Differential olive grove management regulates the levels of primary metabolites in xylem sap

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Abstract

Aims The conventional management adopted in many Mediterranean olive orchards makes them more vulnerable to climate change and attacks by pathogens, due to the decreased chemical plant defenses. In this scenario, a metabolomic analysis was carried out on the xylem sap (X_{sap}) of olive plants (*Olea europaea* L.) grown in the Salento peninsula (Italy).

Methods Trials were carried out in two olive groves, one organically and one conventionally managed (controls), successively both converted to sustainable management (i.e. frequent light pruning, soil and foliar fertilization, cover crops). The X_{sap} was extracted from the shoots of olive plants using a Scholander pressure chamber pressurized with N_2 and gas chromatography-mass spectrometry metabolite profiling was performed in the X_{sap}.

Results An untargeted gas chromatography mass spectrometry (GC-MS) based metabolomic analysis of primary metabolites (including underivatized volatiles) of the X_{sap} revealed relative abundances of 153 identified metabolites and 336 unknown features across the 12 samples from four groups of samples. Among them, more than half were involved in the primary metabolism. Many of the compounds with increased levels under sustainable management (such as amino acids, soluble sugars, sugar alcohols) have a well-known role as osmoprotectants or are involved in plant defense, growth and development during stress or recovery stages.

Conclusions Sustainable management in olive groves can increase the ability of plants to overcome environmental stressors and enhance ecosystem balance.

Keywords: metabolomic profiling; olive xylem sap; plant defence; plant-soil interactions; sustainable soil management.

Abbreviations: GC/MS = gas chromatography/mass spectrometry; PCA = principal component analysis; S_{ctrl} = Squinzano control plot; S_{sust} = Squinzano sustainable plot; V_{ctrl} = Vernole control plot; V_{sust} = Vernole sustainable plot; X_{sap} = xylem sap.

Introduction

The olive tree (*Olea europaea* L.) represents the main crop in the Salento peninsula (Puglia region, southern Italy), which is ranked first in terms of areas suitable for olive cultivation (379,910 hectares, about 32.6% of the overall areas at the national level) and olive oil production (about 26% of the national production) (Palese et al. 2013; Lardo et al. 2018).

For many years, the Salento landowners widely applied on dry-farmed olive groves an approach based on low-cost management techniques (i.e., minimum tillage of the soil) coupled with chemical weed control or they abandoned them, making olive trees more vulnerable to the effects of climate change and to the attacks by pathogens, as in the case of the pest *Xylella fastidiosa* in the recent years (Castellini et al. 2020). By applying sustainable agronomic practices, such as no-tillage, fertigation and internal C-inputs (spontaneous cover crops and pruning residues), light and annual pruning and/or fertigation with treated wastewater, it is possible to obtain benefits in terms of climate change mitigation and increase of soil organic carbon (Lardo et al. 2018), faster C and N turnover (Pascazio et al. 2018) and improvement of soil structure and water storage (Palese et al. 2014). Many studies highlighted that sustainable management of olive grove could have positive effects on environmental impact (water cycle, soil microbiological fertility, biodiversity, productivity, and product quality) (Palese et al. 2013) and natural defense of plants to biotic and abiotic stresses (Bragard et al. 2019; Sofo et al. 2019a). It was revealed that olive groves sustainably managed present a higher microbial diversity and complexity both in the soil and in the phyllosphere compared to conventional management (Sofo et al., 2014; Pascazio et al., 2015). It was also showed that sustainable management affects the composition of soil bacterial communities, favoring those with physiological and protective functions for the plants (Fausto et al. 2018).

Many studies were focused on the key role of some metabolites in a) plant defense responses and plant-pathogen interactions, b) plant tolerance to environmental stresses, and c) the capacity of plants to cope with nutrient deficiencies (López-Bucio et al. 2000; Bolton 2009; Kangasjärvi et al. 2012). Among the primary metabolites, sugars play an important role in the

process of olive ripening, as they provide energy for metabolic changes and serve as a source for the biosynthesis of fatty acids (Marsilio et al. 2001). Višnjevec et al. (Višnjevec et al. 2018) confirmed that the content of oleuropein, sugars and sugar alcohols in leaves and fruits of olive trees ultimately depend on various factors, and not just on drought stress. Furthermore, 76 metabolites were identified in three different tissues of olive, mostly corresponding to distinct types of primary metabolism, with some of them involved in secondary metabolism pathways (Rao et al. 2017).

Primary metabolism has been investigated in studies on olive fruit and leaves under particular agronomic management or specific stress. Martinelli et al. (2012) tested the effects of irrigation on metabolic changes in olive. Their metabolomic analysis by gas chromatography-mass spectrometry (GC-MS) allowed to identify several hundred metabolites in ripe olive mesocarp, 46 of which showed significantly different contents in the rain-fed and irrigated samples. Some of these compounds, involved in primary metabolism (carbohydrates, amino acids, organic acids), appeared to be more abundant when irrigation was performed. A similar study (Martinelli et al. 2013) examined 57 compounds, among which 19 metabolites (organic acids, fatty acids, soluble sugars and terpenes) accumulated differently in the two sets of the sample (pulp + skin) of ripe olives grown under water-stress and irrigated conditions, applied during the last part of the fruit developmental cycle. A reduction in soluble sugars and unsaturated fatty acids was detected in water-stressed samples, suggesting an acceleration of the ripening process.

Despite the tremendous importance of metabolites for the plant, primary metabolism remains poorly characterized, particularly in the xylem sap (X_{sap}). The importance of X_{sap} lives in the fact that, besides transporting water, nutrients, and metabolites, xylem is also involved in long-distance signaling in response to pathogens, symbionts, and environmental stresses (Xylogiannis et al. 2020). While the characterization of compounds of the primary metabolism in olive leaves and fruit has been examined, what happens in the X_{sap} and how compounds change in response to different management remains still a matter of debate (Sofo et al. 2019a). We hypothesize that sustainable olive grove management could influence the primary

metabolites present in the X_{sap} regulating the levels of those involved in plant defense and abiotic stress tolerance. We adopted a sustainable management in two different olive orchards and, through a metabolomic approach, the metabolic profile and natural metabolite variations in the X_{sap} of olive trees were investigated. The aim of this study is providing new insights into the understanding of compounds that could have an important role in the physiological and developmental processes of olive plants under differential management practices.

Materials and methods

Description of the field sites and their agricultural management

The trial has been carried out from 2017 to 2019 at two olive orchards: an organic olive grove in Squinzano municipality (site 1) and a conventional olive grove in Vernole municipality (site 2), in the Salento Peninsula of Apulia Region (Southern Italy). Both areas have a warm and temperature climate, annual precipitation of approximately 742 mm (mean 2017-2019), a mean annual temperature of 16.9 °C, and a mean annual relative humidity of 0.77%. Details on the two sites are reported in Table 1. Olive trees belonging to 'Ogliarola di Lecce' cultivar, were selected for sampling within each site to be of similar age and to be growing with agronomic practices specific for each management. Over the three years, phytosanitary treatments had been carried out by the farmers, according to EU Decision 2015/789 (http://data.europa.eu/eli/dec_impl/2015/789/oj), including the control of the insect vector of *X. fastidiosa* (*Philaenus spumarius*) and the removal of wild plant hosts.

In site 1 (Squinzano), organic management includes the following practices: light plowing one time a year, weed mowing two times a year, spontaneous cover crops, organic fertilization (compost, 2 q ha^{-1}) once a year distributed on the soil surface. Pruning was not carried out for at least ten years and when the trees used to be pruned, the pruning residues were burned. Pyrethrum and copper were used for pest and disease control following the

recommendations for organic crop production according to EU Regulation 2018/848 (http://data.europa.eu/eli/reg/2018/848/oj) relating to organic production and labeling of organic products.

The site 2 (Vernole) was managed according to conventional agronomic practices: severe pruning carried out every 3-4 years with the removal of pruning residues from the field, harrowing one time a year, weed mowing two times a year, spontaneous cover crops, empirical soil fertilization carried out in winter using nitrogen fertilizer and foliar nitrogen fertilization in summer once per year. Pest management was performed with copper and fly control with traps.

Application of sustainable management protocols

Within each site (Squinzano and Vernole), a 0.5-ha plot with 20 treated plants and 10 control plants was selected. The control plants included the olive trees managed organically (site 1, Squinzano; S_{ctrl}) and conventionally (site 2, Vernole; V_{ctrl}) for site 1 and 2, respectively, whereas the treated plants represented the olive tree subjected to sustainable management (site 1, Squinzano; S_{sust}; and site 2. Vernole, V_{sust}). In each sustainably treated plot (S_{sust} and V_{sust}), a severe pruning was carried out at the beginning of the experimental plan in February 2017. Subsequently, plants were lightly pruned twice a year in winter and summer to get a uniform distribution of light in all parts of the canopy and to facilitate air circulation and prevent the increase in relative humidity. Pruning residues were cut and burned, harrowing and weed mowing were carried out twice per year according to EU Decision 2015/789 to prevent the widespread of the infection, in addition spontaneous vegetation crops were left grown on the ground during the growing season.

Historical information on the nutritional management of plants was also asked to farmers, and soil analyses were carried out in both experimental plots. Considering the results of the soil analyses and the information collected, the fertilization plan was determined for S_{sust} and V_{sust}. Therefore, 30 t ha^{-1} $year^{-1}$ of compost were distributed to the soil of S_{sust} and V_{sust} in winter, and 30 nitrogen units (26% ammonium sulfate) per hectare were distributed once in V_{sust}.

In V_{sust}, it was performed a foliar treatment based on biostimulants Kendal® and Megafol® (Valagro, Atessa, Chieti, Italy) that was distributed with a dose of about 250-300 mL for the first two years, while during the third year Activo Rame (Eno Advance S.r.l., Poggibonsi, Siena, Italy) was used, an amino acid complex (mainly glycine, proline, and alanine) containing polypeptides and 5% (w/w) copper. For pest management the insecticide Decis® (Bayer CropScience S.r.l., Filago, Bergamo, Italy) two times a year and Activo Rame three times a year were applied.

No irrigation was carried out because of the unavailability of wells or nearby water supplies in both plots.

Soil sampling and chemical analyses

At the beginning (February 2017) and at the end of the trial (October 2019), soil samples were collected randomly from 10 different points at 0-30 cm depth (7-cm-diameter cores). After plant debris, roots, and stones were removed, they were mixed thoroughly in a clean pail without sieving to give a composite sample. Three composite soil samples were collected in the field for each experimental plot (n = 3). Each composite sample was divided into two parts, a field-moist sample and an air-dried one. The field-moist samples were refrigerated at 4 °C before biochemical analyses, whereas the air-dried samples were used to determine chemical and physical parameters.

Chemical analyses were carried out following the official methods of DM 13/09/1999 SO n. 185, GU n. 248 21/10/1999. Met II.6 and the enzymatic activities of some enzymes involved in the main biogeochemical cycles, such as acid and alkaline phosphatase (Eivazi and Tabatabai 1977), β-glucosidase (Eivazi and Tabatabai 1988) expressed as μg p-nitrophenol g^{-1} dry soil h^{-1}, and urease (Tabatabai and Bremner 1972) expressed as μg NH_4^+-N g^{-1} dry soil h^{-1}, were measured. The enzyme activities were expressed as units per g of dry soil (units g^{-1} soil).

Plant material and xylem sap extraction

The X_{sap} was collected from shoots of olive trees in October 2019 from three control plants and three treated plants for each plot (n = 3). To avoid border interferences, plants in the central part of each plot and far each other, were randomly chosen.

The X_{sap} was extracted using a Scholander pressure chamber (Model 600, PMS Instruments, Corvallis, OR) pressurized with N_2. Two shoots with a length of approximately 15-20 cm were taken from each of the four cardinal points per plant using sterile cutting shears. The plant material was put in plastic bags, transported to the laboratory, and stored at 4 °C before use. For each shoot, a 1-cm wide bark strip was removed in the proximal part with a sharp knife sterilized with 75% ethanol, to prevent external contamination. The cut end of the stem was placed in the pressure chamber facing out. The foliage of the cutting was placed in the pressure chamber and the lid was locked down. Then, high pressure was applied (approximately from 5.0 to 7.0 MPa, *i.e.* 50-70 bar) to exude the X_{sap} from the tissue at the proximal end of the cutting. After discarding the first drops, 400-500 µL of sap were collected into Eppendorf tubes for 15-20 min per shoot and kept at –80 °C until metabolomic analysis was performed.

Metabolomic analysis

Sample derivatization and GC/MS analysis

Samples extraction, derivatization, and analysis were performed using a modified version of the protocol proposed by (Lisec et al. 2006). In particular, 400 µL of X_{sap}, for each sample and replicate, were collected and immediately lyophilized at −40°C. After lyophilization, the samples were newly suspended in 1.4 mL of methanol (at −20°C) and vortexed for 5 min. Then, 60 µL ribitol (0.2 mg mL^{-1} stock in ddH_2O) were added as internal quantitative standard. Samples were shaken for 10 min at 950 rpm in a thermomixer (at 70 °C) and then centrifuged for 10 min at 11,000 g to avoid the eventual presence of debris. After supernatant collection, 750 µL of $CHCl_3$ (−20 °C) and 1500 µL of ultrapure H_2O (4 °C) were

added. Samples were then emulsified by vortexing the vials for 30 sec and successively centrifuged for 15 min at 2,200 g.

The upper polar phase (300 µL for each sample and replicate) were collected, transferred in a 2 mL vial and dried in a speed vacuum at room temperature. To the dried samples, 40 µL methoxyamine hydrochloride (20 mg mL^{-1} in pyridine) were added. Samples were incubated at 37 °C for 2 h in a thermomixer (950 rpm). After methoxyamation, the samples were silylated by adding 70 µL of MSTFA, and then the mixture incubated at 37 °C for 60 min (950 rpm).

The derivatized samples were injected in a gas chromatograph apparatus (Thermo Fisher Scientific; G-Trace 1310) coupled with a single quadrupole mass spectrometer (Thermo Fisher Scientific, ISQ LT). A MEGA-5MS capillary column (30 m × 0.25 mm × 0.25 µm + 10 m of precolumn) was used. Both injectors and sources were settled at a temperature of 250 °C and 260 °C, respectively. Samples (1 µL) were injected in a splitless mode with a helium flow of 1 mL min^{-1} using the following temperature gradient: isothermal 5 min at 70 °C followed by a 5 °C min^{-1} ramp to 350 °C and a final 5-min heating at 330 °C. Mass spectra were recorded in full scan using a 40-600 m/z range with a scan time of 0.2 sec and a solvent delay settled at 9 min. n-Alkane standards (C8-C40, all even), blank solvents, and pooled samples (quality control - QCs) were injected at scheduled intervals for monitoring instrumental performance, shifts in retention indices (RI) and tentative identification.

GC/MS analysis and data acquisition

Data analysis (raw peaks extraction, baseline filtering and calibration, peak alignment, deconvolution, peak identification, and integration) was carried out using the open-source software MS-DIAL, version 4.16 (Tsugawa et al. 2015). The software parameters for data collection, peak detection, deconvolution, alignment, and filtering were settled as successively reported. A minimum peak height of 1,000 amplitudes was applied for peak detection, and a sigma window value of 0.5, EI spectra cut-off of 10 amplitudes was implemented for

deconvolution. The linear weighted moving average was used as a smoothing method with a smoothing level of 3 scans and an average peak width of 20 scans. The identification settings were settled as following: retention time tolerance = 0.5 min, RI tolerance = 30, m/z tolerance = 0.5 Da. Both the EI similarity cut-off and the identification score cut-off was settled at 70%. In the alignment parameters setting process, the retention time tolerance was 0.075 min, with an EI similarity tolerance of 70%, and both retention time factor and EI similarity factor settled at 0.5.

Data annotation was carried out in MS-DIAL using publicly available libraries. Compounds identification was based on the mass spectral pattern as compared to EI spectral libraries such as the MoNA (Mass Bank of North America, (http://mona.fiehnlab.ucdavis.edu/), the Mass Bank, the MSRI spectral libraries from Golm Metabolome Database (Horai et al. 2010). Metabolite annotation and assignment of the EI-MS spectra were achieved following the guidelines for metabolomics standards initiative for compounds identification, i.e., Level 2: identification was based on spectral database (match factor > 80%) and Level 3: only compound groups were known, e.g. specific ions and RT regions of metabolites (Sansone et al. 2007). Additional annotation of unknown EI-MS features that did not match with the existing spectral libraries were annotated using MS-FINDER version 3.44 (Lai et al., 2018).

Statistical analysis

Experiments were carried out using a randomized design with three replications (n = 3), that is three soil composite samples and three plants for X_{sap} extraction. Means of the values of soil chemical data were separated according to Fisher's LSD test at $p \leq 0.01$.

Metabolomic data were analyzed using the software Metaboanalyst 4.0 (Chong et al., 2019). Metabolomics data were normalized using the internal standard and QCs for LOESS based normalization functions available in the MS-DIAL software for batch correction procedures.

Internal standard normalized dataset, obtained as MS-DIAL output, were log2 normalized and square root transformed. Data were then classified through principal component analysis (PCA), where the output comprised score plots to visualize the contrast between different samples and loading plots to explain the cluster separation. To highlight statistical differences among single metabolites and treatments, data were then analysed through the univariate analysis to yield volcano plots that demonstrated the significantly differential metabolites with a P value ≤0.05 and the following fold-change cut-off: FC >1.2 and FC <0.8.

Raw Data Sharing

The raw datasets and the metadata associated with the GC-MS-based metabolomics efforts are deposited at the Mendeley database (DOI: 10.17632/5yxhmnxmks.1, https://data.mendeley.com/datasets/5yxhmnxmks/1) and are freely available for download from 01 March 2021.

Results

Chemical and enzymatic analyses

The soil management practice and the supply of carbon and energy sources provided by compost to the soil have produced an increase in soil organic C in S_{sust} and V_{sust}, compared to S_{ctrl} and V_{ctrl} (Table 2), and an increase in soil C (more than the double in V_{sust}, compared to V_{ctrl}) (Table 2). The content of soil total N in the treated plots (S_{sust} and V_{sust}) (Table 2) was higher than in the controls (S_{ctrl} and V_{ctrl}). Instead, low values of pH and available-P in S_{sust} and V_{sust} than in S_{ctrl} and V_{ctrl} were found (Table 2). The activity of β-glucosidase was significantly higher in the treated plots (S_{sust} and V_{sust}) compared to the controls (S_{ctrl} and V_{ctrl}) (Table 3). The acid/alkaline phosphatase and urease activities were higher in S_{sust} and V_{sust} than in S_{ctrl} and V_{ctrl} (Table 3).

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308 Metabolomic analysis

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310 Untargeted metabolomic analysis of xylem sap using gas chromatography mass
311 spectrometry (GC-MS) revealed individual and grouped metabolites that discriminated samples

312 Using an untargeted GC-MS based metabolomics approach, we obtained identification
313 and relative abundances of 153 annotated metabolites and 336 unknowns EI-MS features shared
314 between all 4 sample groups. The processed data from MS-DIAL are provided for identified
315 metabolites (Supplementary Table S1) and unknown features (Supplementary Table S2),
316 displaying their retention times, quant mass, signal/ noise (S/N), EI-spectrum, and relative
317 abundances. Further, using MS-FINDER, we tentatively assigned annotations to 41 EI-MS
318 features as well (Supplementary Table S3). A KEGG-based metabolic pathway enrichment
319 analysis revealed enrichment of taurine and hypotaurine metabolism, aminoacyl-tRNA
320 biosynthesis, arginine biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis,
321 galactose metabolism, glutathione metabolism, pentose and glucuronate interconversions, D-
322 glutamine and D-glutamate metabolism, nitrogen metabolism, thiamine metabolism, among
323 others (Supplementary Table S4). Most of these annotated metabolites belonged to flavonoids,
324 and lipids, and mostly plant specialized metabolites. The normalized relative abundances
325 (Supplementary Table S5) were used to calculate the fold changes between control and
326 treatment samples (Supplementary Table S6).

327 A 4-way Venn diagram (Figure 1) revealed 9 metabolites that were shared and
328 significantly (p-value< 0.05) increased for S_{sust} and V_{sust} samples, such as ribose-5-phosphate,
329 trehalose, fumarate, 2-phosophoglycerate, taurine, and others (Supplementary Table S7). Eight
330 metabolites that significantly (p value < 0.05) increased in S_{sust} were ribose, UDP-N-
331 acetylglucosamine, urea, 5,6-dihydrouracil, octadecylglycerol and others. Fourteen metabolites
332 significantly (p-value< 0.05) increased in V_{sust} samples were pantothenic acid, gluconolctone,
333 lactic acid, tyrosine, glutamine, maltotriose, xylulose, cystamine and others. Significantly (p-

value< 0.05) decreased metabolites in V_{sust} included ureidopropionate, urocanic acid, cysteinylglycine, benzoic acid, tryptamine, and others (Supplementary Figure 1)

An ANOVA analysis identified 75 significantly differential metabolites mainly belonged to chemical classes of the amino acids, polyamines, organic acids, sugars, volatiles, and sugar alcohols differentially produced in olive xylem sap of control (S_{ctrl} and V_{ctrl}) and treated (S_{sust} and V_{sust}) plots (Supplementary Table S8). ANOVA analysis revealed significantly differential metabolites belonging to organic acids (succinic acid, alpha-ketoglutaric acid, fumaric acid: that belong to the TCA cycle, and pyruvic acid, lactic acid, 2-phosphoglyceric acid, 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, and glycolic acid), amino acids (asparagine, aspartate, beta-alanine, homocysteine, glutamine, tyrosine, and histidine), and sugars (ribose, xylulose, trehalose, and melibiose) and sugar alcohols (palatinitol) (Figure 2).

Besides, data with a FC < 0.8, FC > 1.2 and a *p* value of 0.05 were presented as volcano plot highlighting the significantly altered metabolites (up or down accumulated) by the field management (Figure 3 and Supplementary Table S6). As shown in Figure 3a in the treatment S_{sus}/S_{ctr} three compounds (isobutyl acetate; tagetone<dihydro-> and 4-hydroxybenzoic acid) were down-accumulated in the X_{sap} as a consequence of soil management, whereas other 21 compounds (trehalose; 2-phosphoglycerate, D-ribose, 5-phosphate, fumaric acid, linalool<tetrahydro->, taurine, encecalin, oxamic acid, nootkatinol, UDP-N-acetylglucosamine, senecioic acid, ribose, cyclohexadecanolide, palatinitol, hexadienol, butanoate<2E,4E->, octadecylglycerol, 5,6-dihydrouracil, urea, hexanal dimethyl acetal) were significantly accumulated.

Concerning the treatment V_{sust}/V_{ctrl}, were reduced (tryptamine; urocanic acid; cysteinylglycine; 2-oxoglutaric acid; palatinitol; benzoic acid; ureidopropionate; hexadienol butanoate<2E,4E->), whereas 23 significantly accumulated (glutamine; 4-hydroxybenzoic acid; xylulose; octane<n->; maltotriose; trehalose; 2-phosphoglycerate; D-ribose 5-phosphate; cystamine; fumaric acid; tyrosine; penten-3-ol<1->; linalool<tetrahydro->; ethyl ether; taurine; encecalin; lavandulyl acetate<tetrahydro->; oxamic acid; gluconolactone; N-formylkynurenine; lactic acid; pantothenic acid; nootkatinol) (Figure 3b and Supplementary Table S6). Principal

Component Analysis (PCA) was carried out on all samples analyzed using GC-MS, such as blanks, pooled quality controls (QCs) and all four sample groups together to demonstrate the system suitability. The PCA score plot, revealed a good discrimination of sample groups against QCs and blanks (Supplementary Figure 2). Similarly, a Pearson correlation analysis among samples based on the relative metabolite abundances revealed clustering of samples within the group (Supplementary Figure 3).

Both unsupervised PCA run on identified metabolites (Supplementary Figure 4a) and unassigned/unidentified features (Supplementary Figure 4b) revealed discrimination of sample groups. Further, both supervised PCA analysis (Figure 4a) and unsupervised partial least squares discriminant analysis (PLS-DA) conducted on annotated metabolites (Figure 4b) demonstrated group separation with the first 2 principal components (PCs) explaining 63.4% variance for PCA and 53.6% variance in PLS-DA score plots. PLS-DA derived variable importance of projection (VIP) scores revealed 3-nitro-tyrosine, 2-phosophoglycerate, Ribose-5-phosphate, octadecylglycerol as the ones with the highest VIP scores for the four sample groups (Figure 4c). A random forest analysis revealed octadecylglycerol, glutamine, farnesol, lavandulyl acetate with the highest mean decrease accuracy for the four sample groups (Figure 4d).

A hierarchical clustering analysis (HCA) run on the samples of both identified and unknown/unassigned metabolites reveals clustering of the sample groups and the pooled QCs (Supplementary Figure 4c,d and Supplementary Table S9).

Discussions

Soil management practices

The weed control, fertilization, frequent pruning, and pruning residues management have influenced the ecosystem of both olive groves managed organically (site 1) and conventionally

(site 2). The balanced and rational nutrition provided by treated organic material recycling and additional fertilizers to the experimental plots has induced effective olive trees' effective responses. The chemical properties of soils subjected to sustainable management for three years were significantly improved (Tables 2 and 3). Similarly, Ebabu et al. (2020) found that after three years of sustainable land management practices in three contrasting agro-ecological zones, most soil parameters (such as bulk density, soil organic carbon, total nitrogen, available phosphorus, and potassium) were optimal for supporting plant production.

Comparing the control plots (S_{ctrl} and V_{ctrl}) to the treated plots (S_{sust} and V_{sust}) (Table 2) we confirm that the adoption of sustainable management (aimed mainly at increasing the C inputs) can favour an increase of soil C stock (Fiore et al. 2018). In our study, three years of sustainable management were enough to reveal an increase in soil C (Table 2) , showing that soil chemical properties can be at least partially recovered also after disturbances over a longer time (García-Gil et al. 2000). A conceivable explanation of N content differences between treated (S_{sust} and V_{sust}) and control plots (S_{ctrl} and V_{ctrl}) (Table 2) is a higher activity of the microbial nitrifying population, which is affected by different fertilizer applications (Chao et al. 1996).

The lower values of pH and available-P in treated (S_{sust} and V_{sust}) than in control (S_{ctrl} and V_{ctrl}) plots (Table 2) could mainly be because of the continuous cropping system and to the long term use of mineral nitrogen fertilizers, which substantially decrease the amounts of exchangeable base cations (mostly Ca^{2+} and Mg^{2+}) but on the other side, they increase the H^{+} concentration (Schroder et al. 2011).

Some soil management practices, such as no-tillage or reduction in tillage frequency, increase the activity of β-glucosidase because of improvement in microbial biomass, more substrate availability, and reduced soil disturbance (Sofo et al. 2014). The high β-glucosidase activity occurring in S_{sust} and V_{sust}, compared to S_{ctrl} and V_{ctrl} (Table 3) was significantly related to the increase of soil organic carbon (SOC) (Table 2). The activity of this enzyme has been found to be higher in fertilization treatments with compost than in those without compost, as well as those with synthetic fertilizers and herbicides (Crecchio et al. 2004; Meyer et al. 2015).

Low levels of soil P and pH influence the production of phosphatases in the ecosystem (Acosta-Martínez and Tabatabai 2000). In our analysis, a decrease in pH in S_{sust} and V_{sust} (Table 2) corresponded to a rise of acid phosphatase activity (see S_{sust} and V_{sust}; Table 3). The increase of acid phosphatase is more marked in the V_{sust}, compared to S_{sust}, likely because the difference between the two plots (organic vs sustainable and conventional vs sustainable) was more defined. The increase in phosphatase activity (both acid and alkaline) in soils amended with organic materials (as in S_{sust} and V_{sust}; Table 3) can be attributed to the stimulation of microbial growth and soil organic matter enrichment (Adetunji et al. 2017).

Urease expression is under N regulation, and its production is activated in the presence of urea or alternative N sources (Mobley et al. 1995). The increase of urease activity in S_{sust} and V_{sust} (Table 3) were related to the higher soil total N in sustainable plots compared to controls (Table 2).

Metabolomic profile in olive xylem sap under different management

The established role of X_{sap} is to transfer and distribute, through the xylem vessels, the minerals, and water absorbed by roots to the aerial parts of the plant (Shi et al. 2002). In the past, X_{sap} was thought to be mainly composed of inorganic compounds and water, but relatively recent studies highlighted that X_{sap} also contains a wide range of small, water-soluble, organic substances. These include many primary metabolites, such as polyols and simple sugars, amino acids, organic acids, plant hormones, and secondary metabolites, which play a pivotal role in plant growth and resistance to stresses (Lowe-Power et al. 2018; Sofo et al. 2019b). Several studies recently showed that X_{sap} composition could be influenced by a large variety of factors, such as changes in climatic conditions, biotic issues, and changes in crop management (Sofo et al. 2019a).

In our study, to get more insights into X_{sap} metabolic changes in olive groves under different management in the two sites, a GC/MS-driven untargeted-metabolomic analysis was carried out.

The multivariate analysis, carried out on both annotated and unknown compounds, pointed out a clear separation among all groups (S_{ctrl}, S_{sust}, V_{ctrl}, and V_{sust}), suggesting that either the location or soil management had a significant influence on xylem sap composition. Also, the univariate analysis allowed to identify those metabolites significantly changed in response to the treatments.

As reported in the Venn diagram, and supported by the ANOVA analysis, in the xylem sap of both organically managed fields have been identified 9 common metabolites significantly up-regulated by the treatment. In particular, has been observed an up-regulation of trehalose, fumaric acid, taurine, encecalin, linalool and nootkatinol, which are known mainly to play a pivotal role in protecting plants from both biotic and abiotic stress.

The osmoprotectants and reactive oxygen species (ROS) scavenger role of trehalose has largely been described (Kosar et al., 2019). Kaplan et al. (2004) reported that in plants exposed for few hours to heat stress, trehalose content was increasing two times, whereas in chilled plants, it was eightfold higher than control after a few days of exposition to stress. A microarray analysis pointed out that during abiotic stresses (e.g., cold, UV, salinity) most of the genes involved in trehalose metabolism were significantly activated, supporting the hypothesis that trehalose levels change in response to abiotic environmental fluctuations (Iordachescu and Imai, 2008). As well as trehalose, also taurine has been shown to act as a ROS scavenger since it has been reported that this amino acid promotes plant growth and development, improves the efficiency of the photosynthetic machinery, and protects cell membranes from lipid peroxidation (Hao et al., 2004).

In the X_{sap} of plants, cropped through sustainable management, was also observed an increment in pyruvate and fumaric acid (two Krebbs cycle intermediates), which could be connected to the increment in 2-phosphoglyceric acid. In fact, this metabolite serves as the substrate, during glycolysis, for the con conversion of glucose to pyruvate. The increase in both fumaric acid and pyruvate could be extremely useful for plants during hypothetical stress. In fact, both metabolites are metabolized under stresses to generate energy and carbon skeletons to produce other compounds (Rhodes et al., 1986; Chia et al., 2000). In particular, fumaric acid

could help to maintain cellular pH and turgor pressure and be metabolically accessible as a transient storage form of fixed carbon (Fernie and Martinoia, 2009). Similarly, it has been reported that high content in pyruvate is important in helping plants during water stress (Rhodes et al., 1986).

Concerning the three specialized metabolites, the chromene encecalin, the tropolone nootkatinol, and the terpenoid linalool, several reports showed that these metabolites have antimicrobial, insecticidal, antifungal, and broad antimicrobial activity (Isman and Proksch, 1985; Saniewski et al., 2007; Herman et al., 2016). Encecalin is a chromene characterized by antifeedant and insecticidal properties reducing larval growth and decreasing survivorship of neonate larvae (Isman and Proksch, 1985), whereas linalool is largely known for its repellent activity versus various insect species (Lawal et al., 2014; Pajaro-Castro et al., 2017).

The presence of these three secondary metabolites is extremely interesting in olive trees. In the last years in Apulia, olive has been strongly attacked by a xylematic bacteria (*Xylella fastidiosa*), which is decimating the population of centuries-old olive trees. This pathogen is spread through different insect vectors belonging to the family Aphrophoridae (*Philaenus spumarius, Philaenus italosignus, Neophilaenus campestris,* among others) (Saponari et al., 2019). Therefore, we could speculate that encecalin, nootkatinol and linalool could increase sustainably managed plants' ability to cope with this biotic stress.

Since xylem vessels are formed by non-metabolically active cells, it should be assumed that controlled uptake and secretion by neighboring protoxylem, sugar-rich phloem cells, and parenchyma cells could deplete or enrich X_{sap} with specific metabolites which will be distributed through the aerial parts (Shi et al. 2002). Therefore, it is expected that the composition of X_{sap} could represent a signature of the root status, whose health and activity are strongly influenced by soil management and fertilization. Youssefi et al. (2000) highlighted that a positive correlation exists between N fertilization and amino acid content in X_{sap}. Interestingly, as reported in both ANOVA and volcano plot analysis, in V_{sust} fields, which received a higher

amount of fertilizer and amino acid-based bio-stimulants treatments, a higher amount of several amino acids (ornithine, putrescine, spermidine, among others) has been observed compared to S_{sust} (ST6 Fold Changes). The increment of these compounds could play a pivotal role in ameliorating plants' ability to cope with both biotic and abiotic stress typical of semi-arid environments where olive is cultivated.

It has been largely reported that both polyamines and amino acids, such as ornithine, putrescine, asparagine, and spermidine, play a pivotal role in protecting plants recovery from environmental stresses (Kuznetsov and Shevyakova 2007; Bown and Shelp 2016). Polyamines, particularly putrescine, spermidine and ornithine, can protect plants from several environmental adverse conditions, such as salt, osmotic, chilling, and oxidative stress (Kuznetsov and Shevyakova 2007). Also, several amino acids (e.g., alanine, asparagine, etc.) and soluble sugars (e.g., ribose, trehalose, etc.) and sugar alcohols (e.g., palatinitol), all of them stimulated by the treatments, could alleviate plant stress, acting as osmoprotectants (Singh et al. 2015; Lu et al., 2020). Pagliarani et al. (2019) showed that during drought conditions, similarly to the semi-arid climate in our experiment, there is an alteration between starch and soluble sugars partitioning. In particular, under such type of stress, an increase of soluble sugars is observable in poplar trees, because of starch degradation, and a drop of xylem pH that induces an accumulation of soluble sugars (sucrose, fructose, glucose, etc.). These latter are pivotal for plant protection and for repairing xylem functionality during drought. Soluble sugars could also be a source of carbon for plant maintenance, growth, and development during stresses or recovery stages (Chaves et al. 2002). Besides, biotic factors and/or soil management type could take part in increasing the sugar alcohol concentration (e.g., galactinol and palatinitol) in the X_{sap} of several tree species (Noiraud et al. 2001).

Concerning palatinitol, a sugar alcohol which significantly accumulated in X_{sap} of S_{sust} treated plants, several manuscripts reported its role and/or its involvement in protecting plants from stress (Lu et al., 2020; Lee et al., 2016). It is known that sugar alcohols serve could serve as energy conservation compounds allowing plants sustainment during stress (Sasse et al., 2018).

Besides amino acids, sugars, polyols and polyamine accumulation in response to soil management, also the increase in organic acids, observed in S_{sust} and V_{sust} fields, could play an important role in improving plants performances, and their production seems to be more stimulated in V_{sust} fields than in S_{sust} ones (ST6 Fold Changes). Glucose, organic acids, such as fumaric and succinic acids, which are components of the tricarboxylic acid (TCA) cycle, can be metabolized by stressed plants to generate energy and carbon skeletons for the biosynthesis of other metabolites playing an important role in stress defense (López-Bucio et al. 2000). Ashrafi et al. (2018) found that, in the shrubby species thyme subjected to drought, the tricarboxylic acid intermediates have a prominent role in activating drought tolerance mechanisms. The transport of organic acid along the transpiration stream has also been connected and correlated with the transport of micronutrients. It has been found that the formation of metal-citrate complexes increased copper transport through the excised stem of *Papyrus*, iron in several dicotyledonous, zinc in *Pinus radiata*, and aluminium in *Fagopyrum esculentum* (López-Bucio et al. 2000; Ma and Hiradate 2000). In the X_{sap}, these complexes are more efficiently transported, as they are subjected to a reduction of lateral escape and lower adsorption to the negatively-charged vessel walls.

Conclusions

From the general analysis of the results we can affirm that, compared to classical management, the sustainable soil management of olive orchards had a positive impact on the chemical and biochemical soil properties, improving total C, N levels, the content of available nutrients, as well as regulating soil microbial activities. In this study, a significant change in X_{sap} composition was observed in response to soil management. In the X_{sap} of sustainably managed olive orchards, it was observed the up-regulation of several primary and specialized metabolites (such as amino acids, soluble sugars, sugar alcohols, among others) involved in plant defense against biotic and abiotic stresses.

In conclusion, we suggest that the transition from the low-input traditional management model to an alternative, sustainable and multifunctional one, could be a solution for maintaining soil fertility and increase plant defences in these agricultural systems. The sustainably managed olive groves could better face the environmental challenges related to climate change, including the consequent lack of resources (particularly water and nutrients). In this scenario, the adoption of sustainable agronomic practices could increase both the resistance and resilience to biotic and abiotic stresses in this important tree crop, with clear environmental, economic, social and cultural benefits.

Limitations of the study

Our study has several limitations. The first limitation is connected with the hypothesis that the increase of X_{sap} metabolites with osmoprotectants and defence roles could really increase plant defence and recovery. To confirm this hypothesis plants must be stressed in order to understand if they can effectively increase plant resistance and resilience. Moreover, other analytical techniques such as liquid chromatography-mass-spectrometry, characterized by a less complex sample manipulation and preparations steps (liquid-liquid separation, drying and derivatization) and with wider metabolic coverage, could hallow in the identification and relative quantification of various primary and specialized metabolites belonging to more numbers of pathways and involved in plant stress metabolic responses.

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Conflict of interest

The authors declare that they have no conflict of interest

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Tables

Table 1. Details of the olive orchard sites and agronomic practices applied. a.s.l. = above sea level.

Parameter	Unit	Site 1	Site 2
Variety	-	Cellina di Nardò, Ogliarola di Lecce	Cellina di Nardò, Ogliarola di Lecce, Leccino
Age of trees	(years)	50-60	70-80
Training system	-	Vase	Vase
Layout	(m)	12 × 12	10 × 10
Planting density	(trees ha^{-1})	70	100
Location	-	N 40° 18' 59.02 " E 18° 16' 17.97"	N 40° 27' 9.65" E 18° 3' 4.84"

Elevation	(m a.s.l.)	35	37
Soil texture (USDA)	-	sandy with coarse-texture	loamy-sand with coarse-texture
pH	-	7.9	7.4
Soil management type	-	minimum tillage	minimum tillage
Coverage	-	grass cover	grass cover
Irrigation method	-	none	none

Table 2. Soil chemical analysis in site 1 (control S_{ctrl} and treated S_{sust}) and in site 2 (control V_{ctrl} and treated V_{sust}). For each plot, the average of three replicates (n = 3) at a depth of 0-30 cm is presented. Values followed by different letters are statistically different ($p \leq 0.01$) within columns, according to Fisher's LSD test. The values were validated following the Eurachem guidelines (www.eurachem.org) respecting the validation parameters: LOD (limit of detection), LOQ (limit of quantification), RL (repeatability limit) and MS (measuring range). The Soil organic C was derivated from the formula Soil organic C = Soil total C × 1.724.

Treatment	**Soil organic C (% w/w)**	**Soil total C (g kg^{-1})**	**Soil total N (g kg^{-1})**	**Soil available P (mg kg^{-1})**	**Soil pH**
S_{ctrl}	2.1 b	12.0 ab	1.4 b	5.0 b	7.9 a

S_{sust}	2.5 a	14.2 a	2.1 a	0.9 c	6.7 d
V_{ctrl}	1.3 c	7.0 b	0.3 c	9.0 a	7.4 b
V_{sust}	2.7 a	15.4 a	2.4 a	0.9 c	7.1 c

Table 3. Enzymatic activity analysis in site 1 (control S_{ctrl} and treated S_{sust}) and in site 2 (control V_{ctrl} and treated V_{sust}). For each plot, the average of three replicates (n = 3) ± standard deviation at a depth of 0-30 cm is presented. Values followed by different letters are statistically different ($p \leq 0.01$) within columns, according to Fisher's LSD test.

Treatment	**β-glucosidase (units g^{-1} soil)**	**Acid phosphatase (units g^{-1} soil)**	**Alkaline phosphatase (units g^{-1} soil)**	**Urease (units g^{-1} soil)**
S_{ctrl}	38.67 ± 0.72 c	26.97 ± 3.38 c	59.64 ± 3.51 d	16.92 ± 4.40 d
S_{sust}	242.75 ± 29.69 b	172.12 ± 34.28 b	430.28 ± 79.23 b	20.94 ± 0.61 c
V_{ctrl}	25.89 ± 2.06 c	38.69 ± 1.61 c	83.95 ± 5.52 c	30.50 ± 0.21 b

V_{sust}	309.69 ± 31.49 a	298.86 ± 14.19 a	628.11 ± 39.61 a	46.08 ± 1.01 a

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851 **Figure legends**

852

853 **Figure 1.** A 4-way Venn diagram showing the significantly (*p* value = 0.05) increased and decreased metabolites in

854 S_{sust} (ST) and V_{sust} (VT) samples, as compared to S_{ctrl} (SC) and V_{ctrl} (VC), respectively (n =3).

855

856 **Figure 2.** Significantly differential metabolites accumulated in S_{ctrl} (SC), S_{sus} (ST), V_{ctrl} (VC), and V_{sust} (VT) samples

857 as analyzed using a 3-way ANOVA. Chemical metabolites classes point to amino acids, organic acids, sugars,

858 volatiles and specialized metabolites, and other such as polyamine (n =3).

859

860 **Figure 3.** Volcano plot analysis showing the metabolites significantly up or down accumulated by the field

861 management in the treatment S_{sust}/S_{ctrl} (ST/SC) **(a)** and in the treatment V_{sust}/V_{ctrl} (VT/VC) **(b)** (n =3).

Figure 4. (a) Principal component analysis showing score plots discriminating S_{ctrl} (SC), S_{sus} (ST), V_{ctrl} (VC), and V_{sust} (VT) groups by virtue of the first 2 PCs. **(b)** partial least square discriminant analysis (PLS-DA) showing discrimination of S_{ctrl}, S_{sust}, V_{ctrl}, V_{sust} groups by virtue of the first 2 components. **(c)** PLS-DA derived analysis variable importance of projection (VIP) features for the groups, and **(d)** random forest (RF) analysis displaying the mean decrease accuracies (n =3).

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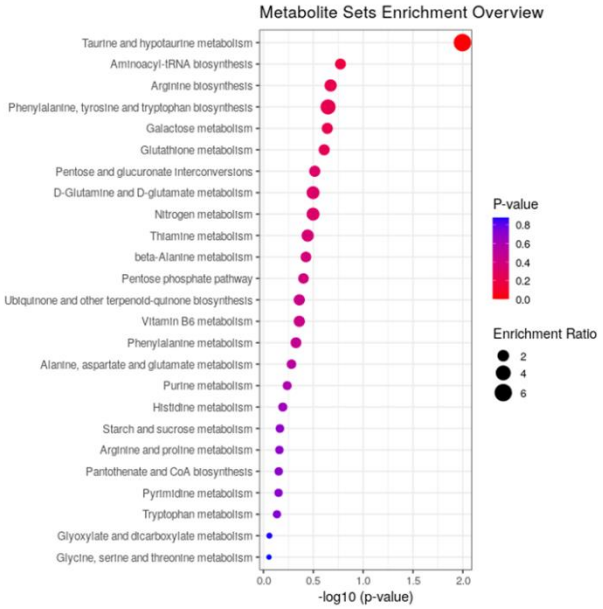
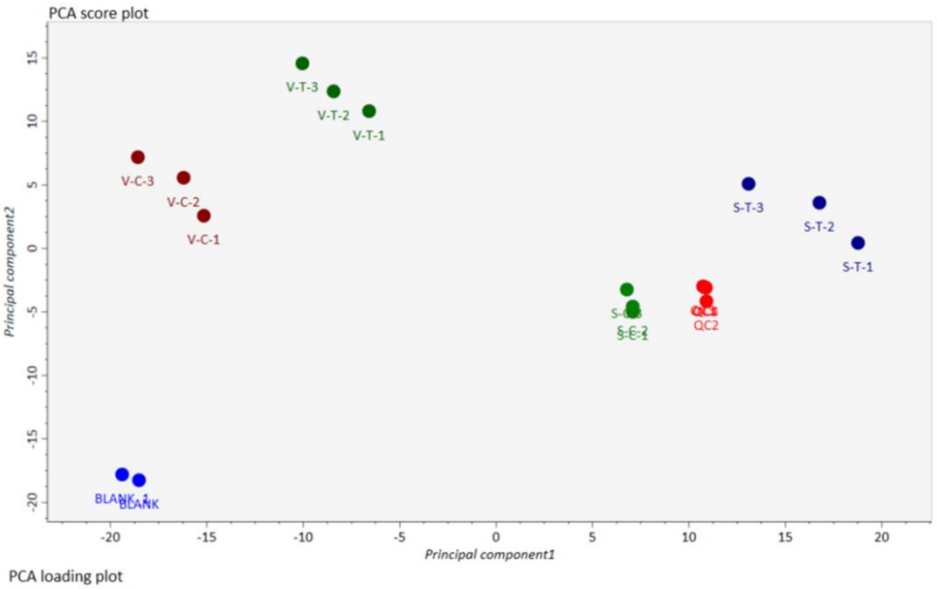
Supplementary material

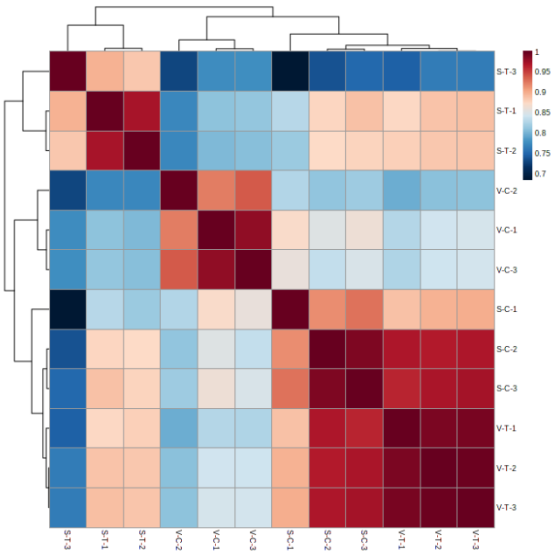
Supplementary Figure S1. Pathway enrichment analysis revealed different metabolic pathways that were enriched, but none were significantly differential (*p* value cut off ≤ 0.05).



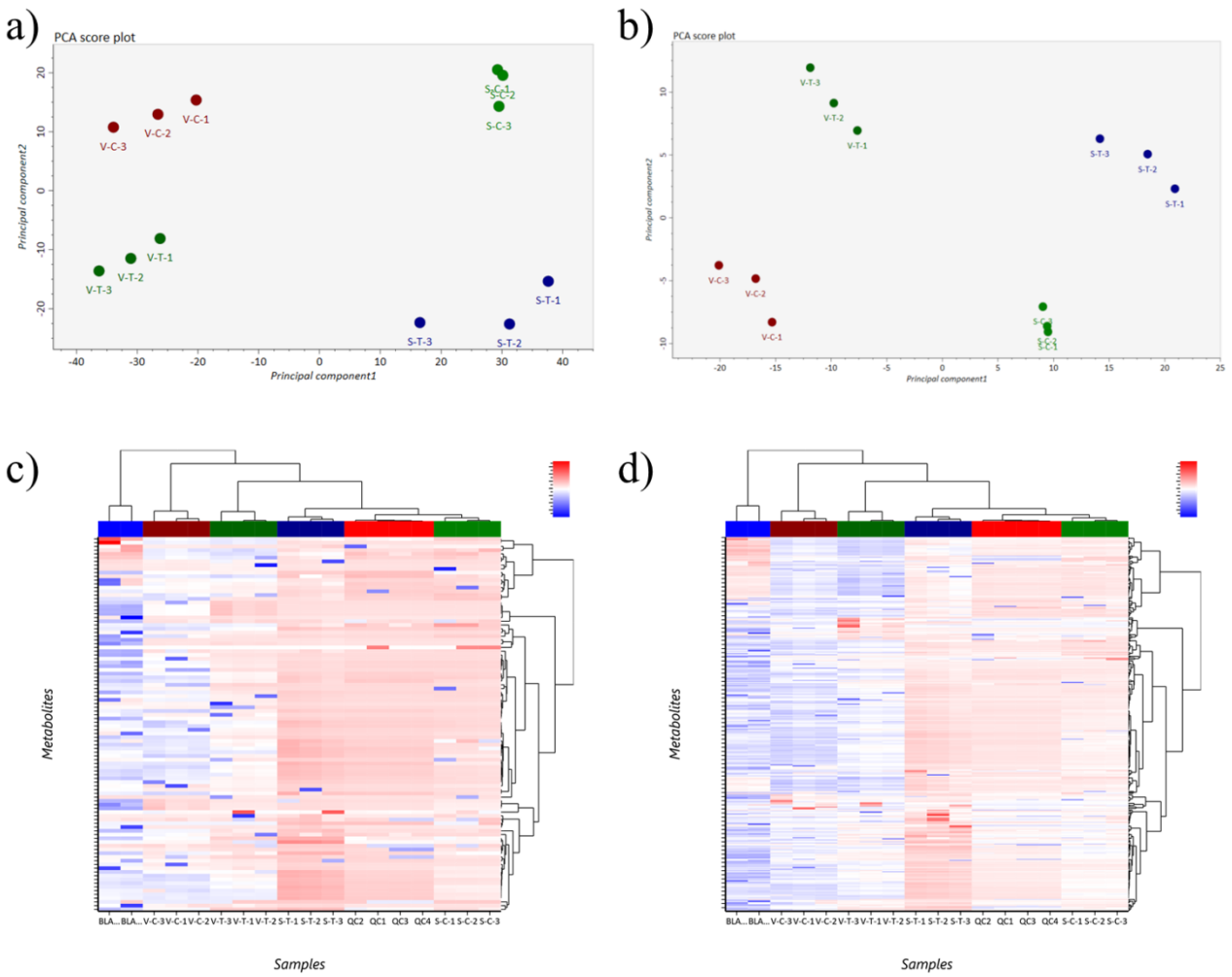
Supplementary Figure S2. A score plot for the principal component analysis (PCA) displaying the first 2 principal components (PC1, PC2) showing the good separation of blanks, QCs, and the 4 sample groups (S_{sust}, S_{ctrl}, V_{sust} V_{ctrl}) thus indicating a good quality assurance of our untargeted GC-MS based platform. Note the S_{ctrl}, S_{sust} groups located in different quadrants than V_{ctrl}, V_{sust} sample groups. (S_{ctrl} = SC, S_{sust} = ST, V_{ctrl} = VC, and V_{sust} = VT) (n =3).



Supplementary Figure S3. Pearson correlation among the samples based on the relative metabolite abundances. (S_{ctrl} = SC, S_{sust} = ST, V_{ctrl} = VC, and V_{sust} = VT) (n =3).



Supplementary Figure S4. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) for the sample groups used in the study. **(a)** PCA showing PC1, PC2 based on relative abundances of all identified compounds. **(b)** PCA showing PC1, PC2 based on relative abundances of all unknown/ unassigned compounds. **(c)** HCA is shown as a heat map displaying the sample groups and QCs for all identified metabolites. **(d)** HCA is shown as a heat map displaying the sample groups and QCs for all unknown/ unassigned metabolites. (S_{ctrl} = SC, S_{sust} = ST, V_{ctrl} = VC, and V_{sust} = VT) (n =3).



Supplementary Figure S5. (a) Pearson correlation showing clustered groups of metabolites in the study samples. **(b)** Hierarchical clustering analysis (HCA) of relative normalized abundances of top 25 metabolites (selected from3-way ANOVA) displayed as a heatmap where columns are individual samples and the rows are metabolites. (S_{ctrl} = SC, S_{sust} = ST, V_{ctrl} = VC, and V_{sust} = VT) (n =3).