Nigro *et al*., 2013). DNA sequences belonging to *Xylella fastidiosa* were detected at the same time from olive trees showing symptoms of decline collected in the same area (Saponari *et al*., 2013). The area affected by the disease was estimated to be from 8000 to 10000 ha (Nigro *et al.,* 2013; Martelli *et al*., 2016). Subsequently, *X. f.* subsp. *pauca* was isolated from diseased olive specimens, and the disease was named as the "olive quick decline syndrome" (OQDS) (Cariddi *et al*., 2014; Loconsole *et al*., 2014). Once colonizing host tissues, *X. fastidiosa* can be ransmitted and spread by insect vectors (Almeida *et al*., 2005), and many wild plants are reservoirs of the pathogen (EFSA PLH Panel, 2018). After possible introduction from abroad (Marcelletti and Scortichini, 2016; Giampetruzzi *et al*., 2017) and the colonization of olive trees, vectors and wild plant species have played fundamental roles in the subsequent spread of *X. f.* subsp. *pauca* from the Gallipoli district to neighboring areas of Salento (Strona *et al*., 2017; White *et al*., 2017).

In recent years, several studies aimed at elucidate the genomic structure of *X. fastidiosa*, its origin and detection, the host range where the bacterium can survive, the role of the insect vectors and the potential distribution of *X. fastidiosa* in the Mediterranean basin (EFSA PLH Panel, 2018) were performed. However, no specific study has verified if abnormal soil or leaf availability of micronutrients is associated with the outbreak of *X. f.* subsp. *pauca* on olive in the Salento peninsula.

The area where the OQDS was first observed (i.e., Gallipoli and nearby municipalities) is characterized by distinctive lithological types. According to the geological map of Italy (i.e., leaf 214), in that area there are four types, namely QP, Q^1S-Q^1C and C^{11-7}. QP indicates the "Salento calcarenite" (i.e., chalky and sandy-chalky substrates), Q^1S and Q^1C indicates the so-called "Gallipoli formation" (i.e., Q^1S, sandy-clay and marly substrates; Q^1C, sandy-clay soils mixed with lithic calcarenite and arenite), whereas C^{11-7} is the calcareous substrates of the "Galatina dolomite" (Largaiolli *et al*., 1969). The soils of this area are also distinct from those of other areas of Apulia (Costantini *et al*., 2012). However, all the Salento and most of Apulia soils are characterized by their calcareous substrates (Costantini *et al*., 2012). The soils of Gallipoli area have good texture (no of silty or very sandy soils), and are from moderate to good depth, with presence of rock fragments, high drainage capacity and low salinity (Ancona *et al*., 2010).

Recent studies have stressed the importance of the element composition in plant tissues and the host ion changes in relation to *X. fastidiosa* virulence and the development of disease symptoms (Cobine *et al*., 2013; De La Fuente *et al*., 2013; Oliver *et al*., 2014; 2015; Navarrete and De La Fuente, 2014; 2015). Consequently, the element content and availability in the soil and in the host leaves could be linked with olive tree infection by *X. fastidiosa*. In the present study, soil and leaf content was assessed for nutrients that have basic physiological roles in plant metabolism. The putative relationships and role of nutrients with *X. f.* subsp. *pauca* infection in Salento area is also discussed.

MATERIALS AND METHODS

Sampling, and soil and leaf analyses

To verify the micronutrient content of soils and nutrient availability to olive trees, soil and leaf samples (from cv. Ogliarola salentina) were taken during summer of 2018, and analyzed. The samples were from the Gallipoli area and from other districts where *X. f.* subsp. *pauca* was found during last 5 years, and where OQDS symptoms were observed since at least 8-10 years. In these areas, the incidence of *X. f.* subsp. *pauca* is very high (Boscia *et al*., 2017). Samples were taken from 23 farms located in the following municipalities: Alezio, Gagliano del Capo, Galatone, Gallipoli, Leverano, Presicce, Sannicola, Trepuzzi (Salento, Lecce province). All farms had trees with visible symptoms putatively attributable to OQDS (leaf scorching, twig and branch dieback). The following elements were analyzed in soil and leaf tissue: magnesium (Mg), iron (Fe), zinc (Zn), copper (Cu), boron (B), manganese (Mn) and molybdenum (Mo). Leaf calcium content and soil pH were also assessed. Soil pH was measured in bi-distilled water using a suspension of 1:5 solid to liquid phase. For each farm, leaf samples (four subsamples from each tree) were taken from three olive trees, following a distribution of a triangle on the farm (Papadia *et al*., 2011) and according to the methods suggested by Sanzani *et al.* (2012). Samples were taken from part of the crown of each tree not showing any visible symptom of OQDS. In addition, for beneath each tree from which leaves were collected, four soil slice samples were taken from within the external limit of the foliage projection (Papadia *et al*., 2011). Samples were taken at 30–35 cm of depth where there was abundance of tree roots (Fernandez and Moreno, 1999), avoiding collection of rocks. This procedure was adopted to ascertain the soil and leaf composition related to the trees assessed.

All samples were placed in plastic bags and transported to the laboratory for preparation. Leaves were washed with distilled water to remove all the soil particles and then dried. Each soil (1 g) and leaf (0.5 g) subsample was analyzed separately at the University of Salento by using the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES), by following the standard procedures. Briefly, samples of known dry weight were mixed with 4 mL H_2O_2 and 6 mL HNO_3 at 180 °C for 10 min, using a microwave digestion system (Milestone START D). They were then cooled, diluted with ultrapure water to a final volume of 20 mL, filtered through Whatman No. 42 filter papers, and measured for elemental content using an inductively coupled plasma atomic emission spectrometer (ThermoScientific iCap 6000 Series). Results were expressed as $mg \cdot kg^{-1}$ dry weight (ppm).

Statistical and principal component analysis

The Shapiro-Wilk test was applied for checking the normality of the data obtained from the ICP-AES analyses, using the R statistical environment, version 3.5.1 (R Development Core Team, 2013), and correlation matrix based on Pearson's coefficient was calculated for all the measured elements using MetaboAnalyst 4.0, a web-based tool for visualization of metabolomics (Xia *et al*., 2009; Xia and Wishart, 2016). This approach was used to assess possible linear associations between the variables of the two datasets considered. Multivariate statistical analyses and graphics were obtained using SIMCA-P software SIMCA 14 software, (Sartorius Stedim Biotech, Umeå, Sweden) (Bro and Smilde, 2014). Exploratory data analyses were carried out using Principal Component Analysis (PCA), applying well-established procedures (Papadia *et al*., 2011), and used to obtain a general overview of the natural data grouping. The original dataset was rearranged in a new multivariate coordinate space where the dimensions were ordered by decreasing variance in the data. The principal components were displayed as a set of scores, which highlights clustering or outliers, and a set of loadings (p), which explain the influence of input variables on principal components. Autoscaling, also known as unit or unit variance scaling, was applied to the data (Van den Berg *et al*., 2006). The PCA models were validated using the internal cross-validation default method (7-fold), and were further evaluated with the permutation test (400 permutations) (Trygg and Wold, 2002; Triba *et al*., 2015). The quality of the models was described by R^2 and Q^2. The R^2 value is a cross validation parameter defined as the proportion of variance in the data explained by the models, while the Q^2 parameter is an internal cross validation parameter, which indicates the predictability of the model. Loadings plots were evaluated to investigate the role of the measured variables in the models (Van den Berg *et al*., 2006; Wheelock and Wheelock, 2013).

Occurrence of Xylella fastidiosa *in olive farms*

The occurrence of *X. fastidiosa* in the sampled olive tree leaves was assessed using real-time PCR (Harper *et al*., 2010) following the procedures described by Modesti *et al*. (2017) and Scortichini *et al*. (2018). For these analyses, sampled leaves were taken from branches not showing disease symptoms (i.e., leaf wilting or twig dieback).

RESULTS

Soil and leaf analyses

In the soil of the farms located in the area where the first sign of OQDS was noticed (i.e., the Gallipoli area) there were low contents of some micronutrients. In particular, according to the indicated normal mean value content in soil of each element assessed (Hodgson, 1963; Alloway, 1995; McLennan and Taylor, 1999; Kaiser *et al*., 2005; Pendias, 2010; Reimann *et al*., 2014; Noulas *et al*., 2018), manganese and molybdenum contents were low in all the farms assessed (Table 1). The contents of all the other micronutrients were within the range of the mean values. In the leaves sampled from farms of this area, copper, boron and molybdenum contents were below the minimum levels required for a normal olive tree growth (Mahler, 2000; Tittarelli *et al*., 2002; Sanzani *et al*., 2012) (Table 2). The analyses for farms located in Galatone district, an area currently showing extensive tree diebacks, also showed general low soil contents of manganese and molybdenum. In addition, there were very low amounts of copper, boron and molybdenum, and, to a lesser extent, of manganese was found into the leaves (Tables 1 and 2). All the other farms located in areas close to Gallipoli, namely Alezio and Sannicola or in districts with olive trees showing the OQDS, namely Gagliano del Capo, Leverano, Presicce, and Trepuzzi, had low amounts of molybdenum in soil and of copper in the leaves. In most of the farms, boron and molybdenum were also in low amount in soil and leaves (Tables 1 and 2).

Soil pH was close to 7.0, or greater than 7.0 or 8.0, for all farms, except for two in Galatone area that had soil pH of approx. 6.0 (Table 1). Real-time PCR showed that *X. f.* subsp. *pauca* was present in all the sampled trees.

Table 1. Magnesium and micronutrient contents, and pH values for soils sampled in Gallipoli, Galatone and other districts of Lecce province, from farms with olive quick decline syndrome and infections by *Xylella fastidiosa* subsp. *pauca*. For each farm, means (± standard deviation) are presented for 12 samples collected in three different sites.

District	Mg (%)	Fe (%)	Mn (ppm)	Cu (ppm)	Zn (ppm)	B (ppm)	Mo (ppm)	pH (H_2O)
Gallipoli 1	0.41±0.03	3.58±0.30	400.89±4.99	48.61±1.44	49.58±0.29	25.00±0.19	0.064±0.004	7.42±0.21
Gallipoli 2	0.32±0.01	1.78±0.06	262.22±3.30	19.22±0.13	34.33±0.23	16.92±0.14	0.123±0003	8.26±0.11
Gallipoli 3	0.25±0.02	1.77±0.14	269.22±3.72	41.24±0.38	26.65±0.16	16.55±0.18	0.065±0.006	7.72±0.21
Gallipoli 4	0.35±0.02	2.39±0.19	294.32±4.51	37.84±0.30	31.24±0.21	20.09±0.11	0.046±0.007	8.13±0.13
Gallipoli 5	0.32±0.02	2.14±0.12	278.12±1.86	88.16±0.75	32.23±0.15	16.36±0.08	0.065±0.006	6.81±0.22
Gallipoli 6	0.30±0.02	2.74±0.19	395.33±5.47	21.48±0.16	34.05±0.21	23.49±0.21	0.033±0.003	7.92±0.16
Gallipoli 7	0.29±0.03	2.67±0.27	275.82±4.30	30.53±0.41	36.08±0.18	19.77±0.13	0.122±0.006	7.93±0.14
Gallipoli 8	0.28±0.02	1.98±0.19	239.39±3.41	32.67±0.25	24.81±0.12	17.20±0.14	0.113±0.006	8.25±0.20
Galatone 1	0.14±0,01	1.79±0.14	141.42±1.18	54.13±0.41	20.38±0.18	12.08±0.10	0.472±0.008	6.12±0.34
Galatone 2	0.45±0.02	4.06±0.20	372.40±5.05	30.62±0.25	47.99±0.29	26.89±0.21	0.476±0.009	6.23±0.24
Galatone 3	0.42±0.03	2.48±0.16	175.43±2.21	46.00±0.51	28.51±0.15	23.94±0.11	0.569±0.002	8.63±0.19
Galatone 4	0.36±0.02	2.64±0.14	335.86±3.13	63.14±0.36	38.14±0.28	21.25±0.18	0.688±0.004	8.04±0.21
Galatone 5	0.35±0.02	2.29±0.13	221.03±2.59	21.11±0.20	37.48±0.14	16.91±0.14	0.218±0.004	8.25±0.34
Galatone 6	0.40±0.01	2.66±0.05	312.99±5.12	30.03±0.25	41.70±0.15	18.33±0.10	0.317±0.009	8.48±0.28
Galatone 7	0.35±0.02	2.07±0.17	256.48±2.15	37.60±0.20	28.21±0.13	16.67±0.13	0.511±0.004	8.41±0.33
Trepuzzi 1	0.36±0.03	1.72±0.19	601.43±66.38	86.43±0.58	32.59±0.11	22.00±0.10	0.027±0.005	8.61±0.29
Trepuzzi 2	0.33±0.02	1.49±0.11	530.67±34.91	62.70±0.27	32.96±0.07	20.01±0.18	0.031±0.006	8.72±0.19
Leverano 1	0.42±0.03	3.14±0.24	1322.25±117.87	31.49±0.13	44.73±0.19	42.29±0.35	0.178±0.009	8.28±0.09
Leverano 2	0.33±0.03	3.52±0.40	1141.06±132.51	18.58±0.06	50.02±0.15	52.65±0.39	0.024±0.011	7.95±0.10
Alezio	0.34±0.04	2.98±0.35	899.04±104.26	24.15±0.13	40.99±0.16	43.70±0.44	0.037±0.001	7.64±0.13
Sannicola	1.06±0.08	3.62±0.31	813.66±74.71	27.28±0.11	59.44±0.18	49.90±0.46	0.302±0.012	8.14±0.16
Presicce	0.29±0.02	3.38±0.32	872.89±76.77	35.61±0.22	55.87±0.23	48.74±0.24	0.081±0.009	8.10±0.21
Gagliano	0.30±0.02	3.60±0.36	1660.86±164.49	43.47±0.37	65.50±0.27	49.38±0.54	0.173±0.008	6.78±0.20

Statistical and principal component analyses

For elements measured in the soil samples, multivariate statistical analysis (PCA) and the related Pearson correlation matrix were carried out. The first two principal components used for the PCA model and explained about 60% of the total variance (R^2 = 0.39 for t[1] and 0.21 for t[2]). A specific trend of clustering among the eight groups was observed. In the t[1]/t[2] PCA score plot (Figure 1a), samples from Trepuzzi and Gallipoli districts were almost clustered at positive values of t[1], while samples collected in Leverano, Alezio, Gagliano del Capo and Presicce districts were grouped at negative values of the same t[1] component. Despite the presence of some outliers, the samples collected in the Galatone district were an homogeneous cluster, located in the upper right of the PCA score plot. In this case, the study of the variables (loading plot of Figure 1b) responsible for the class distribution along the first and the second principal components explained the arrangement of the samples in the PCA score plot of Figure 1a. In particular, Fe, Zn and Mn characterized soil samples from Leverano, Alezio, Gagliano del Capo and Presicce districts, while high contents of Mg, Ca, Mo and Cu were observed in the samples collected in the Galatone district. The cluster of samples from the Gallipoli area showed lower contents of all the measured elements. Pearson correlation matrix for soil samples (Table 3) revealed some positive and negative correlations with a high level of significance ($P < 0.001$) for some nutrients (in particular B with Mn, Zn, Fe and Ca; Mn with Zn, Fe, Ca; Zn with Fe and Ca; and Fe with Ca).

For the leaf samples, PCA gave about 55% of total explained variance (R^2 = 0.37 for t[1] and 0.18 for t[2]). This model was weakly predictive but sufficiently descriptive, showing a degree of separation among groups. Despite the different number of samples for each group, the t[1]/t[2] PCA score plot (Figure 2a) highlighted some differences among the studied samples, coming from eight different sites, especially for the first principal component t[1]. In particular, leaves samples from Leverano and Gallipoli districts were clear and homogeneous clusters, grouped, respectively, at positive and negative values of t[1]. In contrast, samples from the Galatone district were a scattered group in the t[1]/t[2] score plot. The study of the variables (loading plot of

Table 2. Calcium, magnesium and micronutrient contents in olive leaves sampled in Gallipoli, Galatone and other districts of Lecce province, from farms showing the olive quick decline syndrome and infections by *Xylella fastidiosa* subsp. *pauca*. For each farm, data show the means (± standard deviation) of 12 samples collected from three different trees. Data are referred to leaf dry weight.

District	Ca (%)	Mg (%)	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	B (ppm)	Mo (ppm)
Gallipoli 1	1.07±0.13	0.108±0.001	63.40±0.49	24.69±0.20	8.71±0.05	19.33±0.10	8.65±0.05	0.182±0.003
Gallipoli 2	0.60±0.01	0.062±0.001	49.38±0.40	17.34±0.19	5.31±0.04	19.13±0.13	9.68±0.06	0.103±0.003
Gallipoli 3	0.80±0.01	0.079±0.001	61.71±0.34	17.21±0.14	5.38±0.03	12.28±0.07	10.91±0.09	0.106±0.003
Gallipoli 4	0.96±0.06	0.106±0.008	65.46±0.38	24.02±0.22	6.40±0.05	16.04±0.11	11.02±0.09	0.113±0.004
Gallipoli 5	1.12±0.07	0.109±0.006	70.71±0.73	32.71±0.37	4.99±0.07	14.28±0.09	10.32±0.15	0.143±0.006
Gallipoli 6	1.23±0.06	0.040±0.006	109.14±1.17	35.20±0.51	4.91±0.01	15.05±0.09	9.58±0.14	0.063±0.002
Gallipoli 7	0.99±0.04	0.101±0.006	73.45±0.96	24.18±0.40	5.65±0.03	15.40±0.09	8.72±0.12	0.098±0.003
Gallipoli 8	1.00±0.05	0.096±0.005	62.01±0.68	18.49±0.28	5.22±0.02	18.30±0.14	8.40±0.11	0.091±0.003
Galatone 1	0.93±0.04	0.121±0.007	110.10±0.76	48.09±0.38	5.11±0.03	31.93±0.12	10.51±0.05	0.092±0.003
Galatone 2	0.93±0.04	0.130±0.005	72.25±0.59	17.88±0.19	6.88±0.03	32.52±0.18	11.34±0.13	0.174±0.004
Galatone 3	0.94±0.05	0.107±0.006	86.28±0.66	24.03±0.18	5.27±0.04	21.36±0.06	10.40±0.09	0.121±0.002
Galatone 4	1.02±0.07	0.104±0.007	79.24±0.60	23.17±0.15	6.29±0.07	17.75±0.07	11.67±0.11	0.267±0.005
Galatone 5	1.93±0.09	0.171±0.007	106.86±0.54	26.98±0.13	19.96±0.10	32.39±0.18	9.87±0.06	0.128±0.004
Galatone 6	1.07±0.05	0.102±0.004	65.11±0.62	23.23±0.21	13.10±0.11	26.87±0.15	22.62±0.16	0.081±0.003
Galatone 7	1.12±0.06	0.108±0.004	68.96±0.69	17.92±0.18	5.20±0.04	17.30±0.11	10.33±0.11	0.147±0.003
Trepuzzi 1	1.37±0.12	0.134±0.001	74.99±0.49	41.13±0.36	6.37±0.02	24.93±0.14	18.50±0.08	0.219±0.005
Trepuzzi 2	0.11±0.02	0.116±0.001	66.86±0.43	53.99±0.57	7.81±0.04	22.97±0.10	18.11±0.11	0.230±0.003
Leverano 1	1.55±0.09	0.172±0.013	80.35±0.49	60.45±0.45	11.76±0.04	21.30±0.07	11.87±0.05	0.223±0.005
Leverano 2	1.08±0.09	0.157±0.011	84.34±0.43	80.85±0.95	18.22±0.07	13.60±0.04	11.74±0.04	0.176±0.003
Alezio	1.15±0.05	0.123±0.005	71.21±0.68	40.20±0.40	5.80±0.04	18.33±0.11	13.34±0.17	0.406±0.072
Sannicola	1.37±0.13	0.165±0.021	72.96±0.40	39.25±0.25	4.65±0.02	20.30±0.07	15.77±0.12	0.405±0.007
Presicce	1.57±0.11	0.149±0.012	97.72±0.64	45.57±0.26	6.53±0.04	12.80±0.04	11.18±0.07	0.073±0.001
Gagliano	1.32±0.03	0.130±0.004	127.56±0.86	44.76±0.37	5.75±0.05	27.25±0.12	10.06±0.05	0.120±0.003

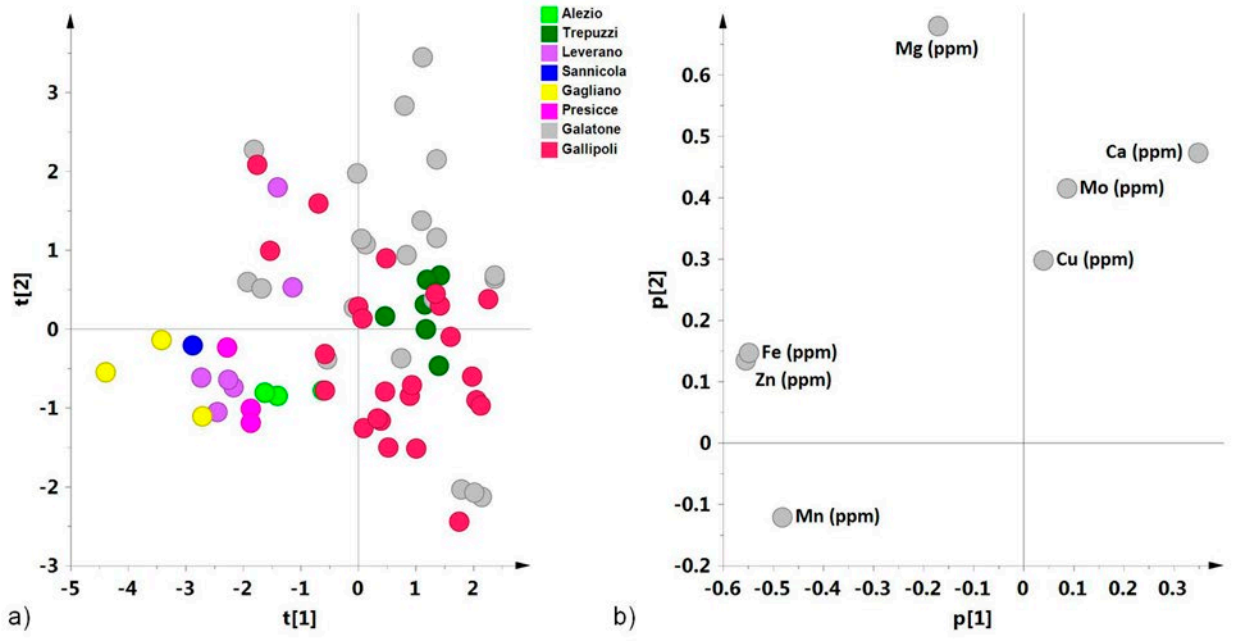

Figure 1. Principal component analysis for soil samples collected from different sites in the Salento area (Southern Apulia). t[1]/t[2] scoreplot **(a)** and corresponding p[1]/p[2] loadings plot **(b).** Two principal components (t[1]/t[2]) explained about 60% of total variance (R^2= 0.37 for t[1] and 0.18 for t[2]), with a predictability value of Q^2 = 0.097.

Table 3. Pearson correlation matrix among the variables for soil (A) and olive leaf (B) samples. *,**,*** indicate significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

	B ppm	Mo ppm	Zn ppm	Fe ppm	Ca ppm	Mg ppm	Cu ppm
A)							
Mn ppm	0.84***						
Zn ppm	0.81***	0.70***					
Fe ppm	0.74***	0.53***	0.81***				
Ca ppm	-0.43***	-0.33**	-0.34**	-0.51***			
Mg ppm	0.15	0.11	0.24*	0.17	0.17		
Cu ppm	-0.21	-0.14	0.02	-0.05	0.07	-0.05	
Mo ppm	-0.15	-0.19	-0.05	0.05	0.15	0.06	0.05
B)							
Mo ppm	0.28*						
Zn ppm	0.18	-0.01					
Fe ppm	-0.13	-0.05	0.20				
Ca ppm	0.15	0.22	0.22	0.50***			
Mg ppm	0.19	0.27*	0.27*	0.45***	0.80***		
Cu ppm	0.17	-0.09	0.29*	0.15	0.40***	0.42***	
Mn ppm	0.19	0.01	-0.03	0.31**	0.32**	0.55	0.34**

Figure 2b) responsible for the class distribution along the first and the second principal components explained the arrangement of the samples in the PCA score plot of Figure 2a. From the loadings plot, samples from the Gallipoli area had low amounts levels of all the measured elements, while samples from Alezio and Trepuzzi districts clustered together, with a greater contents of Mo and B. These two elements (Mo and B) also characterized samples from Sannicola, which, were mostly dispersed in the upper part of the graph. Samples collected in Leverano district were clustered in the right part of the PCA score-plot, with high contents of elements, in particular for Ca, Mg, Mn and Cu content. Pearson correlation matrix among the variables was used to evaluate the inter-element relationships in leaf samples (Table 3). Most of the elements in leaf samples were positively correlated with each other. In particular, Fe showed high positive correlation with Ca, Mg and Mn; Ca was characterized by highly significant correlation ($P < 0.001$) with Mg, Cu and Mn; Mg with Cu and Cu with Mn ($P < 0.01$), and B and Mo also showed correlation ($P < 0.05$).

DISCUSSION

These results indicate that in the Gallipoli area, where the OQDS was first noticed, as well as in districts similarly characterized by extensive damage to olive trees and the relevant occurrence of *X. f.* subsp. *pauca* (Boscia *et al.*, 2017), there is a general lack of some

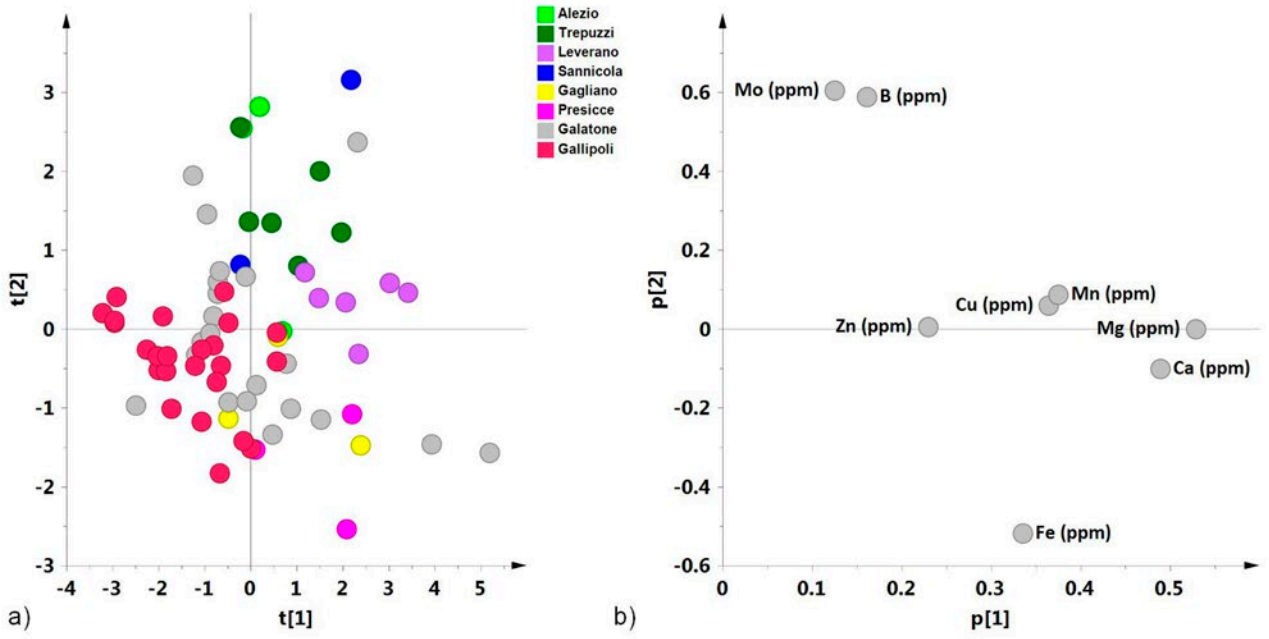

Figure 2. Principal component analysis for leaf samples collected from different sites in the Salento area (Southern Apulia). t[1]/t[2] score-plot **(a)** and corresponding p[1]/p[2] loadings plot **(b).** Two principal components (t[1]/t[2]) explained about 55% of total variance (R^2= 0.39 for t[1] and 0.21 for t[2]), with a predictability value of $Q^2 = 0.105$.

micronutrients (i.e., manganese and molybdenum) in the soils, and low contents of copper, molybdenum and boron in the leaves of olive trees. In the farms of Gallipoli, Galatone and Trepuzzi, manganese contents in the soils were low, although manganese in the leaves was within the range of normal content. This has been previously found in other studies, and emphasizes that low availability of manganese in soils characterized by a high calcium contents did not reduce amounts of this element in olive leaves (Chatzistathis and Therios, 2009).

No previous data can explain the low molybdenum content in the soil, its low content in many olive trees from the areas studied here where OQDS occurs. However, molybdenum availability should be favoured by pH greater than 5.5 (Kaiser *et al*., 2005; Bittner, 2014). This micronutrient is an essential part of nitrogenase, the enzyme catalizying nitrogen fixation of atmospheric nitrogen into ammonia (Hoffman *et al*., 2014). Low boron content within olive leaves is common for olive trees (Rodrigues *et al*., 2012), and its low bioavailability could be related to the calcareous soil matrix (Goldberg, 1997).

PCA confirmed that soil and leaf samples collected from the Gallipoli district had a lower amounts of all the measured elements compared with the other areas samples. In addition, most of the micronutrients in leaf samples showed positive correlations with each other. The significant relationships between these variables indicates similar sources of input (Islam *et al*., 2015; Moreira *et al*., 2016) for these micronutrients. However, detailed investigations in other areas where the OQDS is recorded are necessary to establish robust correlation between individual nutrients. For two farms of Galatone we found soil pHs of approx 6.0, values less than expected for calcareous soils. However, this area is characterized by a high soil salinity (Ancona *et al*., 2010), and that high concentrations of salts in calcareous soils can considerably reduce the pH (Lai and Stewart, 1990; Al-Busaidi and Cookson, 2003).

In agreement with a thorough survey performed on soils throughout Europe, including the Apulia region, and using the same technique herein applied (Ballabio *et al*., 2018), the present study also found adequate amounts of copper in the soils of the districts characterized by the occurrence of *X. f.* subsp. *pauca*. In contrast, the copper contents were low in leaves of olive trees infected by the bacterium. Low availability of copper within the olive leaves is unusual and could be related to the infection. The threshold that indicate low copper content in olive leaves varies according to different authors: Tittarelli *et al.* (2002), for Italian olive orchards, indicated 20 to 36 ppm, whereas De Andrés Cantero (2001) reported values ranging from 6 to 10 ppm. However, also considering the lower limit, the values observed in Salento in olive leaves infected by *X. fastidiosa*, especially in the Gallipoli district, were very often below these thresholds. In addition, leaf copper depletion is rarely found in olive and, apparently, it has never been reported in Italy (Tittarelli *et al*., 2002; Sanzani *et al*., 2012). A study carried out in an olive orchard free from infection of *X. fastidiosa* in Rende, Calabria, Southern Italy, found normal content of copper in soil and in the leaves, as well as normal concentration of molybdenum in soil and in leaves (Buttafuoco *et al*., 2016). The regular spraying of copper-based compounds to olive crowns to control pathogenic bacteria and fungi could explain the normal values of this micronutrient usually observed in Apulia soils (Provenzano *et al*., 2009), thus explaining the unusual observation for leaves in the present study.

Reduced copper content within highbush blueberry (*Vaccinium corymbosum* hybrids) leaves artificially infected by *X. f.* subsp. *multiplex* was found by Oliver *et al.* (2015). Similarly, *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight of rice, removes copper from host xylem, and the decreased concentration of this element leads to increased pathogen survival and virulence (Yuan *et al*., 2010). *Erwinia amylovora*, which causes fire blight, binds copper through exopolysaccharides, so decreasing *in planta* copper toxicity (Ordax *et al*., 2010).

Copper concentrations in the leaf may play an important role in *X. fastidiosa* infection. This pathogen accumulates copper when forming biofilms, a critical physiological state for its *in planta* survival (Cobine *et al*., 2013). When it is in the biofilm phase, *X. fastidiosa* has increased resistant to copper (Rodrigues *et al*., 2008). However, when the copper concentrations are greater than 200 µM, the pathogen loses capability to form biofilms for the relevant toxicity exerted by the element (Cobine *et al*., 2013). Our data indicate that *X. f.* subsp. *pauca* could decrease the copper contents within olive leaf xylem, thus explaining the general low content of this micronutrient found in olive trees infected by *X. f.* subsp. *pauca* in Salento. It is also known that subinhibitory doses of copper can promote the occurrence of *X. fastidiosa* persister cells (i.e., cells that neither grow nor die in the presence of bactericidal agents). These cells can repopulate the xylem after reduction of the stress condition (Muranaka *et al*., 2012). Also zinc leaf concentrations play an important role for *X. fastidiosa* aggressiveness. Zinc detoxification is required for commencement of host tissue colonization (Navarrete and De La Fuente, 2014). Similar to copper, zinc doses greater than

0.25 mM inhibit *X. fastidiosa* biofilm formation (Cobine *et al*., 2013).

The present investigation and previous experimental data corroborate the observed decrease in *X. f.* subsp. *pauca* cell densities and field OQDS symptoms in olive trees repeatedly sprayed with a biocomplex containing zinc (4%), copper (2%) and citric acid (Scortichini *et al*., 2018). Treatments to olive canopies this compound, characterized by a very effective capability to reach the host xylem networks, the leaf content of zinc and copper remained at greater amount than those that *X. f.* subsp. *pauca* can detoxify to increase its aggressiveness. Such a biocomplex also shows an *in vitro* antibacterial activity againsts *X. f.* subsp. *pauca* isolated from olive trees with OQDS in Salento (S. Loreti, N. Pucci, personal communication).

Restoration of the copper and zinc contents in olive trees foliage coupled with improved soil fertility, if performed during some years, could allow co-existence with *X. f.* subsp. *pauca* in orchards where the infections has not yet greatly reduced the crown of the tree. Restoration and possible maintenance of soil fertility contributes to the uptake of micronutrients for olive trees (Chatzisthatis *et al*., 2017). For an integrate disease management strategy aiming at reducing the spread of the bacterium, accurate removal of weeds from olive orchard during periods from the end of winter to spring to decrease the juvenile stages of the insect vector populations and the regular, light pruning of the tree crowns are very important. These strategies are about to be carried out on many olive farms of Salento.

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