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Metabolites response to onion yellow dwarf virus (OYDV) infection in ‘Rossa di Tropea’ onion during storage: a ¹H HR-MAS NMR study.

Running title: **Metabolites response to OYDV infection in ‘Rossa di Tropea’ onion during storage by NMR.**

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Abstract

Background: Plant viral infections induce changes in metabolic components in the host plant, with potential effects on compositional, organoleptic and storability features of agricultural products. Identification of modulated metabolites may provide clues concerning pathways implementing responses in plant-pathogen interactions. A time course study of metabolic fingerprinting of onion yellow dwarf virus (OYDV)-infected vs. healthy ‘Rossa di Tropea’ onion bulbs was performed using

¹H HR-MAS NMR and UPLC, providing an overview of the metabolic state of the bulb in response to OYDV infection during storage.

Results: Metabolites accumulated/depleted upon infection were identified, belonging to flavonoid, saccharide, amino acid and organic acid classes. A decrease in quercetin glucosides content and antioxidant activity was observed in infected bulbs; some amino acids (Arg, Asn, Phe, Val) accumulated, while others were depleted (Leu); for some metabolites, a bimodal time-course was observed during storage (Glc, Lys). Virus interference on metabolic pathways, and the effects of the metabolic shift on edible product storability, organoleptic and nutritional quality were discussed.

Conclusions: OYDV infection induces a metabolic shift in ‘Rossa di Tropea’ onion during bulb storage, involving several pathways and affecting storability and organoleptic and nutritional quality of bulbs at marketable stage.

Keywords: onion, onion yellow dwarf virus, metabolite, HR-MAS NMR, storage.

1. Introduction

Plant metabolites cover a huge range of structures and concentrations, their number being estimated at more than 200,000. The functions of plant metabolism are often divided in “primary metabolism” – common to most species and organs, providing energy, building blocks for growth and development – and “secondary” or “specialized” metabolism, species- or organ-dependent and mainly addressed to interact with environment, with a direct role in response to abiotic and biotic stress¹. Major metabolic alterations in infected plants are particularly marked in case of viral diseases, as viruses use the host’s cellular system to replicate, leading to an altered distribution of photosynthesized compounds within the plant².

Onion (*Allium cepa* L.) is the species within the genus *Allium* (family *Amaryllidaceae*) with the highest global production area and quantity, being rich in health promoting compounds^{3,4,5,6}. Over 10,000 accessions are available in Gene Banks worldwide; among them ‘Rossa di Tropea’ onion is

characterized by high amounts of nutraceutical compounds such as flavonoids⁷. This typical cultivar from Calabria (southern Italy) represents a valuable crop, granted with Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) trademarks. Among the several biotic stresses affecting ‘Rossa di Tropea’ onion, *onion yellow dwarf virus* – OYDV (genus *Potyvirus*, Family *Potyviridae*, ssRNA (+)) is a pathogen known since 1932 and now worldwide spread, reported in Italy in 1993 and in ‘Rossa di Tropea’ in 2005⁸. This virus represents the most limiting biotic stressor in onion, inducing severe symptoms as yellowing, dwarfing and stem twirling. Further, it is reported to reduce bulb’s weight and size up to 40 %, with a seed loss up to 50 %⁹.

Metabolite profiling of onion affected by genetic or environmental factors was recently carried out^{10,11,12}. The effect of storage conditions on saccharides, amino acids and flavonoids has been widely studied^{13,14,15,16}. In fact, variations in metabolites content exert important impact on food-quality related characters, including those involved in storage^{17,18}. Moreover, quercetin concentration was reported as correlated to dormancy, which is an essential phase for onion long-term storability^{19,20}.

Metabolite evaluation in plants takes advantage of Nuclear Magnetic Resonance (NMR)-based techniques, mainly thanks to their analytical capabilities and suitability for both targeted and untargeted metabolomics. NMR either generates a metabolite profile, in which NMR signals are assigned to specific molecules, or a fingerprint, in which the analysis is based on the distribution of intensity in the whole NMR spectrum rather than on the assignment of single signals. NMR is reproducible, high-throughput, has simple sample preparation and can be performed rapidly²¹. Recently, it has been widely used for assessing metabolome shifts in tissues of different plant species in response to treatments, biotic and abiotic stresses^{22,23}. High Resolution - Magic Angle Spinning Nuclear Magnetic Resonance (HR-MAS NMR) is specifically designed to analyze semi-solid samples (e.g. plant tissues); it allows a direct measurement on tissues without any chemical or physical manipulation, avoiding alteration of thermolabile, light-sensitive, unstable compounds and loss of specific fractions during extraction. HR-MAS NMR produces highly resolved spectra and

qualitative/quantitative determination of several molecules can be achieved within the same experiment; these features make HR-MAS NMR a powerful tool for plant metabolomics^{24,25}.

In this work, NMR-based metabolomics was applied to decode the broad amount and diversity of the compounds likely involved in 'Rossa di Tropea' onion-OYDV interaction and in the quality of onion bulbs. Both (i) untargeted and (ii) targeted approaches were applied: (i) uses chemometric tools to extract unpredicted information from the whole NMR spectrum, and (ii) involves the detection of previously annotated candidates, which are likely to be modulated by virus infection in plant.

Furthermore, an absolute quantification of anthocyanins and quercetin and an antioxidant capacity assay were performed by Ultra Performance Liquid Chromatography (UPLC) and UV-vis spectrophotometry on the same samples.

In order to investigate the time course of metabolite changes during storage, the study was carried out on bulb samples collected at three different time points, corresponding to harvesting, post-curing and post-storage stages.

The present work aims to evaluate modulation of 'Rossa di Tropea' onion metabolites upon OYDV infection, hypothesizing a significant alteration of primary and secondary metabolite content in infected bulbs. To confirm this hypothesis, metabolites involved in the defense mechanism, dormancy and senescence processes, and in the biosynthesis of nutritional compounds, including those of nutraceutical interest like anthocyanins and flavonoids, were evaluated.

At the best of our knowledge, there is no previous published investigation on the effects of virus infection on onion metabolome.

2. Materials and Methods

2.1 Plant material

Plant material was collected from a pot experiment, including healthy and OYDV infected plants, each group in triplicate randomized blocks. The experiment was carried out at the Mediterranean University of Reggio Calabria (experimental field located in Gallina, RC, Italy) in two consecutive years, 2016 and 2017. All the processing stages were performed into an insect-proof greenhouse. Healthy seedlings were transplanted in 20 cm pots filled with a substrate obtained mixing 3 parts of silty sand with 1 part of universal substrate, integrated with diammonium phosphate. Each experimental block was spatially separated (50 cm) and constituted by 30 pots (5 rows/6 pots). All the pots included in the trial (180 in total from six blocks) were grown under natural conditions of temperature and light exposure, irrigated (20 l m⁻² in 12 h/round) and fertilized (one application of diammonium phosphate 100 g each pot). In addition, treatments with fungicide were performed (Rizolex[®] - 1 application in pre-transplant, 3 g m⁻² and Signum[®] - 2 applications in post-transplant, the second 10 days after the first, 15 g l⁻¹). In addition, chromotropic (blue and yellow) traps were positioned in the greenhouse. When at least 3/4 hollow, linear leaves were enough elongated, half of the plants (three out of six blocks) were mechanically inoculated with OYDV. Experimental inoculation was accomplished by 10 needle punctures along two different blades per plant with needle previously soaked in sap of OYDV-infected leaves ground with 0.1M phosphate buffer (1:5 w:v). Thirty days post-inoculation, leaf samples from all plants, healthy and inoculated, were assayed for OYDV presence by DAS-ELISA serological test (BIOREBA, Switzerland). The infection rate was about 80 % of inoculated plants, which were tagged according to diagnosis results; only bulbs from ascertained healthy and OYDV-infected plants were harvested and sampled for metabolite analysis. Sampling took place at three different times, namely: t₀) at 60 days post-inoculation (DPI) where plants had completely formed bulbs, leaves were still green and the bulbs were harvested, t₁) at 90 DPI, where foliage was completely dried (post-curing stage), and t₂) at 150 DPI, where samples were collected from bulbs at the end of the storage period, when ready to be marketable. Meanwhile, bulbs were stored in the dark in cardboard boxes in a fresh, airy room.

Eighty-four samples were included in HR-MAS NMR experiments (4 or 5 replicates from each of the 6 blocks - 3 healthy and 3 infected, for 3 time points). Ninety samples were included in UPLC and antioxidant activity experiments (5 replicates from each of the 6 blocks for 3 time points). All samples were freeze-dried immediately after collecting and each one was divided in two aliquots to be assayed by HR-MAS NMR and UPLC/antioxidant activity analyses.

2.2 HR-MAS NMR analysis

Samples were prepared by grinding ca. 4 mg of freeze-dried onion with an agate mortar and pestle with ca. 40 μ L of D₂O 10mM 3-(trimethylsilyl)-propionic-2,2,3,3-d₄ acid sodium salt (TSP) used for chemical shift calibration and as internal standard for estimated quantification. High purity reagents were from Sigma-Aldrich Co. (Saint Luis, MO, USA). The material was then inserted in a 4 mm HR-MAS rotor. ¹H-HR-MAS NMR spectra were recorded at 300.0 °K with a Bruker AVANCE spectrometer operating at a ¹H frequency of 400.13 MHz, equipped with a 4 mm HR-MAS dual channel probehead and spinning the samples at 5 kHz. ¹H HR-MAS NMR spectra were acquired by using a water suppression pulse sequence, zgpr (Bruker library), with 32 K data points over a 4807 Hz spectral width and adding 512 transients. The repetition time was set to 3 s, the duration of the hard pulse was 4.5 μ s with an attenuation of 3 dB. The acquired spectra were processed (Fourier transform 16 K data points, phase correction and baseline adjustment, calibration to TSP $\delta = 0.00$ ppm) by means of the standard routines of the software package Xwinnmr 3.5 and Topspin 3.5 (Bruker Biospin, Germany).

The relative intensity of selected signals was measured using the semi-automatic peak picking routine of Topspin 3.5 software; peak lists were generated for the overall spectrum from 0.80 to 8.00 ppm.

Peak assignment of selected metabolites was performed by literature^{6,26} and database comparison (MeRy-B, Biological Magnetic Resonance Data Bank – BMRB, database of Linkoping Sweden)

and a semi-quantitative estimation of 9 chosen metabolites content was obtained considering the ratio of peak areas of analyte and standard equal to the ratio of respective concentrations, corrected for the number of protons (9 in TSP). Both peak lists and metabolite estimated quantitation data were analyzed by statistical methods reported in Section 2.3.

2.3 Statistical Analysis

Estimated quantitation data were subjected to Student t-test (assuming equal variances for the two groups) to establish statistical significance of differences between healthy and infected groups at each sampling time.

To prepare NMR data for multivariate modeling with an untargeted approach, the collected peak lists were assembled and the resulting data matrix was normalized by constant sum, mean-centered and divided by the standard deviation of each variable (auto scaling), and used as input for statistical analysis. The reduced and normalized NMR spectral data were then analyzed using the Statistical analysis tool in Metaboanalyst software²⁷; no data filtering was applied. Peaks appearing in less than one half of samples in each group were ignored. By default, missing values were replaced by a small value. NMR data for each sampling time were analyzed by Partial Least Squares Discriminant Analysis (PLS-DA). The optimal number of components was determined by evaluating the Variance Captured and Statistics, considering parameters as R^2 , Q^2 and accuracy (see Table I S). The obtained models for each sampling time were based on two components and were validated by the Leave-one-out cross-validation method (LOOCV). Furthermore, permutation test was applied calculating the ratio of the between sum of squares and the within sum of squares (B/W- ratio) for the class assignment prediction of the models with a permutation number of 1000.

For each sampling time and PLS-DA analysis, Variable Important for the Projection (VIP) values were also analyzed in function of the chemical shift; VIP limit of significance value was placed equal to 1.0.

2.4 UPLC and antioxidant analyses

2.4.1 Extraction method

The procedure was the following: 10 g of onion sample were extracted with 25 ml of methanol: water: acetic acid (50:42:8, v: v: v) for 10 minutes. The content was homogenized with IKA T 25 digital ULTRATURRAX[®] (Staufen, Germany), shaken automatically for 800 s in Agitax model SR1CP57 (Madrid, Spain) and centrifuged at 5000 xg for 5 min²⁸. The upper liquid phase was filtered and immediately analyzed to determine antioxidant capacity. Extracts were kept in 1.5 ml vials at -20°C until UPLC analysis.

2.4.2 Determination of anthocyanin content

The total anthocyanin content was determined spectrophotometrically according to the AOAC method 2005.02²⁹ on the diluted extract (1:5 v: v) with 0.025M potassium chloride, pH 1.0 buffer and 0.4M sodium acetate, pH 4.5 buffer and absorbance was determined against blank (distilled water) at 520 and 700 nm on a Perkin Elmer UV-Vis Lambda 2 spectrophotometer (Perkin Elmer, MA, USA). Anthocyanin pigment concentration was expressed as g kg⁻¹ of cyanidin 3-glucoside and calculated as follows:

$$\frac{A * MW * DF}{\epsilon * 1}$$

where:

$A = (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 1.0}} - (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 4.5}}$; MW (molecular weight) = 449.2 g mol⁻¹ for cyanidin 3-glucoside (cyd-3-glu); DF = dilution factor; 1 = pathlength (m); $\epsilon = 269$ molar extinction coefficient (l mol⁻¹m⁻¹), for cyd-3-glu.

2.4.3 Analysis of flavonoids by UPLC

Chromatographic analyses³⁰ were performed on a Knauer PLATINblue series ultra-performance liquid chromatography system (Knauer, Berlin) equipped with a binary pump system using a Knauer BlueOrchid column C18 (100 x 2 mm) coupled with a PDA-1 detector PLATINblue. Optimum separation was achieved with a binary mobile phase gradient at a flow rate of 0.4 ml min⁻¹. The column temperature was 30 °C and the injection sample volume was 5 μ l. Mobile phases were water/acetic acid and acetonitrile. The wavelengths in the PDA detector were set to 210 and 450 nm. Identification of flavonoids was carried out by retention times and UV spectra of available flavonoid standards by UPLC.

2.4.4 Antioxidant capacity

The Trolox equivalent (TE) antioxidant capacity (TEAC) was determined using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation decolorization assay³¹. 2950 μ l of ABTS solution in ethanol and 50 μ l of sample were mixed and 734 nm absorbance was measured for 6 minutes on a Perkin Elmer UV-Vis Lambda 2 spectrophotometer. The antioxidant capacity of extracts was expressed as μ M TE g⁻¹ fresh weight (FW).

3. Results

3.1 HR-MAS NMR analysis

3.1.1 Assignment of ¹H HRMAS-NMR spectrum

An example of ^1H HRMAS-NMR spectrum of 'Rossa di Tropea' onion is reported in Fig. 1 (specifically, a healthy onion at t_2 sampling time) which also shows as inset the high field region 0.80 - 3.00 ppm. Intense peaks in the region 3.00 – 5.70 ppm refer to carbohydrates, which account for most of dry weight in onion bulbs³². Other minor signals highlighted in the inset are due to aliphatic protons, mainly of amino acids; in both regions, peaks were assigned as listed in Table 1.

The doublet signal at 5.42 ppm belongs to the C(1)-H anomeric carbon of sucrose (Scr). The signal at 3.82 due to C(5)-H of α -D-glucose (Glc) is visible; the resonance at 4.57 ppm is due to C(2)-H of β -D-galactose (Gal); for α -lactose (Lac), the signal at 3.66 ppm accounts for C(13)-H proton, while the signal at 3.88 ppm is due to C(6)-H₂. The high field region contains signals belonging to the aliphatic groups of amino acids, organic acids and other compounds: amino acids as L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), L-arginine (Arg), L-lysine (Lys), L-asparagine (Asn), L-glutamine (Gln), L-aspartate (Asp), L-glutamate (Glu), L-phenylalanine (Phe), organic acids as malate (Mal), ascorbate (Asc), citrate (Cit), oxaloacetate (Oxa), γ -hydroxybutyrate (Ghb), pyruvate (Pyr) and succinate (Suc), and other compounds as ethanol (Eth) were identified, their resonances being in accordance with previous literature and matching chemical shifts retrieved from databases cited in Section 2.2.

3.1.2 Multivariate Statistical Analysis

Peak lists generated from spectra produced a matrix containing chemical shifts and NMR intensity values as independent variables, as required by the "untargeted" metabolomics approach (i) in Section 1.

To reduce dimensionality of such datasets, thus allowing an easier representation and classification of samples, they were subjected to multivariate data analysis. PLS-DA was useful to classify

samples according to peak patterns and to identify relevant spectral features responsible for the classification, i.e. the phytopathological status of the sample plant.

Based on R^2 , Q^2 and accuracy values (see Table I S), we determined the optimal number of components for a good-fitting and predictive model, which was based on two components.

Fig. 2 shows the score plots for the two-component model obtained by PLS-DA at each sampling time. The distributions indicate a partial separation of samples according to phytopathological status at all three sampling times; Component 1, accounting for ca. 20% of the captured variance, resulted to be particularly informative in discriminating healthy from OYDV-infected samples. Score plots of the two-component models obtained separately for each of the experimental years of trial are reported in Fig. 1S.

Fig. 3 reports the VIP values for each analyzed sampling time. Reported resonances were chosen according to their VIP value with a threshold of 1.0. This allowed the identification of significant resonances, and the relevant assigned metabolites, leading to observed discriminations, and the sign of this difference (up or downregulation in terms of metabolite content).

In particular, the peak at 3.69 ppm, attributed to Leu, was found lower in OYDV-infected samples with respect to healthy ones at all sampling times; the resonance at 2.94 ppm, belonging to Asn, was found higher in infected samples at t_0 and t_2 ; the resonance at 3.26, due to Phe, indicated an upregulation upon infection at t_1 and t_2 ; the peak at 3.22, due to Arg, indicated an upregulation due to OYDV at t_0 and t_1 . Two metabolites (Lys, 3.02 ppm and Glc, 3.82 ppm) showed a bimodal time course: the first was downregulated by infection at t_1 and upregulated at t_2 , while the second was more abundant in infected samples at t_0 and in healthy samples at t_1 .

3.1.3 Metabolite content estimation

By ^1H HR-MAS NMR we estimated semi-quantitatively the content of 9 chosen metabolites (Table 2), in ‘Rossa di Tropea’ onion, healthy and OYDV-infected, for the three sampling times; this methodology followed the (ii) “targeted” metabolomics approach cited in Section 1.

Metabolites were selected among previously annotated candidates, which were considered, from literature, likely to be modulated by virus infection in plant and whose signals could be easily assigned and integrated: namely they were one saccharide (Gal), 3 aminoacids (Asn, Gln, Val) and 5 organic acids (Asc, Cit, Pyr, Suc, Oxa).

Student's t-test showed significant differences in concentration of 6 out of 9 metabolites at different sampling times (Table 2). In detail, Gal was found downregulated by OYDV infection at t_0 (Fig. 4 panel A); the concentration of Asn, Gln, and Val were all significantly higher in OYDV-infected samples at t_2 , Val being also upregulated at t_0 (Fig. 4 panels B, C, D). Cit and Pyr were found at higher levels in infected samples at t_0 and t_1 , respectively (Fig. 4 panels E, F).

3.2 UPLC and antioxidant analyses

3.2.1 Analysis of flavonoids

Quercetin-3,4'-di-O-glucoside was the most abundant flavonoid compound in our samples; its amount in OYDV-infected samples was significantly lower than healthy ones at t_1 and t_2 ($p < 0.01$); we also observed that its amount increased over sampling time (i.e. upon storage) in both healthy and infected conditions. Quercetin-3-O-glucoside, which was the second most abundant flavonoid assessed, was increased in healthy samples with respect to infected at t_1 , while no significant differences between healthy and infected were recorded at t_0 and t_2 ; similarly, no differences were observed in the concentration of this compound over sampling time, both in healthy and infected groups. Total anthocyanin content was observed to increase with sampling time both in healthy and infected samples, this trend not being affected by OYDV infection (Table 3).

3.2.2 Antioxidant capacity

Antioxidant capacity, measured by TEAC assay, showed the results reported in Table 3: in both groups, healthy and infected, we detected the same trend of increase of antioxidant capacity with

sampling time. Despite this common trend, absolute values were different between the two groups, hence antioxidant capacity in OYDV-infected bulbs was lower than in healthy control, at all three sampling times.

4. Discussion

Responses of onion to OYDV infection have been studied mainly from morphological and epidemiological point of view⁹, but information is lacking at metabolic level. The aim of this study is to elucidate ‘Rossa di Tropea’ onion response to virus infection in terms of metabolites modulation at different post-harvest times. We obtained an overview of the content of nutraceutical compounds such as phenols and antioxidants, and the change in metabolic profile after a late inoculation with OYDV, determining the virus effect on marketable bulbs available to consumers. In addition, it was possible to observe the time course of virus infection and its effect in processing stages and after long-term storage.

The ¹H HR-MAS NMR spectroscopic analysis indicated that viral infection significantly affected onion metabolism: PLS-DA analysis of NMR spectra, performed for each sampling time, indicated a partial separation of healthy from infected groups and, based on VIP values, a set of metabolites were found important for this separation; for 6 of the 9 compounds whose direct estimated quantification was performed, a significant difference was found in infected samples with respect to healthy control. The induced metabolic shift affected different classes of compounds, resulting in their accumulation/depletion upon infection, involving different metabolic pathways. In virus-infected plants we observed modulation of metabolites connected to primary (glycolysis, TCA cycle) and secondary (phenylpropanoids) metabolism pathways. In some cases, the response was transient (i.e. limited to one or two sampling times) or bimodal (one trend of regulation at early stages of storage followed by the opposite trend at later sampling times); this result is in accordance with previous experiments by other authors in different plant-virus systems using genomic,

transcriptomic and metabolomic approaches, suggesting a two-phase defense mechanism^{33,34}; actually, the two compounds showing this bimodal trend in our experiments (Lys, Glc) are both involved in biotic stress defense response, as it will be elucidated later in this discussion.

UPLC and antioxidant activity assays showed an altered antioxidant profile correlated with OYDV infection. For quercetin 3-O-glucoside, the response was displaying again a bimodal time-course.

4.1 Carbohydrates

Onion monosaccharides, disaccharides and fructo-oligosaccharides are major components and key factors in onion physiology, conferring protective properties against cold and drought, post-harvest decay and yielding important quality-related attributes³⁵. The role of primary metabolism in plant-pathogen interaction is to provide energy for plant defense. Nonetheless, recent studies have suggested a direct role of carbohydrates in plant response to potyvirus infection³⁶. The semi-quantitative estimation of Gal content in this work shows a depletion of this saccharide upon infection at t_0 , indicating an early response to OYDV infection, in accordance with what previously observed on potato virus Y-infected tomato leaves³⁷. Untargeted metabolomic analysis indicated an increase of the 3.82 ppm signal of Glc at t_0 and a decrease at t_1 : the downregulation along with progressing infection may be due to carbohydrate uptake for oligosaccharide biosynthesis and/or, to carbohydrate consumption affording energy for the biosynthesis of defensive compounds. This also indicates a high rate of glycolysis (respiration), suggesting that OYDV-infected bulbs could not undergo a marked dormancy period during storage, thus possibly causing sprouting, rooting and loss of storability³⁸; this was confirmed by the visual inspection of samples at t_2 in our experimental trial, showing a remarkably higher incidence of sprouting, rooting and secondary pathogen (fungi) infection on the OYDV-infected samples with respect to healthy control; the hypothesis is also coherent with the observed decrease in quercetin glucosides content in infected samples, as quercetin content was correlated to dormancy^{19,20}. Besides, the decrease of Glc levels

in onion bulbs could be an important drawback also for their sensorial features; in fact, it was established that higher amounts of Glc in two onion cultivars were positively correlated with taste preference, disregarding the amount of other saccharides as sucrose³⁹.

4.2 Amino acids

Amino acids modulation due to virus infection was previously observed in plants but it is still unclear whether such effect is caused by selective protein breakdown or higher rates of biosynthesis⁴⁰.

Our results of targeted and untargeted analysis revealed a decrease in the content of some amino acids and an increase in others in OYDV-infected onion bulbs. Higher levels of Asn, Gln, Phe and Val were induced by virus infection at t_2 ; the same trend was observed also at t_0 for Asn and Val, and at t_1 for Phe. Arg was found upregulated by virus infection at t_0 and t_1 , while returning to same levels of healthy samples at t_2 . Leu was found downregulated at all sampling times in OYDV-infected group, while Lys was in lower amounts at t_1 and in higher amounts at t_2 in infected samples with respect to healthy control.

Generally, the accumulation of free amino acids in pathogen-infected plants is widely reported in literature^{36,41,42} and may reflect an increased demand for carbon, as amino acids can be shuttled into energy-generating pathways such as the TCA cycle.

The recorded accumulation of Asn in OYDV-infected onions is in accordance with previous literature accounting for higher levels of this amino acid in plants infected by pathogens^{43,44}. This may be a direct stress-related response, as in the case of bacterial infection, or an indirect result of the decrease of protein synthesis and/or increased protein degradation in tissues under senescence or programmed cell death. In fact, before being released for remobilization, amino acids are converted to amides. Hence, an increase in Asn suggests that onion bulb tissues could be initiating senescence because of virus infection. Regarding food quality-related characters, Asn

accumulation as a free amino acid in vegetables might also cause drawbacks for food safety, because Asn is a precursor for acrylamide formation at high temperatures⁴⁵.

Pathogen infection has been reported to stimulate the biosynthesis of Glu and Arg, that are both associated with photorespiration⁴⁶.

We observed Gln downregulation in OYDV-infected onion bulbs: Gln has been reported to inhibit priming stress resistance by β -aminobutyric acid⁴⁷, hence low levels of Gln may enhance this function upon virus infection. Another reason for Gln depletion may be the overconsumption of its precursor Glu for the biosynthesis of glutathione, which is reported to be a key intermediate in plant response to stress⁴⁸ and virus infection⁴⁹. Induction of Arg in early stages of infection can be explained by a similar mechanism, as Arg has been proposed as a precursor of nitric oxide and polyamines, both important factors in regulating responses to biotic and abiotic stress. Again, high levels of Arg suggest the demand for sustained synthesis of defense response compounds as nitric oxide and polyamines. Being an essential amino acid, the increased content in Arg in OYDV-infected onions at marketing stage indicated in this work could modify the nutritional profile of the edible product.

The observed accumulation of Phe in infected onion bulbs could be correlated with a suppression of Phenylalanine Ammonia Lyase (PAL), an important plant enzyme catalyzing the deamination of phenylalanine as the first step of the general phenylpropanoid pathway, a step that is common to the production of many metabolites including flavonoids. This explanation is in accordance with the decrease of flavonoid content in infected onion bulbs observed in our study.

Val and Leu are, together with Ile, branched-chain amino acids (BCAAs) classified by their small branched hydrocarbon residues. Unlike animals, plants are able to *de novo* synthesize BCAAs from pyruvate. The early upregulation of Val and Pyr found in our experiments may suggest the induction of this biosynthetic pathway, while the decrease of Leu probably indicates a suppression limited to the part leading to Leu synthesis, which starts out from 2-oxoisovalerate, the last intermediate that is transaminated to form Val.

Lys levels in plants are generally regulated by its biosynthesis; hence, the bimodal trend observed during time is an index of the varying synthesis rate according to infection. As Arg, Lys is an essential amino acid and its levels in OYDV-infected onions at marketable stage could be responsible for an altered nutritional profile of the product.

4.3 Organic acids

Pyruvate is the simplest α -ketoacid and represents a key intermediate in several metabolic pathways throughout the cell: it is the product of the breakdown of glucose via glycolysis for energy production, and its decarboxylation by pyruvate dehydrogenase complex leads to acetyl CoA, whose main function is to deliver the acetyl group to the tricarboxylic acid (TCA) cycle to be oxidized for energy production. The observed accumulation of Pyr content in infected bulbs at t_1 may indicate that Pyr is produced from glycolysis faster than pyruvate dehydrogenase can convert it to acetyl CoA, a situation which has been reported in plants under particularly energetically demanding conditions, as those of pathogen defense³⁶.

Moreover, Pyr concentration has been correlated with the degree of pungency experienced using taste panel evaluation^{50,51,52} (Schwimmer, Crowther, Wall).

The observed accumulation of Cit is also correlated with the biotic stress response: in fact aconitase, converting Cit to cis-aconitate, has been reported to be the most vulnerable of TCA enzymes under oxidative stress conditions⁵³, which are often established upon virus infection; aconitase inactivation leads to Cit accumulation.

4.4 Antioxidant compounds and activity

Flavonoids, particularly quercetin derivatives, are widely distributed in plants and are known to prevent cardiovascular diseases and cancer by their antioxidant activity. Onion is one of the richest vegetables in quercetin³, which is considered a valuable nutraceutical enhancing added value of

the edible product as a dietary element. Quercetin-3,4'-di-O-glucoside was the most abundant flavonoid in our samples, as confirmed by literature⁵⁴. The observed depletion of quercetin glucosides in OYDV-infected onion bulbs can be correlated to a defense response of the plant to virus infection. In addition, overall antioxidant activity was observed to decrease in infected bulbs. This alteration is well documented in literature for several plant patho-systems and has been explained by the oxidative burst following infection. Oxidative burst and controlled accumulation of Reactive Oxygen Species (ROS) are main characteristics of plant defense response and have been shown to induce the expression of a variety of defense genes⁵⁵. Our results showed that OYDV infection led to substantial changes in the antioxidant system in onion bulbs: the reduced redox capacity of infected tissues probably induces the accumulation of ROS, which play a key role in both triggering infected cell death/control (leading to typical necrotic symptoms) and signaling for the induction of defense-related genes in adjacent tissues. The decrease in antioxidant compounds and antioxidant activity in OYDV-infected onion bulbs can be considered a severe concern for the nutritional profile and, hence, for the added value of the edible product.

4.5 Evolution with timing of sampling

The analyses run at three sampling times allowed to study the differences in metabolites content during storage in the healthy control; in fact, it is well known that storage period is critical for the evolution of many primary and secondary metabolites, some of them having a deep impact on storability and overall bulb quality at marketable stage¹³⁻²⁰. Actually almost all the detected metabolites showed a specific trend of increase/decrease through the different sampling times; for example, flavonoids, anthocyanins and organic increased from t_0 to t_2 , in accordance with previous works^{16,17}.

The evolution of metabolites content found in healthy onions was used as a benchmark for observing changes in the trend in OYDV-infected samples, due to the presence of the virus. These changes

involved particularly glucose and ascorbate, whose levels during storage were downregulated in healthy control, while being unaltered in infected samples; and Asn, which was upregulated upon storage in presence of the virus, while being downregulated in healthy control. All these findings, as previously discussed, can give a glance at the significance of phytopathological status of onion crops, in view of the importance of the storage period in the production chain leading to marketable onion bulbs.

4.6 Conclusion

In the present study, the metabolite profiling of ‘Rossa di Tropea’ onion obtained by means of ¹H HR-MAS NMR and UPLC highlighted significant differences in several primary and secondary metabolites between healthy and OYDV-infected samples. NMR data combined with multivariate statistical analysis were able to discriminate samples according to their sanitary status; the metabolites responsible for such discrimination were also identified and were related to biochemical pathways involved in senescence and dormancy processes, important for bulb long-term storability, and biosynthesis of nutraceutical compounds.

This approach proved to be a reliable tool to investigate OYDV-induced metabolic shift in onion, which particularly involves quality-related compounds at harvesting and during storage; due to the high incidence and damages due to OYDV infection in the whole *A. cepa* species, the same investigation could be suitably applied to other onion varieties.

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Table I. ^1H chemical shifts of assigned metabolites, referred to TSP signal $\delta = 0.00$ ppm.

Compound	Assignment	^1H (ppm)	Multiplicity
<i>Carbohydrates</i>			
α -D-Glc	C(5)-H	3.82	
Scr	C(1)-H	5.42	d
β -Gal	C(2)-H	4.57	
α -Lac	C(13)-H	3.66	s
	C(6)-H	3.88	d
<i>Amino acids</i>			
Val	H $_{\gamma}$	1.01	d
Leu	H $_{\beta}$	1.72	
Ile	H $_{\beta}$	1.97	
Arg	H $_{\alpha}$	3.80	
	H $_{\beta}$	1.92	m
Lys	H $_{\epsilon}$	3.02	
Asp	H $_{\alpha}$	2.83	d
Glu	H $_{\beta}$	2.11	m
Asn	H $_{\beta}$	2.87	dd
	H $_{\beta'}$	2.93	
Gln	H $_{\beta}$	2.16	m
<i>Organic acids</i>			
Asc	C(4)-H	4.51	
Mal	H $_{\alpha}$	4.29	dd
Oxa	C(4)-H	2.37	s
Ghb	C(2)-H	1.96	d
Cit	C(2)-H	2.66	d
Pyr	C(1)-H	2.53	s
<i>Other compounds</i>			
Eth	C(2)-H	1.15	t

Table II. Evaluation of regulation of 9 chosen metabolites in healthy vs infected samples by targeted HR-MAS NMR-based metabolomics.

entry	compound	regulation
1	β -D-Galactose	↓ _{t0}
2	L-Asparagine	↑ _{t2}
3	L-Glutamine	↑ _{t2}
4	L-Valine	↑ _{t0, t2}
5	Ascorbic acid	x
6	Citric acid	↑ _{t0}
7	Oxalacetic acid	x
8	Pyruvic acid	↑ _{t1}
9	Succinic acid	x

Table III. Quercetin 3,4' di-O-glucoside, quercetin 3-O-glucoside and total anthocyanin content and antioxidant capacity in healthy and OYDV-infected bulbs at three sampling times. Values are expressed as mean \pm standard error for 15 samples each entry; Student's t test performed between healthy and infected groups at each sampling time: *=significant difference at $p < 0.05$; **=significant difference at $p < 0.01$.

Sampling time	Phyto pathological status	Quercetin 3,4'-di-O-glucoside (g kg ⁻¹ FW)	Quercetin 3-O-glucoside (g kg ⁻¹ FW)	Total anthocyanins (g cyanidin 3-glucoside kg ⁻¹ FW)	Antioxidant capacity (μ mol TE g ⁻¹ FW)
t ₀	healthy	0.0831 \pm 0.0109	0.00451 \pm 0.00057	0.00971 \pm 0.00142	6.35 \pm 1.17*
	OYDV-inf	0.0559 \pm 0.0099	0.00461 \pm 0.00128	0.01106 \pm 0.00307	3.53 \pm 0.49*
t ₁	healthy	0.1659 \pm 0.0262**	0.00757 \pm 0.00224*	0.02296 \pm 0.00322	6.38 \pm 0.71*
	OYDV-inf	0.0868 \pm 0.0438**	0.00287 \pm 0.00037*	0.01963 \pm 0.00318	4.24 \pm 0.44*
t ₂	healthy	0.1933 \pm 0.0129**	0.00444 \pm 0.00023	0.04470 \pm 0.00422	8.20 \pm 0.79*
	OYDV-inf	0.1271 \pm 0.0193**	0.00353 \pm 0.00058	0.03442 \pm 0.00544	4.94 \pm 0.61*

Table 1S. Statistics obtained values (R^2 , Q^2 , accuracy) for the 5-Component-based PLS-DA models for t_0 , t_1 and t_2 sampling times.

Model	Measure	1 Comps	2 Comps	3 Comps	4 Comps	5 Comps
	Q^2	0.0896	0.3175	-0.0412	0.0542	-0.0979
t0	R^2	0.5439	0.6935	0.8459	0.8736	0.9089
	accuracy	0.6071	0.6786	0.5714	0.6429	0.6429
	Q^2	0.0011	0.0189	0.4495	0.5417	0.4162
t1	R^2	0.2968	0.5267	0.7765	0.8649	0.8932
	accuracy	0.4815	0.5556	0.5185	0.5926	0.5556
	Q^2	0.0809	0.0345	-0.0965	-0.1456	-0.0732
t2	R^2	0.3959	0.5867	0.8062	0.8652	0.8991
	accuracy	0.6786	0.6786	0.7148	0.6071	0.6071

Captions to figures

Figure 1. ^1H HR-MAS NMR spectra of ‘Rossa di Tropea’ onion bulbs; peak assignments are reported in Table 1.

Figure 2. Score plots of the matrix obtained by the PLS-DA model built with two components based on ^1H HR-MAS NMR spectra of healthy (h) and OYDV-infected (inf) samples for t_0 , t_1 and t_2 sampling times.

Figure 3. Variable Important in the Projection as a function of the chemical shift, chosen according to their VIP value >1.0 , for t_0 , t_1 and t_2 sampling times; black bars indicate upregulation (infected $>$ healthy), grey bars indicate downregulation (infected $<$ healthy) referred to the intensity of the relevant signal.

Figure 4. Metabolite content estimation expressed in g kg^{-1} dry weight in healthy (h) and OYDV-infected (inf) ‘Rossa di Tropea’ onion bulbs as measured by ^1H HR-MAS NMR for 6 metabolites at different sampling times. Only metabolites regulated at the specific observation times are shown, namely: Gal at t_0 (panel A), Asn at t_2 (B), Gln at t_2 (C), Val at t_0 and t_2 (D), Cit at t_0 (E), Pyr at t_1 (F). Data are presented as means of 15 samples and differences were checked significant by Student’s t-test at $p < 0.05$.

Figure 1S. Peaklist-based PLS-DA for each of the two years of experimental trial. Score plots of the matrix obtained by the PLS-DA model built with two components based on ^1H HR-MAS NMR spectra of healthy (h) and OYDV-infected (inf) samples for t_0 , t_1 and t_2 sampling times in the first (panel A) and second (panel B) of the two years of experimental trial.