Introduction

The importance of grapevine downy mildew – a proteomics approach

Plant pests and disease are a serious threat to food security throughout the world, leading to crop losses within the 10-28% range, a number that is susceptible to increase during the post-harvest period and that is further worsened by climate change[1]. For instance, the oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & De Toni, the causal agent of downy mildew, is one of the most significant grapevine (*Vitis vinifera* L.) pathogens, forcing farmers to apply large amounts of agrochemicals to avoid losses of up to 75% of total yield[2].

Proteomic studies are extremely valuable in the context of plant disease, as they shed light onto the defense mechanisms specific to tolerant and susceptible plants and how those mechanisms are modulated throughout the infection period[2]. Many approaches can be used when analyzing a proteome, but the general workflow requires initial protein separation and subsequent identification. Two-dimensional electrophoresis (2-DE) followed by mass spectrometry (MS) still is one of the most widely used strategies for proteomic screenings, despite its shortcomings regarding sample handling and protein resolution. An approach that somewhat solves these issues is shotgun proteomics, achieved through some form of liquid chromatography followed by tandem MS (LC-MS/MS), increasing sensitivity to proteins present in low amounts and reducing sample handling[3].

The chloroplastidial proteome in plant disease

The chloroplast (Chl) is a crucial organelle for the plant cell. While its predominant biological function is photosynthesis, the Chl is involved in many other processes that aid in cell homeostasis maintenance, especially in the context of plant infection. The formation of ROS is one of the most important contributions from the Chl to plant immunity, as they are relevant in several signaling and defense pathways. Compounds such as apocarotenoids, oxylipins (e.g.: Jasmonic Acid (JA)) and tocopherol are intimately linked to ROS bursts and their absence in the chloroplast impacts the plant's response to infection[4]. The biosynthesis of JA itself begins in the Chl with the oxidation of linolenic acid, and a peak in its accumulation occurs upon grapevine infection with *P. viticola*, which is in line with the proposed role for JA an important phytohormone during plant infection[5,6]. Along the lines of JA synthesis, interesting clues arose regarding the modulation of the Chl lipidome, namely the increase in polyunsaturated fatty acids that make up the chloroplastidial membrane lipids[7,8]. All these processes are dependent on enzymatic reactions mediated by lipoxygenases,

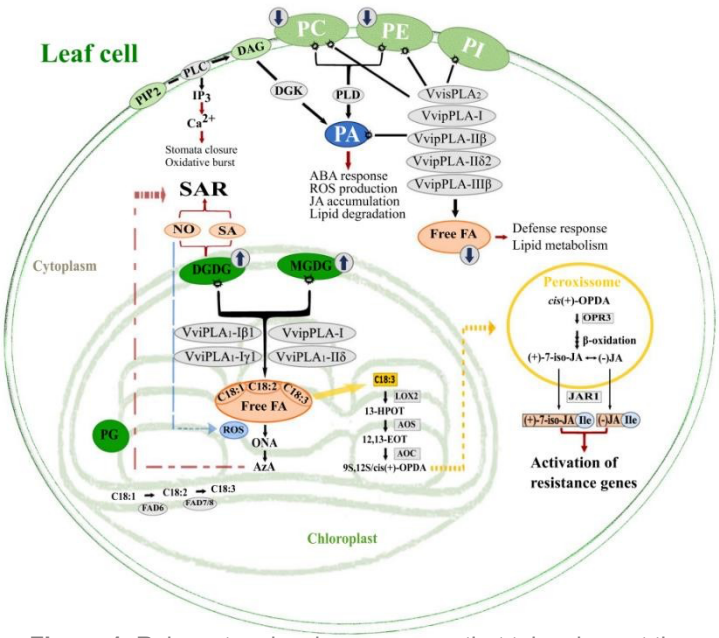

Figure 1. Relevant molecular processes that take place at the chloroplast level during *P. viticola* infection in grapevine. Some interesting highlights are the phospholipase A (PLA) subfamily, the JA biosynthetic pathway, phosphatidic acid (PA) signaling and the modulation of systemic acquired resistance (SAR). Adapted from Laureano et al. 2018

phospholipases, or enzymes in the electron transport chain or in ROS quenching pathways, among others[4].

Additionally, it is known that many plant pathogens interact directly with the Chl, namely through the release of effectors that aid in pathogenicity, many of these being proteins themselves[5]. One interesting example in the grapevine downy mildew context are the RLXR family of effectors, whose function is slowly being uncovered. The RXLR31154 effector was found to interact with PsbP, an oxygen-evolving enhancer protein responsible for ROS production, causing decreased H_2O_2 accumulation and activation of $^1O_2$ signaling pathways, resulting in increased susceptibility[9].

Chloroplast proteomics may provide a snapshot of what takes place at the plastid level and the plastid level alone, as it enriches a protein extract with chloroplastidial proteins. In this experimental design, we propose the extraction and proteome identification of grapevine chloroplasts in the context of *P. viticola* infection, alongside a lipidome analysis. Microscopy imaging will also be obtained to keep track of the infection and to assess ultrastructural changes that may occur to chloroplasts.

Experimental Design

Plant material, inoculation and sampling

Two cultivars will be used in this experiment as representatives of compatible – cv. 'Trincadeira' – and incompatible interactions – cv. 'Regent' – with *P. viticola*. The infection procedure, consisting of spraying a suspension of *P. viticola* sporangia, will be accompanied by a negative stress, which consists of spraying the plants with just water. The timepoints were picked based on prior research in the field, with specific interest in 6hpi, an instance where profound proteomic modulation has been reported in the grapevine downy mildew context[10].

Chloroplast Extraction and Proteomics

Chloroplast extraction and proteomic analysis will be done as described in[11]. This methodology was developed for tomato, which shares methodological analogies to grapevine, and enables high yields and provides extracts that are proteomics-ready.

Because of the large amount of data that proteomics produces, additional attention should be dedicated to their treatment, which will be done as follows:

- Since the proteome modulation is one of the key factors being investigated, the variation between inoculated and control samples for each cultivar will be computed.
- The variations for each cultivar will be compared to each other to access which mechanisms are being modulated differently between the tolerant and susceptible cultivars.

Microscopy

Microscopy assays will be performed with both optical and transmission electron microscopy (TEM). Optical microscopy will elucidate the progression of the disease (i.e., the extent of apoplast colonization, haustoria formation, etc.). Transmission microscopy, on the other hand, will aid in evaluating chloroplast ultrastructure and subcellular localization, which has been reported to be modulated during viral [12] and fungal infections [13].

Lipidome analysis

The chloroplast lipidome will be analyzed in parallel due to the importance of chloroplast membrane lipids in plant immunity and to its relation to the modulation of lipid metabolism-related enzymes that takes place during plant infection [8,14]. Therefore, all lipid and protein extracts will be obtained from the same sample at the given timepoint and cultivar so a more direct link can be established between proteins and lipids at the chloroplastidial level.

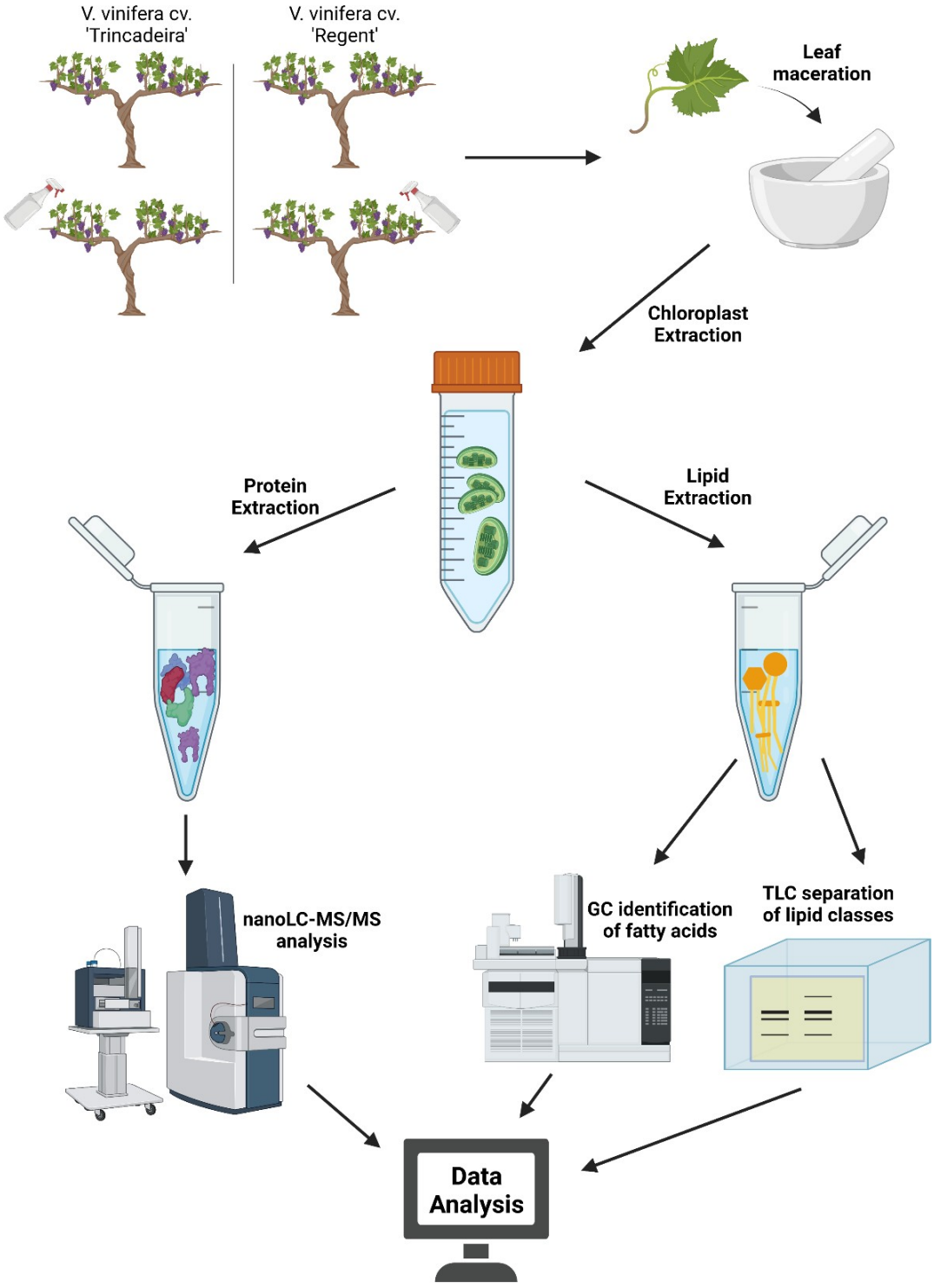

Figure 1. General workflow of the entire experiment. Plants will be divided into 4 groups (2 cultivars, each one with their respective mock and inoculate, 5-10 plants per group) and sampling will be performed at 6 distinct timepoints – 6-, 12-, 24-, 48- and 72-hours post infection (hpi) and 5-days post infection (dpi). Leaves from the 4 groups will be ground for chloroplast extraction which will be used for both protein extraction for proteomics and lipid extraction for lipidome analysis. Proteomic screening will be carried out in nanoLC-MS/MS, while lipid extraction will be accomplished through Gas Chromatography (GC) and Thin Layer Chromatography (TLC) for fatty acid and lipid class profiling respectively. Diagram created with BioRender.

Expected results and concluding remarks

Since some knowledge regarding the chloroplastidial proteome in plant disease exists, several specific proteins will be monitored. These include the above-mentioned endogenous desaturases, phospholipases and enzymes involved in ROS metabolism and cell signaling, alongside proteins in the electron transport chain, as they are representative of photosynthetic function during stress. It can also be interesting to see which *P. viticola* effectors are being detected in the chloroplast, as it would indicate they would act directly with some function that takes place in the organelle.

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