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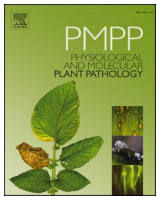

Leaf volatile organic compounds profiles from two citrus genotypes differing in susceptibility to *Phytophthora citrophthora* infection

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ABSTRACT

The leaves of two citrus genotypes, citrange 'Carrizo' and sweet orange 'Washington Navel' were artificially infected by the oomycete *Phytophthora citrophthora*, the causal agent of trunk gummosis and fruit brown rot of citrus, to study the profile of leaf stored terpenoids in the response to the pathogen infection. On 'Washington Navel' leaves the symptoms were more severe and manifested earlier than on 'Carrizo' leaves.

Overall, 35 volatile organic compounds (VOCs) were identified by GC-MS analysis in the not-infected (control) and infected leaf extracts from the two citrus genotypes. Interesting the two volatile profiles differed not in the quality, but in the quantity of each compound. After infection, the amount of 15 out of the 35 identified VOCs varied significantly. The level of five metabolites changed in both species, while two of the compounds changed exclusively in 'Citrange' and eight exclusively in 'Washington Navel'. To understand the potential role of differentially expressed stored volatiles in the citrus response mechanisms to the infection, the inhibitory activity of ten of them volatiles was tested *in vitro* on *P. citrophthora*. Citral, linalool and *trans*-2-hexenal completely inhibited the mycelium growth suggesting the possible involvement of these compounds in the defence mechanisms of citrus against *P. citrophthora*. This study provides new insights into a potential role of stored VOCs in the response mechanisms against the oomycete pathogen *P. citrophthora* in citrus species. Furthermore, it paves the way for future investigations aimed at assessing the potential utilization of VOCs for priming purposes.

1. Introduction

Citrus fruits are one of the major fruit crops worldwide and are grown in more than 140 countries FAO [1]. They are a high value commercial commodity in international trade as both fresh fruit and processed products, such as juices and essential oils; the last ones are widely used in food, perfume, cosmetic and pharmaceutical industries [2]. Moreover, the main byproduct of the citrus juice industry, the pulp, is used for several purposes such as feed for cattle, due to its high nutritive value and good digestibility by ruminant species, organic soil amendment and raw material in the textile industry ([3]; https://orangefiber.it/). Citrus fruits are also well-known for their health benefits associated to the antioxidant properties of some bioactive compounds, such as anthocyanins and carotenoids [4].

However, citrus culture is threatened by several diseases that cause serious yield losses; some of them may be a serious limiting factor for the citrus cultivation in restricted geographical areas or at a global scale [5,6]. Among citrus diseases, the Phytophthora foot and crown rot is

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considered as one of the most relevant because of the damage it causes and its worldwide distribution [7]. The name Phytophthora foot and crown rot is often used in a comprehensive sense to indicate two distinct symptoms often occurring simultaneously on the same tree: root rot and gummous cankers at the base of the trunk, including the collar (crown) [8]. Several species of the oomycete *Phytophthora* are associated to this disease; the two most common are *Phytophthora citrophthora* and *P. nicotianae* [9–11]. Both species are soil-borne and usually infect the basal stem and fibrous roots of citrus trees. *Phytophthora citrophthora* is common in citrus groves of regions with a Mediterranean climate where rainfall occurs prevalently during cool months. In favourable environmental conditions, such as a wet climate and the simultaneous occurrence of rain and wind, this species can temporarily adapt to an aerial lifestyle, causing fruit brown rot and canopy blight [7].

The management of Phytophthora foot and crown rot relies prevalently and almost universally on tolerant rootstocks [8,12]. The 'Carrizo', 'Troyer', and 'C-35' citranges – hybrids of the sweet orange (*Citrus x aurantia var. sinensis)* [13] and the trifoliate orange (*Poncirus trifoliata*) – are among the most widely used rootstock worldwide and are *Phytophthora*-tolerant [14], Conversely, citrus genotypes commonly used as scions, including the most popular sweet orange and lemon cultivars, have been classified from susceptible to very susceptible [5]. The constant and active search for new rootstocks to reach the expectations of citrus producers and to face the emergence of huanglongbing, a destructive disease associated to the phloematic bacterium *Candidatus* Liberibacter asiaticus, made it possible to select new citrus rootstocks [15]. However, the resistance of these recently selected rootstocks to Phytophthora foot and crown rot has not fully investigated. Moreover, the resistance of old and new citrus rootstocks to Phytophthora foot and crown rot could be bypassed through time by the pathogen. For all these reasons, a better knowledge of the biochemical pathogen/host interactions in the pathosystem *Phytophthora*-citrus is of fundamental importance for both citrus breeders and producers, to select tolerant rootstocks and develop effective control methods of the disease.

Secondary carbon compounds are constitutively produced by plants in tissues as the leaves that are potential targets of attacks [16]. In case of attack, plants respond increasing the synthesis of an array of the secondary metabolites that could be cellular or volatiles [17].

Among the volatile organic compounds (VOCs), the volatile terpenoids, such as citral, citronellal and linalool, can be found, as well as green leaf volatiles (GLVs), which are six-carbon (C6) aldehydes, alcohols, and their esters, formed through the hydroperoxide lyase pathway of oxylipin metabolism [18]. These compounds were emitted by tissues exposed to biotic or abiotic stresses, after wounding and/or in plant-to-plant communication [19]. For example, (E)-2-hexenal is produced in many plants [20] and is involved in signalling between plants to prime the immune system of neighbouring plants against the potential infection by pathogens. Depending on its concentration, the (E)-2-hexenal can increase or inhibit the development of phytopathogenic fungi and has been proposed as fungicide to control fungal diseases in crops and fruits [21–25]. However also the stored secondary compounds fraction might also exert a role in the plants defence. Vieira et al. [26] reported increased expression of DREB2 gene and a potential the role of (E)-2-hexenal in the response to water deficit in diploid and tetraploid citrus varieties.

In this study, to understand whether some stored leaf volatile compounds might be involved in the response to *Phytophtora* infection, i) the profile of the stored secondary volatile compounds in control and infected condition of two citrus genotypes with different susceptibility to *P. citrophthora* was investigated, by using organic extraction and GS-MS analysis, for four time points till 10 days from the inoculation; ii) the antifungal activity of some of the differentially expressed volatiles was tested by *in vitro* trials to understand their possible role as antagonistic compounds in citrus.

2. Material and methods

2.1. Phytophthora citrophthora inoculum

A *P. citrophthora* isolate (code 'Tarocco Tapi 4C') obtained from a fruit of sweet orange with brown rot symptoms in Sicily (Mineo, Catania) was used in this study. The isolate was characterized at species level by amplification, sequencing, and analysis of the Internal Transcriber Spacer (ITS) region of the ribosomal DNA performed in accordance with the protocol reported in Ref. [27] (Genbank accession number of the ITS sequence of the isolate 'Tarocco Tapi 4C': PP669816).

The isolate was grown on V8 juice agar (V8A) in Petri dishes for 7 days at 20 °C in the dark. V8A consisted of 100 mL of clarified V8 juice (V8 juice - Campbell Grocery Products Ltd., Ashford, UK - amended with CaCO3 - 30 g/L - and filtered), 15 g agar and 1 L of deionised water [27]. Zoospore production was performed as previously described [28] with slight modifications. Mycelium plugs from 7 days old colonies grown on V8A were flooded in clarified V8 diluted (1:10) with sterile distilled water (sdw) and incubated for 3 days at 23 ± 1 °C with a 16/8 h (light/dark) photoperiod [29]. Sporangia formation was observed during these days, and once mature sporangia were obtained, mycelium plugs were transferred into clean Petri dishes and flooded with sdw. Then, zoospores release was induced by subjecting mycelial plugs to thermic shock (30 min of incubation at 4 °C followed by 30 min of incubation at 30 °C). Finally, zoospores were collected, and their concentration was adjusted to 10^4 zoospores ml^{-1} by using a haemocytometer.

2.2. Inoculation of citrus plants with Phytophthora citrophthora

The experimental design of the inoculation trials comprised commercially produced plants of two citrus genotypes: the sweet orange 'Washington Navel' and the citrange 'Carrizo', *Phytophthora*-susceptible and *Phytophthora*-tolerant, respectively. Sweet orange buds were grafted on citrange 'Carrizo' rootstock 18 months before the experiment and only the leaves of scion were inoculated. Citrange plants were 18-month-old seedlings. Seedlings were grown in 1 L plastic containers in a growth chamber at 22 ± 2 °C, 80 % relative humidity, and a 16/8 h (light/dark) photoperiod. Before inoculation, leaves were disinfected with 1 % sodium hypochlorite and then cleaned with sdw. The inoculation method was performed by following Kalai et al. [30] with slightly modification. Briefly, the leaves were slightly prickled with three sterile entomological needles mounted on a cork at the vertices of an equilateral triangle (three puncture wounds per inoculation site). Each leaf was inoculated on the lamina at two distinct sites straddling the midrib. A drop (15 μl) of *P. citrophthora* zoospore suspension (10^4 zoospores ml^{-1}) was deposited at each inoculation site. Control plants received an equal volume of sdw. After inoculation, all plants were covered with a black plastic bag and incubated for 24 h at temperature of 22 ± 2 °C. Then, plastic bags were removed, and control and inoculated plants were kept in separate climate chambers and maintained at 22 ± 2 °C, 80 % relative humidity, and a 16/8 h (light/dark) photoperiod. Data were recorded 0, 2, 4, 8, and 10-days post inoculation (dpi). The disease severity index (DSI) was assessed visually, since the appearance of first symptoms on the leaves using a 0–4 rating scale (0 = healthy leaf; 1 = < 25 % slight chlorotic halo around the inoculation site; 2 = 26–50 % brown rot of the leaf lamina; 3 = 51–75 % rot of the leaf lamina; 4 = 76–100 % extensive leaf rot. Data were analysed by using a one-way ANOVA, followed by Tukey's HSD test (honestly significant difference) as a post-hoc test (*R* software was used). For each citrus variety and time interval, 12 infected leaves from inoculated plants and three leaves from control plants were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until use in following analyses. All the experimental trials were replicated three times.

2.3. Lipid peroxidation

Lipid peroxidation in terms of malondialdehyde (MDA) equivalents was evaluated using the protocol of Hodges et al. [31]. Briefly, 0.1 g of leaf tissue was homogenized in 0.5 ml of 0.1 % trichloroacetic acid (TCA) (T6399, Sigma- Aldrich, Milano, Italy) using mortar and pestle. The homogenate was centrifuged at 15,000 g for 10 min (4 °C), and 0.5 ml of supernatant was mixed with 1.5 ml of 0.5 % 2-thiobarbituric acid (TBA) (T5500, Sigma- Aldrich, Milano, Italy) dissolved in 20 % of TCA. Samples were incubated in water bath at 95 °C for 30 min. The reaction was ended by incubating the samples on ice. Then the samples were centrifuged at 15,000 g for 5 min (4 °C) and the absorbance was measured at 440, 532, and 600 nm. The absorbance at 440, 600 and 532 nm of the same aliquot of leaf sample without TBA was subtracted to avoid overestimation of MDA. The MDA equivalents were calculated according with the following equations [31]:

$$A = Abs532_{+TBA} - Abs600_{+TBA} - (Abs532_{-TBA} - Abs600_{-TBA})$$

$$B = Abs440_{+TBA} - (Abs600_{+TBA} \times 0.0571)$$

$$MDA\ equivalents\ \left(nmol.ml^{-1}\right) = ((A\ - B)\ /\ 157000) \times 10^{6}$$

2.4. Analysis of VOCs

Leaf samples (0.5 g of fresh material) were frozen and grounded in liquid nitrogen to a fine powder with cold mortar and pestle. The powder was suspended in 2 ml of heptane as extraction solvent in a close glass vial, and slowly stirred in a Gerhardt Thermoshake THO 5 for 24 h at 25 °C. Then after filtration on 0.45 mm Millipore Millex-LCR filter, 1 μl of sample was injected in spitless mode in a 7820 GC-chromatograph equipped with a 5977A MSD mass spectrometer with electron impact ionisation (Agilent Tech., Palo Alto. AC. USA). The chromatographic settings were as follows: injector set at 260 °C; J&W Innovax column (30 m. 0.25 mm i.d. 0.5 μm df); oven temperature program with initial temperature 40 °C for 1 min, then 5 °C min^{-1} until 200 °C, then 10 °C min^{-1} until 220 °C, then 30 °C min^{-1} until 260 °C, then hold time 3 min. The mass spectrometer was operating with an electron ionisation of 70 eV in scan mode in the *m/z* range 29–330 at three scans sec^{-1}. The deconvoluted peak spectra obtained by Agilent MassHunter software were matched against NIST 11 spectral library for tentative identification. Kovats' retention indices were calculated using a C_{10}–C_{20} *n*-alkane series for further compound confirmation and compared with those reported in literature for the chromatographic column used. When available, authentic standards were also injected such to obtain a positive identification.

2.5. Principal component and heatmap analyses

A log2 transformation of the metabolite peak intensities was performed and principal component analysis (PCA) was applied to reduce the dimensionality of the data, using the "prcomp" function from the "Factoextra" package in *R* [32,33]. Then, a cluster analysis using the "k-means" function was conducted to group samples with similar metabolite profiles [34] allowed to identify distinct clusters and gain insights into underlying biological processes. To visualize the metabolite profiles across different conditions, a heatmap was created using the "ComplexHeatmap" package in *R* [33]. First the data were scaled to ensure that each metabolite contributed equally to the heatmap, and then the heatmap was generated using the "Heatmap" function, specifying the number of clusters to identify with the "km = 3" argument. Statistical analysis of the data was performed using the SPSS statistical software. The ANOVA (Analysis of Variance) was employed to assess potential significant differences among the group means. Subsequently, the Tukey's test was conducted to perform multiple comparisons and identify specific group differences at a significance level of 5 %. Additionally, the graphs were plotted using Excel software.

2.6. Antifungal activity of volatiles

The antifungal activity of selected volatiles differentially expressed after inoculation in the two citrus genotypes was tested *in vitro* on *P. citrophthora* mycelium growth. To this aim, Petri dishes were filled with potato dextrose agar, a cross was drawn on the bottom of each dish and a 5 mm-diameter hole was made at the end of one arm of the cross near the edge of the dish. A 3.5 mm mycelial disk, taken from 7-day-old cultures of *P. citrophthora,* was placed in the centre of each Petri dish and 10 μl of pure volatile were pipetted into the hole. Three replications for each volatile and two independent experiments were performed. In the control treatment, sterilized distilled water was pipetted into the hole. The mycelium growth was measured with a cm-graduated ruler twice in a week for two consecutive weeks.

3. Results

3.1. Disease severity in susceptible and tolerant citrus plants inoculated with Phytophthora citrophthora

In the leaves of sweet orange 'Washington navel' inoculated with *P. citrophthora* the symptoms were more severe and appeared earlier than on citrange 'Carrizo' (Fig. 1). On sweet orange leaves, the first symptoms appeared two dpi and consisted in a chlorotic halo around the inoculation site (DSI = 1), while on citrange leaves the same symptom was first noticed only four dpi. Along with the infection course, the symptom severity increased progressively in the leaves of both genotypes. However, on sweet orange the DSI values were slightly higher than in Carrizo as early as four dpi. On sweet orange, the mean DSI peaked 10 dpi (mean value ± SD, 3.1 ± 0.4), while on citrange the mean value ± SD of DSI at 10 dpi was 1.6 ± 0.49, suggesting the leaves of the former variety were more susceptible to infections of *P. citrophthora* (Fig. 1).

3.2. Lipid peroxidation analysis

The MDA concentration strongly increased in the two genotypes after inoculation and the value remained higher than that of control along with the course of the experiment (till 10 dpi). However, after inoculation the values in the sweet orange 'Washington navel' were higher than those measured in citrange 'Carrizo' (Fig. 2).

3.3. Differentially expressed volatiles

Through GC-MS analysis, 35 diverse compounds were detected and identified in the heptane extract of control and inoculated citrus leaves. The volatiles were classified into three main groups: monoterpenes, sesquiterpenes (δ-elemene, β caryophyllene and humulene), and aliphatic aldehydes (*trans*-hexenal and *cis*-hexenol). After infection by *P. citrophthora,* 15 stored volatile compounds significantly changed in their relative abundance during the infection process, at least at one time interval after inoculation. Eight of these compounds changed only in sweet orange, only two in citrange, and five in both citrus genotypes (Fig. 3), among the last ones, the *trans*-2 -hexenal.

3.4. PCA and heat maps

In order to understand the relationship between the metabolite profiles and the different citrus genotypes, PCA analysis was performed using the expression of all the identified metabolites in the control and in the all infection time points. (Fig. 4-A). PC1, representing the highest variance (95.7 %) showed a good correlation within technical replicates mostly those from 'sweet orange' and a clear separation between the metabolites profiles from 'sweet orange' which clustered in the left

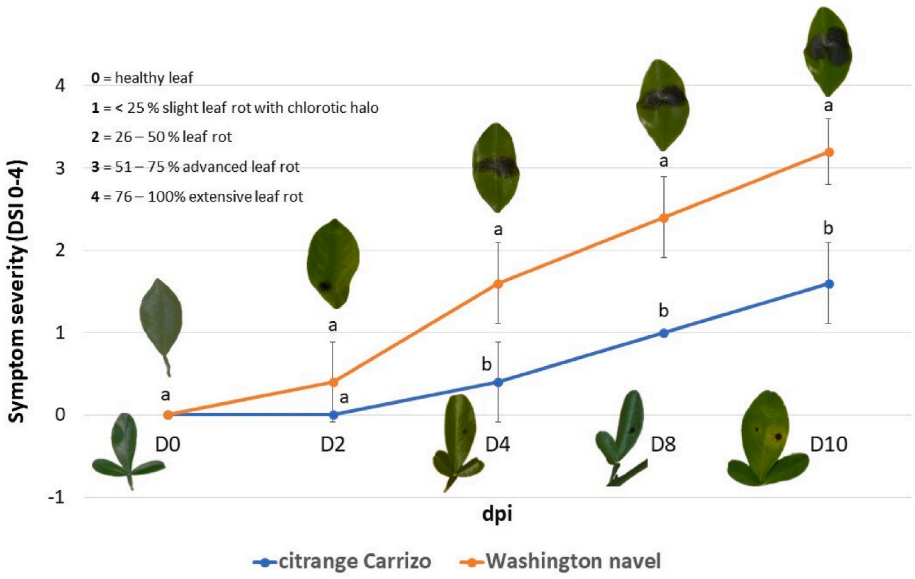

Fig. 1. Symptom progression in citrange 'Carrizo' and sweet orange 'Washington navel' leaves inoculated with *Phytophthora citrophthora*. Symptom severity was expressed as the mean value of the disease severity index (DSI) at 0, 2-, 4-, 8-, and 10-days post-inoculation (dpi). For each time interval, values sharing the same letters are not significantly different according to the Tukey's honestly significant difference (HSD) test ($p \leq 0.05$).

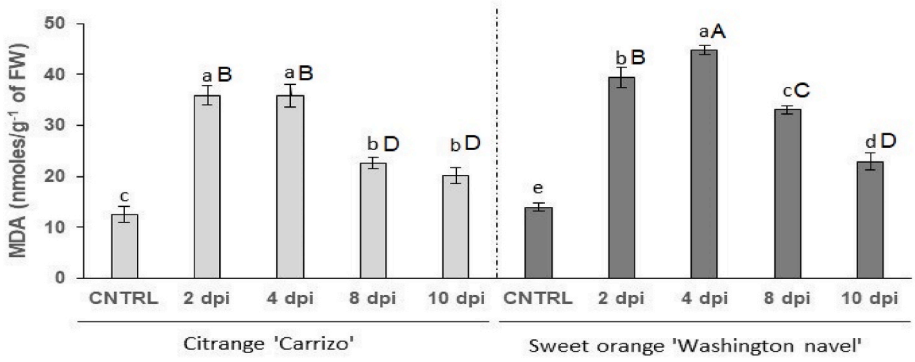

Fig. 2. Lipid peroxidation, expressed as MDA concentration, in the citrange 'Carrizo' and sweet orange 'Washington navel' leaves during *P. citrophthora* time course infection. CNTRL: control; dpi: day post-inoculation. Statistical analysis was made for each citrus cultivar independently. Lower case letters indicate significant differences within the same citrus variety; upper case letters indicate significant differences between the two genotypes according to Tukey's test at $p \leq 0.05$. Bars indicate standard deviation from the mean of three biological replicates for each sampling time.

quadrants and those from citrange, clustering in the right quadrants. PC2, which represents only 1.6 % of the variance, displays the separation among samples from different infection time within each genotype.

Differences between the VOC profiles of the two citrus genotypes were also observed on the heat-map both at control and infected condition (Fig. 4-B). At control condition 20 VOCs, grouping into cluster 3 of the heat-map, were found with a relatively high abundance, as revealed by high color intensity, in resistant citrange compared to the susceptible sweet orange as better evidenced by the comparison of stored volatile organic compound profiles from non-inoculated leaves of citrange 'Carrizo' and sweet orange 'Washington Navel' (Fig. 5). Interestingly, the citrange 'Carrizo', at two dpi, showed a highly different profile compared not only to the profiles of 'Washington Navel' orange (Fig. 4-B), but also to the profiles of the same variety at other time intervals after inoculation (Fig. 4-A), suggesting a specific, but transient metabolic reaction of this citrus variety to the infection.

In citrange, the relative abundance of β-phellandrene increased significantly compared to the control at 2 dpi, but at 4 dpi fell below the control and remained lower than the baseline for the rest of the experiment (Fig. 6). Similarly, the relative abundance of *trans*-geraniol fell below the control at 4 dpi and remained lower than the baseline for the rest of the experiment. Among the eight volatiles expressed differentially only in 'Washington navel' sweet orange after inoculation with *P. citrophthora*, linalool and α-terpineol decreased significantly as early as 2 dpi and remained lower than the control during the entire time course of the infection process, while β-ocymene increased sharply at 2 dpi and decreased progressively until reaching the baseline level at 10 dpi. The relative abundance of citral during the time course of the infection showed two diverse trends, at 2 and 4 dpi was lower, but at 8 and 10 dpi was higher than the control. Finally, the relative abundance of all the other four compounds, including α-pinene, menthone, geranyl acetate and β-caryophyllene increased progressively along the time course of the infection process and at 10 dpi reached a maximum, by far higher than the control. It is noteworthy that among the volatiles, which were modulated by the infection in both citrus genotypes, *trans*-hexenal, a compound of the oxylipin pathway, in citrange leaves was always detected with a significant higher relative abundance than in sweet orange leaves, both in the control conditions, and at diverse time intervals after inoculation. In both citrus genotypes, the relative abundance of this volatile sharply decreased at 2 dpi and then increased progressively to

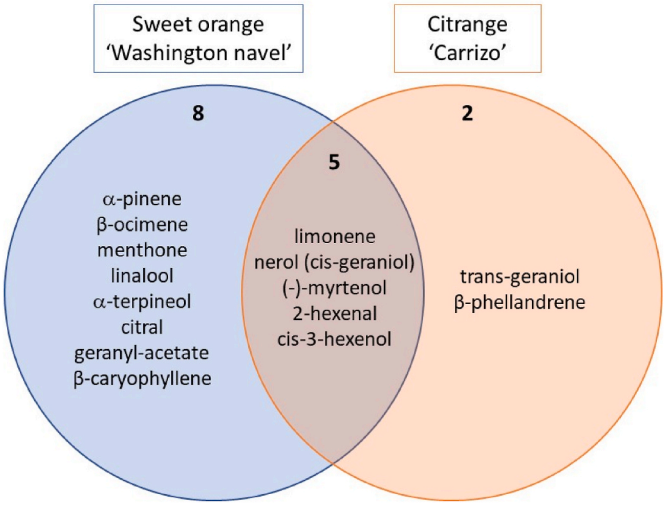

Fig. 3. Venn diagram showing the distribution in the two citrus genotypes of the 15 differentially expressed metabolites after *Phytophthora citrophthora* infection. The relative abundance of 8 metabolites varied exclusively in sweet orange 'Navel' and two in citrange 'Carrizo'.

attain values similar to the baseline (Fig. 6).

3.5. Antifungal activity test

To evaluate their potential antagonistic activity in citrus, twelve among the fifteen differentially expressed volatiles in the two citrus genotypes after the inoculation with *P. citrophthora*, was selected and their inhibitory activity was evaluated *in vitro* following the mycelium growth of the oomycete for two weeks. The criteria used to select the twelve tested volatiles included: i) commercially available high pure quality compound; ii) specifically differentially expressed in one of the two genotypes; or iii) differentially expressed in the two citrus genotypes. Therefore, all the eight volatiles differentially expressed in the leaves of sweet orange 'Washington Navel' and, unfortunately, only one volatile (*trans*-geraniol) differentially expressed in citrange 'Carrizo' leaves and two expressed in both citrus genotypes (*trans*-hexenal and + and – limonene) were tested. Among the 12 tested volatiles, linalool, citral and *trans*-hexenal completely inhibited mycelium, (−) menthone and *trans*-geraniol by 75 %, and geranyl acetate around 50 %, respect to the control growth, while β-caryophyllene and β-ocymene, α-terpineol an α-pinene did not show any inhibitory effects on mycelium growth (Fig. 7).

4. Discussion

In this study, two citrus genotypes known to differ in susceptibility to Phytophthora gummosis, the sweet orange 'Washington Navel' and the hybrid rootstock citrange 'Carrizo', were inoculated on the leaves with *P. citrophthora* to examine comparatively how the infection by this oomycete modulates the profile of VOCs during the host plant colonization process and to identify constitutive or induced metabolites which might play a role in the infection response. Then, twelve among the differentially expressed metabolites were tested with their antagonistic properties against the *Phytophthora* isolate used to inoculate the plants with the aim to identify their possible role as antagonistic compounds in citrus. Symptoms appeared earlier and were more severe in the leaves of *Phytophthora*-susceptible sweet orange 'Washington Navel' than in the *Phytophthora*-tolerant citrange 'Carrizo', indicating this genetically determined character was expressed also in the leaves.

Under pathogen attack, plants produce reactive oxygen species (ROS) due to the imbalance of cell metabolisms [35]; the increased levels of ROS may cause lipid peroxidation of polyunsaturated fatty acids and production of several products. Among these, the malondialdehyde (MDA), a reactive aldehyde commonly known as a marker of oxidative stress, stands out [36]. In a previous study addressing the effect of soil waterlogging on the production of endogenous hormones in citrus plants, it was observed a key role of leaves in regulating the stress tolerance; in this respect, MDA concentration in leaves was assumed as

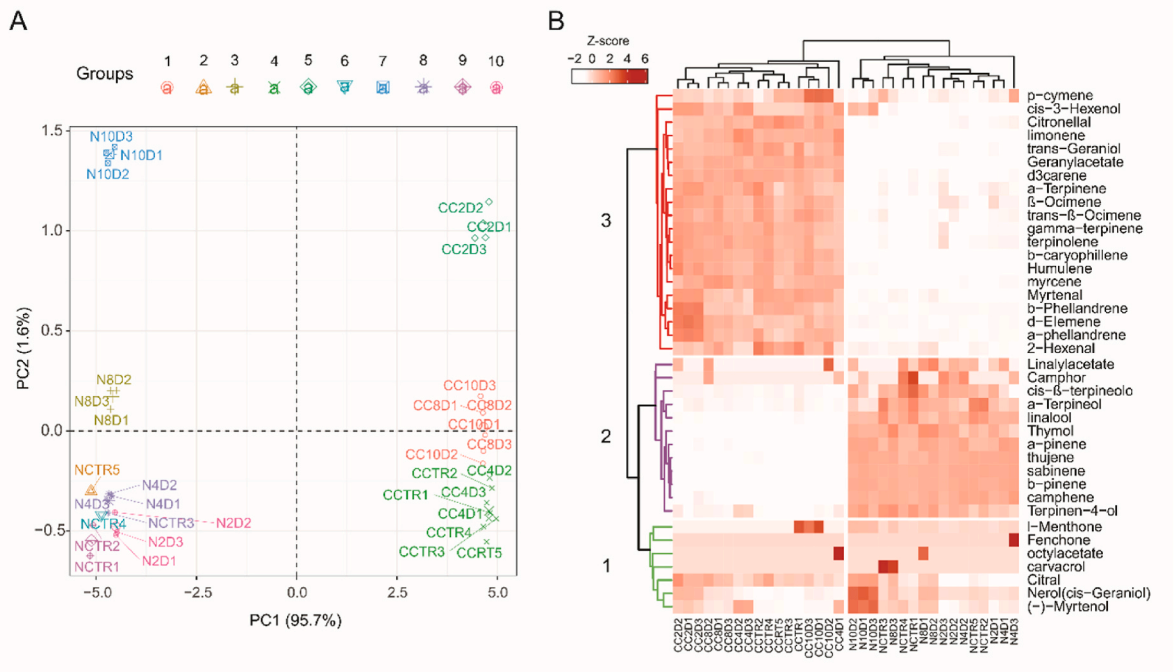

Fig. 4. Principal component analysis (PCA) and heatmap of the metabolomic analysis in citrange 'Carrizo' and sweet orange 'Washington Navel'. CCTR and NCTR indicate the leaves from non-inoculated plants in citrange (CCTR) and Navel (NCTR) respectively; 2D, 4D, 8D, 10D indicates leaves sampled two, four, eight and ten dpi in citrange (CC) or in Navel (N); 1, 2, 3, 4, 5 indicate the number of technical replicates.

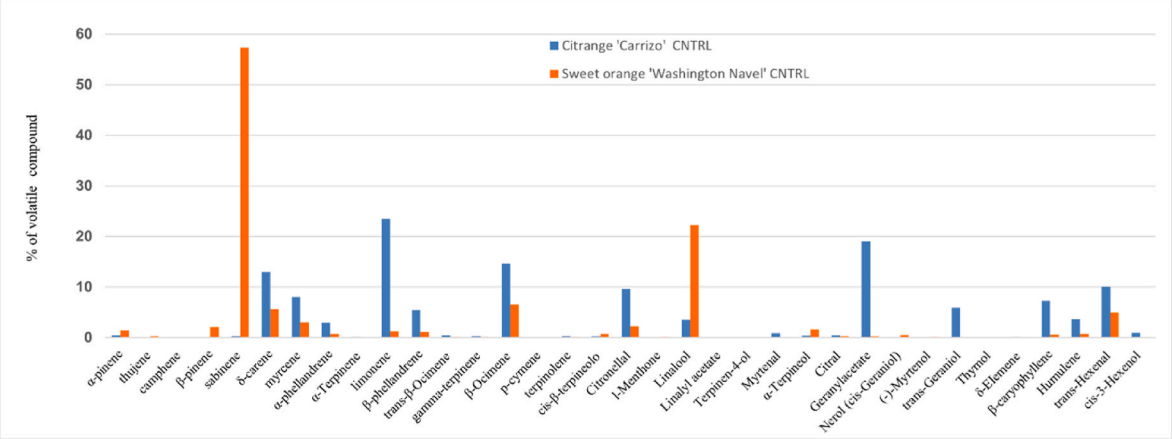

Fig. 5. Comparison of constitutive volatile organic compound profiles from non-inoculated (control CNTRL) leaves of citrange 'Carrizo' and sweet orange 'Washington Navel' categorized in monoterpenoids (from α-pinene to thymol), sesquiterpenoids (δ-elemene, β-caryophyllene, humulene) and aliphatic aldehydes (*trans*-hexenal and cis –3- hexenol). Data are expressed as percentage of the total terpenoids levels.

an indirect marker of oxidative stress Arbona and Gómez-Cadenas, 2008 [37]. Consistently with the literature, in the present study the MDA concentration increased in the leaves of the artificially inoculated citrus plants in both genotypes during the first four days after the inoculation, as a response to the infection, indicating oxidative stress and lipid peroxidation occurred in both genotypes. However, the pattern of MDA accumulation in the leaves of citrange and sweet orange showed substantial differences during the time course of the infection. In sweet orange MDA content remained higher than the control throughout the entire time course of the infection process, while in citrange it decreased gradually and, at 10 dpi, did not differ significantly from the control. This difference in MDA pattern might be correlated with the presence of distinct response mechanisms to the infection [36] still unknown, scavenging the oxidative stress and consequently the lipid peroxidation, in the less susceptible genotypes citrange respect to the sweet orange. A negative correlation between infection and MDA levels has been reported in the leaves of a resistant var. of sunflower upon S. sclerotiorum infection, although both resistant and susceptible varieties showed comparable changes in cell membrane permeability upon infection [38].

Citrus leaf volatiles from the two different citrus genotypes were extracted with heptane and analysed using GC-MS, a method currently used in citrus genetics and breeding as well as to identify the role of stored volatiles in citrus under biotic or abiotic stresses [26]. Several hundred volatile compounds have been reported in the fruit rind, leaves, and flowers of citrus species worldwide, including hydrocarbon terpenes, alcohols, aldehydes, esters, and ketones [39]. It is well known that in citrus species the VOC biosynthesis is under genetic control [40]. Some VOCs are produced exclusively by a single citrus species or a group of species and are so specific that they have been proposed as biomarkers for chemotaxonomic studies. By contrast, others, such as limonene, a hydrocarbon monoterpene, is the most abundant compounds in the volatile fraction of fruit rind and in most cases, account for about 60–95 % of the essential oil in all citrus species and hybrids [41].

According to data from literature, the sweet orange 'Washington Navel' and citrange 'Carrizo' VOC profiles were shown to be constitutively different in present study. Multicomponent analysis also revealed that over 95 % of the total variability in the experiments could be attributed to variations between the two genotypes. In control leaves of 'Carrizo' citrange, the prevalent VOCs were, in the order, δ-limonene, geranylacetate, β-ocimene, δ-carene and trans 2- hexenal. By contrast, in control leaves of 'Washington Navel' sweet orange the prevalent stored VOCs were sabinene and linalool. Despite the numerous studies on VOCs generated by *Citrus* and related genera, the leaf profile of stored volatile compounds of citrange has been characterized for the first time, to the best of our knowledge.

As described in the results chapter, fifteen volatile organic compounds demonstrated a significant variation in their relative abundance after *P. citrophthora* infection, and some of them in dependence of the citrus genotypes. Therefore, in line with the goal of the study, attention was paid to two metabolites of the oxylipin pathway, *trans*-2hexenal and *cis*-hexenol, because they were produced differently by the two citrus genotypes both in non-inoculated and infected leaves.

Oxylipins, among them *trans*-2-hexenal and *cis*-3-hexenol found in this study in both citrus genotypes, are signalling molecules that comprise acyclic or cyclic oxidation products derived from the catabolism of fatty acids, which regulate many defence and developmental pathways in plants (Mirabella et al., 2008; [20]). Oxylipins modulate the expression of numerous genes and influence specific aspects of plant growth, development, and responses to abiotic and biotic stresses [26, 42,43]. Depending on the concentration *trans*-2-hexenal can have different effects on the growth of pathogenic fungi. A low concentration of *trans*-hexenal increased colony growth and spore germination of the fungus *Botrytis cinerea* and the infection of fruits by inducing sulphate assimilation [44]. On the other side high concentration of *trans*- 2-hexenal inhibited fungal disease development [24] and it therefore has been proposed as a fungicide to control fungal diseases in crops and fruits [22, 23]. Moreover, in the *in vitro* inhibition test performed in this study, *trans*-hexenal showed a strong inhibition of the *P. citrophthora* mycelium growth. This metabolite was significantly higher in control leaves of citrange than in control leaves of sweet orange. Its concentration decreased sharply soon after the inoculation, but then returned to the initial levels with a quite similar dynamics in both citrus genotypes. However, the concentration of this volatile in citrange leaves was significantly higher than the corresponding values in sweet orange leaves at all stages of the infection process, suggesting a possible role as a factor of resistance toward *P. citrophthora*. Another possible compound acting as co-factor of resistance in citrange might be *trans*-geraniol whose constitutive level was specifically observed only in this variety. Geraniol is a commercially important terpene alcohol occurring in the essential oils of several aromatic plants. In addition to its pleasant odour, geraniol is known to exhibit insecticidal and repellent properties. Furthermore, it is known to exhibit antimicrobial activity against a wide range of bacteria and fungi [45]. In this study, the *in vitro* antagonistic test showed geraniol inhibited substantially the mycelium growth of *P. citrophthora*, supporting previously published observation indicating a strong antifungal activity of this volatile. As a matter of fact, the antagonistic action of palmarosa oil was mainly attributed to its geraniol content and its solubility in the phospholipid bilayer of cell membranes provoking a combined membrane effects such as increased bilayer disorder and ion leakage which imbalanced the osmotic system of the cell

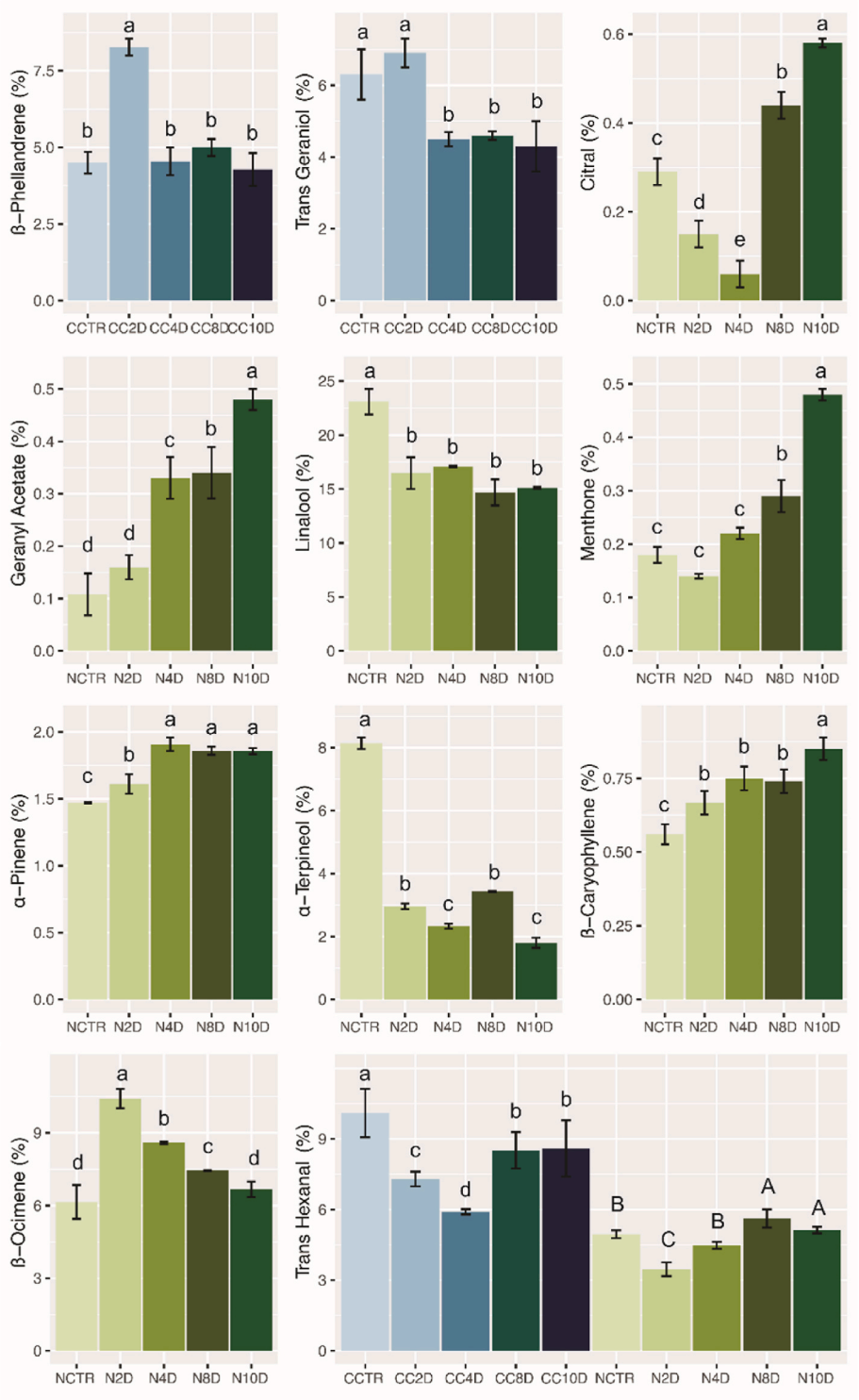

Fig. 6. Modulation during the time-course of the infection process of the differentially expressed volatiles in one the two genotypes and that of *trans*-hexenal which varies in both studied citrus genotypes. CCTR and NCTR indicate the leaves from non-inoculated plants in citrange (CCTR) and Navel (NCTR) respectively; 2D, 4D, 8D, 10D indicates leaves sampled two, four, eight and ten dpi in citrange (CC) or in Navel (N). Bars indicate standard deviation of means of three biological replicates for each sampling time except for control in which five replicates were analysed. Different letters indicate significant differences according to Tukey's test at $p \leq 0.05$.

through loss of ions, and inhibition of cell growth [46]. In this study, the small decrease in *trans*-geraniol content of infected leaves of citrange suggests that the compound might be involved in the response of citrus to *Phytophthora* infection and might support the resistance of the plants to the fungal infection.

In addition, citrange leaves had higher constitutive concentrations of *cis*- 3-hexenol than sweet orange leaves. The content of this volatile increased sharply in citrange leaves following inoculation and remained elevated compared to control leaves during the infection process. Also in sweet orange leaves, the concentration of this volatile increased significantly since two dpi and its level remained higher than in control leaves throughout the infections process, but it reached a level comparable to

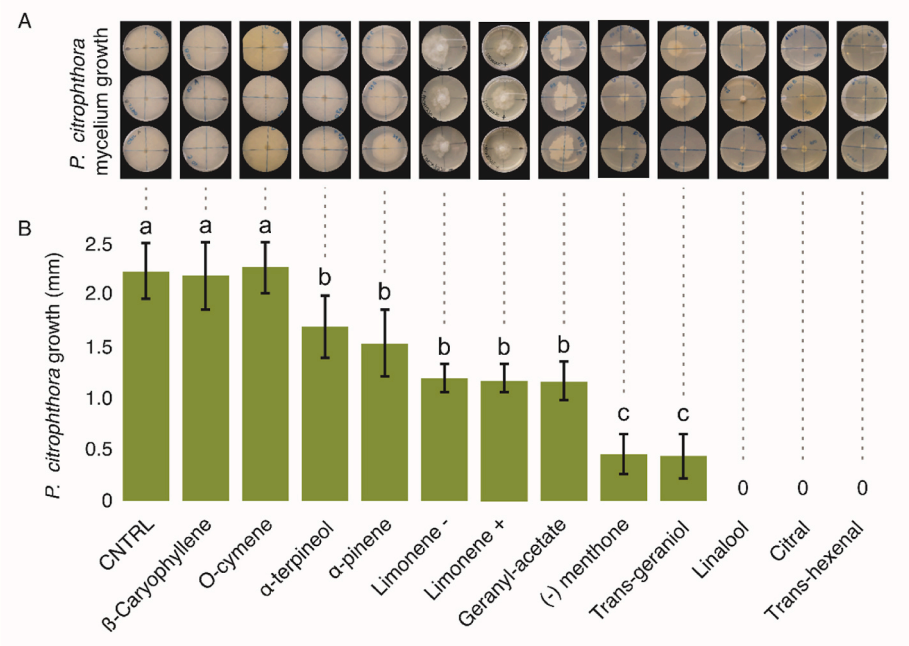

Fig. 7. A. Visual effect of each volatile on the growth of *Phytophthora citrophthora* cultures at the end of the test and **B.** Growth (in mm/day) of *P. citrophthora* exposed to the selected volatiles. CNTRL mycelium treated with sdw. Different letters indicate significant differences according to Tukey's test at $p \leq 0.05$. Error bars represent standard deviation. Data are the mean of three independent biological replicates measured at the end of each test.

that of citrange leaves only 10 dpi. This would support the hypothesis that a delayed response of sweet orange to the infection based on the production of this metabolite may be at least in part responsible for its failure to restrict the colonization of leaf tissues by the pathogen.

On the other hand, a very high level of linalool was measured in 'Washington Navel' sweet orange at the control condition, and such a level decreased along the infection time course. Previous papers reported that linalool, citral, and citronellal emitted by wounded rough lemon leaves significantly inhibited hyphal growth and spore germination of *Alternaria alternata* and induced acquired systemic resistance against fungal diseases Jiang et al. [47,48]. In this study, linalool and citral completely inhibited *P. citrophthora* growth in *in vitro* tests. Considering the results of this study together, it can be hypothesized that both a low constitutive and a delayed production of *cis*-hexenol after pathogen inoculation and the decrease of linalool in sweet orange may be responsible for the susceptibility of this variety to *P. citrophthora*.

In conclusion, by comparing the profiles of leaf stored volatile compounds of 'Washington navel' sweet orange and 'Carrizo' citrange, this study showed for the first time a possible role of stored terpenoids in the response of citrus genotypes to *P. citrophthora* as the levels of several volatiles were modulated after infection. Four of these volatiles, *trans*-hexenal, *cis*-hexenol, *trans*-geraniol and linalool might be involved in the resistance/susceptibility response of citrus plants to the infection by *P. citrophthora*, the causative agent of Phytophthora foot and crown rot, as the *in vitro* test demonstrated that they were able to inhibit the mycelium growth of this oomycete.

This study might provide information that could be exploited in breeding of new rootstocks and more in general for improving the management strategies of *Phytophthora* diseases.

CRediT authorship contribution statement

Biancaelena Maserti: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Marco Michelozzi:** Writing – review & editing, Investigation, Formal analysis. **Gabriele Cencetti:** Formal analysis. **Mario Riolo:** Formal analysis. **Federico La Spada:** Formal analysis. **Francesco Aloi:** Formal analysis. **Antonella Pane:** Supervision. **Paola Bartolini:** Formal analysis. **Francesco Pecori:** Formal analysis. **Edson Mario de Andrade Silva:** Software, Data curation. **Abelmon da Silva Gesteira:** Writing – review & editing, Conceptualization. **Fabienne Micheli:** Writing – review & editing, Conceptualization. **Santa Olga Cacciola:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declared do not have any conflict of interest in submitting the present manuscript and do not have nothing to declare.

Data availability

Data will be made available on request.

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