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Ionomic differences between susceptible and resistant olive cultivars infected by Xylella fastidiosa in the outbreak area of Salento, Italy

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Abstract: *Xylella fastidiosa* causes devastating diseases in numerous plant hosts. One of the most recent and destructive outbreaks is occurring in olive trees in the Salento region of Italy. Olive Quick Decline Syndrome (OQDS) is widespread in the outbreak area, however the 'Leccino' variety of olives has proven to be ~~resistant~~ with fewer symptoms and lower bacterial populations than the 'Ogliarola salentina' variety. We completed an empirical study to determine the mineral contents (viz; ionome) of leaves from infected trees comparing the two varieties, to develop hypotheses related to the resistance of Leccino trees to *X. fastidiosa* infection. All samples from both cultivars tested were infected by *X. fastidiosa*, even if leaves were asymptomatic at the time of collection, due to the high disease pressure in the outbreak area and the long incubation period of this disease. Leaves were binned for the analysis by variety, field location, and infected symptomatic and infected asymptomatic status by visual inspection. The ionome of leaf samples was determined using Inductively-Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) and compared to each other. These analyses showed that 'Leccino' variety consistently contained higher manganese (Mn) levels compared to 'Ogliarola salentina', and these levels were higher in both infected asymptomatic and infected symptomatic leaves. Infected asymptomatic and infected symptomatic leaves within a host genotype also showed differences in the ionome, particularly an increase in calcium (Ca) and Mn levels in the 'Leccino' cultivar, and sodium (Na) in both varieties. We hypothesize that the ionome differences in the two varieties contribute to protection against disease caused by *X. fastidiosa* infection.

Keywords: *Xylella fastidiosa*; Olive Quick Decline Syndrome; ionome; olive; Leccino; Ogliarola salentina; manganese; calcium

1. Introduction

Olive Quick Decline Syndrome (OQDS) is a severe disease that was first reported in September 2013 in Salento, a region in the south of Apulia, Italy. The aetiological agent of the disease is the bacterium *Xylella fastidiosa* [1,2]. *Xylella fastidiosa* is transmitted by xylem-feeding insects and causes well-known diseases such as Pierce's disease (PD) of grapevine and citrus variegated chlorosis (CVC) of citrus. Additional host plants of economic and landscape relevance include almond, oleander, peach, coffee, oak and blueberry [3,4]. The symptomatology, which varies depending on the crop, is generally represented by scorch and marginal necrosis of the leaves, chlorosis and progressive

desiccation of twigs and branches. Although the mechanism by which the bacterium causes disease is not completely understood, the most recognized hypothesis concerns the blockage of the plant xylem vessels that are used to transport water and mineral elements from the soil to the aerial parts. The development of the disease depends on the ability of the pathogen to move from the point of inoculation, proliferate within the xylem vessels and colonize the whole plant [5]. The production of biofilm associated with bacterial multiplication, together with the production of gums and tyloses by the plant as a defence mechanism, could contribute to the obstruction in the vessels [6,7]. Different molecular pathways are induced by infection. Typical defence genes against pathogens, genes for the synthesis of hormones associated with biotic stress, genes known to be involved in the tolerance to water stress, as well as those involved in synthesis of hormones produced during abiotic stress, have all been detected as a response to *X. fastidiosa* infection [8-10]. Genome analysis of different *X. fastidiosa* strains revealed the absence of genes encoding Type III secretion system machinery, as well as the lack of apparent Type III secretion effectors, which are responsible for suppressing host plant defence responses [11,12]. Moreover, *X. fastidiosa* lives in xylem vessels which are mainly composed of dead cells [13,14]. For this reason, a key question regarding plant-pathogen interaction is how the host senses the pathogen and what are the mechanisms triggered by this recognition.

While some hosts show severe disease, other closely related genotypes growing in the same environment show tolerance and/or resistance to the pathogen. These hosts can harbor high populations of *X. fastidiosa*, but show few or no symptoms of the disease [15]. One example is *Vitis rotundifolia* (or Muscadine grape), native to the southeastern region of North America that has evolved tolerance/resistance to the disease. In this region, Pierce's disease is so severe that it prevents the commercial cultivation of many cultivars of *Vitis vinifera* [3]. Ruel and Walker identified as resistant/tolerant the California native *Vitis girdiana* which can support up to one hundred times higher bacterial concentrations without disease [16]. Additionally, *Vitis arizonica*, also from an area of high infection pressure, appear to be very resistant to *X. fastidiosa* [16]. However, the mechanism of resistance is unknown in all these cases.

OQDS is characterized by initial leaf scorch and scattered desiccation of small branches that over time get worse and extend to the whole canopy [17]. Nevertheless, greenhouse and field observations have revealed that plants of *Olea europaea* (olive) 'Leccino', although infected, appear asymptomatic or show very mild symptoms, when compared to the highly susceptible and prevalent variety 'Ogliarola salentina', which is severely damaged and succumbs to infection. Specifically, the canopy of 'Ogliarola salentina' trees affected by the disease show progressive and complete dieback, while infected 'Leccino' trees show a better vegetative growth with less aggressive and more sporadic desiccation around the canopy [2,18-20]. Transcriptome profiling of the two olive cultivars in response to infection by *X. fastidiosa* subsp. *pauca* strain De Donno revealed that the pathogen triggers a differential response strongly involving a re-modelling of cell wall proteins [21]. An up-regulation of genes encoding receptor-like kinases (RLK) and receptor-like proteins (RLP) was observed in 'Leccino', while genes related to drought stress were expressed in 'Ogliarola salentina' [21]. These signalling proteins, whose orthologs are similarly up-regulated in CVC-resistant mandarin [10], are the first barrier that the plant produces against the pathogen. Therefore, it was assumed that 'Leccino' is somehow able to respond to the pathogen. Moreover, trees of 'Leccino' harbour a 100-fold lower bacterial population when compared to 'Ogliarola salentina' [21], similar to resistant *Vitis* species and varieties of citrus in which multiplication and translocation of *X. fastidiosa* is inhibited [5,22-24].

Mineral ~~elements play~~ a role in a myriad of cellular functions acting as protein cofactors in many processes [25]. Therefore, nutrient transport and balance is important for growth and development of plants and also microorganisms, and can be an important factor in disease control and progression [15,25-28]. In host-pathogen interactions the competition to acquire these elements is a phenomenon known as "nutritional immunity" [29,30]. Analysis of the ionome, defined as the total concentrations of the mineral nutrients and trace elements found in an organism [31], represents an approach to investigate the physiological state of the plant [32,33]. A number of experiments have demonstrated important roles of mineral elements for *X. fastidiosa in vitro* and during infection *in planta*. Previous studies on grapevines, citrus and peaches have shown that the concentration of specific mineral

elements of plants infected with *X. fastidiosa* is different from uninfected plants [34-36]. The analysis of the ionome of field-grown grapevines, blueberry and pecan and greenhouse-grown *Nicotiana tabacum* during *X. fastidiosa* infection, revealed that significant changes are present in infected symptomatic and infected asymptomatic leaves, showing a complex interaction between different elements in the host [37]. Part of the complexity is related to the fact that virulence traits of *X. fastidiosa* are modulated by mineral elements. Bivalent cations, such as calcium (Ca) and magnesium (Mg), play a structural role in cell-cell and cell-surface attachment in the host, by acting as a bridge between the negative charges of xylem vessels and the negative charges of bacterial cells [38]. Additionally, *in vitro* assays proved that Ca specifically promotes virulence determinants in *X. fastidiosa*, like biofilm formation, adhesion and twitching motility [39,40]. In fact, *X. fastidiosa* cells from biofilm and planktonic states showed a different profile of mineral elements with biofilm cells accumulating Ca, copper (Cu), potassium (K), magnesium (Mg) and zinc (Zn), when compared to planktonic cells [41].

Here we describe an empirical field survey, carried out in privately-owned olive groves located in the *Xylella*-infected area in southern Italy, where we characterize the leaf ionome of two olive cultivars showing different disease responses to *X. fastidiosa* infection. The very high levels of *Xylella* infections in the sampled fields makes unfeasible to obtain with absolute certainty healthy control olives in these orchards. As we have no samples that predate infection (uninfected control), we adopted an experimental strategy based on the comparison of varieties within the same field and infected symptomatic vs. infected asymptomatic tissues. Our study provides insights into the disease resistance mechanism of the 'Leccino' variety.

2. Results

2.1 Field survey

Leaf samples from naturally infected olive trees were collected from two different olive groves located in the area declared *X. fastidiosa*-infected (Figure 1) [42]. All leaves from the two different cultivars of olives, 'Olgiarola salentina' and 'Leccino', were naturally infected with *X. fastidiosa.* 'Ogliarola salentina' showed severe symptoms, while symptoms on 'Leccino' were less prominent and were often limited to initial discolorations that had not yet advanced to leaf scorching (Figure 2). *fastidiosa* were collected from two fields in the south of the Apulia region (Figure 1)[42]. Leaves designated as infected symptomatic showed initial desiccation, which consists of browning in colour and "rolling" downward of the leaves. Our surveys show no uninfected trees exist in these fields to serve as a control for infection status. Similar results have been reported by ~~other~~ recent studies of field samples from this same region [21,43].

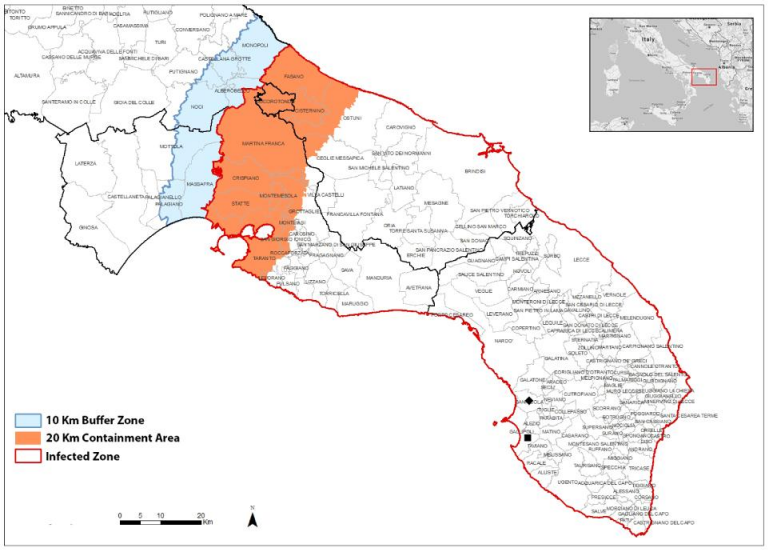

Figure 1. Map of the demarcated areas established in the region of Apulia (Italy), as defined by Commission Implementing Decision (EU) 2018/927 of 27 June 2018, with indication of the

experimental sites used in this study: ■ Field 1 (Gallipoli. Lecce. 40.0233750, 18.0516470); ◆ Field 2 (Sannicola. Lecce. 40.124325, 18.045239). The original map data were from Google Maps (DigitalGlobe, USA). This version was created using Adobe® Photoshop CS6 (Adobe Systems Inc. USA) software.

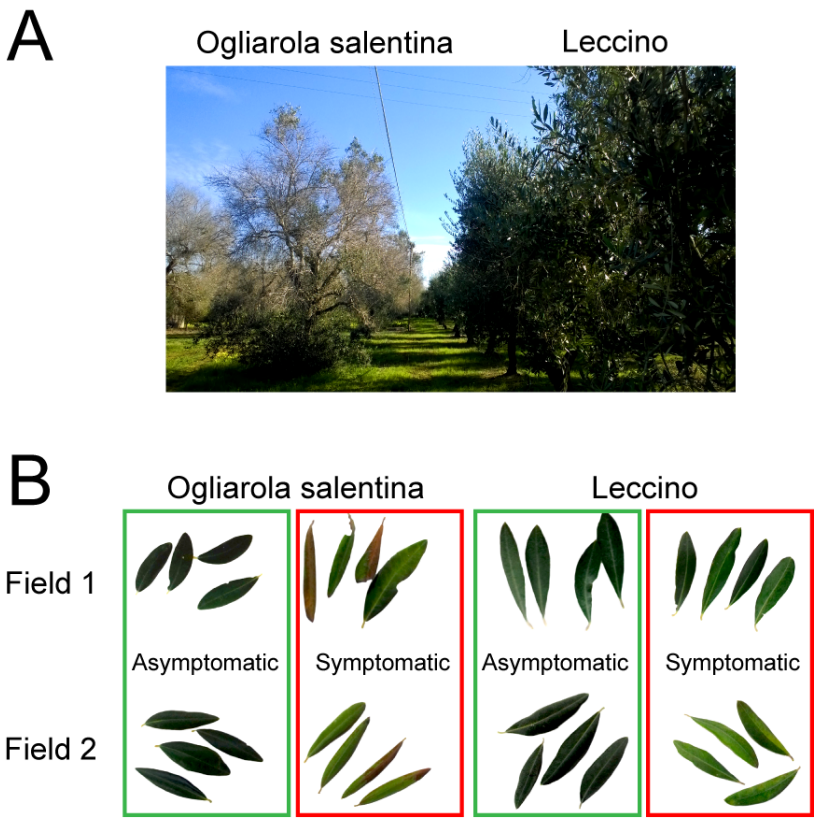

Figure 2. Evidence of different symptomatic response to *Xylella fastidiosa* infection. A) Shows heavily susceptible olive 'Ogliarola salentina' (left), in comparison with resistant 'Leccino' (right), observed in a grove located in the Olive Quick Decline Syndrome-affected area (Alezio, Lecce, Italy). B) A representative sample of the olive leaves used for ionome analysis. The image shows leaves chosen from infected symptomatic (boxed in red) and infected asymptomatic (boxed in green) branches of each of the two varieties, i.e. 'Ogliarola salentina' (left) and 'Leccino' (right) and sampled in each of the two fields surveyed in this study, Field 1 and Field 2.

2.2 Population size of Xylella fastidiosa within the two cultivars

Xylella fastidiosa qPCR detection confirmed that all plants analysed were infected, despite the presence or not of symptoms by visual inspection (Figure 2B). The estimation of bacterial concentration showed that tissues of 'Ogliarola salentina' had an average of 5.1 ± 0.1 log CFU/g in Field 1 and 5.2 ± 0.3 log CFU/g in Field 2, whereas those of 'Leccino' had an average of 3.4 ± 0.1 Log CFU/g in Field 1 and 4.3 ± 0.3 Log CFU/g in Field 2. *Xylella fastidiosa* population was about one order of magnitude higher in the 'Ogliarola salentina' than in 'Leccino', although only Field 1 showed a statistically significant decrease in *X. fastidiosa* population ($P < 0.0001$) (Figure 3).

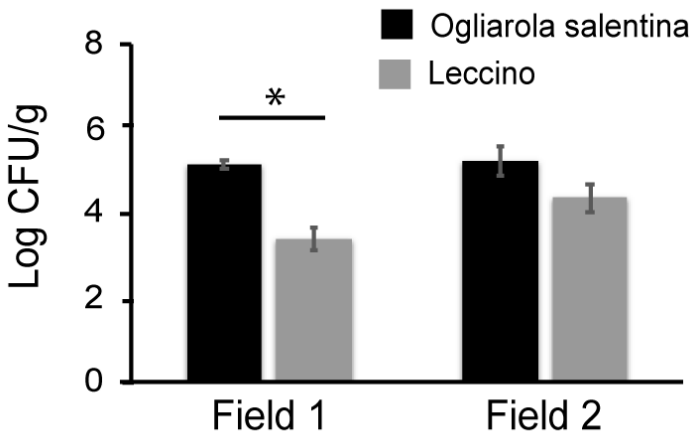

Figure 3. *Xylella fastidiosa* population in leaf samples of olive 'Ogliarola salentina' and 'Leccino', quantified by qPCR assay. Bacterial concentration is expressed as log CFU/g and inferred using a standard calibration curve. Ten biological replicates for each cultivar in Field 1 and four biological replicates for each cultivar in Field 2. A pool of ten leaf petioles representing ten portions of the plant, five infected symptomatic and five infected asymptomatic, was used for bacterial detection and quantification in each plant. Each point represents the average of two independent experiments. ~~Vertical~~ bars represent standard error of the means. * indicates statistically significant differences ($P < 0.0001$) between the two varieties according to two-tailed Student's *t*-test pairwise comparisons.

2.3 Determination of leaf ionome and soil parameters

The total concentrations of mineral elements for the leaves sampled are shown in Table 1. The elemental composition were compared to reference values for leaf content of olive trees [44]. The values for Ca, Mg, S, P, Na and Zn were within these reference ranges. However, K and Fe were considered either low or close to the minimal range. Only K was in the range specified for potential deficiency (K< 4 g/kg) but this was true for all samples independent of cultivar or symptomatology. Soils from Field 1 and Field 2 were analysed to investigate its physicochemical parameters as a potential variable in differences in elemental composition. The region of Field 1 where the 'Leccino' variety was grown, when compared to the 'Ogliarola salentina' region, showed higher Ca (2230 vs. 1601 mg per kg) and lower Mn (26 vs 38 mg per kg). While in Field 2 the soil from where the 'Leccino' variety was grown compared to soil from the 'Ogliarola salentina' region showed lower Ca (2130 vs. 2601 mg per kg) and higher Mn (56 vs 26 mg per kg). The Ca concentration was slightly lower than the reference of 3000 mg per kg while the Mn concentration was higher than the reference of 5-20 mg per kg (Table 2).

Table 1. Elemental analysis of olive leaves expressed in weight per dry weight.

		Ca	**K**	**Mg**	**S**	**P**	**Fe**	**Mn**	**Na**	**Zn**
Reference*	g/kg or **mg/kg**	10-14	8-10	1-1.6	0.8-1.6	1-1.3	**90-124**	**20-36**	**<200**	**4-9**
					Field 1					
'Leccino'	Asymp	13.7	3.9	1.8	1.6	1.3	**41.5**	**43.4**	**33.0**	**9.3**
	Symp	16.2	4.2	1.6	1.5	1.4	**45.6**	**44.3**	**59.1**	**9.5**
'Ogliarola salentina'	Asymp	13.3	3.1	1.6	1.9	1.5	**96.6**	**30.4**	**25.5**	**6.7**
	Symp	12.9	3.1	1.6	1.9	1.3	**95.3**	**31.5**	**61.0**	**8.8**
					Field 2					
'Leccino'	Asymp	6.4	4.2	1.0	1.5	1.2	**63.4**	**44.4**	**15.3**	**7.1**
	Symp	11.3	3.8	1.5	1.6	1.6	**87.6**	**80.5**	**48.9**	**10.2**
'Ogliarola salentina'	Asymp	10.6	3.8	1.5	1.7	1.2	**42.1**	**29.6**	**23.1**	**10.7**
	Symp	14.6	3.4	2.0	1.8	1.7	**60.6**	**40.3**	**126.8**	**18.4**

Field 1 (Gallipoli, Lecce, 40.0233750, 18.0516470); Field 2 (Sannicola, Lecce, 40.124325, 18.045239). Element concentrations are expressed in g/kg, except for bold values (Fe, Mn, Na, and Zn) that are expressed in mg/kg. *Reference concentrations obtained from Kailis and Harris (49).

Table 2. Physico-chemical analysis of soils collected from the area where trees used for ionome profiling were selected.

		Field 1		Field 2	
	Reference range	'Ogliarola' Mean ± St. Dev	'Leccino' Mean ± St. Dev	'Ogliarola' Mean ± St. Dev	'Leccino' Mean ± St. Dev
TEXTURE					
Skeleton (> 2 mm) %	0-10	0	0	1 ± 1.4	0 ± 0.00
Sand (2-0.05 mm) % Fine Fraction	-	51 ± 3	51 ± 3	47 ± 6	37 ± 3
Silt (0.05-0.002 mm)%Fine Fraction	-	25 ± 1	24 ± 4	32 ± 6	34 ± 0
Clay (<0.002 mm) % Fine Fraction	-	24 ± 4	25 ± 1	21 ± 0.7	29 ± 3
CHEMICAL AND PHYSICAL PARAMETERS					
pH in H_2O (1:2.5) mS·cm^2	6.7-7.3	7.05 ± 0.49	8.05 ± 0.07	8.1 ± 0.14	7.3 ± 0.28
MACRO ELEMENTS					
Assimilable phosphorus (mg·kg^{-1} Fine Fraction)	27-32	70 ± 10	34 ± 3	7 ± 1	5 ± 0.9
Exchangeable potassium (mg·kg^{-1} Fine Fraction)	200	310 ± 104	232 ± 52	355 ± 76	102 ± 8
Exchangeable calcium (mg·kg^{-1} Fine Fraction)	3000	1601 ± 126	2231 ± 344	2610 ± 25	2130 ± 632
Exchangeable sodium (mg·kg^{-1} Fine Fraction)	230	63 ± 27	96 ± 11	54 ± 1	37 ± 4
Exchangeable magnesium (mg·kg^{-1} Fine Fraction)	200	211 ± 54	284 ± 104	322 ± 42	427 ± 88
MICRO ELEMENTS					
Assimilable iron (mg·kg^{-1} Fine Fraction)	5-40	16 ± 8	6 ± 0.3	4.4 ± 0.3	7.2 ± 1.3
Assimilable manganese (mg·kg^{-1} Fine Fraction)	5-20	38 ± 7	27 ± 5	27 ± 3	57 ± 7.4
Assimilable copper (mg·kg^{-1} Fine Fraction)	1-5	30 ± 2	22 ± 0.2	11.3 ± 1.7	8.6 ± 0.1
Assimilable zinc (mg·kg^{-1} Fine Fraction)	0.5-2	1 ± 0.3	1.0 ± 0.01	1.5 ± 0.1	0.6 ± 0.04

Values represent averages of two replicate samplings. 'Ogliarola' or 'Leccino' refers to soil collected from the same area where 'Ogliarola salentina' or 'Leccino' trees used in this study were found, respectively. Field 1 (Gallipoli, Lecce, 40.0233750, 18.0516470); Field 2 (Sannicola, Lecce, 40.124325, 18.045239).

2.4 Ionome of 'Leccino' and 'Ogliarola salentina'

A comparison of the ionome of the cultivars in the same field was investigated to identify possible phenotypic markers that affect the susceptibility of the individual varieties. To visualize the ionome data we used a principal component analysis (PCA). This analysis reduces the data for the ten elements tested and presents them in two dimensions. The different varieties separated along both principal component 1 (PC1) and principal component 2 (PC2) in both infected asymptomatic and infected symptomatic leaves (Figure 4A, 4C and Figure 5A, 5C). These PCAs demonstrated that 'Leccino' and 'Ogliarola salentina' have differences in their mineral content independent ~~of disease status~~. The variance explained by each principal component is shown in Table 3 and the component loadings for PC1 and PC2 are shown in Table 4. The PCA component values ~~can be~~ used for hierarchical cluster analysis and generation of a heat map (Figure 4B, 4D and Figure 5B, 5D). In general, the varieties were separated into independent clades with some overlap in ionome characteristics. We did not determine the variables contributing to this overlap/clustering together. These could include severity of symptoms, age of tree or possibly agronomic practices.

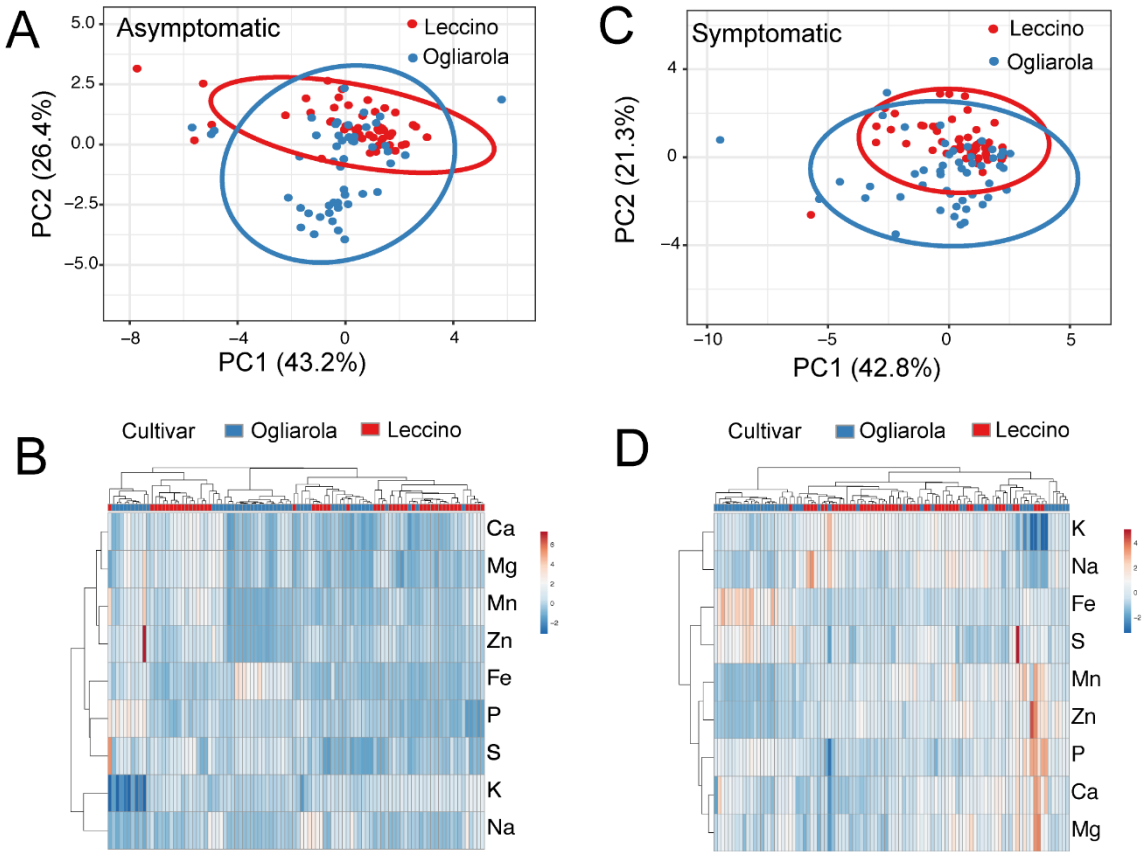

Figure 4. Principal component analysis (A and C) of leaves from Field 1with prediction ellipses that show the probability of 0.95 that a new observation will fall in the same group. In the heat map representation of elemental composition, both rows and columns are clustered using correlation distance and average linkage (B and D). Infected asymptomatic leaves are shown in A and B, and infected symptomatic leaves in C and D. Each point represents an individual sample as outlined in Table 7 (n=100 per cultivar)

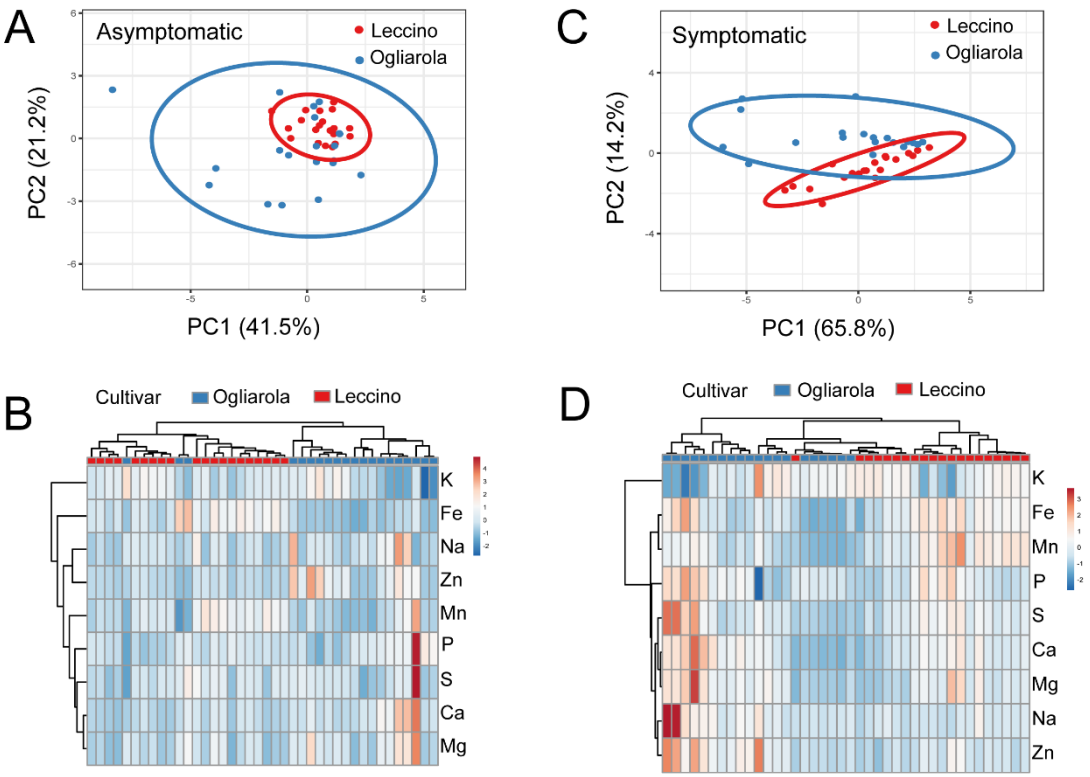

Figure 5. Principal component analysis (A and C) of leaves from Field 2 with prediction ellipses that show the probability of 0.95 that a new observation will fall in the same group. In the heat map representation of elemental composition, both rows and columns are clustered using correlation distance and average linkage (B and D). Infected asymptomatic leaves are shown in A and B and infected symptomatic leaves in C and D. Each point represents an individual sample as outlined in Table 7 (n=40 per cultivar).

Table 3. Variance explained by principal components in PCA of element concentration of infected asymptomatic and infected symptomatic leaves of each olive varieties in different fields.

		PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Field 1	Asymp	0.43	0.26	0.10	0.06	0.05	0.04	0.03	0.02
	Symp	0.43	0.21	0.13	0.10	0.06	0.03	0.03	0.01
Field 2	Asymp	0.42	0.21	0.15	0.09	0.07	0.03	0.02	0.01
	Symp	0.66	0.14	0.11	0.05	0.01	0.01	0.01	0.00

Table 4. Component loadings of principal components 1 and 2 from the PCA of element concentration in infected symptomatic leaves versus infected asymptomatic leaves of each olive varieties in different fields.

			Ca	Fe	K	Mg	Mn	Na	P	S	Zn
Field 1	Asymp	PC1	0.40	0.17	-0.46	0.38	0.22	-0.27	0.46	0.27	0.22
		PC2	0.16	-0.52	0.12	0.24	0.47	0.29	-0.12	-0.31	0.47
	Symp	PC1	-0.37	-0.14	0.37	-0.41	-0.39	0.04	-0.37	-0.32	-0.38
		PC2	0.33	-0.45	0.39	0.23	0.31	0.37	-0.40	-0.18	0.23
Field 2	Asymp	PC1	0.49	0.07	-0.16	0.46	0.29	0.21	0.38	0.42	0.26
		PC2	-0.07	0.44	-0.30	-0.16	0.20	-0.44	0.35	0.17	-0.55
	Symp	PC1	0.38	0.33	-0.33	0.38	0.26	0.28	0.32	0.39	0.32
		PC2	0.04	-0.35	0.19	0.03	-0.49	0.55	-0.21	0.10	0.49

Specifically comparing infected asymptomatic leaves in Field 1, 'Leccino' had higher levels of K (23%), Mn (43%), Na (30%) and Zn (39%) and lower levels of Fe (-47%) and P (-15%) ($P < 0.05$ in all cases) (Table 5) as compared to 'Ogliarola salentina'. In infected symptomatic leaves in Field 1 'Leccino' ~~had~~ higher ~~in the~~ levels of Ca (21%), K (35%), Mn (41%), and lower ~~in~~ Fe (-37%) and P (-23%) ($P < 0.05$ in all cases) as compared to 'Ogliarola salentina' (Table 5). In Field 2 infected asymptomatic leaves of 'Leccino' had higher levels of Fe (51%), Mn (50%), and lower levels of Ca (-40%), Mg (-34%), Zn (-34%) ($P < 0.05$ in all cases) (Table 5) as compared to 'Ogliarola salentina'. The infected symptomatic leaves from Field 2 of 'Leccino' had higher levels of Mn (100%), Fe (45%) and lower levels of Na (-61%) and Zn (-45%) ($P<0.05$ in all cases) (Table 5) as compared to 'Ogliarola salentina'.

Table 5. Percent change of element concentration in leaves of olive 'Leccino' versus 'Ogliarola salentina', separated by visual symptomatology.

		Ca	K	Mg	S	P	Fe	Mn	Na	Zn
Location[#]		**INFECTED ASYMPTOMATIC**								
Field 1	% change	4	**23**	14	-11	**-15**	**-57**	**43**	**30**	**39**
	p-value	0.53	**6E-04**	0.08	0.14	**6E-04**	**1E-07**	**7E-05**	**2E-02**	**6E-03**
Field 2	% change	**-40**	11	**-34**	-5	-10	**51**	**50**	-34	**-34**
	p-value	**6E-03**	0.10	**2E-03**	0.59	0.11	**6E-03**	**2E-03**	0.14	**4E-03**
		INFECTED SYMPTOMATIC								
Field 1	% change	**21**	**35**	-1	4	**-23**	**-52**	**41**	-3	8
	p-value	**2E-02**	**9E-07**	0.93	0.59	**8E-07**	**3E-08**	**2E-03**	0.85	0.56
Field 2	% change	-22	13	-25	-7	-10	**45**	**100**	**-61**	**-45**
	p-value	0.24	0.25	0.17	0.68	0.24	**0.043**	**2E-04**	**1E-02**	**2E-03**

[#]Field 1 (Gallipoli, Lecce, 40.0233750, 18.0516470); Field 2 (Sannicola, Lecce, 40.124325, 18.045239). *Numbers indicate the percent of change in element concentration in the leaves of 'Leccino' compared to 'Ogliarola salentina' olive cultivars. For each comparison either infected asymptomatic or infected symptomatic leaves were used. Numbers in bold indicate significant differences according to Student's t-test (p-values < 0.05).

2.5 Ionome according to symptomatology

To determine if changes in the ionome were occurring in infected symptomatic versus infected asymptomatic samples, we investigated the concentration of elements grouped by cultivar and field. In Field 1 the comparison between infected symptomatic versus infected asymptomatic leaves of 'Ogliarola salentina' ~~suggested~~ no statistical change in elemental composition, except for changes in Na levels that were higher by 139% ($P < 0.001$) in infected symptomatic leaves (Table 6). In Field 2 both Na (449%, $P < 0.001$) and Zn (72%, $P < 0.05$) levels were significantly higher (Table 6). Ionomic profile of infected symptomatic versus infected asymptomatic leaves from 'Leccino' in Field 1 showed statistically significant higher levels of Ca (19%) and Na (79%) ($P < 0.05$ in all cases) (Table 6). In Field 2 'Leccino' shows higher levels of Ca (76%), Fe (38%), Mg (56%), Mn (81%), Na (220%), S (8%) and Zn (45%) ($P < 0.05$ in all cases) (Table 6).

ANOVA analysis of the interaction between three individual elements, Ca, Mn and Na, with the independent variables field, variety, and status (i.e.; symptoms) shows that for Ca there is a significant effect for field (P<0.001), status (P=0.001), and a significant interaction for field*variety (P=0.001) and field*status (P=0.006). ~~For~~ Mn we observed a significant effect for field (P<0.001), variety (P<0.001), status (P<0.001), and a significant interaction for field*variety (P=0.008), field*status (P<0.001), variety*status (P=0.034), field*variety*status (P<0.001). For Na significant effects were observed for variety (P=0.001), status (P<0.001), and significant interaction for field*variety (P=0.008), field*status (P=0.003), variety*status (P<0.001), and field*variety*status (P<0.019). These data support the idea that analysing a control from a different uninfected field outside the outbreak area would not provide a useful comparison for this study as field has an effect on two the major elements that are different.

Table 6. Percent change of element concentration in infected symptomatic leaves versus infected asymptomatic leaves of each olive varieties that differ in response to *X. fastidiosa* infection.

		Ca	**K**	**Mg**	**S**	**P**	**Fe**	**Mn**	**Na**	**Zn**
					Field 1					
'Leccino'	% change	**19***	9	-12	-9	3	10	2	**79**	2
	p-value	**2E-02**	6E-02	1E-01	7E-01	5E-02	2E-01	8E-01	**1E-03**	7E-01
'Ogliarola salentina'	% change	-3	-1	2	0	-12	-1	4	**139**	31
	p-value	7E-01	9E-01	8 E-01	7E-02	9E-01	9E-01	7E-01	**1E-05**	1E-01
					Field 2					
'Leccino'	% change	**76**	-8	**56**	**8**	33	**38**	**81**	**220**	**45**
	p-value	**6E-04**	1E-01	**1E-02**	**6E-03**	1E-01	**7E-03**	**1E-04**	**7E-05**	**5E-03**
'Ogliarola salentina'	% change	37	-10	36	8	36	44	36	**449**	**72**
	p-value	1E-01	6E-01	9E-02	1E-01	7E-01	1E-01	7E-02	**5E-04**	**3E-03**

*Numbers indicate the percent of change in element concentration in infected symptomatic vs. infected asymptomatic leaves of either 'Leccino' or 'Ogliarola salentina' olive cultivars. Comparisons were made separated for each field. Field 1 (Gallipoli, Lecce, 40.0233750, 18.0516470); Field 2 (Sannicola, Lecce, 40.124325, 18.045239). Numbers in bold indicate significant differences according to Student's *t*-test (*p*-values < 0.05).

3. Discussion

Xylella fastidiosa is continuing to emerge as an important and devastating bacterial pathogen for many crops of agronomic relevance, and so far no cure has been identified. The current strategies for management are vector control to limit spread of infection and the use of cultivars which show tolerance/resistance to the pathogen in the field. The latter requires replacing susceptible varieties in current fields, or incorporating the tolerant genotypes into breeding programs. The field survey presented here is the first description of the leaf ionome of two olive cultivars that respond differently to *X. fastidiosa* infection. Our working hypothesis was that ~~mineral~~ profile of each cultivar plays a role in the success of the infection and/or in the development of symptoms. The quantification of the bacterial population in the two varieties grown in the field under high infection pressure confirms the resistance of 'Leccino' to *X. fastidiosa* infection, since the growth of the bacterium is restricted [15,45,46] and this leads to milder symptoms when compared to 'Ogliarola salentina'. Considering that replanting of the varieties is not always a timely or feasible approach, understanding the mechanisms of resistance/tolerance could represent a critical step towards a more rapid response to *X. fastidiosa* outbreaks.

Due to the fact that *X. fastidiosa*-free plants of both varieties were not available in the fields from the outbreak studied here, we were unable to directly assess the changes in ionome in the presence/absence of the pathogen. Other recent published studies have been constrained by this same variable, our review of the literature confirms that no single study conducted in recent years in the area of the Salento outbreak reports conclusive data showing sampling of healthy, uninfected and infected trees in the same field. Most studies take samples from different fields or from different areas [47-51]. Therefore, we focused on the hypothesis that differences in the ionome of cultivar in both infected symptomatic and infected asymptomatic leaves are informative to the resistance of the 'Leccino' variety. We also note that changes in ionome studied here may be affected by different time of infection of the trees analysed, although fields for this study are located in the initial focus of the outbreak ~~so~~ we assume they were infected at similar time points. An internal denominator was used to calculate the concentration of mineral elements for this analysis to account for at least four possible sources of error exist when using direct measurement of dry weight as an external denominator: 1) difficulty making exact measurement of small quantities of leaves, 2) some samples had an unknown substance that remained after acid digestion, the amount was stochastic and variable in leaves, therefore differently contributing to dry weight. It should be noted that no additional mineral elements of interest could be extracted from these insoluble materials with extended and repeated digestions (data not shown), 3) our dry weight was potentially affected by variable extraction of water given the waxy coating on leaves, and 4) potential losses during sample introduction into plasma based on the performance of the instrument.

To assess the importance of mineral content for a potential role in resistance of 'Leccino' our criteria were: 1) the change should be statistically significant in 'Leccino' relative to 'Ogliarola salentina' in both fields, 2) the magnitude of the change should be in the same direction in both fields. Assessing levels by these criteria the element that consistently changed was Mn with an increase in the 'Leccino' plants relative to 'Ogliarola salentina'. The other elements that are different in both fields are Fe and Zn, however, these elements ~~are~~ increased in one field and decreased in the other. The reason for the paradoxical behaviour of accumulating to higher levels in one field but lower levels in the other is unknown, but we speculate that it cannot be protective for disease progression. The analysis of soil compared to leaf ionome suggest that the plants are tightly regulating the levels of these elements, as one field has higher available Mn and the other has lower Mn levels, yet 'Leccino' maintains higher concentrations of the element. Therefore, the only element we can suggest is providing potential protection for 'Leccino' is Mn. In addition, a recent study by Scortichini et al. [43], analysing the soil micronutrient content of farms located in the Gallipoli area, showed ~~a~~ low content of this element in the soil of all the fields investigated, perhaps suggesting a possible reason for susceptibility of trees in the region.

Mn is an essential micronutrient for plant metabolism and development [27]. About 35 different enzymes use Mn, including phenylalanine ammonia lyase (PAL), a key enzyme in the metabolism of

polyphenolic compounds such as flavonoids, phenylpropanoids and lignin [52]. Phenolic compounds are altered during *X. fastidiosa* infection in grapes [53]. Other examples are Mn superoxide dismutase[54], oxalate oxidase [55] and the oxygen-evolving complex of PSII [56-58]. Production of ROS, termed "oxidative burst" is a hallmark of successful recognition of infection and activation of plant defences [59], since it can directly act as antimicrobial agent during the plant defence response [60-63] and/or indirectly contribute in the formation of physical barriers, e.g. lignin deposition at cell wall, that hinder the colonization of the plant by the pathogen [64,65]. Moreover, recent studies showed a significant increase in total lignin in *X. fastidiosa*-infected 'Leccino' compared with the sensitive 'Ogliarola salentina' [51], and increased ROS levels in 'Leccino' compared to susceptible 'Cellina di Nardo' variety [49]. On this basis, we assumed that increased levels of Mn could be protecting the resistant host against increased ROS by providing ample cofactor for antioxidant enzymes thereby reducing oxidative stress damage, and by triggering pathways involved in the production of aromatic amino acids, lignin and flavonoids. Simpson et al. [66] have suggested that the uptake of Fe and possibly other transition metal ions such as Mn, by *X. fastidiosa*, cause the exhaustion of essential micronutrients in xylem, leading, in citrus trees, to symptoms typical of variegated chlorosis. It could then be hypothesized that the availability of higher levels of Mn in 'Leccino' could more effectively counterbalance the deficit triggered by the bacterial infection.

Our previous studies have demonstrated that ionome remodelling occurs before the first signs of disease in the *Nicotiana tabacum* model [37]. The outcome of the present investigation shows that comparing infected asymptomatic and infected symptomatic tissues, the most evident change in all plants is increased Na. This result is in agreement with the significant increase in Na detected in symptomatic leaves of Phony peach diseased-trees also caused by *X. fastidiosa* [34]. *Xylella fastidiosa*-associated diseases are collectively referred to as leaf scorch diseases, based on the common symptom of necrotic tissue at the periphery of pathogen-infected leaves. A long-standing hypothesis, states that leaf scorch is a consequence of obstructed water flow and the production of tyloses and gums by the host, that attempts to hinder the progress of the infection [6]. Although symptoms of water stress induced by *X. fastidiosa* are quantitatively and qualitatively different from water stress induced by non-irrigation, it has been shown both that the pathogen induces water stress and that external water stress conditions enhance the development of the disease [8,67]. Under drought conditions, maintenance of cell turgor may occur by ionic balance and osmotic adjustment, which implies a net accumulation of solutes and ions, in order to maintain a favourable water potential gradient [68,69]. Thus a specific physiological role of Na ion in an osmotic adjustment in infected symptomatic leaves could be proposed. A specific Na uptake is an integral part of *Atriplex halimus* response to water stress [70]. ~~*Atriplex*~~ *halimus* is a xerohalophyte plant species able to cope with low external water potentials [71]. An increased Na concentration in water-stressed plants may be related to an increase in the metabolic requirement of Na to sustain photosynthesis in these conditions. It has been reported that Na is involved in the maintenance of mesophyll chloroplast structure, mainly in relation to granal stacking [72,73]. Sodium may also be involved in the regeneration of phosphoenolpyruvate in mesophyll chloroplasts [74], because a ~~Na^+~~ gradient across the envelope could be an alternative energy source for the active transport of pyruvate [75]. An increase in Na uptake could then be the consequence of a stress-induced decrease in the efficiency of the Na^+/pyruvate cotransport system.

Studies on a tobacco model of infection and initial field studies in grapes, blueberries, and pecans, showed that Ca is higher in samples infected by *X. fastidiosa*, compared to non-infected, and this increase is even greater in the presence of symptoms [35,37]. In fact Ca-oxalate crystals have been detected in the xylem vessels of grapes and coffee plants after *X. fastidiosa* infection [76] and Ca accumulates in vessels obstructed by the bacterium [77]. Data presented here, comparing infected symptomatic and infected asymptomatic leaves, show an increase in Ca content in infected symptomatic tissues in 'Leccino'. We cannot determine whether the increase in Ca at the leaf level corresponds to an increase ~~in the ion~~ in the cytoplasm or in the apoplast. The plant acquires Ca mainly from the soil through the root system before it reaches the shoots through the xylem, as the potential for this ion is favourable to its uptake from the roots [78,79]. Ca is an essential nutrient for the plant, which plays numerous roles and functions. It acts as a counter cation in the vacuole [27], contributes

to the rigidity of cell membranes and primary walls of cells, interacts with different phytohormones for cell division and expansion [80]. In addition, it plays a key role as a second intracellular messenger, able to trigger several downstream signalling pathways. Increases in Ca levels in the cytosol is a known plant defence responses to the attack of a pathogen [81]. Given the global increase in Ca in the leaves we would suggest that this is an uptake response from the extracellular medium rather than export from the organelles. For the reasons previously mentioned for Na, the Ca increase could also be the result of a water-stress response, but no Ca increase has been detected in studies that have analysed the plant's specific responses to water stress [82]. Analyses of grapevine gene expression with *X. fastidiosa* infection show an increase in gene expression for Ca transporters in the infected plants, but not in response to water deficit alone [8]. Similarly, citrus plants infected with *X. fastidiosa* show upregulation of genes involved in Ca signalling [10]. In addition, Rapicavoli et al. [9] explored grapevine responses to early infection by a wild-type *X. fastidiosa* and *wzy* mutant, and RNAseq analysis showed an upregulation of genes related to Ca sensing and signalling, in the wild type strain, that was absent when infected with the *wzy* mutant, unable to cause disease [9,83]. Interestingly, Ca-related genes are differentially regulated in the two olive cultivars under investigation here (Leccino and Ogliarola salentina) based on the avialble dataset of Giampetruzzi et al [21] (Bioproject NCBI: PRJNA316374) . Initial examination of the deposited data show overexpression of CDPK1 and genes encoding calcium-binding proteins (CML19 and CML29) in infected 'Ogliarola salentina' but not infected Leccino which may suggest that this host is more sensitive to the *X. fastidiosa*-induced Ca changes and this is activating a cascade that results in the development of disease symptoms.

This empirical study of two varieties in the field extends our knowledge about the differential response to *X. fastidiosa* infection. Since this is a newly established pathosystem we lack details about host-pathogen interaction and the mechanisms of resistance to the pathogen. The analysis of the ionome reveals differences between the varieties. Although we did not analyse uninfected trees as they are not present in the fields sampled, we did observe that the 'Leccino' variety has higher Mn levels, and this element regulates numerous pathways that could modulate disease symptoms. It is also notable that additional factors such as age of trees, severity of symptoms, specific agronomical practices in the individual fields could be affecting the ionome composition. To mitigate these factors the analysis is restricted to trees from within the same field suggesting they are subject to the same agronomic practices, and we did not compare to uninfected controls from different regions that would be subject to even more of these confounding factors. This information provides a framework for using the ionome to gain insight into varieties that can be grown that are resistant to disease using both the elemental composition of the plant and modulation of the soil characteristics to help slow the progression of this disease. The ionome characterization showed changes with the progression of the symptoms. In particular, the changes in Ca levels in the 'Leccino' show that the previous observed response in tobacco, grapes, pecans and blueberries is conserved in this pathosystem.

4. Materials and Methods

4.1 Field survey samples collection

Leaf samples from naturally infected olive trees were collected in late November 2017 from two different olive groves located in the area declared *X. fastidiosa*-infected (Figure 1) [42], in the south of the Apulia region: Field 1 (Gallipoli, Lecce, 40.0233750, 18.0516470), Field 2 (Sannicola, Lecce, 40.124325, 18.045239) (Figure 1). In each field, olive trees grown under the same agronomic regime, and more than 30 years old, were selected for both cultivars used in our study: 'Leccino' and 'Ogliarola salentina' (Table 7). For each tree, five infected asymptomatic branches and five infected symptomatic branches were selected, and mature leaves were detached from the median part of hardwood cuttings (approx. 2-year-old) and collected for analysis (Table 7). Uninfected olive trees could not be retrieved in the area selected for testing, despite the lack of symptoms. Previous sampling and laboratory tests suggested that the infection rate of these fields was close to 100 % [21] (Boscia, unpublished observations).

Table 7. Summary of olive samples analysed by ICP-OES and qPCR.

FIELD	VARIETY	TREES	REPLICATE SAMPLES	TOTAL SAMPLES
Field1	'Leccino'	10	5 inf. symptomatic – 5 inf. asymptomatic	100
	'Ogliarola salentina'	10	5 inf. symptomatic – 5 inf. asymptomatic	100
Field2	'Leccino'	4	5 inf. symptomatic – 5 inf. asymptomatic	40
	'Ogliarola salentina'	4	5 inf. symptomatic – 5 inf. asymptomatic	40
Total		**28**		**280**

Field 1 (Gallipoli, Lecce, 40.0233750, 18.0516470); Field 2 (Sannicola, Lecce, 40.124325, 18.045239).

4.2 DNA extraction and bacterial quantification

Extraction of total DNA from olive samples was performed according to the CTAB protocol reported by Loconsole et al. (2014), using leaf petioles from the same infected asymptomatic and infected symptomatic tissues used for ionome determination. The presence of *X. fastidiosa* within the plant tissues was assessed by quantitative polymerase chain reaction (qPCR) using the protocol previously described by Harper et al.[84], with TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, USA) on a CXF 96™ Real-Time System (BioRad Laboratories, USA). Reactions were performed ~~on~~ 100 ng/µl total DNA~~s~~. Ten biological replicates for each cultivar in Field 1 and four biological replicates for each cultivar in Field 2. A pool of ten leaf petioles representing ten portions of the plant, five infected symptomatic and five infected asymptomatic, was used for bacterial detection and quantification in each plant. Each sample was run in duplicate and the data were averaged.

To estimate the bacterial population within the tissues, we established a standard curve using known concentrations of *X. fastidiosa* DNA. 10-fold serial dilutions of an inactivated bacterial suspension with an initial OD_{600} of 0.5, corresponding to ca. 10^8 CFU/ml, were prepared to obtain dilutions ranging from 10^7 to 10^1 CFU/ml. Each dilution was spiked in homogenized plant tissues of a healthy olive prior to DNA extraction. Bacterial concentration from cvs 'Leccino' and 'Ogliarola salentina' samples was inferred by the standard calibration curve using Cqs from qPCR.

4.3 Ionome characterization

Characterization of ionome of olive leaves was done as previously described [37], with some modifications. Briefly, leaves were prepared for analysis by drying at 65°C overnight. Dried samples were then crushed to a fine powder by mortar and pestle and sampled at 10 mg of dry weight. Samples were digested for 1 hour at 100°C in 200 µl of mineral-free concentrated nitric acid (OPTIMA, Fisher Scientific International Inc., USA). After dilution with ultra-pure, mineral-free water and centrifugation at 13,000 x g to remove any particulates, samples were analysed by Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES 7300 DV, PerkinElmer, USA) with simultaneous measurement of Ca, Cu, iron (Fe), potassium (K), Mg, manganese (Mn), sodium (Na), sulfur (S), phosphorus (P), and Zn. ~~Mineral element~~ concentrations were determined by comparing emission intensities to a standard curve created by certified standards (CertiPrep, SPEX, USA).

To assess the ionome of the two varieties, an analysis of five samples randomly selected from the two fields were used to identify a mineral element that could be used as an internal denominator for data normalization. Variable dry weights (2.5, 5, 10, 15, 20 mg) were analysed and showed a linear response in total elements measured with correlation for individual samples of $R^2 > 0.95$ (data not shown). When combining data from multiple varieties R^2 varied from 0.71 to 0.95 (data not shown). The higher the R^2, the more the element remains unchanged in variety and field. On this basis, the most reliable elements for detection, with a R^2 greater than 0.95, were P and K. Data were normalized based on these internal denominators (P and K concentration) and converted to dry weight based on the linear regression equation (for K $y=3.98x+0.6972$, for P $y=1.63x-0.5249$).

4.4 Soil sampling and physicochemical analysis

Topsoil samples, 10-15 cm in depth, were randomly collected from four spots at each field used for our survey. Spots were chosen as pair replicates sampled in the areas occupied by trees of each of the two olive varieties. To homogenise and completely remove plant debris and stones, the soil was first mixed and sieved through a 2 mm mesh. The determination of standard chemical and physical properties was conducted according to the accredited Italian soil study methods [85,86]. Total nitrogen content was determined by mineralisation and distillation procedure according to the Kjeldahl method. The content of exchangeable Ca, Mg, K and Na ions, removed from the exchange sites with buffered barium chloride ($BaCl_2$) solution at pH 8.2, was determined by flame atomic absorption spectroscopy (FAAS, Shimadzu Japan). Soluble boron (B) was extracted by treating samples with a dilute aqueous solution of calcium chloride ($CaCl_2$) (ratio 2:1). The content of the element was determined by spectrophotometry using the azomethine-H method. The active limestone content was determined by cold reaction with ammonium oxalate and titration with potassium permanganate ($KMnO_4$).

Cation exchange capacity (CEC) was measured with a buffered $BaCl_2$ solution at pH 8.2 and complexometric titration with triethanolamine. Organic carbon was determined by oxidation with potassium dichromate ($K_2Cr_2O_7$), in the presence of sulphuric acid (H_2SO_4), and titration with iron (II) sulphate heptahydrate, according to Walkley-Black method. Assimilable metals, i.e. iron (Fe), manganese (Mn), Cu and Zn, were extracted with a solution of diethylenetriaminepentaacetic acid/$CaCl_2$/triethanolamine at pH 7.3, following the procedure described by Lindsay and Norwell [87]. Assimilable P content was determined by spectrophotometric FAAS measurement using ascorbic acid (Olsen method). Electrical conductivity and pH were directly determined from aqueous soil extracts (1:2.5 w/v).

4.6 Statistical analysis

Statistical analysis was performed with the analysis toolpack of Microsoft Office Excel® 2016 for Windows software (Microsoft Corporation, USA). Pairwise comparisons among numeric data sets were conducted using the two-tailed Student's t-test. Statistical significance was accepted for *P*-values < 0.05. Principal component analysis, heat maps and hierarchical analysis was performed using CLUSTVIS web tool [88] using unit variance scaling and singular value decomposition (SVD) with imputation. ANOVA analysis was performed to assess interaction of field, status and variety with respect to three elements Ca, Mn and Na. Statistical significance was accepted for *P*-values < 0.05.

Author Contributions: GD, PC, LDL, PS, MS conceived the study and experimental design. GD performed the experiments with the assistance of PC. DB carried out field observations and identified the fields used in this study. AG analysed the transcriptomic data. MM assisted sample preparation and performed qPCR assays. GD, PC, LDL conducted data elaboration. GD, PC performed statistical analyses. GD and PC wrote the paper, with the review of LDL, PS. MM accomplished manuscript and figure editing. PS, MS, VNS, DB performed general oversight of the work. All authors read and approved the final manuscript.

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