Proceeding Paper

Galactolipids as Potential Biomarkers for Early Diagnosis of Esca Complex Disease in Asymptomatic Grapevine [†]

Piebiep Goufo [1,2,*] and Isabel Cortez [1,2,*]

1 Centre for the Research and Technology of Agro-Environment and Biological Sciences, Universidade de Trás-os-Montes e Alto Douro, Quinta de Prados, 5000-801 Vila Real, Portugal
2 Departamento de Agronomia, Universidade de Trás-os-Montes e Alto Douro, Quinta de Prados, 5000-801 Vila Real, Portugal
* Correspondence: pgoufo@utad.pt (P.G.); icortez@utad.pt (I.C.)
† Presented at the 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants, 1–15 December 2021; Available online: https://iecps2021.sciforum.net/.

Abstract: The Esca disease complex seriously affects grapevine yield and longevity. Because of the time delay between wood infection by the fungi and symptom expression, current disease diagnosis relies on destructive sampling of the wood. The goal of this study was to identify metabolites that could be used as biomarkers for developing a noninvasive biochemical method for early diagnosis of the disease. Results from lipidomic analysis showed a positive correlation between the level of leaf necrosis and the levels of galactolipids, suggesting a role for galactolipids in the etiopathogenesis of the disease. Such information could be used to develop a method for identification of Esca-affected grapevines without the need to rely upon symptomatic descriptors.

Keywords: hydraulic failure hypothesis; lipid profile; elicitor-toxin hypothesis; candidate marker; symptom descriptor; prognostic indicator; galactolipid homeostasis

1. Introduction

Plants have developed enzymatic and nonenzymatic mechanisms to maintain cellular integrity in response to pathogen attacks [1,2]. For example, during the establishment of immune responses, several compounds act as signals to trigger and mediate defense responses [1–4]. These plant responses ultimately impact the processes of infection and the expression of symptoms. A growing number of studies conducted during the last decade indicate that lipid-associated molecules play essential roles not only in plant resistance, but also in plant signaling, as mediators of signal transduction [5]. Signal transduction is the process by which all cells constantly receive and act in response to new signals from their environment; the phenomenon has been associated with systemic acquired resistance in plants, and could explain the latency period observed in some diseases [2,6].

A peculiar characteristic of the pathosystem of grapevine–Esca complex disease-associated fungi is an undetermined period of latency within the grapevine (asymptomatic status) [7]. Esca complex is a vascular disease that attacks the perennial organs of grapevine plants and causes extensive wood necroses via the slow and systemic action of fungi such as *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, and *Fomitiporia mediterranea* [8,9]. Leaf symptoms in infected grapevines are known to appear in a discontinuous manner in time and space in the vineyard [6,7], and because of that, diagnosis is difficult [10,11]. The infection status of asymptomatic grapevines can most often only be known after cutting and destroying the grapevines [7]. The complexity of the disease is also related to a lack of efficient and long-term control strategies [10]. A method for early detection of Esca complex could help in preventing its spread in the vineyards, limiting the economic losses that it causes [11].

In this study, it was hypothesized that metabolic responses of the host, and in particular, compounds involved in signal transduction, could allow differentiation of healthy and infected grapevines. Lipid signaling in plants is mediated by an ample range of molecules, such as sphingolipids, glycerophospholipids, fatty acids, oxylipins, and sterols [2,5]. For example, the galactolipids digalactosyldiacylglycerol (DGDG) and monogalactosyldiacylglycerol (MGDG) have a major role in determining the stability of thylakoid membranes [3,12,13]. The importance of lipids in grapevine response to pathogens has been highlighted in recent papers [14–16]. In response to downy mildew, a tolerant grapevine cultivar exhibited a higher galactolipid modulation at the chloroplast level than a susceptible cultivar; with an increase observed in the levels of galactolipids (MGDG in particular), as well as in the levels of α-linolenic acid (C18:3) [14,15]. A higher degree of lipid desaturation, which translated into a high C18:3 content, in response to grey mold and powdery mildew was also shown in grapevine leaves [16]. These studies demonstrate that the difference between tolerance and resistance to diseases is related to how the plants use lipids to defend themselves. Thus, lipid analyses could lead to major insights into the mechanistic basis of the onset and evolution of disease symptoms. The goal of this study was to identify lipid species differentiating healthy, asymptomatic, and symptomatic grapevines which can be used as biomarkers for early diagnosis of Esca complex. The applicability of leaf candidate markers for predicting the onset of diseases is best achieved using unbiased analyzes [5,17]; therefore, samples were submitted for lipidomic analysis, which enabled coverage of a broad range of lipid classes.

2. Materials and Methods

Healthy control (CTL), asymptomatic (ASY) and symptomatic (SY) leaves of the white cultivar Malvasia Fina were collected during the summer of 2019 in the vineyard of Quinta de Nossa Senhora de Lourdes, located in the Douro wine-growing region of Portugal. A detailed description of the vineyard layout and soil characteristics is provided in Goufo et al. [6,18]. The average temperature, rainfall, and relative humidity during the year of sample collection were 13.97 °C, 1131 mm, and 75%, respectively. In the vineyard, four rows were chosen for the study, and constituted of 243 plants grown according to the royal-type trellis system. External symptoms of Esca complex were identified visually, while wood cores were extracted with an increment borer in order to assess internal infections, as fully described in Goufo et al. [6]. Symptomatic leaves were arbitrarily categorized into two groups based on symptom intensity: chlorotic leaves (SY1), and scorched leaves (SY2). For each treatment group (CTL, ASY, SY1, and SY2), 10 leaves were collected from six plants (*n* = 6) after grape maturity had been reached. The leaves were immediately frozen with liquid nitrogen and stored in a freezer at −80 °C.

A platform was developed for lipidomic analyses using an ACQUITY ultra-performance liquid chromatograph (Waters Corporation, Milford, MA, USA), a gas chromatograph (Shimadzu GC-2010 Plus (Kyoto, Japan), and a Q-Exactive Hybrid Quadrupole-Orbitrap high resolution accurate mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The platform was based on the extraction of lyophilized powdered leaves in two steps, followed by four UPLC injections and one GC injection. In the first extraction step, 20 mg of leaf sample was added to 400 μL of methanol. Following centrifugation, the supernatant was collected and split into four aliquots for UPLC analysis. In the second extraction step, 20 mg of leaf sample was added to 4 mL of a mixture of methanol, chloroform, water, and potassium chloride. Following transesterification of the resulting extract, the residue was reconstituted in methanol and used for GC analysis. Chromatographic separation, mass spectrometry analysis, and compound identification parameters were as described by Goufo et al. [18]. Peak signals were acquired using Xcalibur QuanBrowser 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). Detected lipid species were processed and grouped by lipid class using a Laboratory Information Management System (Metabolon, Inc, Morrisville, NC, USA). The raw data were normalized by dividing each sample value by the sample weight and the median value for the metabolite, to obtain the scaled imputed

data. Mean values of scaled imputed data were calculated for each leaf group, and Welch's two-sample *t*-tests were applied to screen for potential biomarker candidates. Where necessary, other classification methods were used, e.g., fold changes, volcano plots, hierarchical clustering, principal component analysis, pathway mapping, and rich factor analysis. All statistical analyses were performed using Array Studio 10.0 (OmicSoft Corporation, Cary, NC, USA).

3. Results

Untargeted analyses in positive and negative modes permitted the detection of 258 molecular lipid species. The raw and processed data for all lipid species was deposited in the Mendeley Data repository (https://doi.org/10.17632/4k49sk6s2w.3; accessed on 25 March 2022) as supplemental information. The lipid species belonged to the following biochemical classes: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, prenol lipids, sterol lipids, hormones, carotenoids, chlorophylls, carnitine metabolics, and choline metabolics. The four leaf groups exhibited distinct lipidomic profiles with clear variations in the levels of lipid species among these classes. The latency period before symptom appearance was associated with 53 lipid species (where 25 were observed to have lower concentrations, and 28 were higher); symptom emergence was associated with 74 lipids species (41 lower and 34 higher); and symptom progression was associated with 124 lipid species (43 lower and 81 higher).

Of all the biochemical classes, the galactolipid class (glycerophospholipids) was the only variable that explained the differences between the experimental groups. Indeed, the levels of 12 galactolipids were increased in the leaves of asymptomatic grapevines, and progressively decreased with symptom emergence and progression (Table 1). The asymptomatic state (wood infection, but asymptomatic leaves) was associated with increases in the abundance of all galactolipids, except for MGDG. Among the strongly changed galactolipids were DGDG (0.25-fold change), 1-palmitoyl-2-linolenoyl-digalactosylglycerol (16:0/18:3) (34:3-DGDG, 0.26-fold change), and 1-palmitoyl-2-linolenoyl-galactosylglycerol (16:0/18:3) (34:3-MGDG, 0.26-fold change). The appearance of symptoms in the leaves was found to be negatively associated with all galactolipids (except for a 0.74-fold increase for MGDG). With symptom progression, levels of galactolipids were depleted in diseased leaves (Table 1).

Table 1. Variations in levels of galactolipids in *Vitis vinifera* L. 'Malvasia Fina' affected by Esca complex disease. CTL = control healthy leaves; ASY = asymptomatic leaves; SY1 = symptomatic leaves with chlorosis; SY2 = symptomatic leaves with scorches. Levels of individual lipid species are expressed as scaled imputed data [18]. Different colors represent different directions and intensities of change (light orange = positive change; light blue = negative change) at $p \leq 0.10$ (Welch's two-sample *t*-test, *n* = 6).

Galactolipids	Abbreviation	CTL	ASY	SY1	SY2
1-palmitoyl-2-linoleoyl-galactosylglycerol (16:0/18:2)	34:2-MGDG	1.10	1.29	0.88	0.85
1-palmitoyl-2-linolenoyl-galactosylglycerol (16:0/18:3)	34:3-MGDG	1.04	1.25	0.97	0.76
1,2-dilinoleoyl-galactosylglycerol (18:2/18:2)	34:4-MGDG	1.02	1.12	0.94	0.93
1-linoleoyl-2-linolenoyl-galactosylglycerol (18:2/18:3)	36:5-MGDG	1.19	1.23	0.83	0.83
1-linolenoyl-2-hexadecatrienoyl-galactosylglycerol (18:3/16:3)	34:6-MGDG	1.12	1.39	0.94	0.75
1,2-dilinolenoyl-galactosylglycerol (18:3/18:3)	36:6-MGDG	1.03	1.13	0.94	0.85
1-palmitoyl-2-linoleoyl-digalactosylglycerol (16:0/18:2)	34:2-DGDG	1.03	1.22	0.85	0.87
1-palmitoyl-2-linolenoyl-digalactosylglycerol (16:0/18:3)	34:3-DGDG	1.01	1.20	0.98	0.80
1,2-dilinoleoyl-digalactosylglycerol (18:2/18:2)	36:4-DGDG	0.97	1.07	0.95	0.93
1-linoleoyl-2-linolenoyl-digalactosylglycerol (18:2/18:3)	36:5-DGDG	1.10	1.16	0.82	0.83
1,2-dilinolenoyl-digalactosylglycerol (18:3/18:3)	36:6-DGDG	1.02	1.15	0.94	0.82
monogalactosylglycerol	MGDG	0.88	0.87	1.46	1.13
digalactosylglycerol	DGDG	1.10	1.30	0.93	0.73

Comparisons between SY1 and SY2 showed that the greater the damage on the leaves, the larger were the decreases in the levels of galactolipids. For example, compared to the control leaves, the level of DGDG fell by 22% (0.25-fold change) with the appearance of chlorosis in the leaves (SY1), and fell by a further 108% (0.59-fold change) with the evolution of symptoms from chloroses to scorches, (Table 1). For lyso-galactolipids, a different pattern of change was observed, with unchanged levels in asymptomatic leaves, and depleted levels in symptomatic leaves with scorches, except for 1-palmitoyl-digalactosylglycerol (16:0) (Table 2).

Table 2. Variations in levels of lyso-galactolipids in *Vitis vinifera* L. 'Malvasia Fina' affected by Esca complex disease. CTL = control healthy leaves; ASY = asymptomatic leaves; SY1 = symptomatic leaves with chlorosis; SY2 = symptomatic leaves with scorches. Levels of individual lipid species are expressed as scaled imputed data [18]. Different colors represent different directions and intensities of change (light orange = positive change; light blue = negative change) at $p \leq 0.10$ (Welch's two-sample *t*-test, $n = 6$).

Lyso-galactolipids	Abbreviation	CTL	ASY	SY1	SY2
1-linolenoyl-galactosylglycerol (18:3)	18:3-LMGDG(1)	1.32	1.24	1.02	0.43
1-palmitoyl-digalactosylglycerol (16:0)	16:0-LDGDG(1)	0.45	0.50	0.61	0.67
1-linolenoyl-digalactosylglycerol (18:3)	18:3-LDGDG(1)	1.06	1.09	1.14	0.59
2-linolenoyl-galactosylglycerol (18:3)	18:3-LMGDG(2)	1.23	1.16	1.22	0.42
2-palmitoyl-digalactosylglycerol (16:0)	16:0-LDGDG(2)	0.75	0.60	0.91	0.47
2-linolenoyl-digalactosylglycerol (18:3)	18:3-LDGDG(2)	1.26	1.17	1.44	0.43

4. Discussion

The remodeling of membrane lipids contributes to the tolerance of several plants to biotic and abiotic stresses [3,5,12,13]. Increments in the levels of galactolipids have been observed in several plants in response to disease pressures and marginal environments [5]. Under heat stress for example, plants exhibit increased levels of 18:2-containing galactolipids, and decreased levels of glycerolipids containing ω-3 trienoic fatty acids (18:3 and 16:3), which are the major constituents of thylakoid membranes [12]. In grapevines, the onset of downy mildew symptoms is characterized by reactive oxygen species accumulation and lipid-associated signaling events related to oxylipin lipids [19]. In this study, accumulation of galactolipids was observed only in the leaves of asymptomatic grapevines, except for MGDG. MGDG and DGDG function non-redundantly to regulate the levels of salicylic acid and systemic acquired resistance in plants [3,13]. Observed increases in the level of galactolipids in the leaves of Esca-asymptomatic leaves, and variations in the MGDG/DGDG ratio with disease progression can be interpreted as supporting the theory that the galactolipid class is involved in the delay of onset of foliar symptoms.

Esca complex is an insidious disease because the first visible foliar symptoms may only develop 5 to 10 years after invasion of wood by the associated fungi [7]. Moreover, symptoms do not develop consecutively, and therefore, the disease status of a grapevine plant cannot be predicted from year to year [7]. During the latency period, the grapevine might lose its vigor and productivity, and predispose other grapevines to the pathogens [20]. For most farmers, detection of the disease is achieved through visual observation of foliar symptoms. Monitoring the disease in asymptomatic grapevines, however, is complicated, since it involves destructive sampling of the wood; i.e., sectioning of trunks or harvesting of pieces of woody tissue in order to observe the characteristic wood necroses [10,20]. Hyperspectral and infrared disease detection models have recently been proposed to provide discrimination between symptomatic and asymptomatic grapevines [21,22]. A biochemical analysis method could provide an easier tool for farmers to monitor the disease in vineyards. In this study, very good correlation (average $r = 0.72$; $p \leq 0.01$) was found between the level of leaf necrosis and the levels of all identified galactolipids. This implies that levels of galactolipids could be used as a prognostic indicator of Esca complex. A simple and fast diagnostic approach may be provided by a biochemical method based

on the measurement of levels of galactolipids in asymptomatic leaves compared with symptomatic leaves.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in the Mendeley Data repository at https://doi.org/10.17632/4k49sk6s2w.3.

References

1. Escudero, V.; Jordá, L.; Sopeña-Torres, S.; Mélida, H.; Miedes, E.; Muñoz-Barrios, A.; Swami, S.; Alexander, D.; McKee, L.S.; Sánchez-Vallet, A.; et al. Alteration of cell wall xylan acetylation triggers defense responses that counterbalance the immune deficiencies of plants impaired in the β-subunit of the heterotrimeric G-protein. *Plant J.* **2017**, *92*, 386–399. [CrossRef] [PubMed]
2. Demidchik, V.; Maathuis, F.; Voitsekhovskaja, O. Unravelling the plant signalling machinery: An update on the cellular and genetic basis of plant signal transduction. *Funct. Plant Biol.* **2017**, *45*, 1–8. [CrossRef]
3. Gao, Q.-M.; Yu, K.; Xia, Y.; Shine, M.B.; Wang, C.; Navarre, D.; Kachroo, A.; Kachroo, P. Mono- and Digalactosyldiacylglycerol lipids function nonredundantly to regulate systemic acquired resistance in plants. *Cell Rep.* **2014**, *9*, 1681–1691. [CrossRef] [PubMed]
4. Goufo, P.; Singh, R.K.; Cortez, I. A Reference list of phenolic compounds (including stilbenes) in grapevine (*Vitis vinifera* L.) roots, woods, canes, stems, and leaves. *Antioxidants* **2020**, *9*, 398. [CrossRef] [PubMed]
5. Tenenboim, H.; Burgos, A.; Willmitzer, L.; Brotman, Y. Using lipidomics for expanding the knowledge on lipid metabolism in plants. *Biochimie* **2016**, *130*, 91–96. [CrossRef] [PubMed]
6. Goufo, P.; Marques, C.A.; Cortez, I. Exhibition of local but not systemic induced phenolic defenses in *Vitis vinifera* L. affected by brown wood streaking, grapevine leaf stripe, and apoplexy (Esca complex). *Plants* **2019**, *8*, 412. [CrossRef]
7. Calzarano, F.; Pagnani, G.; Pisante, M.; Bellocci, M.; Cillo, G.; Metruccio, E.G.; Di Marco, S. Factors involved on tiger-stripe foliar symptom expression of esca of grapevine. *Plants* **2021**, *10*, 1041. [CrossRef]
8. Sofia, J.; Trovão, J.; Portugal, A.; Paiva de Carvalho, H.; Mesquita, N.; Nascimento, T.; Rego, C.; Gonçalves, M. Molecular and phenotypic characterisation of *Phaeomoniella chlamydospora* isolates from the demarcated wine region of Dão (Portugal). *Phytopathol. Mediterr.* **2015**, *54*, 403–413. [CrossRef]
9. Del Frari, G.; Oliveira, H.; Boavida Ferreira, R. White rot fungi (Hymenochaetales) and esca of grapevine: Insights from recent microbiome studies. *J. Fungi* **2021**, *7*, 770. [CrossRef]
10. Pacetti, A.; Moretti, S.; Pinto, C.; Compant, S.; Farine, S.; Bertsch, C.; Mugnai, L. Trunk surgery as a tool to reduce foliar symptoms in diseases of the esca complex and its influence on grapevine wood microbiota. *J. Fungi* **2021**, *7*, 521. [CrossRef]
11. Alessandrini, M.; Rivera, L.C.F.; Falaschetti, L.; Paub, D.; Tomaselli, V.; Turchettia, C. A grapevine leaves dataset for early detection and classification of esca disease in vineyards through machine learning. *Data Brief* **2021**, *35*, 106809. [CrossRef] [PubMed]
12. Higashi, Y.; Saito, K. Lipidomic studies of membrane glycerolipids in plant leaves under heat stress. *Prog. Lipid Res.* **2019**, *75*, 100990. [CrossRef] [PubMed]
13. Yu, C.-W.; Lin, Y.-T.; Li, H.-M. Increased ratio of galactolipid MGDG: DGDG induces jasmonic acid overproduction and changes chloroplast shape. *New Phytol.* **2020**, *228*, 1327–1335. [CrossRef] [PubMed]
14. Laureano, G.; Figueiredo, J.; Cavaco, A.R.; Duarte, B.; Caçador, I.; Malho, R.; Silva, M.S.; Matos, A.R.; Figueiredo, A. The interplay between membrane lipids and phospholipase A family members in grapevine resistance against *Plasmopara viticola*. *Sci. Rep.* **2018**, *8*, 14538. [CrossRef] [PubMed]
15. Laureano, G.; Cavaco, A.R.; Matos, A.R.; Figueiredo, A. Fatty Acid Desaturases: Uncovering Their Involvement in Grapevine Defence against Downy Mildew. *Int. J. Mol. Sci.* **2021**, *22*, 5473. [CrossRef]

16. Cavaco, A.R.; Laureano, G.; Cunha, J.; Eiras-Dias, J.; Matos, A.R.; Figueiredo, A. Fatty acid modulation and desaturase gene expression are differentially triggered in grapevine incompatible interaction with biotrophs and necrotrophs. *Plant Physiol. Biochem.* **2021**, *163*, 230–238. [CrossRef]

17. Goufo, P.; Cortez, I. A Lipidomic analysis of leaves of esca-affected grapevine suggests a role for galactolipids in the defense response and appearance of foliar symptoms. *Biology* **2020**, *9*, 268. [CrossRef]

18. Goufo, P.; Cortez, I. Dataset of levels and masses of lipid species in healthy, asymptomatic and symptomatic leaves of *Vitis vinifera* L. 'Malvasia Fina' affected by Esca complex disease. *Data Brief* **2020**, *33*, 106469. [CrossRef]

19. Figueiredo, A.; Martins, J.; Sebastiana, M.; Guerreiro, A.; Silva, S.; Matos, A.R.; Monteiro, F.; Pais, M.S.; Roepstorff, P.; Coelho, A.V. Specific adjustments in grapevine leaf proteome discriminating resistant and susceptible grapevine genotypes to *Plasmopara viticola*. *J. Proteomics* **2017**, *152*, 48–57. [CrossRef]

20. Fleurat-Lessard, P.; Luini, P.; Berjeaud, J.-M.; Roblin, G. Diagnosis of grapevine esca disease by immunological detection of *Phaeomoniella chlamydospora*. *Aus. J. Grape Wine Res.* **2010**, *16*, 455–463. [CrossRef]

21. Bendel, N.; Kicherer, A.; Backhaus, A.; Klück, H.C.; Seiffert, U.; Fischer, M.; Voegele, R.T.; Töpfer, R. Evaluating the suitability of hyper- and multispectral imaging to detect foliar symptoms of the grapevine trunk disease Esca in vineyards. *Plant Methods* **2020**, *16*, 142. [CrossRef] [PubMed]

22. Levasseur-Garcia, C.; Malaurie, H.; Mailhac, N. An infrared diagnostic system to detect causal agents of grapevine trunk diseases. *J. Microbiol. Methods* **2016**, *131*, 1–6. [CrossRef] [PubMed]