

Proceeding Paper

Metabolites Differentiating Asymptomatic and Symptomatic Grapevine Plants (*Vitis vinifera* ‘Malvasia-Fina’) Infected with Esca Complex Disease-Associated Fungi †

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Abstract: Pathogens are known to affect major physiological processes in plants including the regulation of metabolic networks to maintain homeostasis. Metabolomic analyses generate large datasets that could be analyzed to give insight into the specific metabolic adaptations involved in plant responses to grapevine trunk diseases such as esca complex. The goals of this study were to identify metabolites differentiating asymptomatic and symptomatic grapevine plants infected with esca complex disease-associated fungi, and biosynthetic pathways active during disease progression. Experiments were performed using healthy, asymptomatic and symptomatic leaves of *Vitis vinifera* L. ‘Malvasia-fina’ naturally infected in the vineyard. A global metabolite profile of the samples was obtained using a UPLC + GC-MS/MS² analytical platform. A total of 513 metabolites belonging to 60 pathways were detected. The analysis of the data allowed the elucidation of some of the mechanisms by which grapevine tolerate the presence of pathogens, and the selection of top metabolites worthy of further investigation.

Keywords: grapevine trunk diseases; metabolomic; lipidomic; biochemical pathways; disease onset; symptom appearance; brown wood streaking; white rot; leaf stripe



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1. Introduction

Grapevine trunk diseases, a major biotic stress for plants, have been studied intensively for the process of pathogen infection [1]. A major characteristic of grapevine trunk diseases is the involvement of not one, but several pathogenic species in the etiology of the disease. Esca complex for example is caused by several fungi belonging to the phylum Ascomycota, and to a lesser extent to the phylum Basidiomycota (e.g., *Fomitiporia mediterranea*) [2,3]. Colonization by esca-associated fungi is restricted to the canes, spurs, cordons, and trunks [4]. The perception of these pathogens by the grapevine plant is known to trigger various defense responses e.g., the deployment of anatomical [5], physiological [6,7], and biochemical [8–10] features in order to limit fungal wood invasion and the translocation of fungal toxic metabolites to the leaves. The disease usually exhibits a latent period between wood invasion by fungi and visible foliar symptoms [7,11]. The transition from the asymptomatic state (absence of foliar symptoms) to the symptomatic state is believed to be influenced by biotic, abiotic, and genetic factors [3,6].

According to the literature, photosynthesis and respiration are among the first processes to be impacted upon infection by esca-related pathogens [9,12]. A perturbation

of photosynthesis and respiration processes in grapevine plants is often accompanied by metabolic changes such as up/downregulation of genes that encode enzymes involved in detoxification processes [8,13], modulation of the expressions of antioxidant proteins [10,14], and accumulation of metabolites with different roles in defense [4,11]. Depending on the infection stage, however, distinct metabolic changes have been observed in the tissues of grapevine plants affected by esca complex. In the study by Valtaud et al. [8], the ratio of glutathione disulfide to the total glutathione pool was slightly decreased in asymptomatic leaves compared to healthy leaves but increased with the appearance of visible damage. An alteration of the photosynthetic apparatus could be detected two months before the appearance of foliar symptoms [12], which demonstrated that the disease's causative agents induce pronounced systemic effects in the leaves.

These results show that a perturbation of the central and secondary metabolisms is provoked by the presence of woody fungal pathogens. Despite over 20 years of investigation, however, the precise adaptative mechanisms developed by the grapevine in response to esca complex have not been unraveled. This research gap in the field might be due to the scarcity of studies on plant responses at the transcriptomic, proteomic, and metabolomic levels. Recent untargeted OMIC studies have shown the possibility of identifying specific pathways directly involved in the etiopathogenesis of the disease [12–16]. A global OMIC analysis of grapevine could be a crucial step in efforts to appreciate the mechanisms leading to wood vascular invasion, symptom emergence, and plant tolerance. Thus, the goal of this study was to use a global metabolomic analysis of leaves to (i) identify metabolites differentiating asymptomatic and symptomatic grapevine plants affected by esca complex disease, and (ii) gain an insight into the modulation of biosynthetic pathways with disease evolution.

2. Materials and Methods

The “Quinta de Nossa Senhora de Lourdes” vineyard (465 m, 41°17.12'31" N, 7°44.07'22" W) in Vila Real (Portugal) was used to develop the protocol for sampling. Experiments were performed on *Vitis vinifera* L. ‘Malvasia-fina’ [17]. Field monitoring for six consecutive years allowed for the identification of healthy, asymptomatic, and symptomatic (two levels of severity) plants as shown in Figure 1. Six plants were selected for each group of plants and an average of 10 leaves were collected from each plant.

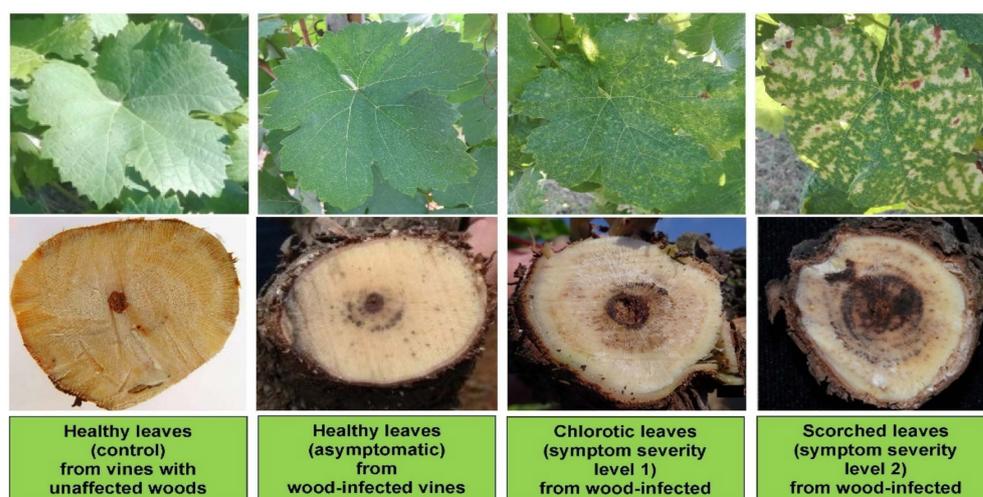


Figure 1. Symptomatic expression of esca complex disease in the leaves and woods (cross-sections) of *Vitis vinifera* L. ‘Malvasia-fina’ plants in a naturally infected vineyard. From left to right are healthy, asymptomatic, chlorotic, and scorched leaves, with a moisture content of ca., 65% [16].

Metabolites were extracted from lyophilized and pulverized leaf samples with methanol using an automated MicroLab STAR[®] system (Hamilton Robotics, Reno, NV, USA). A global

metabolic profiling of the samples was obtained with an Ultrahigh Performance Liquid Chromatography + Gas Chromatography-Tandem Mass Spectroscopy (UPLC + GC-MS/MS²) platform. The analytical platform incorporated four separate UHPLC-MS/MS² injections and one GC-MS/MS²; the UPLC injections were optimized for hydrophilic, hydrophobic, basic, and polar compounds and the GC injection for free fatty acids. The method integrated both relative quantification (full scan MS) and qualitative capabilities (MS/MS) into each analysis without the need for additional data acquisition, as fully described by Goufo et al. [17]. After chromatographic separations and mass spectrometry analyses, raw data were extracted as area-under-the-curve detector ion counts and scaled imputed data were calculated. The identification of metabolites was based on three criteria: retention index within a narrow retention index window of the proposed identification, ± 10 ppm accurate mass match to a library of ca. 10,000 MS/MS spectra of standard compounds, and the MS/MS forward and reverse scores.

Statistical comparisons contrasted leaves from each of the infected plants to those of the control uninfected plants. Following log transformation, samples were subjected to Random Forest analysis [18] in order to identify metabolites that differed significantly among experimental groups and to provide an “importance” rank ordering of these metabolites. A random subset of the data with identifying true class information was selected to build a decision tree; the remaining data were passed down the tree to obtain a class prediction for each sample. The process was repeated thousands of times to produce the forest. The final classification of each sample was determined by computing the class prediction frequency for the remaining data variables over the whole forest. To determine which metabolite made the largest contribution to the classification, the “Mean Decrease Accuracy” (MDA) was computed and a confusion matrix was plotted to express the accuracy of the classifier’s predictions.

3. Results and Discussion

In total, 513 metabolites were identified in the leaves of the grapevine, including 436 compounds of known identity and 77 compounds of unknown structural identity, belonging to nine biochemical families (amino acids, carbohydrates, lipids, cofactors + prosthetic groups + electron carriers, nucleotides, peptides, hormones, secondary metabolites, and xenobiotics) (Figure 2).

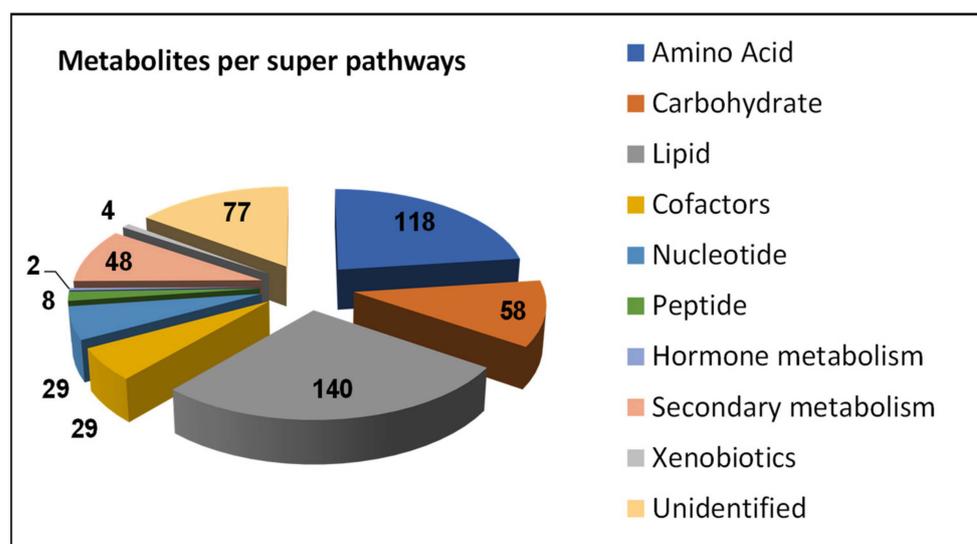


Figure 2. Major classes of metabolites identified in the leaves of the grapevine cultivar Malvasia-fina.

The metabolites were assigned to 60 pathways for better visualization of significantly altered biochemicals and for targeting the pathways of interest (Figure 3). Although a Principal Component Analysis plot showed clear separation from the control only for

symptomatic leaves (data not shown), several metabolites achieved statistical differences in the *t*-test between healthy and asymptomatic samples ($p \leq 0.10$). Several metabolites showed interesting changed patterns related to the modulation of grapevine metabolism. Hormone data, for example, showed that systemic signals are transferred from the infected wood to the leaves [16]. Secondary metabolites data indicated that defense compounds are mostly locally induced following the onset of foliar symptoms [11].

SUB PATHWAYS with significantly altered biochemicals			
Serine family (phosphoglycerate derived)	9	Lyso-galactolipids	6
Aromatic amino acid metabolism (PEP derived)	13	Sphingolipid	6
Aspartate family (OAA derived)	23	Sterols	3
Glutamate family (alpha-ketoglutarate derived)	40	Sulfolipids	3
Branched Chain Amino Acids (OAA derived)	2	CoA metabolism	1
Branched Chain Amino Acids (pyruvate derived)	16	Nicotinate and nicotinamide metabolism	8
Amines and polyamines	5	Oxidative phosphorylation	2
Glutathione metabolism	10	Carnitine metabolism	2
Glycolysis	6	Riboflavin and FAD metabolism	1
TCA cycle	9	Ascorbate metabolism	4
Calvin cycle and pentose phosphate	3	Thiamine metabolism	1
Photorespiration	3	Tocopherol metabolism	3
Amino sugar and nucleotide sugar	18	Vitamin B metabolism (B6 or B12)	5
Inositol metabolism	2	Chlorophyll and heme metabolism	7
Sucrose, glucose, fructose metabolism	15	Purine metabolism	15
C5 branched dibasic acid metabolism	2	Pyrimidine metabolism	13
Fatty acid, free saturated	16	Jasmonic acid metabolism	1
Fatty acid, free unsaturated	20	Dinucleotides	1
Fatty acid, hydroxy	7	Dipeptide	7
Fatty acid amide	1	Dipeptide Derivative	1
Fatty acid, Amino	2	Abscisic acid metabolism	1
Fatty acid, Dicarboxylate	9	Alkaloids	3
Fatty acid conjugate	1	Alkaloid derivative	1
Phospholipids	23	Benzenoids	11
Lyso-phospholipids	11	Flavonoids	13
Phospholipid Metabolism	7	Furan metabolism	1
Choline metabolism	4	Phenylpropanoids	5
Glycerolipid Metabolism	3	Siderophores	1
Glycerolipids - Monoacyl	4	Terpenoids	5
Glycerolipids - Diacyl	3	Orphan secondary metabolites	1
Galactolipids	13	Stilbenoids	4

Figure 3. List of biosynthetic pathways affected by esca complex disease in the leaves of *Vitis vinifera* L. ‘Malvasia-fina’. The numeral values correspond to the number of metabolites belonging to each pathway.

Random Forest analysis (Figure 4) could classify the samples with 96% accuracy, which allowed for the selection of 30 metabolites as potentially differentiating asymptomatic and symptomatic grapevine plants infected with esca complex disease-associated fungi. Most of these metabolites were amino acids or belonged to secondary-metabolism. The levels of several aromatic amino acids such as phenylalanine fell during foliar symptom emergence and the decreases were amplified with symptom progression. This indicated a shift in C partitioning away from the shikimate pathway [19] to other pathways such as the phenylpropanoid pathway. In fact, an abundance of flavonoids with various roles in defense [20] was observed in diseased leaves compared with healthy leaves.

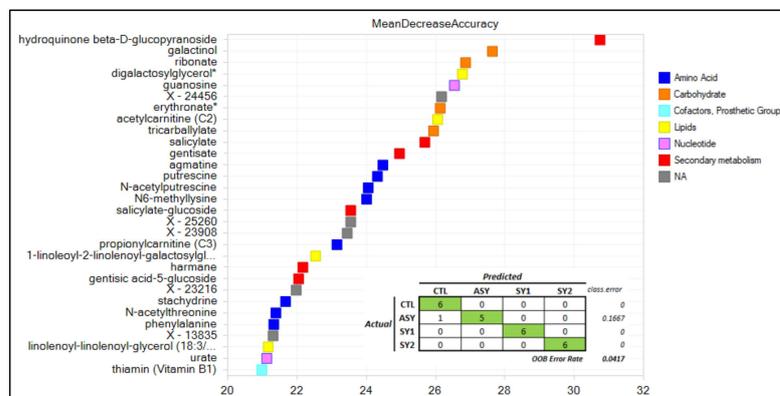


Figure 4. Random Forest results of the analysis of 513 metabolites detected in leaves of *Vitis vinifera* L. ‘Malvasia-fina’ affected by esca complex disease. The insert plot represents the confusion matrix. The forest output for the top 30 metabolites that made the largest contribution to the differentiation of leaf groups: CTL = control healthy leaves; ASY = asymptomatic leaves; SY1 = symptomatic leaves with chlorosis; SY2 = symptomatic leaves with scorches.

Overall, the data show that there are different adaptation strategies developed by the grapevine plant in response to esca attack. A deeper analysis of these data should permit the identification of the pathways active during stress, which could help to establish the structural features essential for selecting or breeding tolerant plants.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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