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Xylella fastidiosa and olive quick decline syndrome (CoDiRO) in Salento (southern Italy): a chemometric ^{1}H NMR-based preliminary study on Ogliarola salentina and Cellina di Nardò cultivars

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Abstract

Background: *Xylella fastidiosa* is a Gram-negative bacterium which lives in the xylem of plants, causing its occlusion and other alterations inducing eventually the death of the infected plants. In Salento, the sub-peninsula in the south-eastern of Apulia Region (southern Italy), the infection of *X. fastidiosa* has been associated with the widespread presence of CoDiRO (complex of parasitic agents that constitute the so-called "olive quick decline syndrome") and currently represents a serious local emergence. The need to adopt specific agronomic measures to contrast the further disease spread has been recently raised. The extensive NMR-based metabolomic approach to study the metabolic effects of CoDiRO on local olive cultivars such as Ogliarola salentina and Cellina di Nardò was used.

Results: In this study, the effects of a CE approved fertilizer containing zinc, copper, and citric acid, known as DENTAMET®, on CoDiRO-exhibiting olive trees infected by *X. fastidiosa* were studied by ^{1}H NMR spectroscopy. The changes in the metabolomic profiles of aqueous extracts obtained from leaves of the two olive cultivars are reported. Upon the DENTAMET® treatments, different and opposite polyphenolic and sugars patterns in the two cultivars, which showed a different incidence and severity of disease before the treatments, were detected.

Conclusions: Differences in the sugars and polyphenols content of treated versus untreated trees could potentially contribute to the syndrome monitoring and might be related to the *X. fastidiosa* presence.

Keywords: *Xylella fastidiosa*, CoDiRO, Olive trees, NMR, Metabolomics, DENTAMET®

Background

Starting from 2010, on the west coast of Salento area (Lecce province, southern Italy), symptoms of the so-called CoDiRO "Olive Quick Decline Syndrome" were observed (i.e., leaf scorching, twig and branch wilting, tree die-back) [1]. Subsequently, the syndrome spread over many hectares of olive trees causing dramatic effects and currently it represents a serious local emergence [2]. *Xylella fastidiosa* is a Gram-negative bacterium member of the *Xanthomonadaceae* (γ-proteobacteria) which colonizes the xylem of host plants and the foreguts of insect vectors [3]. This plant pathogen was recently found associated with the CoDiRO [4, 5]. *X. fastidiosa* infects a wide range of host plants, such as grapevine, almond, blueberry, cherry, peach, coffee, and citrus trees, causing great economic losses mainly in North, Central and South

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America [6]. The pathogen is also known to infect landscape and ornamental trees such as oak, maple, and oleander [4]. The introduction of latently infected ornamental plants from Central America was reported as the venue for the subsequent spread of the pathogen in Salento [7]. The main symptoms associated with infections of *X. fastidiosa* are the marginal and or apical leaf scorching, twig and branch die-back and plant death [3]. The symptoms have been attributed to prolonged water stress caused by bacterium growth and biofilm formation in the xylem vessels [8]. The negative socioeconomic impact of *X. fastidiosa* infection in North and South America (i.e., USA and Brazil) and now in Italy is well known. In the latter case, the negative impact is due not only to relevant economic losses, but also to the dramatic effect on the typical cultural heritage represented by olive trees. Apulian 1000-year-old olive trees are protected as local patrimony and considered a symbol of the local identity, also for their massive presence in the territory. Moreover, six over a number of 60 mln of olive trees were classified as monumental, as resulted from the first olive tree census in the whole Apulia region [9]. For these reasons, when implementing a specific control method in order to contain the further disease spread, it is important to study also its social and cultural acceptability as well as its socioeconomic impact [10]. Therefore, the need to adopt specific agronomic and phytosanitary measures to improve the vegetative state of the plants has been recently raised. For these reasons, the study of the CoDiRO effects on symptomatic olive trees by using the NMR-based metabolomics approach was carried out. In this study, we analyzed the effects of a CE approved fertilizer, known as DENTAMET® (i.e., a mixture of zinc and copper complex with hydracids of citric acid [11]) sprayed on symptomatic olive trees located in Salento. This fertilizer can be considered as a product with a dual action formulation by which the correction of Zn and Cu deficiencies occurs very quickly, as well as a resistance is induced by the cyclic peptide resembling the chemical structure of several antimicrobial substances released from the plant in response to different stresses. Thanks to the low environmental impact and few restrictions on its use, DENTAMET® is allowed for organic farming and commercialized in more than 30 countries in the world [11].

The changes in the metabolomic profiles of aqueous extracts obtained from leaves of Ogliarola salentina and Cellina di Nardò olive cultivars assessed by ^{1}H NMR spectroscopy and MVA are reported.

Methods

Sample collection and treatment procedures

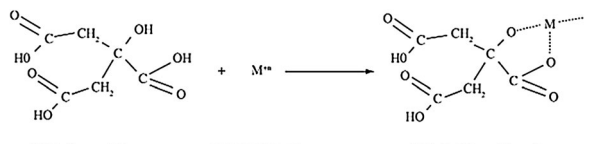
All samples were obtained by collecting, in mid June 2015, leaves from olive trees, strictly located in the same pedoclimatic areas, which were previously treated with DENTAMET® (i.e., two treatments in April, followed by one in May). This foliar fertilizer based on zinc (4.0% w/w) and copper (2.0% w/w) salt is obtained by an electrolytic process, and then complexed with hydracid of citric acid obtained through a process of fermentation similar to those who occurs in nature by certain soil fungi, [11] (Scheme 1). Although the reported metal ion binding of the citrate molecule in DENTAMET® is according to Scheme 1, the possible presence of other coordination mode and/or solvolytic species in the patented commercial product could not be excluded.

In addition, leaves from olive trees showing symptoms of CoDiRO (i.e., twig wilting and branch die-back) with any kind of control measures were collected and served as control samples (i.e., untreated).

A total of 55 leaf samples, each one containing 15–20 apparently healthy, mature leaves, were collected from three different CoDiRO-exhibiting olive tree orchards, located in the districts of Veglie, Galatina, and Galatone (Lecce province, Salento peninsula), where the presence of *X. fastidiosa* was previously ascertained by PCR detection. Briefly, the plants were tested for the bacterium presence using 5 µL of DNA template obtained after extraction with Qiagen kit from 1 g of leaf midribs in PCR with primers and under conditions reported in literature [12]. In the district of Veglie, a total of 16 samples (eight from two treated olive cv. Ogliarola salentina and eight from two untreated cv. Ogliarola salentina trees) were collected. In Galatina district, a total of 19 samples (eight from two treated and eleven from four untreated trees) of olive cv. Cellina di Nardò were processed, while in Galatone district 20 samples (ten from treated and ten from untreated trees) were employed from olive cv. Cellina di Nardò. Where possible, the leaf samples were collected from the four cardinal points in each plant, and, in some cases, from the sucker in the lower part of the plant (Table 1).

Sample preparation for NMR analysis

Olive trees leaves were plunged into liquid N_2 before freeze drying and ground to a fine powder with a stainless steel blender. Freeze-dried plant material (15 mg) was weighted into an autoclaved 2 mL Eppendorf tube.



Scheme 1 "Metal-hydracid" complexation scheme of foliar fertilizer based on Zn and Cu salt

Table 1 Summary of the experimental trials with DENTAMET® Diagro

Farm	La Duchessa	Cosimo Pinca	Cosimo Pinca
Location	Veglie (LE), C.da Duchessa	Galatone (LE), C.da 3 Pietre	Galatina (LE)
GPS point	N 40°20′50.31″ E 17°54′24.55″	N 40°7′22.92″ E 18°1′27.91″	N 40°9′55.96″ E 18° 6,48.26″
Crop	*Olea europea* cv. Ogliarola salentina	*Olea europea* cv. Cellina di Nardò	*Olea europea* cv. Cellina di Nardò
Age of plants	~70 years old	~60 years old	~60 years old
Plant distance	~10 m × 10 m	~10 m × 10 m	~10 m × 10 m
Dose of product and time of spray treatment	5 kg/ha (3.9 L of product) for foliar application by atomizer in April (2) and May	5 kg/ha (3.9 L of product) for foliar application by atomizer in April (2) and May	5 kg/ha (3.9 L of product) for foliar application by atomizer in April (2) and May
Plot area (blocks of trees)	20 treated and 20 untreated trees	15 treated and 15 untreated trees	20 treated and 20 untreated trees
Mean incidence and severity of disease (as recorded before the treatment)	20% of trees showing symptoms with a severity of 10%	50% of trees showing symptoms with a severity of 20%	60% of trees showing symptoms with a severity of 20%

All the plant material was made inert by chemical and physical processes, including 200 °C overnight treatment, according to standard procedures

A D_2O:CD_3OD (1 mL, 80:20) mixture containing 0.05% w/v TSP-*d*4 (sodium salt of trimethylsilylpropionic acid) was added to each sample. The contents of the tube were mixed thoroughly with vortex mixer and then heated at 50 °C in a water bath for 10 min. After cooling at room temperature, the samples were spun down in a microcentrifuge at 10,000*g* for 5 min; then, 700 µL of the supernatant were filled into a 5 mm NMR tube.

NMR spectroscopy and data processing

All measurements were performed on a Bruker Avance III 600 Ascend NMR spectrometer (Bruker, Germany) operating at 600.13 MHz for ^{1}H observation, equipped with a *z* axis gradient coil and automatic tuning-matching (ATM). Experiments were run at 300 K in automation mode after loading individual samples on a Bruker Automatic Sample Changer, interfaced with the software IconNMR (Bruker). For each sample, a one-dimensional ZGPR and NOESY experiment (referred to as 1D-NOESY), including water signal saturation during relaxation, mixing time, and a spoil gradient, was performed. All spectra were referenced to the TSP signal ($\delta = 0.00$ ppm). NMR data were processed using TopSpin 2.1 (Bruker) and visually inspected using Amix 3.9.13 (Bruker, BioSpin). ^{1}H-NMR spectra were segmented in rectangular bucket (0.04 ppm width) and integrated. The data table generated with all the spectra was submitted to multivariate statistical analysis, using Simca-P version 14 (Umetrics, Sweden).

Chemometric data analysis

Multivariate analyses were applied to mean-centered data. The Pareto scaling method, which is performed by dividing the mean-centered data by the square root of the standard deviation, was then applied to the variables (the bucket-reduced NMR spectra). Unsupervised (principal component analysis, PCA) and supervised (partial least squares discriminant analysis, PLS-DA and orthogonal partial least squares discriminant analysis, OPLS-DA) pattern recognition methods were performed to examine the intrinsic variation in the data.

Principal component analysis is the mostly used unsupervised dimensionality reduction method to get an overview of the multivariate profiles and for identifying patterns in data. PLS-DA and OPLS-DA were used for the discrimination of samples with different characteristics (such as cultivars and/or geographical origin) as shown in several recent studies of metabolomics [13–16]. The robustness and predictive ability of the statistical models for discrimination purposes were tested by cross-validation default method (7-fold) and further evaluated with permutation test (400 permutations) of SIMCA 14 software, Umetrics, Umea, Sweden [17, 18]. The R^2 (cum) and Q^2 (cum) are the two parameters that describe the goodness of the models. The former (R^2) explains the total variations in the data, whereas the latter (Q^2) is an internal cross validation parameter, which indicates the predictability of the model [19].

Results and discussion

Multivariate statistical analyses on NMR data

Typical 600 MHz ^{1}H NMR spectra for treated and untreated aqueous extracts obtained from olive leaves are reported in Fig. 1. Sugars and organic acids characterized the alkyl and hydroxyl-alkyl region (middle and low frequencies, from 5.5 to 0.5 ppm), whereas phenolic compounds are typical for the aromatic region (high frequencies, 9.0–6.0 ppm). Relevant ^{1}H NMR data are reported in Table 2. The metabolites were assigned on

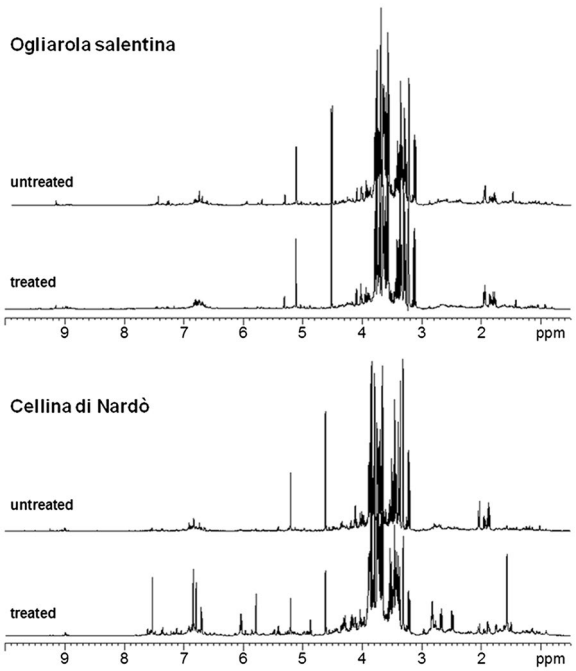


Fig. 1 600 MHz ^{1}H-NMR spectra of the aqueous extracts from the leaves of Ogliarola salentina and Cellina di Nardò olive trees (DENTAMET® treated and untreated)

Table 2 Chemical shifts (*δ*) and assignment of relevant metabolite resonances in the ^{1}H NMR spectrum of *X. fastidiosa* leaves extracts

Metabolites	*δ* (ppm)
Alanine	1.48 (d)
Choline	3.20 (s)
α-Glucose	5.22 (d), 3.50 (dd)
β-Glucose	4.62 (d), 3.22 (t)
Sucrose	5.41 (d), 3.53 (dd)
Hydroxytyrosol	6.90 (d), 6.81 (d), 6.70 (d), 3.78 (t), 2.76 (t)
Tyrosol	6.84 (d), 6.70 (d), 3.78 (t), 2.76 (t)
Oleuropein	6.04 (q), 5.78 (s), 3.89 (sugar protons), 2.68 (d), 2.5 (dd), 1.55 (d)
Other aldehydic and dialdehydic forms of oleuropein and ligstroside	9.22 (s) 9.18 (s), 6.04 (d), 5.74

A typical 600 MHz ^{1}H NMR spectrum with the assignment of the metabolite peaks was reported in Additional file 1: Figure S1

Letters in parentheses indicate the peak multiplicities

s singlet, *d* doublet, *t* triplet, *dd* doublet of doublet, *q* quartet

the basis of 2D NMR spectra analysis (2D ^{1}H *J*res, ^{1}H COSY, ^{1}H–^{13}C HSQC, and HMBC) and by comparison with published data [20–22].

In order to reveal a possible general data grouping of the samples, an unsupervised PCA analysis was applied considering separately the untreated and the DENTAMET® treated samples of the two classes, Ogliarola salentina and Cellina di Nardò cultivars (Fig. 2). The first PCA model is built with all the untreated Ogliarola salentina and Cellina di Nardò samples (Fig. 2a). In this model, the first two components give $R^2 = 0.71$ and $Q^2 = 0.55$, $t[1]$ and $t[2]$ accounting for 56 and 15% of the explained variance, respectively. The PCA model of Fig. 2b is built with all the treated Ogliarola salentina and Cellina di Nardò samples, and the first two components give $R^2 = 0.71$ and $Q^2 = 0.59$, describing the samples distribution in the bidimensional space defined by $t[1]$ and $t[2]$ (in this case accounting for 53 and 18% of the explained variance, respectively). Analysis of the PCA ($t[1]/t[2]$) score plots showed that only in the case of samples treated with DENTAMET®, a clear partition of data was observed with a good grouping according to the original cultivar (Ogliarola salentina and Cellina di Nardò). On the other hand, when all the CoDiRO-exhibiting plants (the untreated samples) were submitted to PCA analysis, no differences of metabolomic profiles appeared among samples. These results suggest that the effect of the presence of a pathogen, such as *X. fastidiosa* on the metabolic profile of CoDiRO symptomatology exhibiting plants samples, could be predominant with respect to the differences normally observed among the olive cultivars. Nevertheless, the presence of other external factors, abiotic or biotic, responsible for the lack of discrimination observed in PCA score plot (Fig. 2a) could not be excluded. On the other hand, the further observed discrimination after the treatment strongly suggests that CoDiRO complex could be responsible for the metabolic uniformity observed in Fig. 2a.

All the CoDiRO-exhibiting plants (considering at the same time treated and untreated samples and the two cultivars, Ogliarola salentina and Cellina di Nardò) have been studied by unsupervised PCA and supervised OPLS-DA analyses. The explorative unsupervised method (PCA) used for the whole data did not give clear group separation (data not shown), while the OPLS-DA model based on treated vs. untreated category as discriminating class (Fig. 3) produced a good descriptive but weak predictive model [one predictive and four orthogonal components give R^2X (cum) = 0.82, R^2Y (cum) = 0.60, and Q^2 (cum) = 0.24]. A first level of discrimination was also observed on the basis of the treatment applied to the samples (DENTAMET® treated vs untreated). The study of the variables responsible for the class separation observed in Fig. 3a could be determined by the analysis of the p(corr) in the S-line plot (Fig. 3b). Interestingly, by examining the loadings of the original variables a higher relative content of polyphenols, such as oleuropein and ligstroside and their derivatives (tyrosol

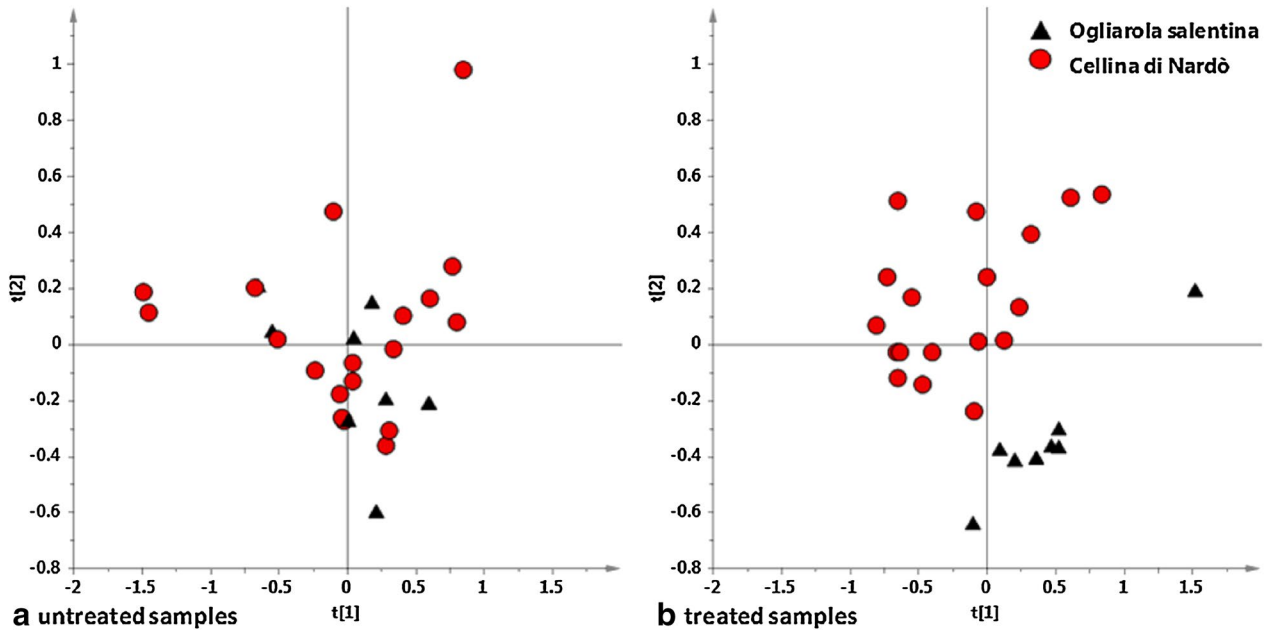


Fig. 2 PCA (*t*[1]/*t*[2]) score plots for cv. Ogliarola salentina (*black triangle*) and cv. Cellina di Nardò (*red circle*) cultivars, considering separately (**a**) the untreated and (**b**) the treated samples

and hydroxytyrosol) was observed for the treated sample. This resulted from the presence of signals in the aromatic region, at frequencies corresponding to tyrosol and hydroxytyrosol (6.84, 6.70, 3.78, 2.76 ppm and 6.90, 6.81, 6.70, 3.78, 2.76 ppm) and oleuropein and its aldehydic derivatives (6.04, 5.78, 3.89, 2.68, 2.5, 1.55 ppm and 9.22, 9.18, 6.04, 5.74 ppm). On the other hand, a higher relative content of sugars was observed for the untreated samples. This resulted from the presence of signals of anomeric protons of α- and β-glucose (doublets at 5.22 and 4.62 ppm, respectively) [20–22].

In order to deeply analyze the response of the CoDiRO-exhibiting plants to the treatment, the metabolic profile of treated and untreated plants was better characterized for each cultivar. In the first case, the unsupervised PCA analysis, applied to Cellina di Nardò samples resulted in no data clustering observation for the first two components, PC1 and PC2. Indeed, inspection of further components other than the first two was required (PC2 vs. PC4), in order to observe in the scoreplot a certain degree of samples clustering (see Additional file 1: Figure S2). Therefore, the supervised OPLS-DA analysis gave a good model [1 + 2 + 0, R^2X (cum) = 0.725, R^2Y (cum) = 0.728 and Q^2 (cum) = 0.53] with a clear partition between DENTAMET® treated and untreated samples (Fig. 4a). By examining the loadings of the original variables, the molecular components distinctive for each class could be determined. CoDiRO-exhibiting samples showed a lower polyphenol content for untreated with respect to treated samples. Interestingly, a relatively higher polyphenol content (with respect to other Salento cultivars) was observed for the for Cellina di Nardò EVOOs samples originating from healthy trees [23]. In the case of Ogliarola salentina samples, the chemometric analysis of a matrix composed by a reduced number of ^{1}H spectra showed a clear partition between DENTAMET® treated and untreated samples, as reported in the OPLS-DA score plot (Fig. 5a). The unsupervised method (PCA) gave unclear results (see Additional file 1: Figure S3), while the OPLS-DA analysis gave a good model [1 + 2 + 0, R^2X (cum) = 0.786, R^2Y (cum) = 0.837 and Q^2 (cum) = 0.489], showing a clear partition between DENTAMET® treated and untreated samples along the first predictive component (Fig. 5a). By examining the loadings of the original variables in the S-line plot (Fig. 5b), the molecular components distinctive for each class could be defined. In particular, infected Ogliarola salentina untreated plants showed a metabolic profile characterized by a higher content of polyphenol molecules. On the other hand, the DENTAMET®-treated infected plants were characterized by a higher sugar content. In this case, the observed polyphenols decrease, in treated with respect to control trees, is in accord with the polyphenols production associated to drought stress [24, 25], notwithstanding the levels of phenols are characteristic for each cultivar such as abiotic stress responses [26–28]. Interestingly, when unsupervised exploratory method (PCA) was applied a good grouping according

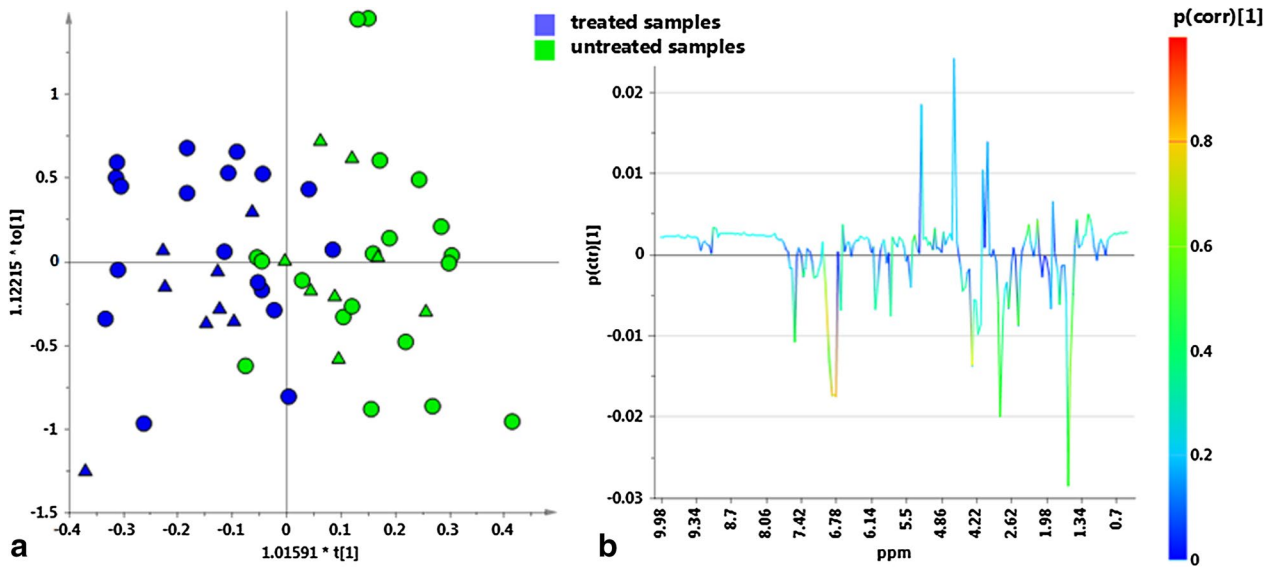


Fig. 3 **a** OPLS-DA *t*[1]/*t*[2] scoreplot cv. Ogliarola salentina (*triangle*) and cv. Cellina di Nardò (*circle*), (one predictive and four orthogonal components give [R^2X (cum) = 0.82, R^2Y (cum) = 0.60 and Q^2 (cum) = 0.24]. **b** S-line plot for the model displaying the predictive loadings colored according to the correlation scaled loading [*p*(corr)]

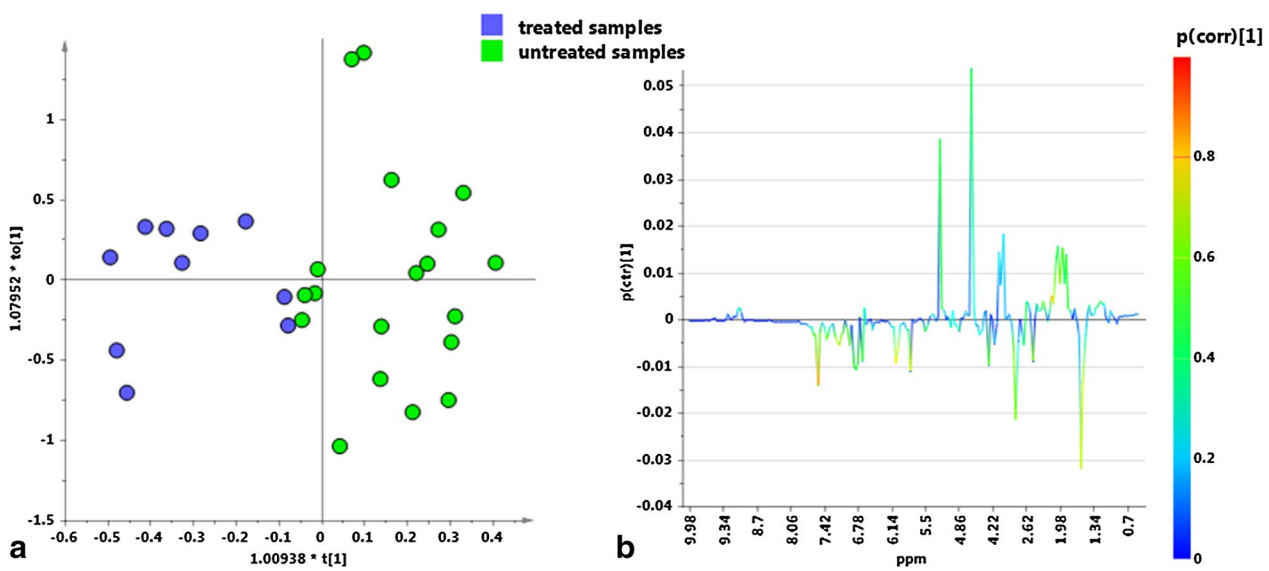


Fig. 4 **a** OPLS-DA *t*[1]/*t*[2] scoreplot cv. Cellina di Nardò (one predictive and two orthogonal components) give [R^2X (cum) = 0.725, R^2Y (cum) = 0.728 and Q^2 (cum) = 0.53]. **b** S-line plot for the model displaying the predictive loadings colored according to the correlation scaled loading [*p*(corr)]

to the original cultivar (Ogliarola salentina and Cellina di Nardò) resulted only in the case of DENTAMET®-treated samples while the differences normally observed according to the olive cultivars were not predominant in the case of untreated CoDiRO-exhibiting plants. Considering all the CoDiRO-exhibiting samples (obtained from both the Ogliarola salentina and Cellina di Nardò cultivars), supervised methods (in particular OPLS-DA analysis) were required to obtain the discrimination between untreated and DENTAMET®-treated samples. The different olive cultivars would seem to differently respond to the DENTAMET® treatments by altering their metabolic profiles in the sugars and polyphenols content. In particular, OPLS-DA analyses revealed that Cellina di Nardò

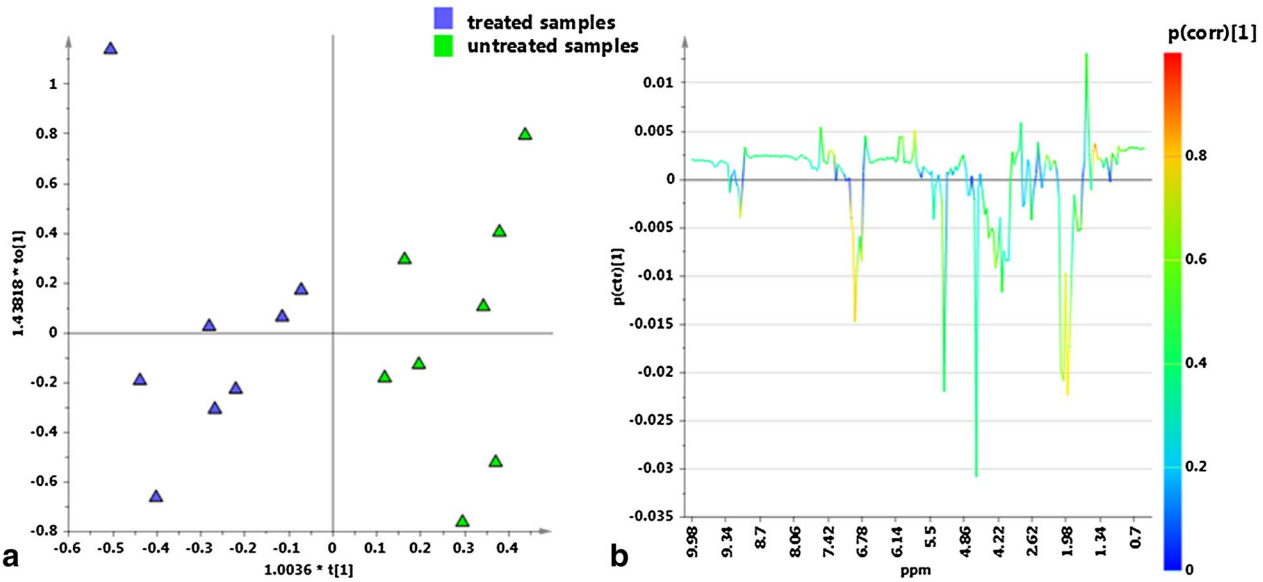


Fig. 5 **a** OPLS-DA *t*[1]/*t*[2] scoreplot cv. Ogliarola salentina (one predictive and two orthogonal components) give [R^2X (cum) = 0.786, R^2Y (cum) = 0.837 and Q^2 (cum) = 0.489]. **b** S-line plot for the model displaying the predictive loadings colored according to the correlation scaled loading [*p*(corr)]

CoDiRO samples showed a lower polyphenols and a higher sugar content for the untreated with respect to the treated ones. In contrast, in the case of Ogliarola salentina CoDiRO-exhibiting trees, DENTAMET®-untreated samples showed a higher content of polyphenol molecules while samples from treated infected plants were characterized by a higher sugar content. As already reported in literature [24, 25], physiological and biochemical responses to stress are closely cultivar-dependent. In the present case, since studied Ogliarola salentina and Cellina di Nardò cultivar trees were also characterized by a different level of pathogen attack, the observed changes in metabolic profiles due to DENTAMET® treatment could be also related to such a specific factor. Moreover in plants, both polyphenols and carbohydrates can play a relevant role during the pathogen infection. In fact, the downregulation of polyphenol-oxidase expression dramatically increased the susceptibility of tomato plants to *Pseudomonas syringae* pv. *tomato* [29]. In olive, polyphenols could also play a direct and significant role in protecting the tree towards pathogen infections. In particular, oleuropein, as extracted from olive waste water, has been shown to be effective towards *Pseudomonas savastanoi* pv. *savastanoi*, the causal agent of olive knot disease, by inhibiting its growth [30]. Also carbohydrate activation can be related to a number of stress that can disturb or subvert to normal plant metabolism such as a wound or a pathogen attack [31, 32]. It should be said, however, that the interplay occurring between polyphenols and carbohydrates during a pathogen infection has not been studied in detail. The metabolomic approach here preliminary applied to olive trees showing the CoDiRO symptoms could shed light into their interrelationships upon a pathogen attack.

Conclusions

In the present work, non-targeted ^{1}H NMR fingerprinting, in combination with unsupervised (PCA) and supervised pattern recognition techniques (in particular OPLS-DA), was used to analyze the response of the CoDiRO-exhibiting olive trees cvs Ogliarola salentina and Cellina di Nardò, grown in the Salento peninsula, to the DENTAMET® treatments. In both cultivars, the occurrence of *X. fastidiosa* was ascertained. Metabolic profiles obtained by ^{1}H NMR spectra were able to differentiate the two cultivars for treated with respect to untreated samples. On the other hand, the effect of DENTAMET® treatment resulted specific and different for each of the two studied cultivars. In particular, treated Cellina di Nardò trees showed a higher polyphenols content, whereas treated Ogliarola salentina trees showed a higher sugar content. It should be stressed that a different incidence and severity of disease was observed for the Cellina di Nardò with respect to the Ogliarola salentina CoDiRO-exhibiting trees, with the former showing a more severe infection. Therefore, further research is needed to determine if the different metabolic responses are correlated to olive cultivars or to pathogen attack

levels or both factors. In particular, in order to more precisely assess the relationships between *X. fastidiosa* infection and the polyphenol and carbohydrates trend upon the infection, future studies can be performed by using precise dose(s) of bacterial inoculum to be inoculated in pot-cultivated olive plants. Then the relative content of polyphenols and carbohydrates into the leaves together with the multiplication trend of the pathogen could be monitored during the seasons by the metabolomic approach applied here.

Additional file

Additional file 1: Figure S1. Typical 600 MHz ^{1}H NMR spectrum of olive leaf extracts. Diagnostic peaks of some metabolites are indicated in the two expansions of the spectrum. **Figure S2.** PCA (a) *t*[1]/*t*[2] and (b) *t*[2]/*t*[4] score plots for Cellina di Nardò cultivars, considering DENTAMET® treated and the untreated samples [six components, R^2X (cum) = 0.935, Q^2 (cum) = 0.816]. **Figure S3.** PCA (a) *t*[1]/*t*[2] and (b) *t*[2]/*t*[4] score plots for Ogliarola Salentina cultivars, considering DENTAMET® treated and the untreated samples [six components, R^2X (cum) = 0.964, Q^2 (cum) = 0.815].

Abbreviations

CoDiRO: Olive Quick Decline Syndrome; CE: European community; MVA: multivariate statistical analysis; PCA: principal component analysis; OPLS-DA: orthogonal partial least squares discriminant analysis.

Authors' contributions

CRG and LDC: prepared the samples for NMR analysis, performed the NMR experiments, analyzed and interpreted the NMR and statistical data, wrote and reviewed drafts of the manuscript, prepared the final writing; MS collected leaf samples, reviewed drafts of the paper, and contributed to the final writing; MP, LZ, FM collected leaf samples and performed the PCR analysis; GC collected leaf samples; AB reviewed drafts of the paper and contributed to the final writing; GD and NC collected leaf samples and performed the PCR analysis; DM contributed to prepare the samples and treated the plant material; FPF supervised NMR experiments and statistical analysis, wrote and reviewed drafts of the paper and contributed to the final writing. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Additional Data attached in the Additional file 1. Other data and materials could be requested from the corresponding author.

Consent for publication

The authors agreed the publication of the manuscript in this journal.

Ethics approval and consent to participate

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