University of Zagreb

Faculty of Science
Department of Biology

Emanuel Gaši

THE EFFECT OF ARBUSCULAR MYCORRHIZAL FUNGI ON VIRUS - INFECTED GRAPEVINE (*Vitis vinifera* L.)

DOCTORAL DISSERTATION

Zagreb, 2025

University of Zagreb

Faculty of Science
Department of Biology

Emanuel Gaši

THE EFFECT OF ARBUSCULAR MYCORRHIZAL FUNGI ON VIRUS - INFECTED GRAPEVINE (*Vitis vinifera* L.)

DOCTORAL DISSERTATION

Supervisor:

Tomislav Radić, PhD, Senior Scientist

Zagreb, 2025

Sveučilište u Zagrebu

Prirodoslovno-matematički fakultet
Biološki odsjek

Emanuel Gaši

UTJECAJ ARBUSKULARNIH MIKORIZNIH GLJIVA NA VINOVU LOZU (*Vitis vinifera* L.) ZARAŽENU VIRUSIMA

DOKTORSKA DISERTACIJA

Mentor:

Dr. sc. Tomislav Radić, znanstveni savjetnik

Zagreb, 2025.

Emanuel Gaši

THE EFFECT OF ARBUSCULAR MYCORRHIZAL FUNGI ON VIRUS - INFECTED GRAPEVINE (*Vitis vinifera* L.)

Doctoral dissertation

The doctoral dissertation was carried out as a part of the Doctoral Programme in Biology at the ***University of Zagreb, Faculty of Science, Department of Biology****. This doctoral dissertation was made at the* ***Institute for Adriatic Crops and Karst Reclamation****, under the supervision of* ***Tomislav Radić****, PhD.*

*The research presented in this dissertation was supported by the Croatian Science Foundation projects: "**Arbuscular mycorrhiza potential to modify grapevine defense against viruses**" (MycoGrape, IP-2020-02-8397, PI: Tomislav Radić, PhD) and "**Young Researchers' Career Development Project – Training New Doctoral Students**" (DOK-2021-02-2381).*

SUPERVISOR INFORMATION

Tomislav Radić (October 18th 1973), Ph.D., Senior Scientist at the Institute for Adriatic Crops and Karst Reclamation.

Tomislav Radić, PhD, is actively working in the agroecosystem microbiology field at the Department of Plant Sciences, Institute for Adriatic Crops and Karst Reclamation, since 2008. He has received his PhD in biology, from the Faculty of Science, University of Zagreb, in 2005. His early scientific interests were focused on marine microbial ecology including the role of plankton in production and transformation of organic microparticles. Later, he becomes involved in the terrestrial microbial communities, particularly agronomically important beneficial organisms, with a focus on arbuscular mycorrhizal fungi. Recently, he is active in studying influence of beneficial and pathogenic microorganisms' interaction on plant physiology. To date, he has led four national and three international scientific projects and additionally collaborated on 25 projects. The research presented in this dissertation stems from the Croatian Science Foundation project: "Arbuscular mycorrhiza potential to modify grapevine defense against viruses" (MycoGrape), led by PhD Radić. He has completed six research mobilities and training programmes, actively participated in numerous scientific conferences, and co-authored forty-five peer-reviewed articles and two academic books.

University of Zagreb
Faculty of Science
Department of Biology

Doctoral thesis

THE EFFECT OF ARBUSCULAR MYCORRHIZAL FUNGI ON VIRUS - INFECTED GRAPEVINE (*Vitis vinifera* L.)

Emanuel Gaši

Institute for Adriatic Crops and Karst Reclamation

Grapevine hosts many pathogenic viruses, but arbuscular mycorrhizal fungi (AMF) can modify a plant's defence response. Experimental treatments with one or multiple AMF species and viruses were designed to investigate the ability of AMF to modify grapevine defence response to virus infection, through the analysis of the different aspects of grapevine physiology and relative quantification of GRSPaV, GLRaV-3 and GPGV. The main findings point to more detrimental effect of multiple virus infections, but also more beneficial effect when grapevine is inoculated with multiple AMF species. The beneficial effect of AMF on grapevine health was seen through increased growth, photosynthesis rate and phosphorus content. Further, AMF inoculation modified plants defence response through changes in ROS homeostasis and hormonal profile. Simultaneously, AMF changed virus titre in the roots and leaves, depending on the virus, plant tissue and phenological phase. In conclusion, AMF are able to modify grapevine defence response and potentially make grapevine more tolerant to virus-induced biotic stress.

(127 pages, 3 figures, 0 tables, 208 references, original in: English)

Keywords: AMF; biotic stress; phytohormones; oxidative homeostasis; grapevine leafroll-associated virus 3; grapevine rupestris stem pitting-associated virus

Supervisor: Tomislav Radić, PhD, Senior Scientist

Reviewers:

Sandra Radić Brkanac PhD, Full Professor

Katarina Hančević, PhD, Senior Scientist

Marin Ježić, PhD, Associate Professor

Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek

Doktorski rad

UTJECAJ ARBUSKULARNIH MIKORIZNIH GLJIVA NA VINOVU LOZU (*Vitis vinifera* L.) ZARAŽENU VIRUSIMA

Emanuel Gaši

Institut za jadranske kulture i melioraciju krša

Vinova loza je domaćin mnogim patogenim virusima, ali arbuskularne mikorizne gljive (AMF) mogu modificirati obrambeni odgovor biljaka. Eksperimentalni tretmani s jednom ili više vrsta AMF i virusa osmišljeni su kako bi se istražila sposobnost AMF-a da modificira obrambeni odgovor vinove loze na virusne infekcije, kroz analizu različitih aspekata fiziologije vinove loze i relativnu kvantifikaciju GRSPaV, GLRaV-3 i GPGV. Glavni rezultati ukazuju na štetniji učinak koinfekcija virusima, ali i povoljniji učinak kada je vinova loza inokulirana s više vrsta AMF-a. Koristan učinak AMF-a na zdravlje vinove loze vidljiv je kroz poticanje rasta, stope fotosinteze i sadržaja fosfora. Nadalje, inokulacija AMF je modificirala obrambeni odgovor biljaka promjenama u homeostazi ROS-a te profilu fitohoromona. Istovremeno, AMF je promijenila titar virusa u korijenu i listovima, ovisno o virusu, biljnom tkivu i fenološkoj fazi. U zaključku, AMF mogu modificirati obrambeni odgovor vinove loze i potencijalno povećati tolerantnost na biotički stres uzrokovan virusima.

(127 stranica, 3 slike, 0 tablica, 208 literaturnih navoda, jezik izvornika: engleski)

Ključne riječi: AMF; biotički stres; fitohormoni; oksidativna homeostaza; virus uvijenosti lista vinove loze 3; virus jamičavosti drva vinove loze Rupestris

Mentor: Dr. sc. Tomislav Radić, znanstveni savjetnik

Ocjenjivači:

Prof. dr. sc. Sandra Radić Brkanac

Dr. sc. Katarina Hančević, znanstveni savjetnik

Izv. prof. dr. sc. Marin Ježić

This doctoral thesis contains the following original scientific papers:

1. Gaši, Emanuel; Radić, Tomislav; Čarija, Mate; Gambino, Giorgio; Balestrini, Raffaella and Hančević, Katarina (2023) **Arbuscular mycorrhizal fungi induce changes of photosynthesis-related parameters in virus infected grapevine**, *Plants*, 12: 1783

2. Gaši, Emanuel; Radić, Tomislav; Gambino, Giorgio; Čarija, Mate; Matić, Frano; Balestrini, Raffaella and Hančević, Katarina (2024) **Arbuscular mycorrhizal fungi modify temporal virus accumulation and distribution in different grapevine tissues**, *Phytobiomes Journal*, 9(1): 139 -148

3. Radić, Tomislav; Vuković, Rosemary; Gaši, Emanuel; Kujundžić, Daniel; Čarija, Mate; Balestrini, Raffaella; Sillo, Fabiano; Gambino, Giorgio and Hančević, Katarina (2024) **Tripartite interactions between grapevine, viruses, and arbuscular mycorrhizal fungi provide insights into modulation of oxidative stress responses**, *Journal of Plant Physiology*, 303: 154372

4. Gaši, Emanuel; Likar, Matevž; Arbona, Vicent; González-Guzmán, Miguel; Hančević, Katarina; Balestrini, Raffaella; Čarija, Mate; Regvar, Marjana; Gambino, Giorgio; Sillo, Fabiano and Radić, Tomislav (2025) **Hormonal changes associated with arbuscular mycorrhizal fungi indicate defense-like alterations in virus-stressed grapevine**, *Physiologia Plantarum*, 177(1): e70136

1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most important perennial crops, both culturally and economically. A testament of its significance can be found in the rich domestication history and cultivation tradition, with a large number of cultivars distributed worldwide (Reynolds, 2017). Viticulture is influenced by all aspects of the environment affecting grapevine's growth, yield and overall health. Major factors that could lead to serious decrease in grapevines vigour and induce stress response are studied extensively. Salt stress, drought, waterlogging, suboptimal temperature and different pollutants have been used to study physiological changes of the grapevine through abiotic stress response. However, one 'long-standing' problem, causing major losses in crop production, have been pathogens (Armijo *et al.*, 2016).

Biotic stress can be caused by a broad range of pathogens and pests invading the host plant. Pathogenic microorganisms are classified as necrotrophic, biotrophic and hemibiotrophic, based on the lifecycle and infection strategy. Necrotrophic pathogens promote host cell death in order to feed and reproduce, but biotrophs and hemibiotrophs infect a living tissue and utilise metabolism of the host for their needs (Glazebrook, 2005). Grapevine plants are facing multiple pathogenic microorganisms that are causing a disturbance in the vineyard health and longevity, directly impacting the quality and quantity of the grapes and wine. Some of the most noticeable pathogens in grapevine are fungi such as *Botrytis cinerea*, *Erysiphe necator* and fungal like organisms (FLO) *Plasmopara viticola* (Armijo *et al.*, 2016). Additionally, grapevine ranks among the most virus-susceptible horticultural crops, hosting more than one hundred viruses (Fuchs, 2025). Viruses are obligate biotrophic pathogens, parasitizing and exploiting grapevines cell processes for replication and assembly, while simultaneously inducing damage and reducing plant vigour. While only minor portion of viruses are associated with symptom appearance and disease development in the grapevine host, they are still a cause of major economic losses worldwide. Both latent virus infections and those that trigger symptoms can induce undesired physiological changes and even shorten the grapevine's lifespan. The defence response of grapevine against biotic stress caused by viruses are mostly not effective due to the compatible virus - host interaction scenario, leading to a systemic spread of the virus infection. Grapevine leafroll associated virus 3 (GLRaV-3) is the main causative agent of one of the most widespread grapevine virus diseases, with negative impact on viticulture, comparable with dangerous fungal diseases (Burger *et al.*, 2017). Further,

some of the more recently discovered viruses, such as grapevine pinot gris virus (GPGV), could have significant impact on grapevine physiology, but are still underexplored (Saldarelli *et al.*, 2017). Fortunately, majority grapevine viruses do not cause any visible symptoms or disease development. For example, while grapevine rupestris stem-pitting associated virus (GRSPaV) can be found globally and is probably the most widespread grapevine virus (Meng and Rowhani, 2017), a few factors need to be fulfilled in order for GRSPaV to induce symptoms. Contrary to this, in some instances, GRSPaV can even be beneficial to the grapevine host (Gambino *et al.*, 2012). Due to the ubiquity, complex phytopathology and the lack of comprehensive knowledge about GRSPaV, it needs to be investigated in more detail, both when occurring alone or in co-infection with other important viruses, such as GLRaV-3 and GPGV.

The main tactics for combating virus diseases in the vineyard are focused on the disease prevention through planting of healthy material, controlling vectors which transmit plant viruses and elimination of infected material. In some parts of the world, the growers are advised to eliminate the infected grapevine by roguing and replacing it with certified planting material, free from most dangerous viruses (Pietersen *et al.*, 2017). Recently, newly emerging RNA silencing technologies are being developed, aiming to produce transgenic plants highly resistant to virus infection, but much work is need to optimise such technology (Tarquini *et al.*, 2023). A promising tool for combating different pathogens, or in alleviating their effects to the host, especially in sustainable agriculture, has been seen with application of microbial biostimulants to the host plant, such as mycorrhizal fungi.

Arbuscular mycorrhizal fungi (AMF) are widespread soil microorganisms able to interact with the root systems of majority of terrestrial plant species by forming common, yet crucial symbiotic relationship. This holds true for many plant hosts, including grapevine (Balestrini *et al.*, 2010; Radić *et al.*, 2014). AMF have already been used in helping grapevine mitigate the negative effect of different abiotic stresses (Trouvelot *et al.*, 2015), such as water stress (Torres *et al.*, 2021) and high temperature (Torres *et al.*, 2016). Further, AMF have proven to be capable of inducing heightened defence and faster response to subsequent pathogen infection, named ‘priming’. During mycorrhiza establishment, modulation of plant defence responses occurs

systemically, leading to a primed state of the plant that is characterised by faster or stronger activation of physiological mechanisms as a defence response to pathogen infection (Jung *et al.*, 2012). This primed state is playing a central role in physiological changes connected to heightened pathogen resistance known as mycorrhiza induced resistance (MIR; Pozo and Azcón-Aguilar, 2007). However, AMF influence on plants facing biotic stress caused by viruses is still underexplored. So far, this multitrophic interaction between plants - AMF - viruses have been studied in different host species, e.g. tomato (Miozzi *et al.*, 2020), potato (Deja-Sikora *et al*., 2023) and cucumber (Metwally *et al.*, 2024). Woody perennial crops have been used rarely as model organisms for researching this interaction, with exception of sour oranges, where mycorrhizal inoculation did not minimise the pathogenic effects caused by citrus tristeza virus (CTV) (Nemec and Myhre, 1984). In grapevine, virus - AMF interaction was studied indirectly, through investigating AMF influence on the nematode vector of the grapevine fanleaf virus (Hao *et al.*, 2018). Overall, these research observations have led to inconsistent results and conclusions varying from protective to potentially detrimental role of AMF in plant diseases caused by viruses. In some experiments MIR effects of AMF were found in virus-infected plants, while other studies on AMF-virus interactions have reported 'negative' effect of AMF on plants infected with viruses, reported as faster virus replication or pronounced symptom development, a phenomenon named mycorrhiza induced susceptibility (MIS; Miozzi *et al.*, 2019). Since there is a lack of a common trend and a high variability in the observations, based on the identity of each member of this tripartite interaction, there is a need for an in-depth investigation of grapevine - virus - AMF interaction. Harnessing the power of the grapevine microbial relationship to mitigate negative influence of viral pathogens would potentially be of great use in agricultural practice, boosting the productivity while having little environmental impact.

In this dissertation, investigation was focused on a widely occurring interactions in the vineyards: The Merlot grapevine cultivar in combination with GRSPaV, either alone or in co-infection with GLRaV-3 and GPGV, along with the ubiquitous AMF species *Rhizophagus irregularis*, either alone or in a three-species consortium. Given its prevalence, this system served as a valuable model for studying grapevine-virus-AMF interactions.

The main objective of this dissertation was assessing the effect of AMF inoculation with one or multiple fungal species on changes in grapevine response to virus infection by GRSPaV solely or in combination with GLRaV-3 and GPGV. It is hypothesized that physiological indicators of virus-induced biotic stress will be reduced in grapevine inoculated with AMF. Further, GRSPaV relative concentration in different grapevine tissues is hypothesized to vary depending on the presence or absence of AMF symbiosis. Last hypothesis is that multiple species inoculums, both virus and AMF, have a stronger combined effect on grapevines' physiological response than the 'one-species' inoculum. The following theoretical framework presents a detailed literary overview of crucial aspects of grapevine physiology influenced either by pathogenic viruses, or beneficial AMF. Further, research papers containing novelties in the field of widespread multipartite interactions grapevine – virus – AMF and grapevine biotic stress mitigation are presented and discussed in detail.

2. TEORETICAL FRAMEWORK

2.1. **Variety of grapevine (*Vitis vinifera* L.) interactions with viruses**

Grapevine is prone to virus infections, as it is a host to more than a hundred viruses (Martelli, 2017; Fuchs, 2020, 2025). Considering different known viruses, viroids, phytoplasmas and bacterial pathogens that infect grapevine, it is thought to be a woody crop with the highest number of known intracellular infectious agents (Maliogka *et al.*, 2015). Additionally, development of high-throughput sequencing as a detection method resulted in discovery of novel viruses hosted by grapevine (Bertazzon *et al.*, 2020; Shvets *et al.*, 2022; Belkina *et al.*, 2023; Dahan *et al.*, 2023). Among described viruses, about one-third are connected with the onset of four major grapevine disease complexes present around the world. Infectious degeneration and decline is caused by viruses from the genus *Nepovirus*, leafroll disease is connected to the viruses from the family *Closteroviridae*, including grapevine leafroll-associated virus 3 (GLRaV-3; *Ampelovirus trivitis*), rugose wood disease is caused by viruses from the *Betaflexiviridae* family, including grapevine rupestris stem pitting-associated virus (GRSPaV; *Foveavirus rupestris*), and fleck disease is caused by the viruses from *Maculavirus* genus (Martelli, 2017). Other viruses are not ascribed to major disease complexes, but nevertheless, can induce detrimental diseases to the grapevine, such as grapevine leaf mottling and deformation disease caused by grapevine pinot gris virus (GPGV, *Trichovirus pinovitis*) (Saldarelli *et al.*, 2017).

GLRaV-3 is the most important causative agent of grapevine leafroll disease (GLD) (Burger *et al.*, 2017). This single-stranded RNA virus has the longest and most complex genome among related viruses. The genome of GLRaV-3 consists of 13 open reading frames (ORFs), with each ORF encoding for some aspect of virus infectivity, replicability, assembly or movement inside a host (Maree *et al.*, 2013; Burger *et al.*, 2017). By achieving compatible interaction with the grapevine, this virus ensures systemic spread and disease development with characteristic symptomatology. The leafroll disease can cause an array of different phenotypes, depending on the host cultivar identity. Additionally, rootstock identity and coinfections with other viruses could be influential factors in symptom development (Naidu *et al.*, 2015). In *V. vinifera*, red-berried cultivars have the characteristic interveinal reddening and green veins of the leaves at the berry ripening stage, while white cultivars have more subtle chlorotic

changes of the leaves (Figure 1). Both red- and white-berried cultivars have characteristic downward rolling of symptomatic leaves (Naidu *et al.*, 2014; 2015). Thus, GLD may cause considerable decline in the yield and quality of grapes (Maree *et al.*, 2013).

On the other hand, GRSPaV is a virus with still unresolved pathology. It is a positive-sense RNA belonging to genus *Foveavirus* (family *Betaflexiviridae*) with its genome consisting of five ORFs (Meng and Rowhani, 2017). GRSPaV has a less severe impact on the grapevine and viticulture than GLRaV-3, with predominantly asymptomatic and latent infections present in *V. vinifera*. Nevertheless, GRSPaV is a ubiquitous virus associated with stem pitting of *V. rupestris* rootstock and vein necrosis, but definitive proof linking this virus to the disease development is not fully elucidated (Meng and Rowhani, 2017). Interestingly, GRSPaV is even debated to be in a potentially mutualistic relationship with a grapevine facing abiotic stress (Gambino *et al.*, 2012; Perrone *et al.*, 2017). Still, strong correlation of different GRSPaV strains to a disorder like stem pitting and vein necrosis is indicative of etiological involvement of this virus (Meng and Rowhani, 2017). Rugose wood predominantly manifests when GRSPaV susceptible *V. rupestris* is used in the vineyard (Martelli, 2017). So far, nine divergent variants of GRSPaV are identified, making it one of the most molecularly diverse grapevine viruses. The symptoms of this disease are complex and dependent on different factors (Figure 1), such as rootstock and scion grafting compatibility, GRSPaV variant, presence of coinfections, climatic conditions and cultivar identity. Rugose wood is the main symptom while basipetal pitting in the form of a band stretching downwards from the inoculation point can be seen in *V. rupestris*. Overall vines are less vigorous and may even decline and die few years after planting (Martelli, 2017).

The third grapevine virus important for familiarising with, in the context of this dissertation, is grapevine pinot gris virus (GPGV). This virus has a filamentous particle and belongs to *Betaflexiviridae* family like GRSPaV, but is placed within *Trichovirus* genus. (Martelli, 2017). GPGV was discovered recently in Italy (Giampetruzzi *et al.*, 2012) and later confirmed across the world, causing leaf mottling and deformation disease in grapevine (Martelli, 2017; Saldarelli *et al.*, 2017). Similarly to GRSPaV, the infection with GPGV is frequently present in symptomless grapevine and different

GPGV genotypes are not correlated with a specific symptom, making the visual diagnosis of GPGV infection unreliable (Saldarelli *et al.*, 2017; Kaur *et al.*, 2023). However, viruses can have serious impact on grapevine even in asymptomatic infections, through disturbing the host health on a physiological level (Hančević *et al.*, 2023). Nevertheless, symptoms associated with leaf mottling and deformation disease, apart from the ones in the name, are short and stunted shoots and internodes with reduced vigour and yield, especially evident in the early phase of seasonal vegetative growth (Saldarelli *et al.*, 2017).

These three viruses have been the main infectious agents used in this study to investigate AMF influence on grapevine defence response to virus-induced stress. Short introduction to each virus identity and disease aetiology is crucial for gaining perspective into the importance of the research presented in this dissertation. In the next segments, emphasis is put on the different aspects of grapevine physiology that are most influenced by virus-induced biotic stress.

2.1.1. Impact of virus infection on grapevines nutrition, development and vigour

Grapevine viruses are mostly phloem-restricted, single-stranded RNA viruses that are put into contact with the plant vascular system either directly, through wounding and grafting with already infected material, or through insect vector feeding. The former path of coming into contact with phloem is a direct one, while the later one entails a cell-to-cell movement via a symplastic route before entering phloem for a long-distance transport (Lough and Lucas, 2006; Heinlein, 2015; Malinowski *et al.*, 2024). Phloem is a nutrient dense environment crucial for transporting key elements along the root-to-shoot axis, necessary for the development of new plant organs. In this nutrient-packed environment, microorganisms can thrive while viruses are able to sustain systemic infection of the entire plant.

Viruses, such as GLRaV-3, are unevenly distributed along the phloem and induce cytopathological modifications in phloem cells, sieve tubes, companion cells, and parenchyma cells (Kim *et al.,* 1989). Those cytopathological changes are in the form of RNA-filled vesicles originating from the membrane of mitochondria, a

replication site of GLRaV-3. Additionally, loosely or firmly packed virus particles can be found along the lumen of sieve tubes, often surrounded by a membrane (Faoro *et al.*, 1992; Faoro and Carzaniga, 1995). However, GRSPaV particles are aggregated in phloem parenchyma cells but likely present in all other grapevine tissues (Meng and Rowhani, 2017). Intense virus replication is able to provoke callose deposition as a defence tactic, with grapevine trying to limit the speed and range of virus spread, resulting in blockage of phloem nutrient trafficking (Keller, 2015). This virus-induced phloem blockage is affecting grapevine physiology, and is mostly seen through GLRaV-3 symptomatology. In GLRaV-3 infected grapevine, callose deposition and reduced transport results in restricted export of photosynthesis derived products, especially sugars. Decreased sugar synthesis in berries and increased sugar concentration in the leaves is related to the modified anthocyanin synthesis, creating characteristic leaf reddening symptoms in red grapevine varieties and less sweet fruits. Onset of foliar anthocyanin synthesis results in the increase of osmotic pressure in parenchymal cells, possibly resulting in downward rolling of the leaf blade, a characteristic symptom of GLRaV-3 disease. (Gutha *et al.*, 2010; Naidu *et al.*, 2014; Halldorson and Keller, 2018). The perturbed nutrient sink results in reduced net photosynthesis rate, stomatal conductance and transpiration rate in the grapevine (Bertamini *et al.*, 2004). Overall, plant viruses modify the normal metabolism of the host, resulting in perturbed photosynthesis through inducing damage to crucial organelles like chloroplasts or mitochondria (Anikina *et al.*, 2023). However, as all other processes required for plant survival, viral synthesis requires energy as well, and photophosphorylation is not drastically inhibited in virus-infected plants to ensure non-disturbed virus proliferation (Ertunç, 2020).

Symptom development and virus-induced physiological changes in the host plant can be viewed as a direct consequence of virus proliferation in the host cells, disturbing the normal cellular machinery and replicating to such an extent as to induce physiological alterations and symptom development. On the other hand, direct virus impact on host physiology can be viewed through interaction of specific virus and plant components (Hull, 2014). In that context, protein modification and relocalisation of host proteins are an important part of virus-induced physiological changes. Host proteins are modified by viruses through posttranslational processes including phosphorylation, acetylation and glycosylation, affecting host protein processing mechanisms (Culver

and Padmanabhan, 2007). The ability to control host protein activity is connected with ensuring undisturbed synthesis of functional viral proteins such as movement proteins, replicases and capsid proteins. For example, a decrease in the amount of rubisco (ribulose-1,5-bisphosphate carboxylase oxygenase), the most abundant plant protein, is caused by the infection with the viruses that cause yellowing and mosaic diseases (Hull, 2014). On the other hand, host physiology is influenced by viral components through protein reallocation. For example, tobacco mosaic virus (TMV) infection in *Arabidopsis thaliana* causes modification of auxin-responsive pathways, resulting in symptom development. In particular, replicase proteins are inhibiting auxin proteins localised in nucleus, resulting in auxin-responsive genes being reprogrammed, thus inducing disease symptoms (Padmanabhan *et al.*, 2006).

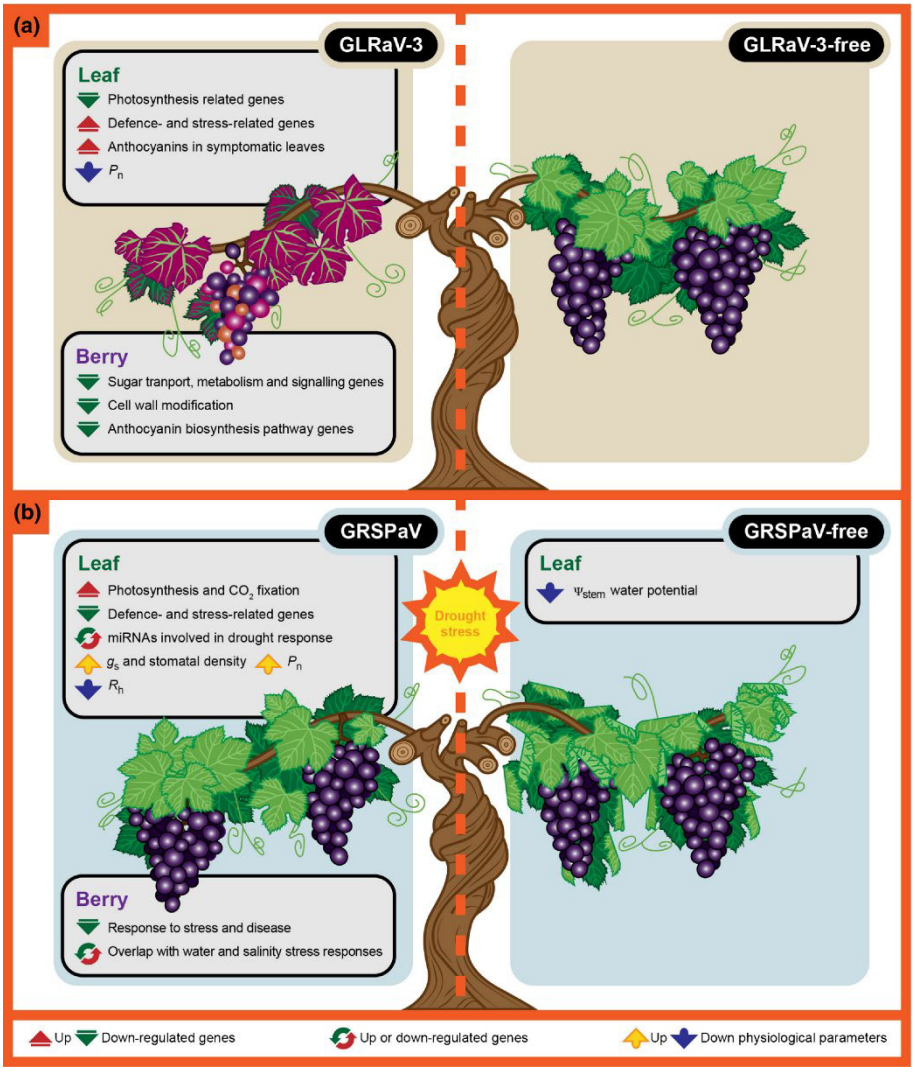


Figure 1. Comparison of virus-infected and virus-free physiological and molecular changes in grapevine leaf and berry. Changes are shown for two distinct grapevine

viruses: grapevine leafroll associated virus 3 (a) and grapevine rupestris stem-pitting associated virus (b) (Perrone *et al.*, 2017).

Nutrient partitioning and low-molecular-weight compounds also play a major part in virus-induced changes of host physiology. However, due to the high specificity of virus and host interactions, there is a high variability in reported observations. For example, nitrogen plays an important role in photosynthesis, amino acid synthesis, and respiration (Foyer *et al.*, 2011). Therefore, the synthesis of nitrogen-demanding amino acids can be related to the reduction of soluble nitrogen in the periods of intense synthesis of viral proteins (Hull, 2014). On the other hand, adequate nutrient availability is crucial for the induction of defence-related processes that are intensified after pathogen invasion (Tripathi *et al.*, 2022). Phosphorus is another macronutrient crucial for plant health and defence response. Generally, as a building element of DNA, RNA and ATP, phosphorus is linked to almost all processes in the host. The role of nitrogen in ATP synthesis is proposed to be a crucial step in jasmonic acid-mediated defence response through activation of hormone signalling defence (Tripathi *et al.*, 2018). Other macronutrients, such as potassium, calcium, sulphur and magnesium, as well as micronutrients, have a crucial role in the normalcy of plant metabolism as well and can be affected by virus infection to an extent. For example, the application of boron reduces the severity of the tomato mosaic virus-induced disease (Graham and Webb, 1991).

In summary, the most common physiological and biochemical changes in virus-infected grapevine are related to the decrease of photosynthesis as a result of reduction in pigment concentration, the number of chloroplast ribosomes and the amount of rubisco. Further, rates of respiration and activity of certain enzymes are often increased in infected plants. In particular, leaves of infected grapevine show reduced net photosynthetic rate, stomatal conductance and transpiration rate (Bertamini *et al.*, 2004) and modification of cellular redox state (Hančević *et al.*, 2023). Additionally, virus infection can induce dramatic hormonal profiling changes in infected plants, a defence response that will be discussed in the further chapters, in detail.

2.1.2. Virus infection through the lens of oxidative homeostasis

When plant cells come into contact with microbial pathogens, including viruses, few rapid defence responses can be expected, depending on the host susceptibility. An interaction of a host and a pathogen is taking place at a molecular level. In susceptible grapevine, the first response to a cell invasion by a virus is a transient increase concentration of calcium ions (Ca^{2+}), serving as messengers for the propagation of a defence response (Aldon *et al.*, 2018). Ca^{2+} ions are perceived by calcium receptors in the cell, namely calmodulin, calmodulin-like proteins or calcium-dependent protein kinases, all of which have a crucial role for activating a cascading response to biotic and abiotic signals (Aldon *et al.*, 2018). However, for a strong and effective defence, second wave of prolonged induction of Ca^{2+} is happening in resistant hosts, inducing a cascading effect and cell genetic reprogramming, leading to incompatible virus - host interaction. Unfortunately, the second Ca^{2+} influx is absent in grapevine - virus interaction, leading to local and systemic spread of virus infection, as is the case with all compatible plant - virus interactions (Whitham *et al.*, 2006).

One of the main defence responses at a cellular level, is disturbance of redox homeostasis and induction of reactive oxidative species (ROS) production. ROS are highly reactive molecules that, except for hydrogen peroxide, contain unpaired electrons. They are by-products of normal cell processes, produced by successive reduction of molecular oxygen by degree of one electron. Under low ROS productivity, they act as messengers for inducing gene expression and affecting translation process, but also as inducers of enzymatic activity (Foyer and Noctor, 2003). ROS includes superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$) (Riedle-Bauer, 2000). The main sites of ROS production are chloroplasts, mitochondria, peroxisomes and apoplast (Mittler *et al.*, 2022). The ROS production intensifies in plants facing stress, with prolonged oxidative stress causing damage to cell molecules and structures, resulting in disturbed oxidative homeostasis of the cell (Sharma *et al.*, 2012). Antioxidative enzymes are crucial for maintaining ROS balance and avoiding excessive cell damage. However, in resistant plants, ROS-mediated defence is an important step in limiting virus spread. Virus infection of an incompatible host will drastically increase ROS production and concentration in the cell, named ‘oxidative burst’, necessary for induction of hypersensitive response resulting in death

of the infected cells (Foyer and Noctor, 2003). On the other hand, compatible interaction of grapevine with its viruses is not characterised by increased ROS production. In fact, ROS accumulation is only a transient reaction to early infection, but becomes controlled, ensuring normal cell functioning necessary for the production of virus particles and virus spread (Hernández *et al.*, 2016). However, ROS production might pose beneficial adaptation in a long-term viral disease, with transient increase of ROS accumulation in leaves promoting senescence and normal nutrient cycling (Espinoza *et al.*, 2007a). Additionally, virus-induced damage promotes an increase in ROS production. In plants, chloroplasts and mitochondria are damaged by virus replication leading to inhibition of electron transport chain and ROS accumulation. This ROS accumulation in compatible interactions is counterbalanced by enzymatic and non-enzymatic antioxidative reactions. Therefore, basal defence is activated in compatible plant - virus interactions, in the form of ROS production, but is unsuccessful in halting the disease progression. This was demonstrated in the interaction between grapevine and GLRaV-3 coinfection with other viruses, where significantly elevated levels of H_2O_2 and superoxide dismutase activity were related to virus-induced stress in grapevines (Sgherri *et al.*, 2013; Hančević *et al.*, 2023). These oxidative parameters have even been proposed as general indicators of viral infection (Hančević *et al.*, 2023). However, despite the documented oxidative burst, the authors confirmed the viruses' spread and its systemic infection within the plant. Additionally, grapevine genes associated with ROS accumulation and callose deposition are upregulated with GLRaV-3 infection (Espinoza, *et al.*, 2007b; Espinoza, *et al.*, 2007a).

2.1.3. Plant hormonal response to virus infection

Plant defence is based on the molecular crosstalk between host and the invading pathogen named the 'zig-zag' model (Jones and Dangl, 2006). The induction of a basal defence response is founded on the ability to detect the presence of a pathogen. For that purpose, plant cells have pathogen- or pattern-recognition receptors that are able to detect the presence of proteins or elicitors of pathogenic origin. Those elicitors are called pathogen-associated molecular patterns (PAMP). The recognition of PAMPs prompts a defence response termed pattern-triggered immunity. Pathogens are able to suppress this first line of defence with elicitors. The host

response to this attack is effector-triggered immunity through the use of resistance gene (R-gene) encoded resistance proteins (Dangl and Jones, 2001; Jones and Dangl, 2006). These R-genes are crucial for coding proteins with the ability to promptly recognise a pathogen, induce a strong defence response and ensure an incompatible interaction, without a systemic spread of the pathogen (Ngou *et al.*, 2022). Among diverse physiological changes in compatible infections, plant hormones play a crucial role in balancing the ROS homeostasis, but also in disease defence response throughout the entire plant.

Plant hormones are a diverse group of structurally unrelated small molecules that are involved in the regulation of a wide range of plant physiology processes. Plant hormones are defined as extracellular molecules acting as signalling cues for propagation of a response in target cells, away from their production site (Keller, 2015). Auxins, abscisic acid (ABA), cytokinins, gibberellins, ethylene, brassinosteroids, jasmonic acid (JA), salicylic acid (SA), nitric oxide and strigolactones are included in the list of molecules referred to as phytohormones. They are involved in different stages of cell life cycle, plant growth and development, organogenesis, response to environmental changes and pathogen invasions, seed germination and many other events during plant life (Pieterse *et al.*, 2009; Collum and Culver, 2016; Zhao and Li, 2021).

In virus infection scenario, SA is often the main phytohormone leading the response to the infection. If the infected plant possesses the R-gene, the SA response is connected with the induction of ROS production, callose deposition and hypersensitive response, leading to host resistance to the pathogen invasion (Alazem and Lin, 2015; Ngou *et al.*, 2022). Additionally, the RNA-interference mechanism (RNAi) important for halting the process of viral replication and stopping its cell-to-cell spread is related to SA accumulation (Campos *et al.*, 2014). In compatible interactions, including grapevine, the host does not possess necessary R-genes for proper defence. Therefore, the plant is able to recognise the pathogen invasion and start the defence process, but molecular interaction leaves grapevine defence suppressed and weighs in favour of the systemic viral infection. One way the virus-derived proteins could avoid defence response is through suppression of NONEXPRESSER OF PR GENES 1 (NPR1). The NPR1 is crucial for SA-based defence. Plant viruses' proteins suppress

NPR1 by reducing its concentration through induction of proteasomal degradation of NPR1, resulting in disturbed balance between SA and JA (Zhang *et al.*, 2023).

Resulting imbalance can greatly influence plant response to biotic stress, since SA and JA are regarded as the main hormones orchestrating the response to biotrophic and necrotrophic pathogens (Glazebrook, 2005). SA signalling leads to a hypersensitive response followed by the establishment of Systemic Acquired Resistance (SAR) to biotrophic pathogens (Mishra *et al.*, 2024), whereas JA is required for induced systemic resistance (ISR) against necrotrophic pathogens (Yu *et al.*, 2022). However, distinction based solely on pathogen lifestyle is not always the rule. Thus, JA signalling in grapevine has been implicated in resistance against biotrophs, such as various species of the *Erysiphales* order and the *Peronosporaceae* family (Hamiduzzaman *et al.*, 2005). Interestingly, SA-mediated antiviral and proviral effect has been noted in compatible interactions. SA can be utilised by viruses in order to self-regulate to avoid excess damage to the host cells, that would otherwise stop the virus spread (Murphy *et al.*, 2020). The complex interaction of different phytohormones with one another and with other parts of the plant defence response is curated based on multiple variables defining plant-pathogen interaction (Alazem and Lin, 2015; Islam *et al.*, 2019; Aerts *et al.*, 2021).

The role of different hormones, conventionally not connected to plant defence, has recently been investigated in pathogen response scenarios. For example, ABA has been linked to abiotic drought stress (Ferrandino and Lovisolo 2014) and to biotic stress, in the context of defence against fungal diseases (Asselbergh *et al.*, 2008; Ton *et al.*, 2009). Additionally, induction of indole-3-acetic acid (IAA) has been reported in both biotrophic (*Erysiphe necator*) and necrotrophic (*Botrytis cinerea*) fungus infecting susceptible grapevine host. In both instances, interplay between SA and auxin (IAA) has been noted and the influence of hormonal reprogramming in defining susceptibility or tolerance of the grapevine host to a fungal pathogen has been proposed (Coelho *et al.*, 2019; Amaro *et al.*, 2023). However, hormonal crosstalk shaping the outcome of viral infection is complex, species-specific, and still not fully elucidated. Apart from SA - JA crosstalk, different hormonal interactions have been noted in virus-infected plants. ABA signalling acts inhibiting on SA-based plant immunity, and synergistically with JA-based plant immunity, in the context of herbivory (Pieterse *et al.*, 2012; Alazem and

Lin, 2017). However, in the virus infection scenario, indigenous ABA levels were reported to be decreased, increased or not affected, when comparing resistant with susceptible plants (Zhao and Li, 2021). This once again points out the fluidity and complexities of hormonal homeostasis that is context dependent and can be strongly influenced by the internal factors, such as plant age, or external factors, e.g., different stress combinations or environmental cues affecting plant physiology (Berens *et al.*, 2017; Nobori and Tsuda, 2019). Recently, RNA silencing suppression protein (p24) of grapevine leafroll-associated virus 2 has been found to undermine the SA-mediated defence response through silencing of the transcription factor belonging to the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily (Zhang *et al.*, 2022), highlighting the complex interactive network between grapevine and virus, centred around phytohormones.

2.2. **Formation and functioning of plants and arbuscular mycorrhizal fungi interaction**

AMF are obligate symbionts forming mutually beneficial relationships with a wide variety of plant hosts. AMF association between fungus and plant is a type of low specificity symbiosis, since it is estimated that mycorrhizal symbiosis can be formed by more than 70% of all vascular plants (Brundrett and Tedersoo, 2018). Phylogenetically, AMF belong to a separate phylum of *Glomeromycota* within the fungal kingdom (Zhao *et al.*, 2023; Wijayawardene *et al.*, 2024). They were formerly named vesicular-arbuscular mycorrhiza based on the characteristic structures they are forming, a name which has been abandoned because not all AMF species form vesicles. Vesicles are bladder-like storage structures, while arbuscules are branched, tree-like structures and main sites of nutrient exchange processes (Figure 2). The entire fungus is stretching from the rhizosphere, with its extraradical mycelium, to the plant root, via intraradical hyphal network and finally, into the cortical cells, where arbuscules are formed (Smith and Read, 2008a).

The grapevine is among the wide range of suitable hosts of AMF, and frequent symbiosis has been reported with different cultivars and even wild grapevines

(Trouvelot *et al.*, 2015; Radić *et al.*, 2021). In grapevine, as in other hosts, the interaction starts by chemical signalling. The grapevine root exudates-molecules called strigolactones, which stimulate AMF spore germination and hyphal branching near the root surface (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). The germination of spores can be induced in the absence of host plants, but AMF are obligate biotrophs, meaning they cannot complete their life cycle and produce the next generation of spores without a living photoautotrophic partner (Parniske, 2008). After the germination and hyphal branching, the fungus starts to produce diffusible signals, as a mixture of lipochitooligosaccharides called Myc factors and chitin oligomers (Oldroyd, 2013). Perception of fungal diffusible signals by the root cells triggers the calcium (Ca^{2+}) spike in the rhizodermis and leads to activation of the common symbiosis signalling pathway (CSSP or CSP). This pathway is characterised by transcriptional changes and modification of symbiosis-related gene expression in epidermal cells. As a result, the host plant is prepared for the symbiotic relationship (Parniske, 2008; Schmitz and Harrison, 2014; Choi *et al.*, 2018). The formation of the symbiosis is facilitated through the formation of the fungal attachment structure called hyphopodium on the outer surface and prepenetration apparatus in the plant cells, to accommodate the fungus. The fungal hypha, that developed from the hyphopodium, enters the prepenetration apparatus and grows through the cell and into the apoplast where intraradical hyphal network spreads laterally along the axis of the roots. Intraradical hyphae branching induces the development of the prepenetration apparatus of the inner cortical cells, where hyphal structure penetrates the cells and creates the arbuscules (Parniske, 2008; Smith and Read, 2008b; Choi *et al*., 2018). Mark of a functional symbiosis is the formation of the arbuscules inside of the root cortex, which enable carbon uptake by the fungus, and mineral nutrients delivery to the grapevine. The fungal structures are not directly in contact with the cell, rather the periarbuscular membrane is created as a continuation of the cell membrane which houses the finely branched arbuscules. For successful arbuscule branching, massive cell restructuring is taking place, including reduction in the vacuole size, position of the nucleus and overall dense compaction of endoplasmic reticulum and other organelles within the cytoplasm (Balestrini *et al.*, 1992; Pumplin and Harrison, 2009). Between the periarbuscular membrane, created by the host cell, and the fungal membrane is the periarbuscular interface. This interface is specialised in the molecular content being released into it through the surface

membrane transporters, and plays a crucial part in the nutrient exchange process (Balestrini and Bonfante, 2005; Smith and Read, 2008a; Choi *et al.*, 2018).

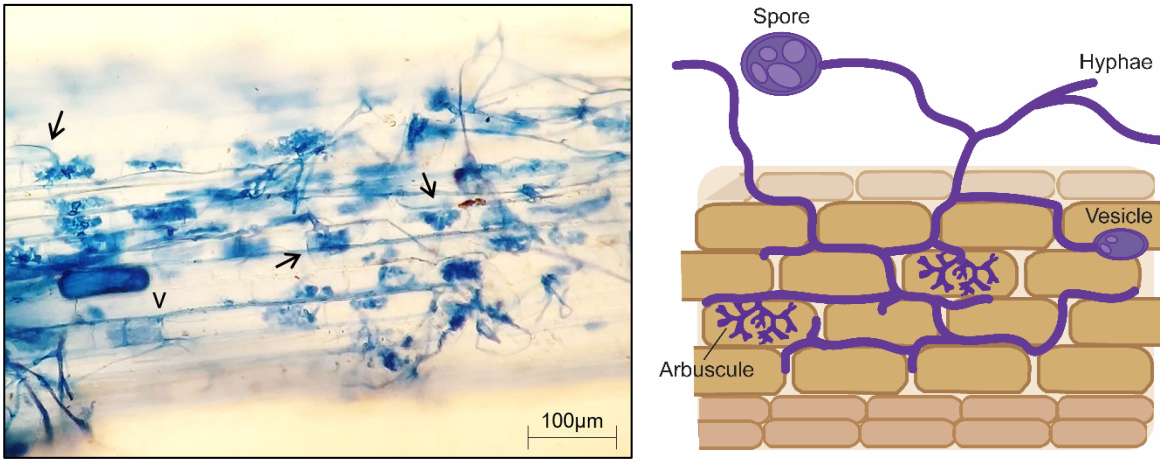


Figure 2. Detailed view of the main structural elements of AMF. On the left, microscopic view (×200, light microscope) of AMF structures in fine grapevine roots treated with Trypan blue (own photography), showing arbuscules (arrows) and vesicle (v). On the right, detailed schematic overview of the mycorrhizal structures (own illustration).

2.2.1. Effects of AMF colonisation on growth and nutrient acquisition

The relationship between the AMF development and increase in plant growth has been long known and studied across different hosts (Smith and Read, 2008c). The positive effect that AMF has on growth was also noted in the grapevine (Linderman and Davis, 2001; Ridgway *et al.*, 2006). However, the induction of growth parameters differs with the type of the mycorrhizal inoculum, grapevine genotype, soil nutritional status and the identity of a rootstock-scion union (Schreiner *et al.*, 2007; Ozdemir *et al.*, 2010; Trouvelot *et al.*, 2015; Moukarzel *et al.*, 2023). Nevertheless, plants inoculated with AMF have higher biomass and growth rates than the uninoculated counterparts. This physiological manifestation is strongly correlated with better nutrient status of the plants even in nutrient-deficient soils (Smith and Read, 2008c). Since grapevine root hairs are too thick to grow inside the narrow spaces of the soil particles, AMF extraradical hyphae, which are much thinner and are able to pass through the fine interstitial pores, (Smith and Read, 2008c), thus AMF are highly beneficial to their grapevine host in nutrient acquisition through increasing the volume of the soil that the

plant is able to have access to. This makes mineral nutrition acquisition one of the main beneficial attributes of AMF-inoculated plants. The AMF-plant symbiosis results in enhanced assimilation of different macronutrients, such as phosphorus (P), nitrogen (N), potassium (K) and sulphur (S). Additionally, concentrations of micronutrients such as zinc (Zn) and copper (Cu) are higher in AMF-inoculated plants as well (Smith and Read, 2008d; Trouvelot *et al.*, 2015).

In AMF-plant interaction, P is the most important element, since AMF symbiosis has the greatest effect on P acquisition. P is a macroelement that is needed by both, the grapevine and the fungal partner. It is present in the soil in poorly mobile and insoluble form, thus, with an overall low concentration of dissolved P in the soil, it is not readily available to plants (Smith and Read, 2008c). Many soils can have high P content, found in the complex forms, which is therefore unavailable to the plant for direct uptake (Harrison *et al.*, 2010). Therefore, a depletion zone around the roots is formed quickly, after all available soluble phosphate is used up from the soil. On the other hand, AMF presents an alternative route through which plant could receive more mineral nutrients, including P. Improved P nutrition in AMF-inoculated plants has been well described (Neumann and George, 2010). Recently, heightened P uptake was demonstrated in nutrient poor soils or soils with insoluble P, with half of all P coming from the symbiotic interaction, allowing uninhibited plant growth without prioritising the need for below-ground biomass development (Yang *et al.*, 2012; Qi *et al.*, 2022). The main advantage of AMF symbiosis is increased P uptake, therefore in adequate P conditions plants rarely invest in this symbiosis. However, because of quick depletion of P resources, plants that are growing in soils with suboptimal nutritional status react by releasing strigolactones and initiating the AMF symbiosis (Kretzschmar *et al.*, 2012). Once the symbiosis is established, the P can be transferred into the plant. The mechanism for P uptake is based on the organic acid secretion, use of phosphatases and transporters of inorganic P in order to solubilise and uptake otherwise unavailable P from the soil. The orthophosphate from the soil is moved into the extraradical mycelium using high-affinity transporters on the fungal cell wall, where it is converted into the polyphosphates that are able to be stored and transported to the intraradical mycelium. Finally, polyphosphate is hydrolysed and released from the arbuscules into the periarbuscular interface as inorganic P, ready for the plant to uptake it through the cortical cells (Harrison *et al.*, 2010; Smith and Smith, 2011; Choi *et al.*, 2018).

AMF can enhance the supply of N, another macronutrient crucial for plant development. In grapevine, adequate N in the soil is important for rapid shoot development of young vines in the spring and one of the most important soil nutrients for grape composition (Trouvelot *et al.*, 2015). AMF can accelerate the organic matter decomposition and uptake of N directly from the organic matter (Hodge *et al.*, 2001). While N can be taken up by the AMF extraradical mycelium in the form of organic amino acids or inorganic NO_3^-, the preferred form is NH_4^+ (Tanaka and Yano, 2005; Ngwene *et al.*, 2013). The inorganic N is directly taken up from the soil by ammonium transporters and integrated into the amino acid arginine in the extraradical mycelium. Arginine is transferred to the intraradical mycelium, where it is hydrolysed, releasing urea. Urea is transferred as ammonium into the periarbuscular interface where it is taken up by the plant (Govindarajulu *et al.*, 2005). However, the growth response of AMF-inoculated plants based on N uptake has not been fully understood, with opposing observations for plant growth depending on AMF species (Smith and Smith, 2011). Few studies have made the association of beneficial physiological effects of AMF-derived N acquisition (Cavagnaro *et al.*, 2006).

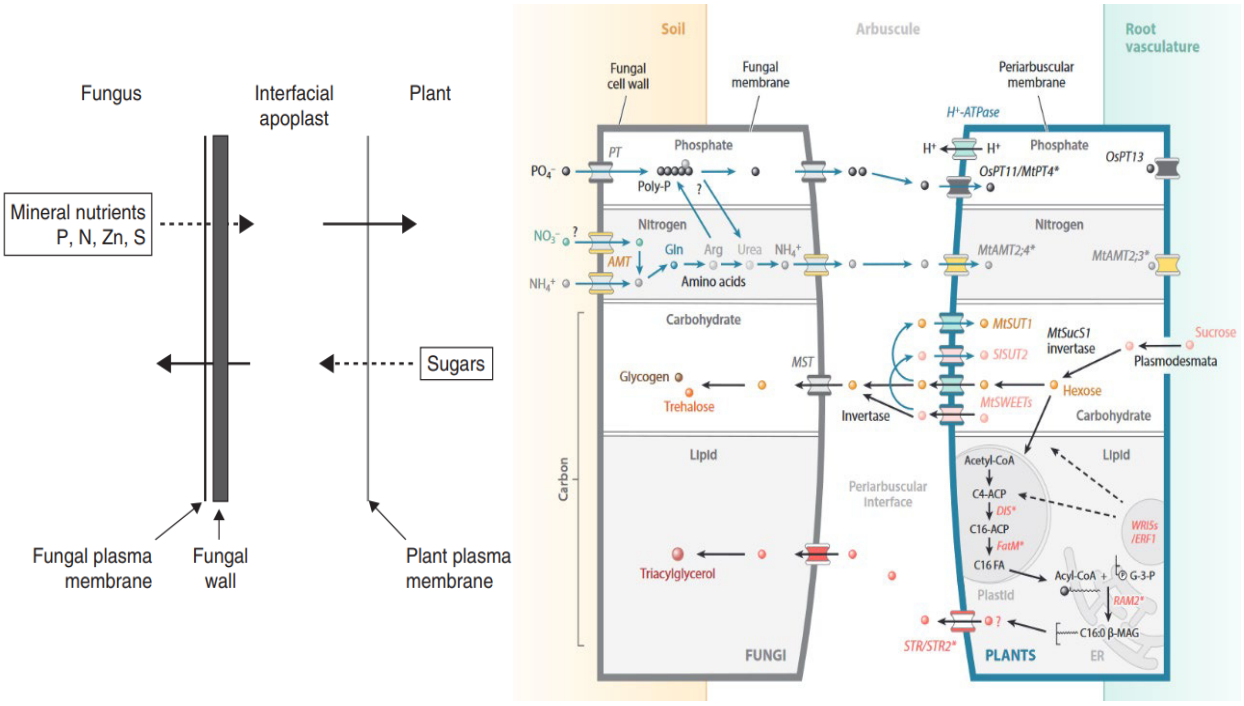


Figure 3. - Simplified mechanism of nutrient exchange between the fungal and plant cell membranes in the periarbuscular space (left) and detailed overview of transporters

involved for nutrient uptake from the soil and transfer to the plant cell in exchange for carbohydrates and lipids (right) (Smith and Read, 2008c; Choi *et al.*, 2018).

In addition to P and N, mycorrhizal fungi also markedly improve the uptake of other mineral nutrients. For example, S is taken up and transported as amino acids cysteine and methionine, or as sulphate, to the plant host (Allen and Shachar-Hill, 2009). Additionally, AMF colonisation results in increased K concentrations in plants (Garcia and Zimmermann, 2014). It has been noted, depending on AMF species present in the soil, that AMF, most notably *Claroideoglomus* spp., influence grapevine chlorophyll content through increase in uptake of specific nutrients such as magnesium (Mg), Zn and K (Moukarzel *et al.*, 2023). Even in abiotic stress conditions AMF modify nutritional status of plants by, e.g., decreasing Na concentration of salt stressed plants (Huang *et al.*, 2023).

Beneficial effects of mycorrhizal symbiosis in relation to grapevine growth and nutrition is similar in other crop species (Schreiner, 2005; Trouvelot *et al.*, 2015). It has been shown that inoculation of cover crops with *Funneliformis mosseae* results in indirect colonisation of grapevine roots, leading to higher photosynthetic activity after heat stress (Nogales *et al.*, 2021). However, the beneficial responses are species-specific and isolate-specific (Koch *et al.*, 2017). This species-specific beneficial effect is noted in a study on the grapevine, where biomass was greater in plant inoculated with *F. mosseae* than with *Rhizophagus intraradices*, despite the latter resulting in higher root colonisation percentage (Cangahuala-Inocente *et al.*, 2011). Similarly, in Kober 5BB rootstock, *F. mosseae* enhanced shoot growth, while *R. intraradices* primarily affected root growth along with P and Zn concentrations (Ozdemir *et al.*, 2010). Regardless, AMF-mediated growth and nutrition benefits are well noted in the grapevine, resulting in physiologically healthier plants.

2.2.2. Influence of AMF on oxidative balance in plants

Oxidative homeostasis is important in two particular instances. Firstly, changes in cell oxidative status are important for facilitating symbiotic interaction. Plant reactive oxygen species (ROS) are produced during normal physiological state and used as

signalling molecules in different aspects of cellular processes including plant - AMF interaction development. Secondly, AMF symbiosis is not only responsible for beneficial nutritional changes, but is also involved in the plant response to biotic and abiotic stresses. In that context, AMF are affecting the changes in oxidative status and antioxidative reactions linked to plant response to stressful conditions.

During mycorrhizal symbiosis, plant tend to acquire 'primed' defence status characterised as the state of induced responsiveness to pathogen invasion (Pozo and Azcón-Aguilar, 2007; Jung *et al.*, 2012; Cameron *et al.*, 2013). AMF inoculation results in systemic primed state characterised by changed transcriptional and hormonal modifications of the entire plant, called mycorrhizal induced resistance (MIR), which shares similarities with induced systemic resistance caused by beneficial bacteria (Pieterse *et al.*, 2014; Comby *et al.*, 2017). In order for MIR to be established, AMF have to avoid initial host response. Since plants are unable to distinguish between pathogenic and beneficial microorganisms in the early stages of invasions, there are some commonalities in the host defence response (Pel and Pieterse, 2013). In the early stages, proteins of AMF cell wall structures (e.g., chitin) are being recognised as elicitors of response and are able to induce first line of defence called pattern-triggered immunity that is based on recognition of microbe-associated molecular patterns that are conserved between pathogenic and beneficial fungi (Zamioudis and Pieterse, 2012; Comby *et al.*, 2017; Ngou *et al*., 2022). After the initial local defence response, AMF are able to excrete the effector proteins which suppress the plant defence response, making the symbiotic partnership possible (Kloppholz *et al.*, 2011). The ROS are generated at multiple points during symbiosis formation. In the first line of defence, recognition of chitin is marked by a transient burst of intracellular ROS and accumulation of salicylic and jasmonic acid (Song *et al.*, 2011). Additionally, during the formation of symbiosis, cortical cells where arbuscules are formed increase their H_2O_2 content in the early stages of arbuscule formation, followed by a gradual decrease with the plant growth (Zhang *et al.*, 2013).

In grapevine exposed to biotic stresses, ROS production and the activity of antioxidative systems are crucial for deciding the fate of the infection, as discussed in Chapter 2.1.2. Once the AM symbiosis is well established, the production of ROS transitions from being a response to the formation of the symbiosis to a more

pronounced response to the pathogen-induced stress (Comby *et al.*, 2017). Under stressful conditions, ROS accumulation causes post-translational modifications of methionine residues and cysteine thiol groups, resulting in structurally altered proteins and activation of transcription factors capable of transiently change the expression of the defence genes (Waszczak *et al.*, 2015). Further continuation of ROS accumulation and inadequate ROS scavenging leads to molecular and cellular damage including irreversible damage of nucleic acids, lipids and proteins (Sahu *et al.*, 2022). Grapevine defence, under different stress scenarios, is being stimulated through AMF-mediated oxidative response (Carvalho *et al.*, 2015). AMF induction of oxidative defence response during abiotic stress is reported in a recent meta-analysis, with increased activity of antioxidative enzymes superoxide dismutase, catalases, peroxidase and ascorbate peroxidase by roughly a quarter, and reduction of H_2O_2 concentration relative to uninoculated controls (Chandrasekaran and Paramasivan, 2022). Similarly, tomatoes under salinity stress had improved activity of antioxidative enzymes when inoculated with AMF (Huang *et al.*, 2023). In the recent study, AMF inoculated wheat showed decrease in concentrations of H_2O_2, malondialdehyde and superoxide during drought period, pointing to mitigated effects of drought (Abdelaal *et al.*, 2024)

Nevertheless, elements of oxidative homeostasis are only one part of AMF mediated defence response. Particularly, ROS as signal molecules, are interconnected with the hormonal crosstalk.

2.2.3. Plant hormonal changes influenced by AMF

Influence of the virus infection on grapevine hormonal profile was discussed in the Chapter 2.1.3. The influence of the mycorrhizal symbiosis on hormones is complex, with web of interactions depending on many factors, such as the host species, environmental properties and microbiome of the host. Advances in understanding of AMF-induced hormonal changes stems from the use of transgenic plants and mutants (Ludwig-Müller, 2010). Importance of the phytohormones as regulators of physiological processes in grapevine is well known, but the complexities of the crosstalk between ROS and phytohormones, as well as the signalling function of phytohormones in AMF symbiosis development, are under debate (Gutjahr, 2014; Carvalho *et al.*, 2015).

As described in the former chapter, plants activate an early defence reaction in response to the AMF colonisation process before it is being suppressed. However, phytohormones are involved throughout the entire colonisation, from the pre-symbiotic stage and the early recognition processes, up to the development of fungal structures inside of the root cortical cells and beyond (Bedini *et al.*, 2018). Apart from the already described strigolactone role in spore germination and initial pre-symbiotic events, other phytohormones are involved in the formation of AMF symbiosis.

Abscisic acid (ABA), in general, has an enhancing role in AMF symbiosis (Bedini *et al.*, 2018). ABA promotes root colonisation and an early-stage arbuscule formation (Herrera-Medina *et al.*, 2007). AMF have the ability to increase the endogenous ABA content during colonisation (Ludwig-Müller, 2010). Also, the gene for ABA synthesis is differentially expressed in the mycorrhizal roots, indicating the importance of the ABA phytohormone for this relationship (Jahromi *et al.*, 2008). In AMF-inoculated plants, multiple crosstalk mechanisms were uncovered starring ABA. Interaction of ABA with gibberellin (GA) is antagonistic, with downregulation of GA-expressing genes and enhanced catabolic activity (Martín-Rodríguez *et al.*, 2016). However, crosstalk between ABA and ethylene (ET) was not found to be crucial for AMF establishment, yet levels of both phytohormones individually are important for AMF development (Fracetto *et al.*, 2017). AMF inoculation in combination with exogenously applied ABA results in enhanced colonisation and plant growth (Lou *et al.*, 2021). However, in the context of pathogen invasion, AMF-ABA interaction is complex, and a detailed description of AMF-mediated defence response is discussed in Chapter 2.3. Additionally, AMF can modify ABA concentrations and reduce stress caused by drought (Das and Sarkar, 2024), a very common type of stress experienced by grapevine, in which ABA has a particularly important role (Carvalho *et al.*, 2015).

Jasmonic acid (JA), and jasmonate derivatives, are lipid metabolites involved in the activation of genes and the synthesis of proteins related to defence against both biotic and abiotic stresses (Bari and Jones, 2009). In AMF-inoculated plants, exogenous application of JA resulted in either promotion or inhibition of symbiosis, with low (5 µmol) or high (5 mmol) concentration of JA, respectively (Regvar *et al.*, 1996; Ludwig-Müller *et al.*, 2002). This observation is reflected in multiple studies that shows

JA, as well as JA-precursor and JA-conjugate (JA-isoleucine) being detected in the AMF-colonised roots (Meixner *et al.*, 2005; Stumpe *et al.*, 2005; Das and Gutjahr, 2020). However, JA could also suppress calcium spiking and inhibit AMF development under higher concentrations, possibly making JA homeostasis important for proper AMF formation (Das and Gutjahr, 2020; Qu *et al.*, 2021; Dai *et al.*, 2024). Additionally, antagonistic relationship between SA and JA might be an explanation for the somewhat beneficial JA role in AMF symbiosis (Bari and Jones, 2009; Pieterse *et al.*, 2009).

On the other hand, SA is an important phytohormone for combating biotic stress. Under the AMF formation scenario, the effect of SA is mostly inhibitory (Das and Gutjahr, 2020). It was noted that a transient increase of SA concentration happens in the early AMF symbiosis, but is not observed in the later stages of colonisation (Bedini *et al.*, 2018). This is expected, since plants cannot distinguish beneficial from pathogenic microorganisms in the early stages of colonisation. Additionally, SA plays a main role in defence against biotrophic pathogens. Therefore, SA might be a defence response that quickly gets suppressed during AMF - plant interplay. The SA will be discussed in more detail in the next chapter.

Apart from SA and JA that play a central role in plant defence tactics, all other phytohormones are deeply embedded in processes relevant to AMF development, AMF shaping of plant physiology through priming and involved in defence response through hormonal crosstalk. Auxins, hormones that are crucial for cell elongation, organogenesis and fine-tuning of the root architecture, also have a role in AM development and arbuscule formation, but the evidences are still scarce (Das and Gutjahr, 2020). However, increased auxin concentrations in AMF-inoculated roots is frequently observed (Wang *et al.*, 2021). Hormonal profile of *R. irregularis* spores, contained auxin, but whether it is AMF-derived and its potential impact on the colonisation process and plant physiology is not fully elucidated (Pons *et al.*, 2020).

Not all phytohormones have positive or neutral effect on AMF symbiosis. Gibberellins (GA) can inhibit arbuscule formation at low doses, or completely suppress symbiosis at high doses, through interaction with the DELLA protein complex that sustains symbiotic interaction (Fonouni-Farde *et al.*, 2016; Das and Gutjahr, 2020). ET

and cytokinins (CK), both have a complex relationship with AMF. Their inhibitory role has been frequently documented, but is often conflicting (Bedini *et al.*, 2018; Das and Gutjahr, 2020). ET concertation might need to surpass a certain threshold in order to suppress AMF formation (Foo *et al.*, 2016), while the proposed mechanism of symbiosis suppression is through targeting the signalling factors of calcium spiking in the cells and inhibition of the epidermal entry of the hyphae (Das and Gutjahr, 2020). However, CK have dubious role in AMF formation and sustaining of the symbiosis. For example, CK has been detected in the spores, indicating that AMF either contain or produce CK (Pons *et al.*, 2020), while AMF possibly possess unconfirmed receptors of CK (Mongès *et al.*, 2023). However, mechanism of action and deeper understanding of this hormone in the context of AMF-grapevine symbiosis still needs to be uncovered.

2.3. **AMF as modulators of a defence response to biotic stresses**

So far, few aspects of AMF plant interaction have been discussed and defence-related properties of this symbiosis have been briefly mentioned in multiple instances. However, apart from the already described nutritional benefits acquired from the AMF symbiosis, another crucial advantage present in the mycorrhizal plants is the induction of the defence systems. The already mentioned priming effect is an integral part of heightened physiological readiness to combat pathogen invasion. However, plant response to biotic stress is highly dependent on the type of the pathogen the plant is facing. Generally, plants could have compatible or incompatible interactions with invading pathogen, based on the pathogens ability to spread systemically (Glazebrook, 2005). Depending on the compatibility, plant defence response differs. Similarly, the beneficial effect of AMF on host defence mechanisms depends on the type of biotic stress that the host is facing and differs with the pathogen-host compatibility.

Shared between both compatible and incompatible interactions of the host and the pathogen is the recognition of PAMP (or MAMP - microbe-associated molecular patterns) through presence of pathogen-derived elicitors of defence. The elicitors are microbe-dependent and can be different molecules: carbohydrates (e.g. chitin from the fungal cell wall), peptides (e.g., flagellin found in bacterial cells), double-stranded RNA (dsRNA) or capsid proteins (ssRNA genome replication intermediary or parts of viral

particles, respectively) and others. Each elicitor has recognition receptor capable of detecting the pathogen (or beneficial microorganism) and triggering a defence reaction (Ngou *et al.*, 2022). The recognition mechanism for beneficial microorganisms, such as AMF, is described in Chapter 2.2.

After successful recognition, incompatible interactions are defined through timely influx of calcium in the cell and ROS accumulation, leading to activation of transcription factors and expression of defence-related genes. These genes encode for the synthesis of antimicrobial molecules and defence-related phytohormones. Timely reaction of the host and the presence of defence-related genes are crucial for limiting the pathogen spread. On the other hand, compatible interactions are characterised by the absence of defence-related genes. Therefore, pathogen recognition results in activation of stress response, but not strong enough to suppress invasion of the host. (He *et al.*, 2020; Pruitt *et al.*, 2021; Ngou *et al.*, 2022). However, in both types of interactions, AMF can induce MIR defence response. MIR is manifested through increased specialised metabolites concentration, different hormonal profile, structural changes of cell walls and priming of immune response (Fiorilli *et al.*, 2024). Effectiveness of MIR is affected by different factors including the host-pathogen-beneficial AMF genotypes (Comby *et al.*, 2017). Additionally, common trend with microbe-mediated defence response is the complex relationship with abiotic factors such as the soil nutrient availability and environmental factors that have the ability to influence the outcome of MIR (Fiorilli *et al.*, 2024).

In the context of MIR to biotic stress induced by viruses, there are conflicting observations (Singh *et al.*, 2024), i.e., effects on virus infection could be either beneficial or detrimental (Miozzi *et al.*, 2019). The beneficial effects (MIR) are defined through reduced virus titre, reduced symptom development and an overall more tolerant phenotype of the plant (Maffei *et al.*, 2014; Thiem *et al.*, 2014). On the contrary, the AMF could have a more synergistic effect with the virus, resulting in increased virus titre, more pronounced disease phenotype and reduced vigour of the plant (Sipahioglu *et al.*, 2009; Rúa *et al.*, 2013). This phenomenon is termed mycorrhiza-induced susceptibility (MIS) (Miozzi *et al.*, 2019). More recent studies on AMF-induced changes of defence response in virus-infected plants have shown more of these beneficial effects of AMF in the context of MIR. The mycorrhizal tomato plants have primed

physiological state when combating cucumber mosaic virus, shown through limited symptom development and mitigation of the effects of downregulated photosynthesis-related genes (Miozzi *et al.*, 2020). In potato plants, potato virus Y infection has been potentially masked by AMF inoculation through reduced oxidative stress (Deja-Sikora *et al.*, 2020). However, comparative transcriptome analysis has revealed upregulated differentially expressed genes encoding for pathogenesis-related proteins, confirming the beneficial effect of AMF on virus-infected potato plants (Deja-Sikora *et al.*, 2024). Finally, the protective AMF effect has also been demonstrated in cucumber plants infected with cucumber mosaic virus. The protective effect is based on reduced symptom development, but also altered oxidative status, antioxidative enzyme activity and expression of pathogenesis-related proteins (Metwally *et al.*, 2024).

Nevertheless, both MIR and MIS are observed in AMF-inoculated virus-infected plants and are an important indicator of the complexity of the disease development in the plants. This tripartite interaction requires further experiments to draw clearer conclusions, particularly regarding woody plants, for which there are insufficient data to rely on. The newly proposed ‘health triangle’ is accounting for the AMF and all other microbiota (beneficial and pathogenic) that are influencing the dynamic state of the plant health (Leveau, 2024).

2.4. **Objectives and hypothesis**

Objective: assessing the effect of arbuscular mycorrhizal fungus *Rhizophagus irregularis* alone or its combination with other AMF species on changes in grapevine response to infection with GRSPaV alone or in combination with other viruses.

Hypothesis:

1. Selected physiological indicators of biotic stress in grapevine point to reduced stress caused by virus disease if the AMF symbiosis is established.

2. Grapevine rupestris stem-pitting associated virus concentration in different grapevine tissues is affected by the presence of AMF in the grapevine roots.

3. Mixed mycorrhizal and virus inoculums used in the study have stronger effect on grapevine's physiological response than one-specie inoculums.

3. ORIGINAL SCIENTIFIC PAPERS

3.1. **Publication I.** : "*Arbuscular mycorrhizal fungi induce changes of photosynthesis-related parameters in virus infected grapevine*"

plants

MDPI

Article

Arbuscular Mycorrhizal Fungi Induce Changes of Photosynthesis-Related Parameters in Virus Infected Grapevine

Emanuel Gaši [1], Tomislav Radić [1,*], Mate Čarija [1], Giorgio Gambino [2], Raffaella Balestrini [2] and Katarina Hančević [1]

[1] Institute for Adriatic Crops, Put Duilova 11, 21000 Split, Croatia; emanuel.gasi@krs.hr (E.G.); mate.carija@krs.hr (M.Č.); katarina.hancevic@krs.hr (K.H.)
[2] Institute for Sustainable Plant Protection, National Research Council (IPSP-CNR), Strada delle Cacce 73, 10135 Torino, Italy; giorgio.gambino@ipsp.cnr.it (G.G.); raffaella.balestrini@ipsp.cnr.it (R.B.)
* Correspondence: tomislav.radic@krs.hr; Tel.: +385-21434469

Abstract: The negative effects of viruses and the positive effects of arbuscular mycorrhizal fungi (AMF) on grapevine performance are well reported, in contrast to the knowledge about their interactive effects in perennial plants, e.g., in grapevine. To elucidate the physiological consequences of grapevine–AMF–virus interactions, two different AMF inoculum (*Rhizophagus irregularis* and 'Mix AMF') were used on grapevine infected with grapevine rupestris stem pitting virus, grapevine leafroll associated virus 3 and/or grapevine pinot gris virus. Net photosynthesis rate (A_N), leaf transpiration (E), intercellular CO_2 concentration (C_i) and conductance to H_2O (g_s) were measured at three time points during one growing season. Furthermore, quantum efficiency in light (Φ_{PSII}) and electron transport rate (ETR) were surveyed in leaves of different maturity, old (basal), mature (middle) and young (apical) leaf. Lastly, pigment concentration and growth parameters were analysed. Virus induced changes in grapevine were minimal in this early infection stage. However, the AMF induced changes of grapevine facing biotic stress were most evident in higher net photosynthesis rate, conductance to H_2O, chlorophyll a concentration, total carotenoid concentration and dry matter content. The AMF presence in the grapevine roots seem to prevail over virus infection, with *Rhizophagus irregularis* inducing greater photosynthesis changes in solitary form rather than mixture. This study shows that AMF can be beneficial for grapevine facing viral infection, in the context of functional physiology.

Keywords: GRSPaV; *Rhizophagus irregularis*; *Funneliformis mosseae*; *Funneliformis caledonium*; GLRaV-3; GPGV; net photosynthesis rate; chlorophyll

Citation: Gaši, E.; Radić, T.; Čarija, M.; Gambino, G.; Balestrini, R.; Hančević, K. Arbuscular Mycorrhizal Fungi Induce Changes of Photosynthesis-Related Parameters in Virus Infected Grapevine. *Plants* **2023**, *12*, 1783. https://doi.org/10.3390/plants12091783

Academic Editors: Carlos Garrido, Victoria E. González-Rodríguez and Hernando José Bolivar Anillo

Received: 15 March 2023
Revised: 12 April 2023
Accepted: 25 April 2023
Published: 26 April 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important perennial crops globally, with viral pathogens posing a great threat to the viticulture through major economic loses [1]. With more than 80 viral species associated with the grapevine host, it represents one of the most virus-prone crops [2]. The effects of viral diseases on grapevine is a complex research topic, including changes in primary and secondary metabolites, photosynthesis, oxidative stress, antioxidative metabolism and cellular alterations [3–6]. Therefore, grapevine photosynthesis remains the point of interest for virus-induced damage across different virus–grapevine cultivar systems [7]. The severity of photosynthetic perturbations in the grapevine is dependent on the viral species, with pernicious grapevine viruses (e.g., grapevine leafroll-associated virus 3, GLRaV-3) accounting for more detrimental consequences on photosynthesis [8–12]. Some new emerging viruses, such as grapevine pinot gris virus (GPGV) may cause severe consequences, but its influence on the host physiology, such as photosynthesis, is underexplored [13]. However, many grapevine viruses are

latent without triggering apparent phenotypic changes and with underexplored influence on grapevine physiology [1]. Grapevine rupestris stem-pitting associated virus (GRSPaV) is considered ubiquitous with seemingly asymptomatic infection [14], but recent works point to possible beneficial role of this virus on grapevine [15,16]. The presence of GRSPaV positively influences grapevine growth regardless of lower net photosynthetic rate and CO_2 assimilation induced by virus infection [17]. Therefore, GRSPaV, despite being one of the most widely spread, represents a virus with unique and still unclear pathology.

The grapevine, however, tends to form mutualistic relationship with arbuscular mycorrhizal fungi (AMF) in the rhizosphere [18,19]. The mycorrhizal association greatly contributes to grapevine growth and nutrition [20,21]. Moreover, positive impact of AMF has been reported in grapevine exposed to numerous abiotic stresses through improvement of leaf water status, photosynthetic activity and chlorophyll concentration [22]. In addition, the remedial properties of AMF are described in grapevine facing biotic stresses. [23]. So far, the induction of defense response has been shown in grapevine inoculated by *Rhizophagus irregularis* and with subsequent infection by *Plasmopara viticola* or *Botrytis cinerea* [24]. The authors observed changes in stilbenoid biosynthesis pathways and argue that mycorrhizal fungi could enhance defense response against aerial pathogens [24]. Similarly, bioprotective effects of AMF has been shown against grapevine attacking ectoparasitic nematode *Xiphinema index*, where local and systemic defense processes were activated in the grapevine as a consequence of previously established mycorrhizal symbiosis [25]. The indirect mycorrhizal protection against the grapevine fanleaf virus (GFLV), born by aforementioned nematode species, has been shown in the mycorrhizal grapevine through inhibition of nematode transmission [26]. For investigating AMF alleviation of biotic stress induced by virus infection most works have been done on herbaceous crops, while studies on perennial plants, e.g., grapevine are fairly obscure. Nevertheless, a significant progress has been made in unraveling this complex interaction. So far, there have been reports of mycorrhiza induced resistance (MIR) based on induction of plant defense pathways [27], but also mycorrhiza induced susceptibility (MIS) defined through higher viral replication and intensified symptom development [28]. Few comprehensive reviews have systematically summarized research involving different plant hosts, AMF and virus species [28–30]. Recent studies showed AMF stimulated priming effects in virus infected tomato plants through mitigating physiological discrepancies and symptom development caused by viral infection [31]. Further, interesting study using same AMF species and host plant, but different viral species showed differential response regarding viral accumulation [32]. Therefore, the host response to the viral infection is not simply dependent on the relationship with AMF, rather the properties of each individual partner, e.g., lifestyle, species and genotype [29,33].

The physiological processes of the grapevine, in the light of multiple interactions regarding viral pathogens and symbiotic fungi (e.g., arbuscular mycorrhizal fungi), are vastly under-investigated, despite being predominantly present in agroecosystems in vineyards worldwide. Since grapevine is increasingly gaining status of a model organism for all fruit trees species, it serves as a perfect candidate for investigating above described complex interactions influencing plant physiology [1]. Therefore, the aim of this paper is to explore the physiological changes in the grapevine induced by arbuscular mycorrhizal fungi in the light of different severities of viral biotic stress. For that purpose, the GRSPaV will be used as a less pathogenic stress inducer, and GRSPaV coinfection with GLRaV-3 and/or GPGV will be used as a source of stronger pathogenic stress induction in the grapevine. The grapevine photosynthetic physiology processes and growth parameters will be the main interest in evaluating the effects of this multi-interactive biosystem.

2. Results

2.1. Root Colonization with AMF

Prior to AMF inoculation, grapevine plants were subjected to detection of virus presence and virus combinations used are presented in the Table 1. Inoculation of selected grapevine treatments with AMF resulted in high total root colonization and also in high arbuscules and variable vesicles colonization as shown in Table 1. High level of total AMF colonization was a prerequisite for evaluating AMF influence on grapevine photosynthesis, which was the aim of the study.

Total arbuscular mycorrhizal colonization and colonization by arbuscules, vesicles and hyphae did not depend on virus inoculum, but varied with type of AMF inoculum to a statistically significant level, as expected. This was confirmed by two-way ANOVA which gave no virus × AMF interaction ($p < 0.05$, Table 1) but strong dependence on AMF status. Application of two different AMF inoculums resulted in significantly higher colonization of arbuscules and vesicles and of total AMF colonization in treatments with only *Rhizophagus irregularis*, compared to treatments inoculated with mix of AMF species. Presence of microscopic intersections with hyphae only showed the opposite pattern, being more abundant in treatments with mix AMF species applied.

Table 1. Basic description and root AMF colonization percentages of the treatments used in the research. The colonization is shown as an average percentage ± standard deviation.

Treatment	Type of Inoculum (Factor)		Colonisation Percentage			
	Virus Status	**Mycorrhizal Status (AMF)**	**Arbuscules (%)**	**Vesicles (%)**	**Hyphae Only (%)**	**Total%**
T1	No virus	No AMF	Ø ^{a}	Ø ^{a}	Ø ^{a}	Ø ^{a}
T2		*Rhizophagus irregularis*	66.1 ± 13.2 ^{b}	44.3 ± 24.1 ^{bcd}	12.5 ± 4.8 ^{bc}	78.6 ± 8.4 ^{b}
T3		Mix *	75.6 ± 15.6 ^{b}	14.8 ± 6.1 ^{bcd}	15.8 ± 9.9 ^{bc}	92.4 ± 4.7 ^{b}
T4	GRSPaV	No AMF	Ø ^{a}	Ø ^{a}	Ø ^{a}	Ø ^{a}
T5		*Rhizophagus irregularis*	88.7 ± 12.4 ^{b}	76.8 ± 19.9 ^{cd}	5.7 ± 6.2 ^{abc}	94.3 ± 6.2 ^{b}
T6		Mix *	55.1 ± 10.8 ^{b}	9.7 ± 4.9 ^{b}	26 ± 7.4 ^{c}	81.4 ± 9 ^{b}
T7	GRSPaV + GLRaV-3	No AMF	Ø ^{a}	Ø ^{a}	Ø ^{a}	Ø ^{a}
T8		*Rhizophagus irregularis*	93.7 ± 4.7 ^{b}	82.4 ± 9.7 ^{cd}	3.1 ± 3.1 ^{abc}	97.5 ± 1.1 ^{b}
T9		Mix *	68.8 ± 17.5 ^{b}	18.2 ± 7.1 ^{bc}	18.2 ± 9.5 ^{bc}	87.6 ± 10.7 ^{b}
T10	GRSPaV + GPGV	No AMF	Ø ^{a}	Ø ^{a}	Ø ^{a}	Ø ^{a}
T11		*Rhizophagus irregularis*	86.8 ± 10.1 ^{b}	65.4 ± 15.7 ^{cd}	3.9 ± 3.7 ^{ab}	90.6 ± 8.2 ^{b}
T12		Mix *	85 ± 7.9 ^{b}	28.3 ± 12.1 ^{bcd}	10.7 ± 6.4 ^{bc}	96 ± 2.7 ^{b}
T13	GRSPaV + GLRaV-3 + GPGV	No AMF	Ø ^{a}	Ø ^{a}	Ø ^{a}	Ø ^{a}
T14		*Rhizophagus irregularis*	86.1 ± 11.8 ^{b}	71.5 ± 11.7 d	5.8 ± 3.7 ^{bc}	94.3 ± 5 ^{b}
T15		Mix *	85.8 ± 8.6 ^{b}	34.1 ± 14.4 ^{bcd}	7.4 ± 1.9 ^{bc}	93.4 ± 8.2 ^{b}
Main Effects	Virus	No virus	47.5 ± 38.6	20.0 ± 25.3	5.0 ± 4.2	18.6 ± 10.5
		GRSPaV	60.0 ± 30.3	31.3 ± 32.0	8.3 ± 3.8	44.8 ± 5.3
		GRSPaV + GLRaV-3	61.2 ± 41.7	38.0 ± 38.3	3.6 ± 4.2	29.0 ± 8.5
		GRSPaV + GPGV	70.4 ± 33.3	40.3 ± 30.8	3.8 ± 3.7	44.9 ± 5.7
		GRSPaV + GLRaV-3 + GPGV	70.9 ± 32.3	46.1 ± 31.4	4.7 ± 2.5	44.7 ± 5.1
		p	ns	ns	ns	ns
	AMF	No AMF	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}
		Rhizophagus irregularis	85.5 ± 13.0 ^{c}	69.1 ± 19.9 ^{c}	5.9 ± 4.9 ^{b}	92.1 ± 8.0 ^{c}
		Mix *	70.1 ± 18.0 ^{b}	20.9 ± 13.8 ^{b}	16.7 ± 10.1 ^{c}	87.2 ± 11.0 ^{b}
		p	<0.001	<0.001	<0.001	<0.001
	Virus × AMF	F	0.732	0.486	0.776	1.11
		p	ns	ns	ns	ns

* *Rhizophagus irregularis*, *Funneliformis mosseae* and *Funneliformis caledonium*; GRSPaV—grapevine rupestris stem pitting virus, GLRaV-3—grapevine leafroll associated virus 3, GPGV—grapevine pinot gris virus; lowercase letters indicate significant difference based on two-way ANOVA ($p < 0.05$).

2.2. Photosynthesis Analysis

By comparing treatments with viruses only (T4, T7, T10 and T13), we could estimate if virus combinations caused changes in grapevine's measured parameters compared to control (T1) and how it relates to the treatment inoculated with AMF (Figure 1). In first and second measuring point there was no difference in net photosynthesis rate of virus infected plants (T4, T7, T10 and T13) compared to virus free control (T1). However, decreased values of net photosynthesis rate were observed at third measurement where GRSPaV (T4) and GRSPaV + GPGV (T10) were present, while GRSPaV + GLRaV-3 (T8, T9) treated plants had lower, but insufficiently significant, net photosynthesis rate. For the conductance to H_2O, only GRSPaV + GLRaV-3 + GPGV (T13) infected plants had significantly higher values than virus free control, evident only at the third measurement. GRSPaV + GLRaV-3 infected plants along with GRSPaV infected plants expressed faster response to virus infection (first measuring point), through reduced transpiration rate and intercellular CO_2 concentration compared to virus-free control.

These observations were used to estimate whether AMF addition would change virus effect by performing two-way ANOVA. For the net photosynthesis rate, the positive effect of AMF was the most obvious out of all gas exchange parameters (Figure 1). At all three measuring points, this photosynthetic parameter was significantly higher in treatments were *R. irregularis* (T2, T5, T8, T11 and T14) or Mix AMF (T3, T6, T9, T12 and T15) were added, compared to the treatment where only viruses were present. During the first measurement net photosynthesis rate was significantly enhanced, mostly in *R. irregularis* inoculated, virus infected grapevine plants (T5, -8, -11, -14). During the following months, Mix AMF also caused significant increase compared to non-AMF controls, especially in the second measuring point for treatments involving GLRaV-3 (T9, T15). In the final measuring point, GRSPaV and GRSPaV + GPGV infected plants, had the most significant induction of net photosynthesis rate regardless of AMF inoculum used. Repeated measures ANOVA revealed that there were significant changes between the measurements in observed parameters during the studied period, particularly for net photosynthetic activity, which decreased from first and second to the third measurement in all treatments ($p < 0.001$). Conductance to H_2O was also significantly influenced by the added AMF, especially at the first measurement (May), at the point of early virus and AMF infection, where GRSPaV (T4) and GRSPaV + GPGV (T10) treated plants were most responsive to *R. irregularis* inoculation (Figure 1). Also, for transpiration and intercellular CO_2 concentration two-way ANOVA revealed significant interaction between two independent factors: virus and AMF status. Regarding transpiration, this interaction ($F = 3.150$, $p = 0.01$) pointed out that this parameter was significantly higher in GRSPaV and GRSPaV + GLRaV3 treatments when *R. irregularis* was inoculated, compared to treatments where mix AMF inoculum was added or to treatments without AMF. For intercellular CO_2 concentration, although significant interaction ($F = 4.24$, $p = 0.02$) was found, only one treatment stands out (GRSPaV + GLRaV3, without AMF) being lower from all the others.

Three months after AMF inoculation additional measurements of photosynthetic parameters were performed on three leaves per plant: old-basal leaf, mature-medium leaf and young-apical leaf. Three-way ANOVA revealed no interaction virus × AMF × leaf type ($F = 1.764$, p = ns) for the net photosynthesis rate where this parameter was related to the leaf type ($F = 22.367$, $p < 0.001$) and AMF status of the treatment ($F = 63.586$, $p < 0.001$) but not to the type of virus combination (non-significant; Figure 2). On the other hand, for the quantum efficiency in light (Φ_{PSII}) and electron transport rate (ETR) significant interactions virus × AMF × leaf type was found ($F = 1.828$, $p = 0.035$ and $F = 1.93$, $p = 0.023$, respectively). For both of these parameters AMF was the factor that influenced them the most ($F = 76.78$, $p < 0.001$ and $F = 13.61$, $p < 0.001$ respectively), followed by the type of the leaf ($F = 11.93$, $p < 0.001$ and $F = 12.91$, $p < 0.001$ respectively). For all three parameters in Figure 2, the lowest values were measured in old basal leaf. No significant differences were found between two types of AMF inoculum, but both were generally represented with values higher from the non-AMF controls. Although independent factor of virus status gave no significant

effects in three-way ANOVA, significantly increased parameters' values in mycorrhized vs. non-mycorrhized treatments were found in treatments GRSPaV + GPGV and GRSPaV + GLRaV3 + GPGV.

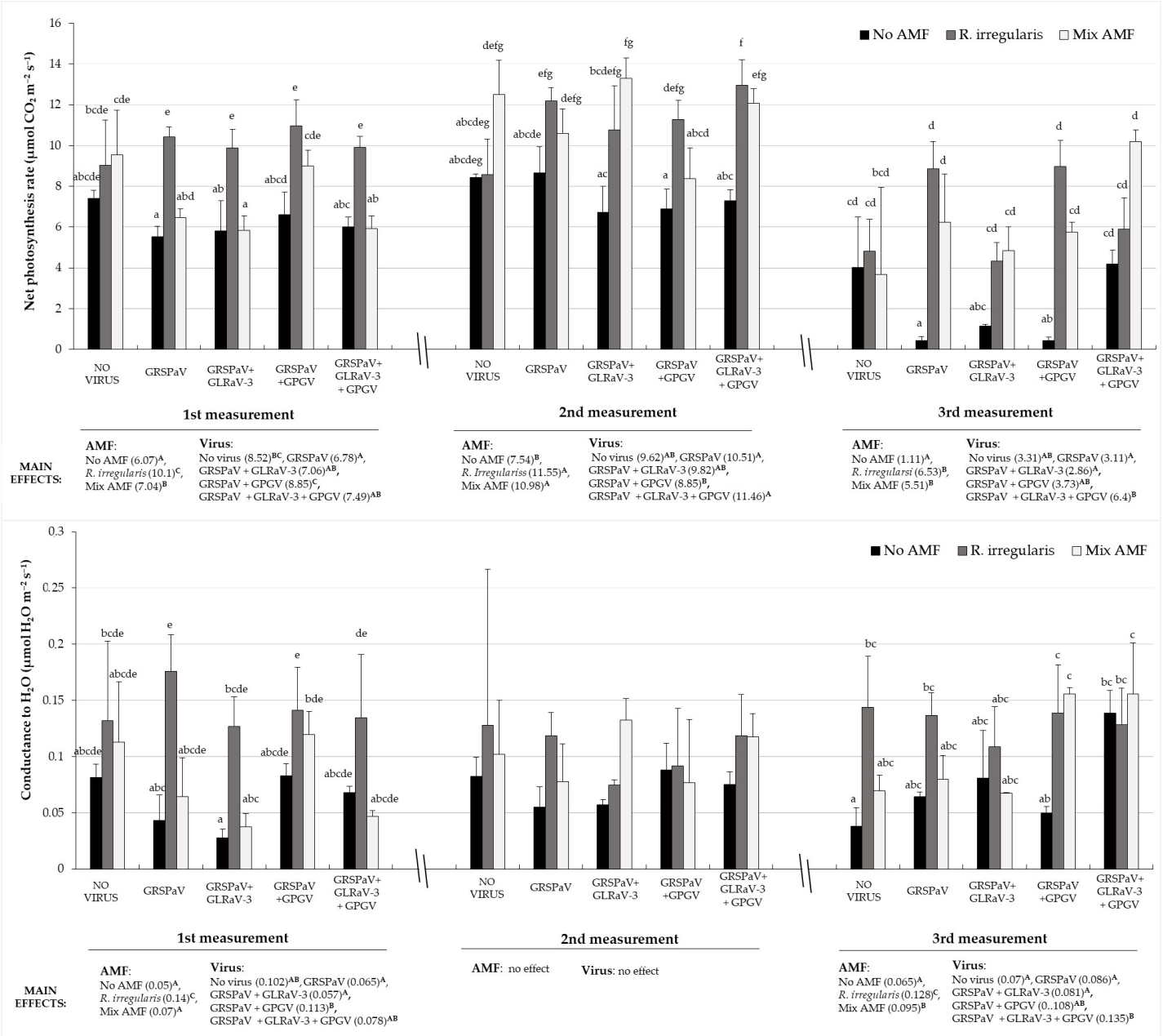


Figure 1. Effects of AMF inoculation on net photosynthesis rate (**top**) and conductance to H_2O (**bottom**) shown in three measuring points during the growing season of grapevine infected by different combinations of GRSPaV, GLRaV3 and GPGV viruses. Measuring was done in May (1st), June (2nd) and September (3rd). Two-way ANOVA was made for each measurement with uppercase letters indicating statistically significant difference in main effects with means in brackets. Treatments with distinct lowercase letters indicate a statistically significant difference in each measurement ($p < 0.05$) determined by the Bonferroni post-hoc test.

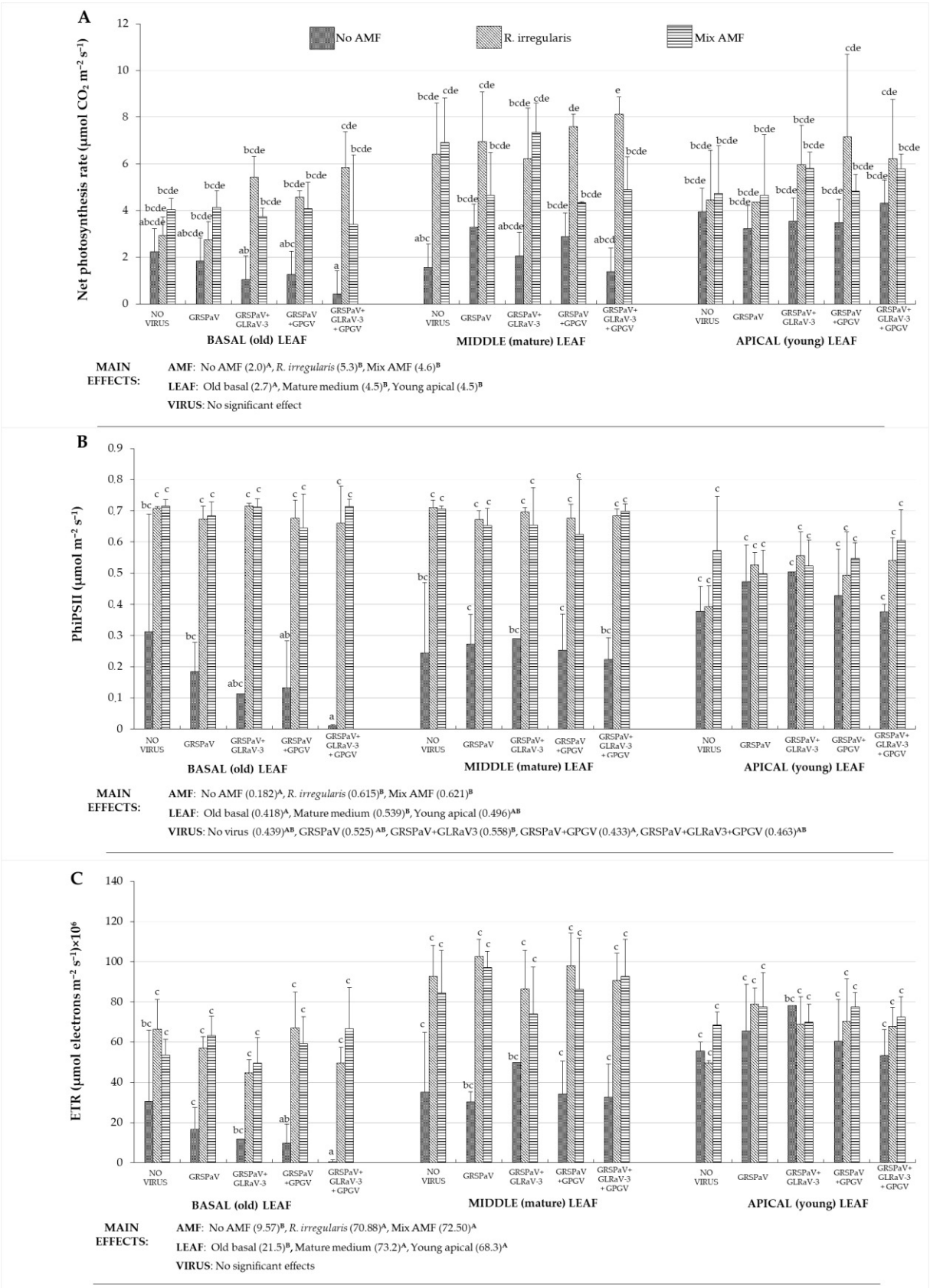


Figure 2. Effects of AMF inoculation on net photosynthesis rate (**A**), quantum efficiency in light (**B**), and electron transport rate (**C**) in the grapevine leaves of different maturity infected with GRSPaV, GLRaV3 and/or GPGV viruses. Parameters were analyzed by three-way ANOVA and statistically significant differences in main effects are indicated by distinct uppercase letters. Distinct lowercase letters represent statistically significant difference ($p < 0.05$), made with Bonferroni post-hoc test.

2.3. Pigment Concentrations

"NO AMF" treatments (T4, -7, -10, -13), containing only viruses, showed no significant difference compared to the healthy control. However, addition of AMF brought significant increase above their non-AMF control, particularly for the treatment GRSPaV + GLRaV-3 (Table 2). Contrarily to LiCor parameters, pigments concentrations revealed higher values when Mix AMF were in inoculum than when *R. irregularis* alone was added. Two-way ANOVA revealed significant interactions between AMF and virus compositions influencing chlorophyll a (F = 2.270, $p = 0.045$) and total chlorophyll (F = 2.263, $p = 0.046$). However, majority of pigment accumulation, mainly chlorophyll a and carotenoids, was significantly increased due to AMF inoculum, particularly in treatment with Mix AMF.

Table 2. Measurement of leaf chlorophyll a and b, total leaf chlorophyll and carotenoids concentration, as well as ratios of chlorophyll a and b, and total chlorophyll and carotenoids of grapevine.

Treatment	Virus StaTUS	AMF Status	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Total Carotenoids	Chlorophyll a/ Chlorophyll b	Chlorophyll/ Carotenoids
T1	NO VIRUS	NO AMF	1.43 ± 0.10 ^{ab}	0.85 ± 0.10	2.28 ± 0.19 ^{ab}	0.52 ± 0.07 ^{abc}	1.68 ± 0.08	4.39 ± 0.19
T2		*R. irregularis*	1.76 ± 0.40 ^{ab}	0.56 ± 0.32	2.32 ± 0.72 ^{ab}	0.76 ± 0.10 ^{abc}	4.09 ± 1.62	2.97 ± 0.55
T3		MIX AMF	1.96 ± 0.43 ^{b}	1.04 ± 0.16	3.00 ± 0.59 ^{ab}	0.73 ± 0.24 ^{abc}	1.87 ± 0.12	4.29 ± 0.59
T4	GRSPaV	NO AMF	1.65 ± 0.38 ^{ab}	1.22 ± 0.45	2.87 ± 0.83 ^{ab}	0.54 ± 0.01 ^{abc}	1.44 ± 0.22	5.32 ± 1.44
T5		*R. irregularis*	1.59 ± 0.31 ^{b}	1.28 ± 0.64	2.87 ± 0.91 ^{b}	0.43 ± 0.14 ^{abc}	1.59 ± 0.77	8.34 ± 5.29
T6		MIX AMF	2.15 ± 0.47 ^{b}	1.21 ± 0.47	3.35 ± 0.89 ^{b}	0.79 ± 0.15 ^{bc}	1.94 ± 0.50	4.28 ± 1.10
T7	GRSPaV + GLRaV-3	NO AMF	0.71 ± 0.09 ^{a}	0.36 ± 0.05	1.07 ± 0.14 ^{a}	0.37 ± 0.08 ^{abc}	1.96 ± 0.04	2.93 ± 0.24
T8		*R. irregularis*	1.88 ± 0.27 ^{b}	1.34 ± 0.61	3.22 ± 0.84 ^{b}	0.54 ± 0.14 ^{abc}	1.69 ± 0.73	6.49 ± 2.63
T9		MIX AMF	2.65 ± 0.37 ^{b}	2.06 ± 0.16	4.71 ± 0.21 ^{b}	0.70 ± 0.29 ^{abc}	1.31 ± 0.29	8.01 ± 3.01
T10	GRSPaV + GPGV	NO AMF	1.40 ± 0.11 ^{ab}	1.00 ± 0.31	2.40 ± 0.42 ^{ab}	0.45 ± 0.09 ^{abc}	1.50 ± 0.36	5.67 ± 2.02
T11		*R. irregularis*	1.88 ± 0.35 ^{b}	1.04 ± 0.34	2.92 ± 0.60 ^{b}	0.65 ± 0.19 ^{abc}	1.91 ± 0.43	4.85 ± 1.83
T12		MIX AMF	2.24 ± 0.45 ^{b}	1.11 ± 0.29	3.35 ± 0.65 ^{b}	0.82 ± 0.21 ^{bc}	2.11 ± 0.49	4.29 ± 1.08
T13	GRSPaV + GLRaV-3 + GPGV	NO AMF	1.37 ± 0.10 ^{ab}	1.23 ± 0.27	2.61 ± 0.37 ^{ab}	0.28 ± 0.08 ^{a}	1.15 ± 0.16	9.52 ± 1.28
T14		*R. irregularis*	1.58 ± 0.11 ^{ab}	0.96 ± 0.13	2.53 ± 0.23 ^{ab}	0.57 ± 0.06 ^{abc}	1.67 ± 0.17	4.52 ± 0.60
T15		MIX AMF	2.32 ± 0.61 ^{b}	1.29 ± 0.59	3.61 ± 1.18 ^{b}	0.82 ± 0.15 ^{c}	1.92 ± 0.31	4.39 ± 1.17
Main Effects	Virus	No virus	1.7 ± 0.4	0.8 ± 0.3	2.5 ± 0.7	0.7 ± 0.2	2.5 ± 1.6	3.9 ± 0.9
		GRSPaV	1.8 ± 0.5	1.2 ± 0.6	3.1 ± 1.0	0.6 ± 0.2	1.7 ± 0.6	5.9 ± 3.9
		GRSPaV + GLRaV-3	1.6 ± 0.9	1.3 ± 0.8	3.0 ± 1.6	0.5 ± 0.2	1.7 ± 0.6	5.9 ± 3.3
		GRSPaV + GPGV	1.8 ± 0.5	1.1 ± 0.3	2.9 ± 0.7	0.7 ± 0.2	1.9 ± 0.5	4.8 ± 1.8
		GRSPaV + GLRaV-3 + GPGV	1.8 ± 0.6	1.2 ± 0.5	3.1 ± 1.1	0.7 ± 0.2	1.7 ± 0.4	5.2 ± 2.2
		p	ns	ns	ns	ns	ns	ns
	AMF	No AMF	1.3 ± 0.4 ^{a}	0.9 ± 0.4	2.2 ± 0.8 ^{a}	0.4 ± 0.1 ^{a}	1.5 ± 0.3	5.6 ± 2.7
		Rhizophagus irregularis	1.7 ± 0.3 ^{b}	1.1 ± 0.5	2.8 ± 0.8 ^{ab}	0.6 ± 0.2 ^{a}	2.0 ± 1.1	5.8 ± 3.7
		Mix *	2.2 ± 0.5 ^{c}	1.3 ± 0.5	3.5 ± 1.0 ^{b}	0.8 ± 0.2 ^{b}	1.9 ± 0.5	4.7 ± 1.8
		p	<0.001	ns	<0.001	<0.001	ns	ns
	Virus × AMF	F	2.270	2.067	2.263	1.560	1.781	2.299
		p	0.045	ns	0.046	ns	ns	ns

* *Rhizophagus irregularis*, *Funneliformis mosseae* and *Funneliformis caledonium*; GRSPaV—grapevine rupestris stem pitting virus, GLRaV-3—grapevine leafroll associated virus 3, GPGV—grapevine pinot gris virus; Lowercase letters indicate the statistically significant difference revealed by two-way ANOVA ($p < 0.05$)

2.4. Plant Growth

Six months after virus inoculation, there was no significant influence of only viruses on grapevines, compared to virus-free control. Similarly, addition of AMF inoculum had no significant effect on plant growth. However, content of dry matter in total fresh weight was significantly influenced both by viruses and AMF inoculation (F = 2.73, $p = 0.016$).

Regarding AMF inoculum, *R. irregularis* treated plants have higher dry mater content than Mix AMF treated plants, while treatments without AMF had the lowest dry matter content. For data on plant growth and tissue weight ratios refer to Table S1.

3. Discussion

In this paper, effects of AMF on grapevine photosynthesis in simultaneous coinfection with virus have been investigated. So far, the negative effects of grapevine viruses, particularly GLRaV-3 [3,10,11,34] and the positive effects of AMF on grapevine photosynthesis and photosynthesis-related parameters have been reported [35–37]. However, there is a gap in research of their interactive effects in perennial plants and up to now no investigation on virus—AMF interactions with grapevine physiology was reported.

During this study we hypothesized that AMF have the potential to modify effects of viruses of different pathogenicity on photosynthesis in grapevine hosts. To verify this hypothesis, we observed plants infected with only viruses and the corresponding treatments with added AMF. Regarding the former ones [15,17], the latest measurement revealed only significantly reduced net CO_2 assimilation. Interestingly, in this case grapevine solely infected with GRSPaV had lower net photosynthesis rate than any other virus combination. Further, concentration of chlorophyll *a*, chlorophyll *b* and total carotenoids were not affected with GRSPaV, with no difference between treatments with or without AMF. This strong effect of GRSPaV on decreasing the net photosynthesis rate while having almost no effect on leaf chlorophyll content was already shown [15]. The underlying reason for that could be due to potential beneficial role of GRSPaV, that was proposed by some authors [15,16].

In accordance to described virus induced changes, further estimations were performed on the effects of AMF in selected treatments. This study proved that the presence of AMF associations greatly influenced grapevine response in parameters linked to photosynthesis. The net photosynthesis rate has been repeatedly higher in AMF inoculated plants compared to virus infected, AMF free plants. Furthermore, AMF inoculum composition seems to play an important role since single species AMF inoculum (*R. irregularis)* induced greater changes than inoculum composed of three species (*R. irregularis, F. mosseae, F. caledonium*). Similar results have been reported with grapevine facing water stress, where AMF contributed to greater photosynthetic rate, but also conductance to H_2O and transpiration rate [22]. The discrepancies in first measurement of net photosynthesis rate between one-species and mix mycorrhizal inoculum may be due to possible competition interplay or simply prolonged phase of symbiosis establishment for mixed mycorrhizal inoculum as seen from significantly fewer arbuscular and vesicular structures present in the roots inoculated by mixture of AMF. There have been reports of different influence of single versus mixed AMF inoculum on plant growth and physiology in the context of functional complementarity or competition regarding relatedness of AMF species used [37,38] Different influence of single vs. mix AMF on plant physiology is still topic to be further elucidated. However, our results indicate that effects of *R. irregularis* and mix AMF species is primarily significant during first measurement and diminished over time. Although their total colonization rates were similar, higher arbuscular and even more vesicular abundances in *R. irregularis* treatments, found in our study, indicate the possibility of different rates of symbiotic association establishment.

Concurrent appearance of GRSPaV and AMF in the grapevine is present in vineyards worldwide, frequently coinfected with GLRaV-3 and GPGV. Hence, GRSPaV–AMF–grapevine interactions may be observed as a model multipartite biosystem for investigating different variations of virus–AMF relationship with the grapevine. It would be interesting to explore, on transcriptomic level, if the synergistic interplay between GRSPaV and a specific mycorrhizal specie exists that could be utilized in agricultural regions heavily infected with viruses.

In this study, the treatments containing GLRaV-3 had the most severe depletion of chlorophyll *a* and total carotenoid concentration, the observation that was reported in

published literature and explained by heightened chlorophyllase activity [39]. However, the net photosynthesis rate did not reflect severe effect of GLRaV-3 coinfection more than with other viral treatment. The coinfection of GRSPaV with detrimental viruses such as GLRaV-3 or GPGV was intended to provoke more severe host reaction, but the response was similar across viral treatments. The reason for that could be a short infection period or no underlying interaction among viral species, as pointed out for closely and distantly related viruses [39–41]. Moreover, regarding pigment concentrations, grapevine colonized with mixed AMF performed better than those inoculated with single AMF, *R. irregularis*.

The analysis of different leaf age regarding photosynthetic parameters revealed that basal, oldest leaves had most perturbed net photosynthesis rate. This observation is in contrast to field grown grapevine where basal leaves maintain photosynthetic ability over long period of time [42]. This trend is connected to the favorable conditions, whereas in grapevine challenged with virus induced stress, photosynthetic perturbances could occur more easily in older leaves than the younger ones since the accumulation of viral titer is expectantly highest in older leaves [43], which is confirmed by our results. AMF caused increased net photosynthesis rate and electron transport rate, again the least intensively in oldest leaves. Maximum photosynthetic performance of the leaves is found to be reached with the onset of chlorophyll content decrease [44]. Since AMF inoculum has an impact on pigment concentration, the delayed response and discordance of net photosynthesis rate between treatments could result in basal leaves maintaining photosynthetic activity longer into the growing season than the basal leaves of AMF free grapevines. Even though viral induced stress did not significantly disturb quantum efficiency in light or electron transport rate, those two parameters were significantly upregulated in the presence of mycorrhizal fungi.

In summary, this study presents first insight into the complex interplay between viruses, AMF and grapevine as a host. The results contribute to the efforts to elucidate complex and underexplored niche of AMF mediated plant response to viral induced stress. Viral influence on grapevine photosynthesis and photosynthesis related parameters is shown to be mitigated by AMF colonization. Different levels of viral stress inducers through the use of selected viral infections, only partially produced differential effect on grapevine photosynthesis and photosynthesis related parameters, possibly due to short period of vine exposure to viruses. However, the addition of arbuscular mycorrhizal fungi, especially of mono species inoculum (*R. irregularis)*, resulted in induction of net photosynthesis rate, transpiration, conductance to H_2O, quantum efficiency in light and electron transport rate, as well as increased chlorophyll and carotenoids concentrations and dry matter content in some cases. The beneficial role of AMF was especially seen in cases when only GRSPaV was present as a source of stress and in cases of GRSPaV coinfection with GLRaV-3 or GPGV. In virus infected grapevine mixed AMF inoculum reduced loss of leaf pigments more than *R. irregularis* alone. The presented results indicate that arbuscular mycorrhizal fungi can be beneficial for grapevine facing viral infection, in the context of functional physiology and cause enhanced photosynthesis, which is the basis for its growth and development.

4. Materials and Methods

4.1. Experimental Setup

The Kober 5BB rootstock (*Vitis berlandieri* Planch. × *Vitis riparia* Michx.) was grafted with Merlot (*Vitis vinifera* L.) scions (both of Vitipep's, Sarrians, France) and rooted in 6L pots in the greenhouse. Substrate mixture was autoclaved two times at 121 °C for 30 min prior to transplanting. Mixture consisted of soil, perlite, peat and quartz sand in 1:1:1:1/3 ratios, respectively. For the successfully developed plants, leaves were sampled for RNA isolation and detection of GLRaV-1, -2, -3, GVA, GVB, GFkV, GFLV, ArMV, GRSPaV [45], and GPGV [46]. The uninfected grapevines and those which harbored only GRSPaV were

used in further steps. Plants that tested positive for any of the other viruses were excluded from the subsequent experimental setup. The two grapevine groups ('GRSPaV positive' and 'no virus') were infected with desired viruses through "chip budding" method with buds of known viral status in early February. Each plant received two buds from grapevine originating from collection vineyard (Institute of Adriatic Crops and Karst Reclamation). The buds were used as a source of GLRaV-3, GPGV or had no viruses. First indication of successful viral transmission by chip budding came after the grafted buds started growing [47]. To confirm the successful transmission of viruses from infected buds into the grapevine plant, virus detection of GLRaV-3 and GPGV was carried out as explained in the section 'virus detection'. Up to that juncture, five grapevine groups were formed based on their virus status. Each group was subsequently treated with three mycorrhizal inoculums. Inoculation was carried out using one AMF species *Rhizophagus irregularis* (Symplanta LLC, Darmstadt, GE), mixture of *Rhizophagus irregularis*, *Funneliformis mosseae* and *Funneliformis caledonium* (Inoq LLC, Schnega, Germany) or autoclaved inactive AMF inoculum for mock inoculation. In described way 15 treatments were created in total (Table 1). Two months later (late March) mycorrhizal presence was checked to confirm successful colonization of AMF inoculated plants and lack of AMF presence in mock inoculated plants (Figure 3). The AMF detection was done in order to set up the treatments for analyzing the interactive effects of AMF and viruses on grapevine photosynthesis-related parameters. The final treatments were distributed inside a greenhouse using randomized complete block design and each treatment was composed of six biological replicates. Plants were watered regularly, and nutrition was supplemented every 3 or 4 weeks during the duration of the experiment with half strength Hoagland solution [48]. Regular procedures of grapevine protection against pests and diseases were performed as needed, without using copper-based fungicides for the leaves [49]. Three biological replicates per treatment were measured for analysis of the selected gas exchange, plant growth and pigment concentration variables.

4.2. Virus Detection

For virus detection, 100 mg of leaf tissue per sample was used to extract total RNA [45]. The quality and amount of RNA was assessed with Nanodrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by determining the spectrophotometric absorbance and ratios of A_{260}/A_{230} and A_{260}/A_{280}. Complementary DNA was synthesized using M-MLV Reverse Transcriptase (Thermo Fisher Scientific, USA) following manufacturers guidelines. Detection of GRSPaV, GLRaV-3 and GPGV was done by using one technical replicate of each sample and amplifying using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.25 µM of each primer (Table 3), and cDNA sample diluted 1:10. Cycling conditions consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C/15 s, and 60 °C/1 min (CFX96 Touch Real-Time PCR, Bio-Rad, USA). The samples with Ct < 35 and with proper melting temperature data were considered positive. The final detection resulted in treatments setup as described in the Table 1.

Table 3. Primers used for virus detection

Target	Primer	Primer Sequences 5′–3′	Reference
GLRaV-3	Forward	TTGGTGGATGAGGTGCACAT	[50]
	Reverse	GTTGCGAAGACGCCTAGTTGT	
GRSPaV	Forward	GTGATCCATGTCAAAGCACATATG	[50]
	Reverse	CTCAGCGCCCAAAATTGC	
GPGV	Forward	GAATCGCTTGCTTTTTCATG	[51]
	Reverse	CTACATACTAAATGCACTCTCC	

4.3. Mycorrhizal Root Colonization Assessment

The detection of mycorrhizal association present in the roots was done two months after the inoculation, using Trypan blue as a coloring agent [52]. Fine grapevine roots were sampled and rinsed in water, cut to 1 cm segments and autoclaved at 121 °C for 5 min in 10% KOH. Subsequently, the roots were rinsed in distilled water and left for 5 min in 1% HCl. After that, roots were rinsed and stained with Trypan blue overnight. Finally, roots were rinsed, kept in 50% glycerol and 20 segments were mounted on slide. Under a compound microscope the total root colonization was estimated by examination of ~150 fields including assessment of arbuscules, vesicles and only hyphae according to the magnified intersections method [53]. Roots without cortex were excluded from the assessment.

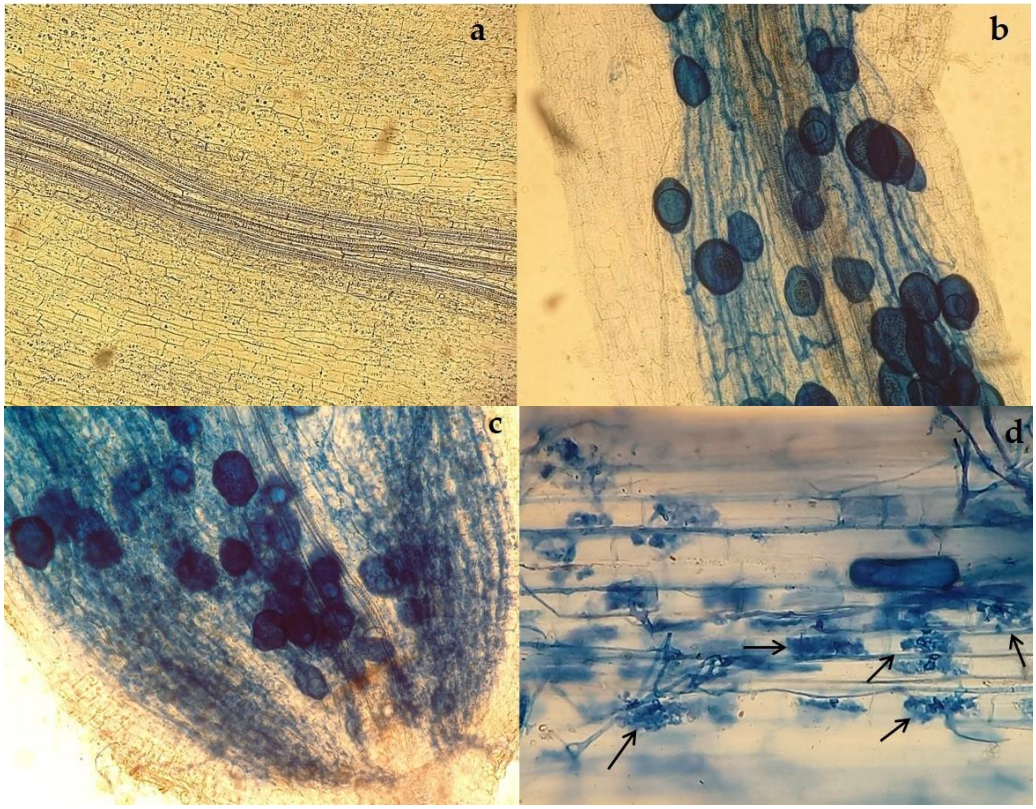


Figure 3. Microscopic view (×200) of grapevine roots treated with Trypan dye. Photos are representative of three different inoculums. Treatments 1, 4, 7, 10 and 13 are inoculated with unviable AMF inoculum (**a**), treatments 2, 5, 8, 11 and 14 are inoculated with *R. irregularis* (**b**) and treatments 3, 6, 9, 12 and 15 are inoculated with 'MIX AMF' consisting of *R. irregularis*, *F. mosseae* and *F. caledonium* (**c**). Arbuscules are indicated with arrows (**d**).

4.4. Gas Exchange

Gas exchange was measured on upper fully developed leaf between 09:00 a.m. and 11:00 a.m. in vivo, using non-destructive method with an open gas exchange system (Li-6400; Li-Cor. Inc., Lincoln, NE, USA). The variables measured were net photosynthesis rate (A_N), leaf transpiration (E), intercellular CO_2 concentration (C_i) and conductance to H_2O (g_s). The measurements were performed with device parameters as follows: CO_2 leaf chamber concentration was set at 400 ppm, saturated red light (500 μmol m^{-2} s^{-1}) with addition of 10% blue light, relative air humidity of 50% and block temperature of 30 °C. Photosynthetic parameters were measured three times after the final inoculation with AMF (PI—post inoculation), as follows: two-, three- and five-months post inoculation, 2PI, 3PI, 5PI, respectively. Additionally, quantum efficiency in light (Φ_{PSII}) and electron transport rate (ETR) were measured using compact porometer with pulse-amplitude modulation fluorometer Li-600 Porometer/Fluorometer (Li-Cor. Inc., Lincoln, NE, USA).

Light-adapted leaf measurement was chosen, with auto gsw+F configuration. After enabling stability of the instrument, plants were surveyed under ambient conditions. Measurement of Φ_{PSII}, ETR and gas exchange parameters were done three months post inoculation (3PI) for three leaves per plant differing in age and developmental phase. The measurements were made for the basal leaf (from the lower part of the plant), upper fully developed leaf (middle part of the plant) and apical-not fully developed leaf (upper part of the plant).

4.5. Pigment Analysis

Pigment concentrations were measured once, at 3PI, using fully developed leaves from three biological replicate per each treatment. The powder of freeze-dried fully-grown grapevine leaves was used for pigment analysis. Pigments were extracted from 10 mg of the plant material with 95% ethanol (overnight at room temperature in dark). Absorbances were measured spectrophotometrically at 470 nm, 647 nm and 663 nm. Chlorophyll *a*, chlorophyll *b* and total carotenoids were quantified using empirical equations, as well as chlorophyll a/chlorophyll b and total chlorophyll/total carotenoids ratios [54].

4.6. Grapevine Growth Parameters

At 3PI, shoot length and number of internodes of the grapevine plants were measured. The mean internode length was calculated by dividing total shoot length with number of internodes. Prior to pigment analysis, fresh and dry leaf weight were measured in order to calculate dry matter content in total weight. Leaves were freeze-vacuum dried at −50 °C, under 200 mbar vacuum.

4.7. Statistical Analysis

For statistical analysis two-way and three-way ANOVA as well as repeated measures ANOVA were performed in the Statistica 14.0.1. software (Tibco, Arlington, VA, USA), using Bonferroni post-hoc test ($p < 0.05$). Prior to statistical analysis data was transformed using natural logarithm in order to follow normal distribution.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants12091783/s1, Table S1: Growth parameters and dry content of grapevine interacting with AMF and viruses.

Author Contributions: Conceptualization, T.R., E.G., K.H., and M.Č.; methodology, T.R., E.G., K.H., M.Č., R.B. and G.G.; validation, T.R., K.H., R.B. and G.G.; formal analysis, T.R., M.Č. and E.G.; investigation, E.G., T.R., K.H. and M.Č.; resources, T.R. and K.H.; writing—original draft preparation, E.G. and T.R.; writing—review and editing, T.R., K.H., M.Č., R.B., G.G. and E.G.; visualization, E.G.; supervision, T.R.; project administration, T.R.; funding acquisition, T.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Croatian Science Foundation/Research project: IP-2020-02-8397.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We want to thank Radojka Plećaš and Amedeo Moine for their help during the experimental setup and technical assistance in parameter analysis. We also thank Maja Jukić Špika, for her advices in performance of statistical analyses.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Perrone, I.; Chitarra, W.; Boccacci, P.; Gambino, G. Grapevine–virus–environment interactions: An intriguing puzzle to solve. *New Phytol.* **2017**, *213*, 983–987. https://doi.org/10.1111/nph.14271.
2. Fuchs, M. Grapevine viruses: A multitude of diverse species with simple but overall poorly adopted management solutions in the vineyard. *J. Plant Pathol.* **2020**, *102*, 643–653. https://doi.org/10.1007/s42161-020-00579-2.
3. Pereira, G.E.; Padhi, E.M.T.; Sudarshana, M.R.; Fialho, F.B.; Medina-Plaza, C.; Girardello, R.C.; Tseng, D.; Bruce, R.C.; Erdmann, J.N.; Slupsky, C.M.; et al. Impact of grapevine red blotch disease on primary and secondary metabolites in 'Cabernet Sauvignon' grape tissues. *Food Chem.* **2021**, *342*, 128312. https://doi.org/10.1016/j.foodchem.2020.128312.
4. Hančević, K.; Čarija, M.; Radić Brkanac, S.; Gaši, E.; Likar, M.; Zdunić, G.; Regvar, M.; Radić, T. Grapevine Leafroll-Associated Virus 3 in Single and Mixed Infections Triggers Changes in the Oxidative Balance of Four Grapevine Varieties. *Int. J. Mol. Sci.* **2022**, *24*, 8. https://doi.org/10.3390/ijms24010008.
5. Sgherri, C.; Ranieri, A.; Quartacci, M.F. Antioxidative responses in *Vitis vinifera* infected by grapevine fanleaf virus. *J. Plant Physiol.* **2013**, *170*, 121–128. https://doi.org/10.1016/j.jplph.2012.09.016.
6. Laliberté, J.F.; Sanfaçon, H. Cellular Remodeling During Plant Virus Infection. *Annu. Rev. Phytopathol.* **2010**, *48*, 69–91. https://doi.org/10.1146/annurev-phyto-073009-114239.
7. Barón, M.; Flexas, J.; DeLucia, E.H. Photosynthetic responses to biotic stress. In *Terrestrial Photosynthesis in a Changing Environment: A Molecular, Physiological and Ecological Approach*; Cambridge University Press: Cambridge, UK, 2012; pp. 331–350. https://doi.org/10.1017/cbo9781139051477.026.
8. Sampol, B.; Bota, J.; Riera, D.; Medrano, H.; Flexas, J. Analysis of the virus-induced inhibition of photosynthesis in malmsey grapevines. *New Phytol.* **2003**, *160*, 403–412. https://doi.org/10.1046/j.1469-8137.2003.00882.x.
9. Reynard, J.-S.; Brodard, J.; Dubuis, N.; Zufferey, V.; Schumpp, O.; Schaerer, S.; Gugerli, P. *Grapevine red blotch virus*: Absence in Swiss Vineyards and Analysis of Potential Detrimental Effect on Viticultural Performance. *Plant Dis.* **2018**, *102*, 651–655. https://doi.org/10.1094/pdis-07-17-1069-re.
10. Moutinho-Pereira, J.; Correia, C.M.; Gonçalves, B.; Bacelar, E.A.; Coutinho, J.F.; Ferreira, H.F.; Lousada, J.L.; Cortez, M.I. Impacts of leafroll-associated viruses (GLRaV-1 and -3) on the physiology of the Portuguese grapevine cultivar "Touriga Nacional" growing under field conditions. *Ann. Appl. Biol.* **2012**, *160*, 237–249. https://doi.org/10.1111/j.1744-7348.2012.00536.x.
11. Montero, R.; El aou Ouad, H.; Pacifico, D.; Marzachì, C.; Castillo, N.; García, E.; Del Saz, N.F.; Florez-Sarasa, I.; Flexas, J.; Bota, J. Effects of Grapevine leafroll-associated virus 3 on the physiology in asymptomatic plants of *Vitis vinifera*. *Ann. Appl. Biol.* **2017**, *171*, 155–171. https://doi.org/10.1111/aab.12356.
12. Endeshaw, S.T.; Sabbatini, P.; Romanazzi, G.; Schilder, A.C.; Neri, D. Effects of grapevine leafroll associated virus 3 infection on growth, leaf gas exchange, yield and basic fruit chemistry of *Vitis vinifera* L. cv. Cabernet Franc. *Sci. Hortic.* **2014**, *170*, 228–236. https://doi.org/10.1016/j.scienta.2014.03.021.
13. Meng, B.; Martelli, G.P.; Golino, D.A.; Fuchs, M. *Grapevine Viruses: Molecular Biology, Diagnostics and Management*; Springer International Publishing: Berlin/Heidelberg, Germany, 2017; ISBN 9783319577067.
14. Mannini, F.; Digiaro, M. The effects of viruses and viral diseases on grapes and wine. In *Grapevine Viruses: Molecular Biology, Diagnostics and Management*; Springer Nature: New York, NY, USA, 2017; pp. 453–482. https://doi.org/10.1007/978-3-319-57706-7_23.
15. Gambino, G.; Cuozzo, D.; Fasoli, M.; Pagliarani, C.; Vitali, M.; Boccacci, P.; Pezzotti, M.; Mannini, F. Co-evolution between Grapevine rupestris stem pitting-associated virus and *Vitis vinifera* L. leads to decreased defence responses and increased transcription of genes related to photosynthesis. *J. Exp. Bot.* **2012**, *63*, 5919–5933. https://doi.org/10.1093/jxb/ers244.
16. Pantaleo, V.; Vitali, M.; Boccacci, P.; Miozzi, L.; Cuozzo, D.; Chitarra, W.; Mannini, F.; Lovisolo, C.; Gambino, G. Novel functional microRNAs from virus-free and infected *Vitis vinifera* plants under water stress. *Sci. Rep.* **2016**, *6*, 20167. https://doi.org/10.1038/srep20167.
17. Tobar, M.; Fiore, N.; Pérez-Donoso, A.G.; León, R.; Rosales, I.M.; Gambardella, M. Divergent molecular and growth responses of young "Cabernet Sauvignon" (*Vitis vinifera*) plants to simple and mixed infections with Grapevine rupestris stem pitting-associated virus. *Hortic. Res.* **2020**, *7*, 2. https://doi.org/10.1038/s41438-019-0224-5.
18. Likar, M.; Hančević, K.; Radić, T.; Regvar, M. Distribution and diversity of arbuscular mycorrhizal fungi in grapevines from production vineyards along the eastern Adriatic coast. *Mycorrhiza* **2013**, *23*, 209–219. https://doi.org/10.1007/s00572-012-0463-x.
19. Holland, T.C.; Bowen, P.; Bogdanoff, C.; Hart, M.M. How Distinct Are Arbuscular Mycorrhizal Fungal Communities Associating with Grapevines? *Biol. Fertil. Soils* **2014**, *50*, 667–674. https://doi.org/10.1007/S00374-013-0887-2/FIGURES/6.
20. Trouvelot, S.; Bonneau, L.; Redecker, D.; van Tuinen, D.; Adrian, M.; Wipf, D. Arbuscular mycorrhiza symbiosis in viticulture: A review. *Agron. Sustain. Dev.* **2015**, *35*, 1449–1467. https://doi.org/10.1007/s13593-015-0329-7.
21. Nerva, L.; Giudice, G.; Quiroga, G.; Belfiore, N.; Lovat, L.; Perria, R.; Volpe, M.G.; Moffa, L.; Sandrini, M.; Gaiotti, F.; et al. Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs. *Biol. Fertil. Soils* **2022**, *58*, 17–34. https://doi.org/10.1007/s00374-021-01607-8.
22. Ye, Q.; Wang, H.; Li, H. Arbuscular Mycorrhizal Fungi Improve Growth, Photosynthetic Activity, and Chlorophyll Fluorescence of *Vitis vinifera* L. Cv. Ecolly under Drought Stress. *Agronomy* **2022**, *12*, 1563. https://doi.org/10.3390/AGRONOMY12071563/S1.
23. Dowarah, B.; Gill, S.S.; Agarwala, N. Arbuscular Mycorrhizal Fungi in Conferring Tolerance to Biotic Stresses in Plants. *J. Plant Growth Regul.* **2022**, *41*, 1429–1444. https://doi.org/10.1007/s00344-021-10392-5.

24. Bruisson, S.; Maillot, P.; Schellenbaum, P.; Walter, B.; Gindro, K.; Deglène-Benbrahim, L. Arbuscular mycorrhizal symbiosis stimulates key genes of the phenylpropanoid biosynthesis and stilbenoid production in grapevine leaves in response to downy mildew and grey mould infection. *Phytochemistry* **2016**, *131*, 92–99. https://doi.org/10.1016/j.phytochem.2016.09.002.
25. Hao, Z.; Fayolle, L.; Van Tuinen, D.; Chatagnier, O.; Li, X.; Gianinazzi, S.; Gianinazzi-Pearson, V. Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode Xiphinema index involves priming of defence gene responses in grapevine. *J. Exp. Bot.* **2012**, *63*, 3657–3672. https://doi.org/10.1093/jxb/ers046.
26. Hao, Z.; van Tuinen, D.; Fayolle, L.; Chatagnier, O.; Li, X.; Chen, B.; Gianinazzi, S.; Gianinazzi-Pearson, V. Arbuscular mycorrhiza affects grapevine fanleaf virus transmission by the nematode vector Xiphinema index. *Appl. Soil Ecol.* **2018**, *129*, 107–111. https://doi.org/10.1016/j.apsoil.2018.05.007.
27. Pozo, M.J.; Azcón-Aguilar, C. Unraveling mycorrhiza-induced resistance. *Curr. Opin. Plant Biol.* **2007**, *10*, 393–398. https://doi.org/10.1016/j.pbi.2007.05.004.
28. Miozzi, L.; Vaira, A.M.; Catoni, M.; Fiorilli, V.; Accotto, G.P.; Lanfranco, L. Arbuscular Mycorrhizal Symbiosis: Plant Friend or Foe in the Fight Against Viruses? *Front. Microbiol.* **2019**, *10*, 1238. https://doi.org/10.3389/fmicb.2019.01238.
29. Comby, M.; Mustafa, G.; Magnin-Robert, M.; Randoux, B.; Fontaine, J.; Reignault, P.; Lounès-Hadj Sahraoui, A. Arbuscular Mycorrhizal Fungi as Potential Bioprotectants Against Aerial Phytopathogens and Pests. In *Arbuscular Mycorrhizas and Stress Tolerance of Plants;* Springer: Singapore, 2017, pp. 195–223. https://doi.org/10.1007/978-981-10-4115-0_9.
30. Hao, Z.; Xie, W.; Chen, B. Arbuscular Mycorrhizal Symbiosis Affects Plant Immunity to Viral Infection and Accumulation. *Viruses* **2019**, *11*, 534. https://doi.org/10.3390/v11060534.
31. Miozzi, L.; Vaira, A.M.; Brilli, F.; Casarin, V.; Berti, M.; Ferrandino, A.; Nerva, L.; Accotto, G.P.; Lanfranco, L. Arbuscular Mycorrhizal Symbiosis Primes Tolerance to Cucumber Mosaic Virus in Tomato. *Viruses* **2020**, *12*, 675. https://doi.org/10.3390/v12060675.
32. Khoshkhatti, N.; Eini, O.; Koolivand, D.; Pogiatzis, A.; Klironomos, J.N.; Pakpour, S. Differential Response of Mycorrhizal Plants to *Tomato bushy stunt virus* and *Tomato mosaic virus* Infection. *Microorganisms* **2020**, *8*, 2038. https://doi.org/10.3390/microorganisms8122038.
33. Miozzi, L.; Catoni, M.; Fiorilli, V.; Mullineaux, P.M.; Accotto, G.P.; Lanfranco, L. Arbuscular Mycorrhizal Symbiosis Limits Foliar Transcriptional Responses to Viral Infection and Favors Long-Term Virus Accumulation. *Mol. Plant Microbe Interact.* **2011**, *24*, 1562–1572. https://doi.org/10.1094/MPMI-05-11-0116.
34. El Aou-Ouad, H.; Montero, R.; Medrano, H.; Bota, J. Interactive effects of grapevine leafroll-associated virus 3 (GLRaV-3) and water stress on the physiology of *Vitis vinifera* L. cv. Malvasia de Banyalbufar and Giro-Ros. *J. Plant Physiol.* **2016**, *196–197*, 106–115. https://doi.org/10.1016/j.jplph.2016.04.003.
35. Wu, Q.-S.; Xia, R.-X. Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-watered and water stress conditions. *J. Plant Physiol.* **2006**, *163*, 417–425. https://doi.org/10.1016/j.jplph.2005.04.024.
36. Lu, Y.; Xu, H.; Tong, S. Effects of arbuscular mycorrhizal fungi on photosynthesis and chlorophyll fluorescence of maize seedlings under salt stress. *Emir. J. Food Agric.* **2018**, *30*, 199–204. https://doi.org/10.9755/ejfa.2018.v30.i3.1642.
37. Crossay, T.; Majorel, C.; Redecker, D.; Gensous, S.; Medevielle, V.; Durrieu, G.; Cavaloc, Y.; Amir, H. Is a Mixture of Arbuscular Mycorrhizal Fungi Better for Plant Growth than Single-Species Inoculants? *Mycorrhiza* **2019**, *29*, 325–339. https://doi.org/10.1007/S00572-019-00898-Y.
38. Roger, A.; Colard, A.; Angelard, C.; Sanders, I.R. Relatedness among arbuscular mycorrhizal fungi drives plant growth and intraspecific fungal coexistence. *ISME J.* **2013**, *7*, 2137–2146. https://doi.org/10.1038/ismej.2013.112.
39. Bertamini, M.; Muthuchelian, K.; Nedunchezhian, N. Effect of Grapevine Leafroll on the Photosynthesis of Field Grown Grapevine Plants (*Vitis vinifera* L. cv. Lagrein). *J. Phytopathol.* **2004**, *152*, 145–152. https://doi.org/10.1111/j.1439-0434.2004.00815.x.
40. Čarija, M.; Černi, S.; Stupin-Polančec, D.; Radić, T.; Gaši, E.; Hančević, K. Grapevine Leafroll-Associated Virus 3 Replication in Grapevine Hosts Changes through the Dormancy Stage. *Plants* **2022**, *11*, 3250. https://doi.org/10.3390/plants11233250.
41. Velasco, L.; Bota, J.; Montero, R.; Cretazzo, E. Differences of Three Ampeloviruses' Multiplication in Plant May Explain Their Incidences in Vineyards. *Plant Dis.* **2014**, *98*, 395–400. https://doi.org/10.1094/pdis-04-13-0433-re.
42. Knoll, M.; Redl, H. Gas exchange of field-grown Vitis vinifera L. cv. zweigelt leaves in relation to leaf age and position along the stem. *OENO ONE* **2012**, *46*, 281–294. https://doi.org/10.20870/oeno-one.2012.46.4.1524.
43. Monis, J.; Bestwick, R.K. Detection and Localization of Grapevine Leafroll Associated Closteroviruses in Greenhouse and Tissue Culture Grown Plants. *Am. J. Enol. Vitic.* **1996**, *47*, 199–205. https://doi.org/10.5344/ajev.1996.47.2.199.
44. Sitko, K.; Rusinowski, S.; Pogrzeba, M.; Daszkowska-Golec, A.; Gieroń; Kalaji, H.M.; Małkowski, E. Development and aging of photosynthetic apparatus of *Vitis vinifera* L. during growing season. *Photosynthetica* **2020**, *58*, 186–193. https://doi.org/10.32615/ps.2019.107.
45. Gambino, G. Multiplex RT-PCR Method for the Simultaneous Detection of Nine Grapevine Viruses. *Methods Mol. Biol.* **2015**, *1236*, 39–47. https://doi.org/10.1007/978-1-4939-1743-3_4.
46. Morelli, M.; de Moraes Catarino, A.; Susca, L.; Saldarelli, P.; Gualandri, V.; Martelli, G.P. First report of Grapevine pinot gris virus from table grapes in southern Italy. *J. Plant Pathol.* **2014**, *96*, 439. https://doi.org/10.4454/JPP.V96I2.039.
47. Food and Agriculture Organization of the United Nations. *Graft-Transmissible Diseases of Grapevines: Handbook for Detection and Diagnosis*; Martelli, G.P., Ed.; FAO: Rome, Italy, 1993; 263p.
48. Hoagland, D.R.; Arnon, D.I. The Water-Culture Method for Growing Plants without Soil. *Circular. Calif. Agric. Exp. Stn.* **1950**, *347*, 1–32.

49. Klanjac, J.; Grozić, K.; Goreta Ban, S.; Ban, D.; Ivić, D.; Radić, T.; Pasković, I. Kompatibilnost fungicida i arbuskularnih mikoriznih gljiva u proizvodnji rajčice na otvorenom. *Glasnik Zaštite Bilja* **2018**, *41*, 28–39. https://doi.org/10.31727/gzb.41.5.4.
50. Gambino, G.; Gribaudo, I. Simultaneous Detection of Nine Grapevine Viruses by Multiplex Reverse Transcription-Polymerase Chain Reaction with Coamplification of a Plant RNA as Internal Control. *Phytopathology* **2006**, *96*, 1223–1229. https://doi.org/10.1094/phyto-96-1223.
51. Bianchi, G.L.; De Amicis, F.; De Sabbata, L.; Di Bernardo, N.; Governatori, G.; Nonino, F.; Prete, G.; Marrazzo, T.; Versolatto, S.; Frausin, C. Occurrence of Grapevine Pinot gris virus in Friuli Venezia Giulia (Italy): Field monitoring and virus quantification by real-time RT-PCR. *EPPO Bull.* **2015**, *45*, 22–32. https://doi.org/10.1111/epp.12196.
52. Phillips, J.M.; Hayman, D.S. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* **1970**, *55*, 158–161, https://doi.org/10.1016/s0007-1536(70)80110-3.
53. Mcgonigle, T.P.; Miller, M.H.; Evans, D.G.; Fairchild, G.L.; Swan, J.A. A new method which gives an objective measure of colonization of roots by vesicular—Arbuscular mycorrhizal fungi. *New Phytol.* **1990**, *115*, 495–501. https://doi.org/10.1111/j.1469-8137.1990.tb00476.x.
54. Lichtenthaler, H.K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods Enzymol.* **1987**, *148*, 350–382. https://doi.org/10.1016/0076-6879(87)48036-1.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

3.2. **Publication II**. : "*Arbuscular mycorrhizal fungi modify temporal virus accumulation and distribution in different grapevine tissue*"

OPEN ACCESS
phytobiomesjournal.org
Phytobiomes Journal
A Transdisciplinary Journal of Sustainable Plant Productivity

RESEARCH

Arbuscular Mycorrhizal Fungi Modify Temporal Virus Accumulation and Distribution in Different Grapevine Tissues

Emanuel Gaši,[1] Tomislav Radić,[1,†] Giorgio Gambino,[2] Mate Čarija,[1] Frano Matić,[3] Raffaella Balestrini,[4] and Katarina Hančević[1]

[1] Institute for Adriatic Crops and Karst Reclamation, Department of Plant Sciences, Put Duilova 11, 21000 Split, Croatia
[2] Institute for Sustainable Plant Protection, National Research Council (IPSP-CNR), Strada delle Cacce 73, 10135 Torino, Italy
[3] University of Split, Department of Marine Studies, Ruđera Boškovića 37, 21000 Split, Croatia
[4] Institute of Biosciences and Bioresources, National research Council (IBBR-CNR), Via Amendola 165/A 70126 Bari, Italy

Accepted for publication 20 September 2024.

Abstract

Arbuscular mycorrhizal fungi (AMF) have been shown to improve plant host tolerance to biotic stresses. However, AMF-mediated protection against virus diseases has been highly variable and poorly investigated in perennial crops. In this study, we investigated the influence of AMF on virus concentration and distribution in grapevine coinfected with three common viruses, grapevine rupestris stem-pitting associated virus (GRSPaV) solely or in coinfection with grapevine leafroll-associated virus 3 (GLRaV-3) and grapevine Pinot Gris virus (GPGV). Two different types of AMF inocula were used: (i) *Rhizophagus irregularis* and (ii) *R. irregularis*, *Funneliformis mosseae*, and *F. caledonium* (Mix). Sampling for quantitative RT-PCR was performed three times in a 1-year period in four distinct grapevine tissues for the assessment of GRSPaV concentration in AMF-inoculated grapevine. AMF influence on GRSPaV was the most significant in the first sampling, 2 months postinoculation, with the virus accumulating predominantly in the roots. Simultaneously, GRSPaV concentration in young grapevine leaves was low, possibly due to modified source–sink dynamics. Quantification of GLRaV-3 and GPGV was performed in coinfected grapevine in the third sampling. After a year, both viruses exhibited accumulation in the roots of mycorrhizal plants. However, GLRaV-3 displayed accentuated accumulation and GPGV decrease in foliage, indicating a differential effect on the virus in coinfected grapevine. Regarding AMF symbiosis, generally, the Mix inoculum induced more pronounced virus concentration changes than *R. irregularis* alone. In summary, this study shows differences in the virus load of AMF-inoculated and AMF-free grapevine and adds nuance to the complex multitrophic interactions that are shaping grapevine health.

Keywords: crop, endophytes, plant pathology, symbiosis, virology

Arbuscular mycorrhizal fungi (AMF) are mutualistic symbionts across terrestrial plant species with low specificity and a broad range of compatible hosts, making this form of interaction ubiquitous across ecosystems (Brundrett and Tedersoo 2018). Grapevine is a perennial crop shown to nurture this interaction (Possingham and Obbink 1971; Schubert and Cravero 1985), with AMF providing numerous benefits to the grapevine host, including improved water and nutrient acquisition (Balestrini et al. 2018; Schreiner 2007), an indirect influence on the rhizosphere to support development of other beneficial microorganisms (Chen et al. 2019; Hao et al. 2021), and a reduced effect of biotic and abiotic stressors on grapevine (Alagna et al. 2020; Trouvelot et al. 2015). Mitigating stress induced by a virus infection is a field of extensive research, although there is a lack of studies for grapevine hosts and woody fruit species. This poses a significant gap in understanding multivariate interactions of perennial crops and microbiota. In the context of viral disease, grapevine is one of the most virus-prone, economically important crops (Fuchs 2020). Significant progress has been made

†Corresponding author: T. Radić; Tomislav.Radic@krs.hr

Funding: Support was provided by the Next Generation EU (INOMED-21 [09-207/1-23]) and Croatian Science Foundation (DOK-2021-02-2381 and IP-2020-02-8397).

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

PUBLIC DOMAIN The author(s) have dedicated the work to the public domain under the Creative Commons CC0 "No Rights Reserved" license by waiving all of his or her rights to the work worldwide under copyright law, including all related and neighboring rights, to the extent allowed by law, 2025.

in the control of virus spread through implementing certification programs, massive detections, replacement of infected with certified material, and reduction of vector populations (Almeida et al. 2013; Maliogka et al. 2015). In monitoring practices, virus dynamics is an important factor that has to be considered when testing for virus presence in grapevine throughout the year (Čarija et al. 2022a; Osman et al. 2018; Shabanian et al. 2020; Tsai et al. 2012). For that purpose, a focus on virus distribution and accumulation in different grapevine tissue is crucial when testing for infected individuals. So far, studies on grapevine virus distribution have not considered the influence of beneficial microorganisms, such as AMF, and its potential ability to modify virus distribution throughout the plant. Untangling this complex network is of importance for potentially implementing a more holistic approach in virus diagnostics, considering real-life scenarios. Furthermore, understanding the three-partner relationship is of interest for plant protection by utilizing the naturally occurring microbiome to reduce the impact of biotic and abiotic stress events on grapevine health (Bettenfeld et al. 2022). So far, there is no clear consensus for AMF influence on plant virus disease, and published literature reports unique responses varying with the plant host, AMF species, and virus of interest (Deja-Sikora et al. 2019; Hao et al. 2019; Miozzi et al. 2019).

Grapevine forms numerous associations with the microbial community, shaping its health and overall homeostasis while also creating a space for emergence of complex multivariate interactions between microorganisms (Bettenfeld et al. 2022). Because grapevine rupestris stem-pitting associated virus (GRSPaV, family *Betaflexiviridae*) is not included in certification programs, it spreads freely and presents possibly one of the most ubiquitous grapevine viruses (Meng and Rowhani 2017). GRSPaV has been associated with the onset of Syrah decline, rupestris stem-pitting, and vein necrosis (Al Rwahnih et al. 2009; Bouyahia et al. 2005; Meng and Gonsalves 2007). However, there have also been reports of commensal nature of grapevine–GRSPaV association (Gambino et al. 2012), deepening the complexities of microbial interaction. GRSPaV is generally considered a less harmful grapevine virus, leading to its exclusion from the certification programs in the European Union. However, its biology and pathology are not fully understood, making the influence on viticulture hard to define (Meng and Gonsalves 2007). Because of the high incidence of GRSPaV and AMF presence in grapevine and their possible co-occurrence on a global scale, they represent a potentially valuable system for exploring multitrophic interactions and their impact on the grapevine host. Furthermore, grapevine is a perennial plant and a host to more than 80 described viruses (Fuchs 2020), with reportedly frequent coinfections with multiple viruses (Čarija et al. 2022b; Eichmeier et al. 2018; Hančević et al. 2021; Rivadeneira et al. 2022; Xiao et al. 2018). Although not fully understood for grapevines, plant viruses can exhibit a spectrum of interacting effects, from synergism to antagonism (Moreno and López-Moya 2020; Singhal et al. 2021). However, the three-way interaction of grapevine-virus-AMF is underexplored, and studies with multiple coinfections are completely lacking from the literature. Considering the high prevalence of grapevine viruses, especially GRSPaV, the beneficial potential of mycorrhizal symbiosis in biotic stress, and the unclear relationship between viruses and AMF in perennial plant hosts, the aim of this study was to uncover changes in GRSPaV relative concentration through time and tissues of grapevine depending on its AMF status. Furthermore, the relative quantification of grapevine virus coinfection, consisting of GRSPaV, grapevine leafroll-associated virus 3 (GLRaV-3, family *Closteroviridae*), and grapevine Pinot Gris virus (GPGV, family *Betaflexiviridae*), widely present in Mediterranean Croatia (Hančević et al. 2021), was investigated to explore the possibility of their specific responses to mycorrhizal presence.

Materials and Methods

Grapevine material, Merlot scion (*Vitis vinifera* L.) and Kober 5BB rootstock (*Vitis berlandieri* Planch. × *Vitis riparia* Michx.), was commercially acquired (Vitipep's, Sarrians, France). Merlot was grafted onto Kober 5BB and planted in an autoclaved (121°C for 30 min) substrate mixture containing soil, peat, perlite, and quartz sand. Plants were kept under greenhouse conditions, not interfering with the temperature and humidity. An insect-proof net was used to control the presence of vector insects. Plants were automatically irrigated twice a week, depending on the seasonal needs, and fertilized with "half-strength" Hoagland solution to ensure phosphate-deficient conditions (Hoagland and Arnon 1950).

Preliminary virus screening of the starting grapevine material

Wood scrapings were collected from successfully rooted plants for examination of the phytosanitary status of starting grapevine material. About 100 mg of collected tissue was used for RNA extraction using the CTAB method (Gambino 2015) and quantitative RT-PCR (RT-qPCR) screening of most common grapevine viruses, grapevine leafroll-associated virus 1, 2 (GLRaV-1, GLRaV-2), GLRaV-3, grapevine fleck virus, Arabis mosaic virus, grapevine fanleaf virus, GRSPaV, grapevine virus A (GVA), grapevine virus B, and GPGV (Supplementary Table S1). Complementary DNA was synthesized using M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, U.S.A.) following the manufacturer's guidelines. The cycling conditions of RT-qPCR were described in the prior work of Gambino et al. (2011). Plant material that was positive for any listed virus except GRSPaV was excluded from the experimental setup, leaving only GRSPaV-positive plants for the subsequent virus infection and AMF inoculation.

Treatments

Grapevine was subsequently infected with GLRaV-3 and GPGV through the "chip-budding" method (Fig. 1) to investigate the influence of AMF on interplant virus interaction. The buds from grapevine of a previously determined sanitary status (Hančević et al. 2021) were acquired from the Institute of Adriatic Crops and Karst Reclamation germplasm collection vineyard and used for virus transmission. Following leaf development from infected buds, successful virus transmission was confirmed by RT-qPCR virus detection in leaf tissue originating from Merlot scion. Once the targeted sanitary status of grapevine was met, four virus-specific groups were created as follows: "R" (GRSPaV), "RL" (GRSPaV + GLRaV-3), "RP" (GRSPaV + GPGV), and "RLP" (GRSPaV + GLRaV-3 + GPGV). Two types of AMF inoculum were introduced to the sterile grapevine substrate after virus infection. The "Ri" inoculum consisted of only *Rhizophagus irregularis* DAOM197198 (Symplanta, Darmstadt, Germany), and the "Mix" inoculum consisted of *R. irregularis*, *Funneliformis mosseae*, and *F. caledonium* (Inoq, Schnega, Germany). Each inoculum was added in roughly the same concentration of 8,000 spores per 6-liter pot, as specified by the manufacturer (1 g contains 2,700 spores for Inoq and 10,000 spores for the Symplanta inoculum). Grapevine designated as "AMF free" was also inoculated with a mixture of both inocula, sterilized by a two-time autoclaving process at 121°C/20 min and used as the "No AMF" treatment. Each virus group was divided into subgroups by AMF status, resulting in 12 distinct treatments represented with six biological replicates (Supplementary Table S2). Plants were left for 2 months after AMF inoculation to sample the first time point (2 months after AMF inoculation),

allowing for symbiosis establishment with the roots. Mycorrhizal status was confirmed through microscopic investigation of Trypan blue-treated roots (Brundrett et al. 1996) with the methodology described in previous work (Gaši et al. 2023). Briefly, root samples were rinsed with water and treated with 10% KOH. Samples were then autoclaved (121°C for 5 min), treated with HCl 1% (wt/vol) for 5 min followed by a quick rinse, and left for staining overnight in Trypan blue (0.05% in lactoglycerol) at room temperature. Samples were inspected under a microscope using the magnified intersections method (McGonigle et al. 1990). Sampling of the grapevine tissue for virus quantification was made at three time points throughout one calendar year, with intervals being 2, 3, and 12 months post-AMF inoculation. Four distinct tissue types were sampled: roots, undeveloped leaves (young leaves) from the top half of the plant, petioles, and fully developed leaves from the bottom half of the plant (mature leaves). Tissue was properly labeled and immediately put on dry ice before storage at −80°C.

RNA extraction, cDNA synthesis, and virus quantification

Total RNA was extracted from four different tissues using the CTAB protocol (Gambino 2015). The purity and concentrations of the extracts were assessed spectrophotometrically by measuring absorbance at 230, 260, and 280 nm on Nanodrop One (Thermo Fisher Scientific). DNA contamination was removed using the TURBO DNA-free Kit (Thermo Fisher Scientific) following instructions from the manufacturer. Absence of DNA contamination was confirmed by RT-qPCR using a reference gene (ubiquitin) and with purified RNA sample serving as a template. The SuperScript II

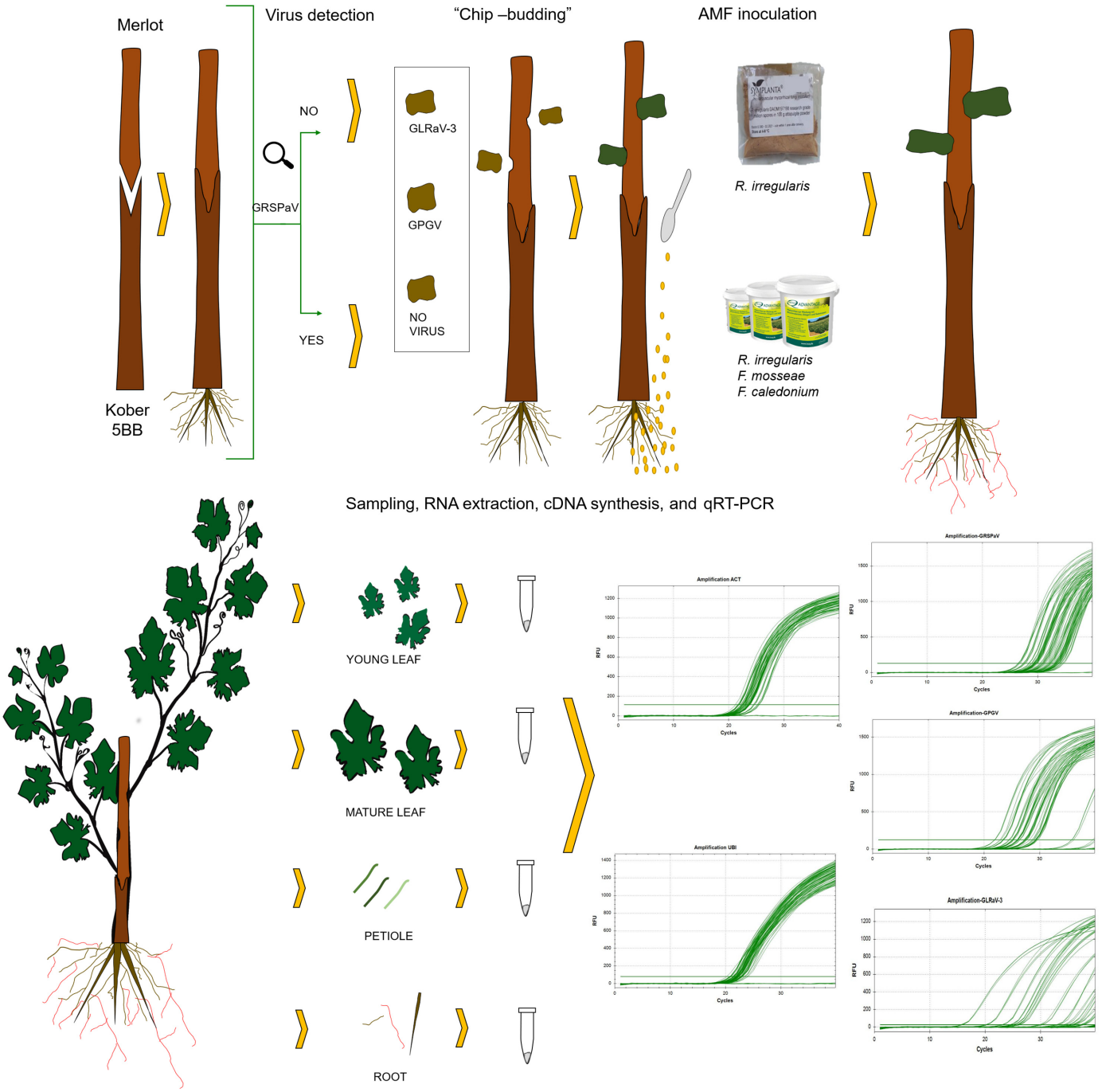


Fig. 1. Schematic overview of experimental design and workflow of key steps included in this study.

Reverse Transcriptase kit (Thermo Fisher Scientific) was used for first-strand cDNA synthesis of 250 ng of purified RNA extracts, following the manufacturer's instructions. For the RT-qPCR, two endogenous genes, ubiquitin (UBI) and actin (ACT), were used as reference genes with stable expression across different tissue samples and different time points (Gambino et al. 2011). Relative quantification was performed with CFX96 Touch Real-Time PCR (Bio-Rad, Hercules, CA, U.S.A.) on three technical replicates for each biological replicate, and the expression of transcripts was quantified after normalization to the geometric mean of two reference genes. The PCR Mix (10 µl) contained 5 µl of iTaq Universal SYBR Green SuperMix (Bio-Rad), 0.2 µM of each primer pair (Supplementary Table S1), and 1 µl of cDNA diluted 1:5. Cycling conditions consisted of initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C/15 s and 60°C/30 s. Specific annealing of the primers was controlled on dissociation kinetics performed at the end of each PCR run. For statistical analysis of GRSPaV tissue-specific distribution, a modified ΔΔCt method was used (Livak and Schmittgen 2001). Modification was made in calculating the ΔCt value by subtracting the Ct value of the "gene of interest" from the geometric mean of the two reference genes (e.g., ΔCt = GEOMEAN [Ct(ACT) + Ct(UBI)] − Ct(virus)), making data interpretation more intuitive. Furthermore, the ΔΔCt of each sample was calculated using the mean ΔCt value of the treatment containing only GRSPaV without AMF for each tissue and sampling point separately. For statistical analysis of differences in relative quantification in GRSPaV, GLRaV-3, and GPGV, ΔCt was calculated for coinfected grapevine treatments (RL and RP). The statistical methods used in this study were two-way analysis of variance (ANOVA), repeated measures ANOVA, and K-means clustering. A Bonferroni post-hoc test was performed for statistically significant interactions ($P < 0.05$). For K-means clustering, the Elbow and Silhouette methods were used to define the optimal number of clusters in a 10-cluster range, with three clusters being the optimal result from both methods. Three clusters were chosen for the analysis, with a maximum number of iterations of 100. To examine variability of GRSPaV concentration through time, ΔΔCt GRSPaV values were used for clustering, and each treatment and tissue type fell into one of the three clusters, approximating GRSPaV temporal changes in that specific treatment and tissue. Statistical analysis was performed using the software Statistica 14.0.1 (Tibco, Arlington, VA, U.S.A.).

Results

In this study, the impact of AMF symbiosis on virus concentration was examined in different coinfected grapevine tissues. The experimental setup generated virus-infected grapevine plants as confirmed by the RT-qPCR 4 months after the chip-budding procedure (cycle threshold < 35; Supplementary Table S3), which were further used for AMF inoculation. The mycorrhizal symbiosis was inspected using light microscopy, and root inoculation was highly efficient, with total colonization greater than 80%. Detailed microscopic analysis was published in prior work (Gaši et al. 2023). The determined preliminary results were the initial basis for the final setup of treatments (Supplementary Table S2) and for the planned samplings of different grapevine tissues for the analysis of relative virus concentration.

GRSPaV relative concentration depends on the AMF inoculum and virus coinfections

To investigate differences in GRSPaV concentration over time, repeated measures ANOVA was used at three sampling points during 1 year. Based on the mycorrhizal status of the treatment (No, Ri, and Mix), general trends of GRSPaV relative concentration in different grapevine tissue were examined. Repeated measures ANOVA revealed a significant decrease in GRSPaV relative concentration in roots from the first sampling made in May to the third sampling performed 1 year later (Fig. 2). Grapevine roots with the Mix inoculum had a tenfold increase in virus concentration compared with No AMF in the first sampling, whereas Ri plants revealed a similar GRSPaV concentration to the No AMF grapevine. A similar trend with respect to GRSPaV continued through the second sampling, with the concentration being 20-fold higher in the roots of Mix plants, followed by Ri and No having similar GRSPaV concentrations. Quantification in the third sampling revealed no difference in root GRSPaV relative concentration regarding the AMF inoculum, and the concentration variability plateaued over time (Fig. 2). Conversely, the virus titer in young leaves showed an increase over time, with GRSPaV relative concentration detected in first sam-

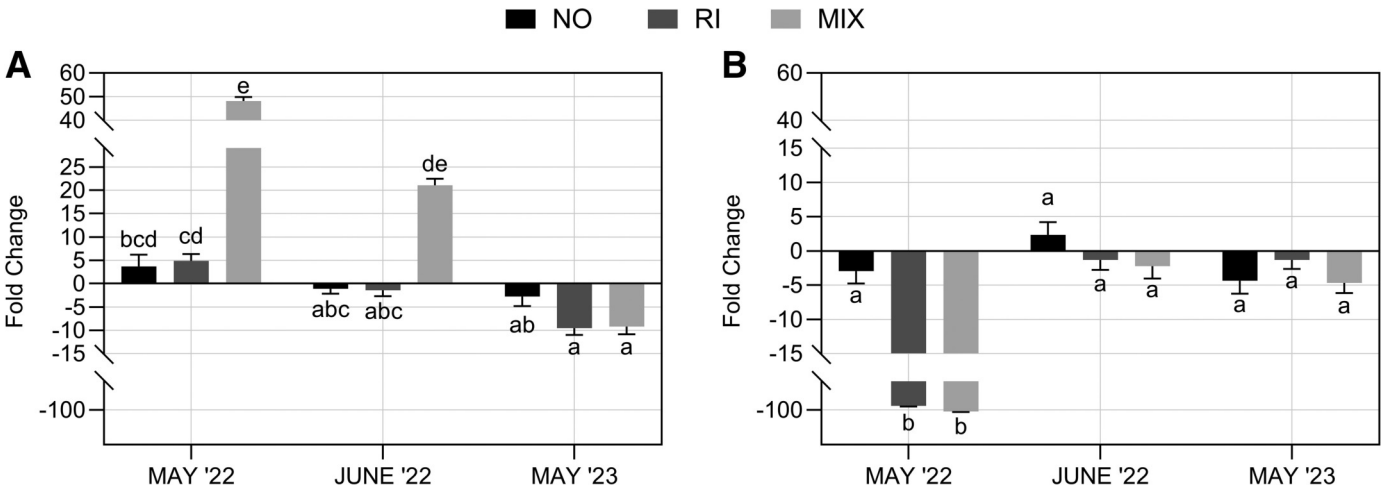


Fig. 2. Relative grapevine rupestris stem-pitting associated virus (GRSPaV) quantification in **A,** roots and **B,** young leaves measured in May and June 2022 and May 2023. GRSPaV concentration data are pooled based on arbuscular mycorrhizal fungi (AMF) inoculum type. Data are presented as fold changes, and vertical bars denote standard error. Repeated measures analysis of variance for three sampling points was calculated with ΔΔCt values, and statistically significant differences ($P < 0.05$) in GRSPaV concentration between AMF inoculum types determined via a Bonferroni post-hoc test are represented with different lowercase letters. $N = 12$. No, inactive AMF inoculum; Ri, *Rhizophagus irregularis*; Mix, *R. irregularis*, *Funneliformis mosseae*, and *F. caledonium*.

pling for Ri and Mix plants being almost 100 times lower than in No AMF. This low relative concentration in the young leaves was observed in the first sampling but was not present in the second sampling. After 1 year, there was no difference in GRSPaV concentration in young leaves between No, Ri, and Mix grapevine. The remaining two tissue types (petioles and mature leaves) had more stable GRSPaV relative concentrations along the investigated time period, with no significant alterations caused by the AMF inoculum (Supplementary Fig. S1).

In tissues with observed modified temporal GRSPaV relative concentration in the presence of different AMF inoculum types, we investigated the GRSPaV–AMF interplay in different virus coinfection scenarios. For that purpose, root and young leaf tissues shown to have modified GRSPaV concentrations were analyzed based on virus treatments (R, RL, RP, and RLP) and tested against both AMF inoculum treatments. The differences in GRSPaV relative concentration in different coinfections was inspected using two-way ANOVA, predominantly showing a 15-fold increase in GRSPaV concentration in the roots of only R-infected grapevine inoculated with Mix compared with No and Ri in the first sampling. However, there was no increase in the roots (Ri or Mix) of other coinfected grapevine (Fig. 3A). Interestingly, in grapevine roots, the results of the second sampling are in contrast to the first. For the second sampling, root GRSPaV concentrations were greater only in Mix grapevine coinfected with other viruses, with $\Delta\Delta$Ct being 6.19, 4.43, and 4.38 for RL, RP, and RLP, respectively (Fig. 3C). Two-way ANOVA revealed that the reduced GRSPaV concentration in the young leaves of mycorrhizal plants in the first sampling was primarily in RP coinfection (Fig. 3B). This AMF effect in young leaves remained in the second sampling in RLP coinfection (Fig. 3D), although it is not statistically significant when all viral treatments are pooled together (Fig. 2).

To investigate the modified GRSPaV relative concentration pattern simultaneously in relation to different virus/AMF combinations, four grapevine tissues, and three sampling points, GRSPaV quantification data were cluster analyzed (Fig. 4A). Based on the GRSPaV concentration of four tissue types and different treatments, K-means cluster analysis revealed three clusters represented with distinct patterns of GRSPaV relative concentration changes over time (Fig. 4B). Most treatments are represented with all three distinct clusters, with GRSPaV concentrations being similar in mature leaves and petioles and differing from roots and young leaves. Exceptionally, the RP and RLP treatments were divided into two clusters, both without mycorrhizal symbiosis. In the RP coinfection, GRSPaV concentration changed similarly over time in roots, petioles, and young and mature leaves. In the RLP treatment, roots and mature leaves shared a similar trend of GRSPaV concentration changes over time, whereas petioles and young leaves showed

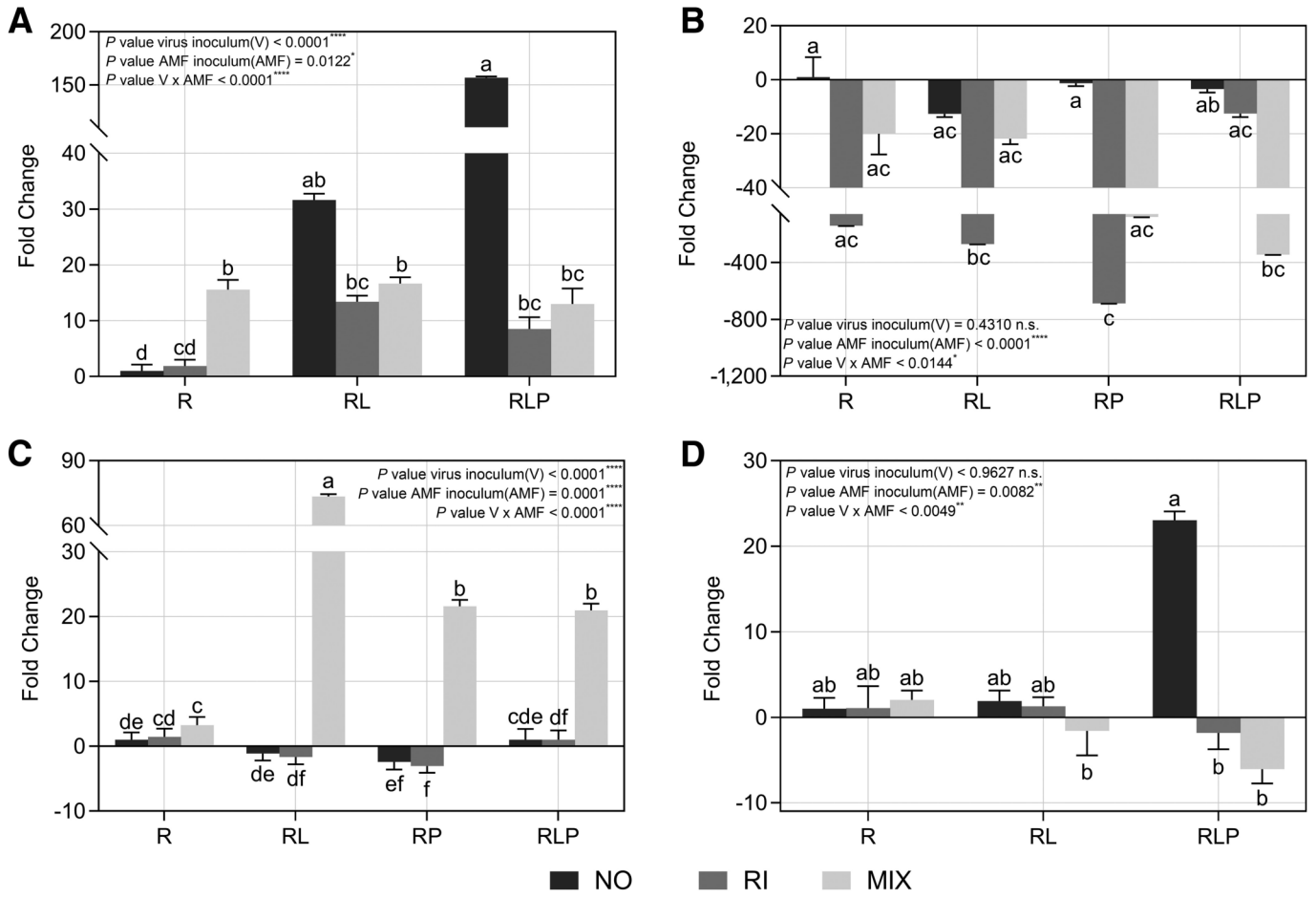


Fig. 3. Grapevine rupestris stem-pitting associated virus (GRSPaV) relative expression of arbuscular mycorrhizal fungi (AMF)-inoculated grapevine **A and C,** roots and **B and D,** young leaves in different virus combinations at A and B, first and C and D, second sampling points. Data are presented as fold changes, and vertical bars denote standard error. GRSPaV concentration against the AMF inoculum was tested with two-way analysis of variance. Different lowercase letters denote significant differences between virus groups, according to a Bonferroni post-hoc test ($P < 0.05$), $n = 3$. R, GRSPaV; L, grapevine leafroll-associated virus 3; P, grapevine Pinot Gris virus; No, inactive AMF inoculum; Ri, *Rhizophagus irregularis*; Mix, *R. irregularis*, *Funneliformis mosseae*, and *F. caledonium*.

virus concentration changes over time defined by the second cluster (Fig. 4A). Nonetheless, general trends of GRSPaV concentration grouped the majority of roots and young leaves in distinct clusters, showing a decrease and increase throughout the experiment, respectively (Fig. 4A and B). Mature leaves and petioles, similarly to the roots, showed a steady decrease over time. However, virus concentration was repeatedly lower in mature leaves and petioles in all samplings, grouping them in separate clusters.

Differential AMF impact on GRSPaV, GLRaV-3, and GPGV relative concentrations 1 year after AMF inoculation

In the third sampling, additional relative quantification was performed for treatments harboring coinfections to investigate different virus-AMF interplays and investigate the presence of differential virus responses to different AMF inocula used. GRSPaV in RL coinfection (Fig. 5A) exhibited accumulation in different tissues than in RP coinfection (Fig. 5B). In RL, GRSPaV relative concentration was lowest in Ri roots ($\Delta Ct = -11.08 \pm 0.6$), with significantly lower concentrations than in petioles ($\Delta Ct = -8.09 \pm 0.3$) and young leaves ($\Delta Ct = -7.06 \pm 0.4$). In the absence of the mycorrhizal inoculum, the GRSPaV concentration was uniform across tissues in RL coinfection, whereas in the presence of Mix, its concentrations significantly decreased in mature leaves (from $\Delta Ct = -7.53 \pm 0.5$ in No to $\Delta Ct = -10.9 \pm 0.35$ in Mix) (Fig. 5A). In RP treatment, the GRSPaV relative concentration decreased, particularly in the roots of No grapevine. However, both Ri and Mix increased the GRSPaV relative concentration in roots (from $\Delta Ct = -14.13 \pm 0.7$ in No to $\Delta Ct = -7.95 \pm 0.3$ and $\Delta Ct = -7.51 \pm 0.9$ in Ri and Mix, respectively) (Fig. 5B). Furthermore, the GLRaV-3 concentration (in RL treatment) was shown to be stable and low across different tissues of No plants. However, in Ri ($\Delta Ct = -4.72 \pm 0.2$) and Mix ($\Delta Ct = 1.10 \pm 0.04$) plants, the virus concentration increased in mature leaves, with significantly higher virus concentrations than in No AMF ($\Delta Ct = -13.63 \pm 1.1$) (Fig. 5C). The GPGV concentration in RP grapevine showed a unique response. A differential effect of AMF symbiosis on GPGV concentration in different grapevine tissues was noted. In particular, the GPGV relative concentration in Ri was highest in roots ($\Delta Ct = -1.56 \pm 0.15$) and differed significantly from the GPGV relative concentration in petioles ($\Delta Ct = -4.61 \pm 0.2$) and mature leaves ($\Delta Ct = -5.6 \pm 0.3$). A similar pattern was also noted for Mix plants, with high GPGV concentrations present in the roots ($\Delta Ct = 0.6 \pm 0.06$) and low GPGV concentrations present in the foliage ($\Delta Ct = -10.63 \pm 2.5$ for young and $\Delta Ct = -10.2 \pm 1.7$ for mature leaves) of RP coinfected plants. It is important to point out that the highest GPGV relative concentrations for No plants were in mature leaves and lowest in roots, whereas for Mix, the opposite holds true (Fig. 5D). Petioles were the only tissue without a difference in relative concentration of GRSPaV, GLRaV-3, or GPGV across different AMF inocula of studied grapevine coinfections (RL and RP), making them the most robust grapevine tissue in regard to AMF influence on virus replication.

Discussion

Grapevine with established AMF symbiosis has numerous benefits making them more resilient to environmental stresses (Trouvelot et al. 2015). On the other side, biotic stress induced by viruses can have a noticeable impact on grapevine health and, in the context of agriculturally important crops, food and wine production (Fuchs 2020). However, little attention has been given to investigating AMF interaction with virus-infected grapevine. Here, two distinct AMF inocula were used to investigate the influence on virus relative concentration in infected grapevine. The nature of AMF–host interaction is variable and dependent on the genotype of both parties and environmental conditions (Berger and Gutjahr 2021). Accordingly, the Mix inoculum showed a drastically different effect than Ri alone, with a robust increase in root GRSPaV accumulation in the first 2 months of inoculation. Deja-Sikora et al. (2023) showed a difference in the *R. irregularis* and *F. mosseae* effect on potato infected with potato virus Y (PVY), with a significant influence on PVY concentration present only for *F. mosseae*. Similarly, the Mix inoculum containing *F. mosseae* in our study had a significant effect on the GRSPaV concentration. However, *F. mosseae* reduced the PVY titer in roots and increased it in leaves, an effect that was the opposite for GRSPaV in this study, where initially, a significant increase was measured in the roots, whereas virus accumulation was decreased in young leaves of Mix grapevine.

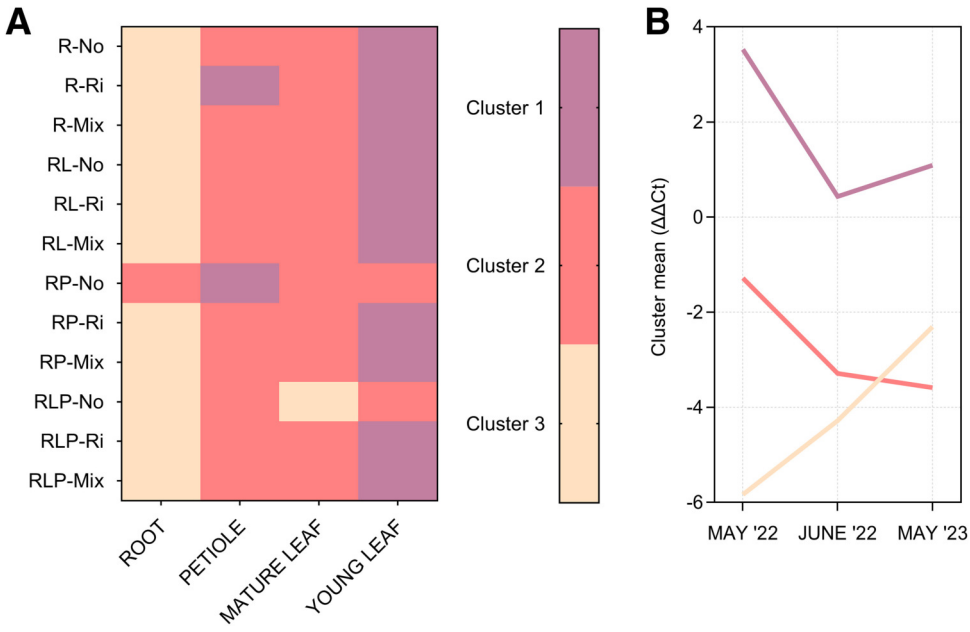


Fig. 4. K-means cluster analysis of temporal variability in grapevine rupestris stem-pitting associated virus (GRSPaV) concentrations in four grapevine tissue types. **A,** Association of treatments and tissue type with distinct cluster group based on virus concentration changes at three time points. **B,** Plot of means for each cluster based on mean ΔΔCt values calculated in reference to the R-No (only GRSPaV, no arbuscular mycorrhizal fungi [AMF]) treatment. R, GRSPaV; L, grapevine leafroll-associated virus 3; P, grapevine Pinot Gris virus; No, no AMF inoculum; Ri, *Rhizophagus irregularis*; Mix, *R. irregularis*, *Funneliformis mosseae*, and *F. caledonium*.

Mycorrhizal grapevine exhibits modified GRSPaV accumulation in roots and young leaves

Distribution of viruses is highly variable across different host tissues during a vegetative growing season, and a similar trend is repeatedly reported across different grapevine–virus systems (Gasparro et al. 2019; Montero et al. 2017; Nuzzo et al. 2022; Osman et al. 2018; Shabanian et al. 2020). Furthermore, studies on the interactions of AMF, plant host, and viruses are limited and predominantly conducted on annual plants (Hao et al. 2019; Miozzi et al. 2019). These studies point out the variable nature of this three-part system, with scarce information on its influence on virus multiplication dynamics and almost no data for grapevine. Here, an attempt was made to describe the impact of AMF on virus multiplication in GRSPaV-infected grapevine in different coinfections. The changes were monitored throughout 1 year, and the extent of mycorrhizal influence on the GRSPaV relative concentration was limited to the first 2 months after the application of the inoculum. Establishment of AMF symbiosis led to an increase in the GRSPaV relative concentration in the roots of infected grapevine while simultaneously leading to the decrease in apical, young leaf tissue. There have been reports of decreased virus concentrations in AMF-infected plants (Maffei et al. 2014; Miozzi et al. 2020), characterized as beneficial. However, few studies have focused on temporal changes in the virus titer of AMF-inoculated plants. Miozzi et al. (2011) pointed out increased tomato spotted wilt virus concentrations in mycorrhiza-inoculated tomato as a long-term effect of mycorrhizal symbiosis, as concluded from RT-qPCR of young leaves on a 2-month scale. Similarly, a significantly higher GRSPaV concentration was measured in the young leaves of Ri and Mix AMF plants as the interaction progressed, although there was no

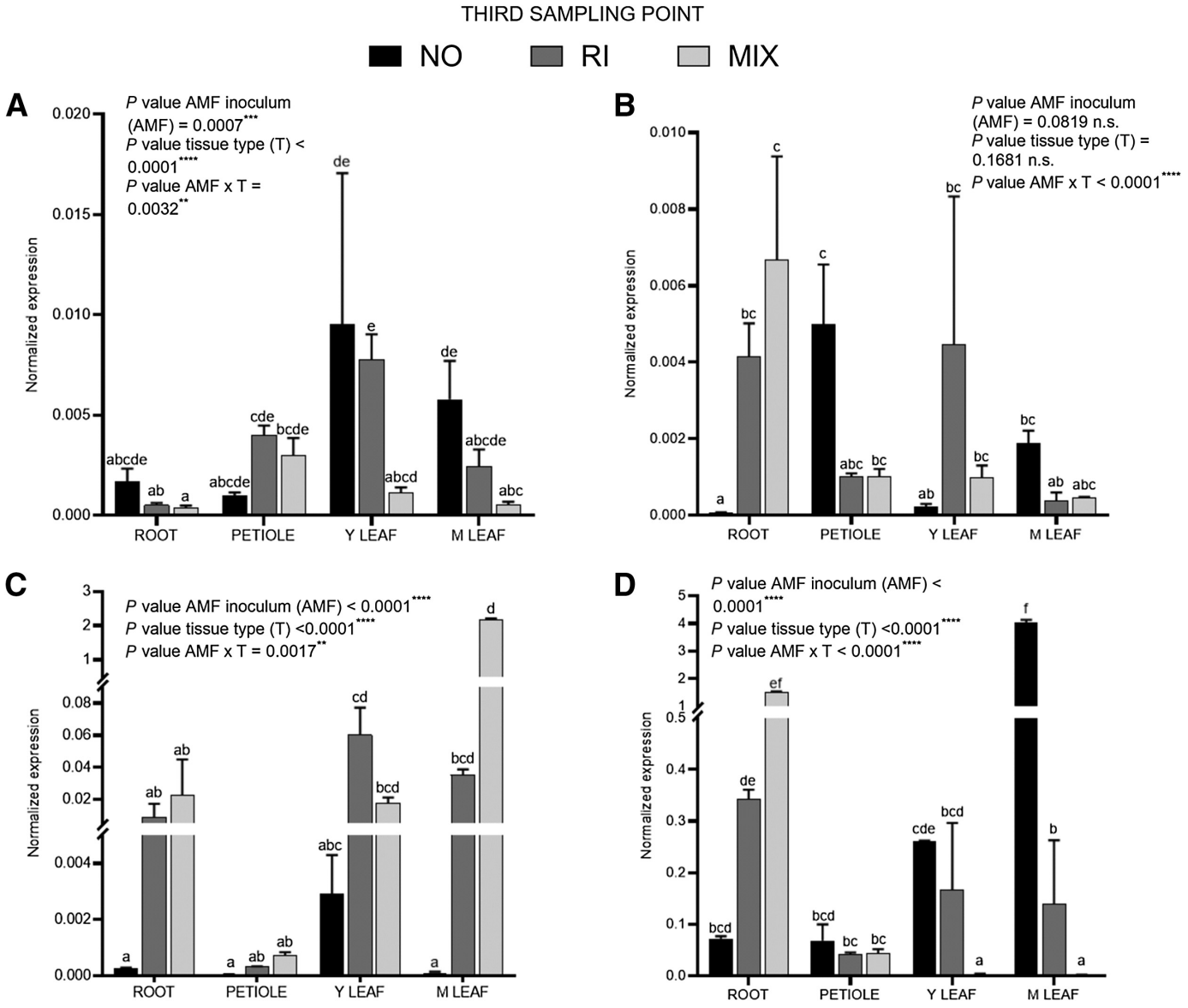


Fig. 5. Relative virus expression of coinfected grapevine treatments at the third sampling point. **A,** Grapevine rupestris stem-pitting associated virus (GRSPaV) relative expression in grapevine coinfected with grapevine leafroll-associated virus 3 (GLRaV-3, RL treatment). **B,** GRSPaV relative expression in grapevine coinfected with grapevine Pinot Gris virus (GPGV, RP treatment). **C,** GLRaV-3 relative expression in RL. **D,** GPGV relative expression in RP. Data are presented as the mean normalized expression of three biological replicates calculated from three technical replicates ($n = 3$). Bars are the standard error of the mean, and different letters indicate statistically significant differences ($P < 0.05$) determined via two-way analysis of variance and a Bonferroni post-hoc test. No, inactive arbuscular mycorrhizal fungi (AMF) inoculum; Ri, *Rhizophagus irregularis*; Mix, *R. irregularis*, *Funneliformis mosseae*, and *F. caledonium*; Y leaf, young leaf; M leaf, mature leaf.

difference between non-mycorrhizal plants and mycorrhizal plants after 1 year of established symbiosis. Moreover, on a longer time scale, AMF-inoculated grapevine had a significant reduction in the GRSPaV concentration only in the first sampling of the mycorrhizal relationship and exclusively in the young leaves. Similarly, Elsharkawy et al. (2012) showed that *Funneliformis mosseae* significantly reduced the accumulation of cucumber mosaic virus in cucumber in the first week of infection, followed by a loss of the effect in the following weeks. However, there is a lack of studies conducted on a longer temporal scale as presented here, especially on perennial woody crops such as grapevine. Moreover, a number of published studies used an AMF pre-inoculation strategy to induce a priming effect before establishing the pathogen infection (Metwally et al. 2024; Miozzi et al. 2020). Khoshkhatti et al. (2020) detected a lower expression of pathogenesis-related genes in virus-infected tomato pre-inoculated with AMF compared with postinoculated ones. Discrepancies in the GRSPaV relative concentration due to AMF inoculation as the form of priming effect cannot be discussed here because the mycorrhizal inoculum was added after the plants were exposed to biotic stress to simulate more accurately the real-life scenario of using infected grapevine propagation material. Therefore, the results of this study should be considered in the appropriate context, and it would be interesting to employ metagenomics studies to clarify the observed effects. However, the lower virus concentration in young leaves could potentially stem from the AMF bioprotective capabilities (Weng et al. 2022). The observed increase in root GRSPaV concentrations could stem from enhanced activity of the roots because establishment of AMF symbiosis involves release of diffusible factors capable of inducing lateral root formation (Chiu et al. 2022; Maillet et al. 2011), and AMF-inoculated grapevine is proven to have more developed root systems (Krishna et al. 2005). On the other hand, virus titer is variable in young leaves in the early vegetative growth season depending on virus transport ability, and lower concentrations are common (Crespo-Martínez et al. 2023). It is worth noting that the young leaves of AMF-inoculated grapevine have further reduced GRSPaV concentrations. This raises the question of whether mycorrhizal plants could have modified the source–sink relationship between roots and "shoots" in an AMF-dependent manner because it has been observed that a significant portion of assimilates is allocated to the roots to facilitate symbiosis and novel root formation (Goddard et al. 2021; Kaur and Suseela 2020). In this scenario, once the symbiosis is established, the dynamic balance of assimilate translocation could change, favoring shoot development and resulting in the GRSPaV concentration in the young leaves rising as the experiment progresses. Thus, the dynamic balance of nutrient allocation and simultaneous phloem-limited virus transport could be modified by the presence of AMF in the early stages of symbiosis formation. A deeper understanding of the AMF-virus interplay is needed to clarify these assumptions.

Virus coinfections influence the dynamics and long-term GRSPaV accumulation of mycorrhizal grapevine

The present studies on AMF-host-virus mostly focus on one or more AMF species versus one virus (Deja-Sikora et al. 2020, 2023; Ebrahimi et al. 2020; Elsharkawy et al. 2012; Maffei et al. 2014; Miozzi et al. 2020). Interactions of a higher number of pathogenic organisms in the AMF-host system are poorly investigated, although it is usually a common situation in real agroecosystems. Therefore, an attempt was made to quantify GRSPaV by RT-qPCR in a multi-pathogenic grapevine system as a first step toward understanding the complex interplay of the grapevine virome in the presence of mycorrhizal fungi. We showed that different virus composition had a significant effect on early AMF induction of GRSPaV root accumulation. Interestingly, the GRSPaV concentration was highest in the first sampling in sole infection of Mix grapevine, whereas in the second sampling, the GRSPaV concentration was higher only in coinfected (RL, RP, and RLP) Mix grapevine. The reason for this sudden drop in GRSPaV concentration in sole infection is unclear, but some underlying causes may lie in possible virus–virus antagonistic interactions (Syller and Grupa 2016), the specificity of the virus strains (Perrone et al. 2017) that were not tested in this study, or the possibility of unique AMF species interactions (Mix inoculum). Nevertheless, in the third sampling (May 2023), additional quantification was performed for all viruses in RL and RP combinations. For different viruses, however, a different trend of accumulation was noted. Similarly, a contrasting effect of AMF on virus accumulation was noted for two important tomato viruses in young tomato leaves (Khoshkhatti et al. 2020). Interestingly, the increased GRSPaV concentration in the early stages of RL for Ri and Mix roots was no longer present in the late stage of symbiosis, yet a drastic decrease was evident in the foliage of RL for Mix grapevine at the third sampling point. On the other hand, root GRSPaV accumulation was still high in RP-infected mycorrhizal plants. This observation suggests that GRSPaV does interact with other grapevine viruses in a meaningfully different way in the presence of AMF. Similarly, GLRaV-3 in the presence of GVA has a synergistic effect with reportedly increased GVA concentration (Rowhani et al. 2018), and Čarija et al. (2022a) suggested an antagonistic effect of GLRaV-3 with GVA, GPGV, and GRSPaV in long-term coinfections. However, the influence of beneficial microorganisms is rarely taken into account when exploring the virus coinfection relationship, and extensive research is needed to clarify this type of microbiome interaction. In this study, the mycorrhizal inoculum had a profound effect on the GLRaV-3 concentration in almost every tissue tested. This effect was observed 1 year after grapevine inoculation, which raises a concern that this effect could be long-lasting, and AMF inoculation could make grapevine more susceptible to one of the most economically disruptive viruses. In contrast to GLRaV-3, AMF caused a higher GPGV concentration only in root tissue, whereas foliage showed a reduced concentration for AMF-inoculated grapevine. Many studies focusing on AMF and virus interactions observed higher virus accumulation in the host (Daft and Okusanya 1973; Deja-Sikora et al. 2023; Miozzi et al. 2011; Rúa et al. 2013), with recent studies also detecting the opposite effect (Aseel et al. 2019; Metwally et al. 2024; Miozzi et al. 2020). However, long-term studies on economically important perennial crops are limited, and future research should address possible lasting effects AMF could have on grapevine harboring biotrophic pathogens while also considering cost-benefit balance for grapevine health and vineyard productivity.

Conclusion

In summary, tissue-specific distribution of GRSPaV is influenced by AMF in a way that is time, tissue, and AMF- and virus-composition dependent. AMF-induced GRSPaV accumulation during the first 2 months of symbiosis is apparent in grapevine roots. The increase in roots is overlapped with reduced virus titer in young leaves, indicating the possibility of changed source–sink dynamics in mycorrhizal plants. Moreover, the Mix inoculum consisting of *R. irregularis*, *F. mosseae*, and *F. caledonium* has a more pronounced impact on virus concentration than *R. irregularis* alone. Furthermore, AMF-inoculated plants showed a long-lasting effect of increased virus concentration in a virus-specific manner, with GLRaV-3 and GPGV being significantly accumulated in roots. Interestingly, these two viruses exhibit opposite dynamics in mature leaves of mycorrhized grapevine, with accumulation of GLRaV-3 and a reduced titer for GPGV. In this complex system, virus–virus

interaction is also possible; however, a multivariate interplay of more than one virus and AMF symbiosis should be addressed in more detail. In conclusion, AMF may have a major influence on grapevine virus concentration, in terms of both its increase and decrease throughout the growing season and host tissue. However, viral disease symptom alleviation or exacerbation by AMF symbiosis and the comprehensive physiological and molecular impact of grapevine–virus–AMF interactions in real agroecosystems remain to be estimated.

Literature Cited

Alagna, F., Balestrini, R., Chitarra, W., Marsico, A. D., and Nerva, L. 2020. Getting ready with the priming: Innovative weapons against biotic and abiotic crop enemies in a global changing scenario. Pages 35-56 in: Priming-Mediated Stress and Cross-Stress Tolerance in Crop Plants. M. A. Hossain, F. Liu, D. J. Burritt, M. Fujita, and B. Huang, eds. Academic Press, Cambridge, MA, U.S.A.

Almeida, R. P. P., Daane, K. M., Bell, V. A., Blaisdell, G. K., Cooper, M. L., Herrbach, E., and Pietersen, G. 2013. Ecology and management of grapevine leafroll disease. Front. Microbiol. 4:00094.

Al Rwahnih, M., Daubert, S., Golino, D., and Rowhani, A. 2009. Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology 387:395-401.

Aseel, D. G., Rashad, Y. M., and Hammad, S. M. 2019. Arbuscular mycorrhizal fungi trigger transcriptional expression of flavonoid and chlorogenic acid biosynthetic pathways genes in tomato against *Tomato mosaic virus*. Sci. Rep. 9:9692.

Balestrini, R., Chitarra, W., Antoniou, C., Ruocco, M., and Fotopoulos, V. 2018. Improvement of plant performance under water deficit with the employment of biological and chemical priming agents. J. Agric. Sci. 156:680-688.

Berger, F., and Gutjahr, C. 2021. Factors affecting plant responsiveness to arbuscular mycorrhiza. Curr. Opin. Plant Biol. 59:101994.

Bettenfeld, P., Cadena i Canals, J., Jacquens, L., Fernandez, O., Fontaine, F., van Schaik, E., Courty, P.-E., and Trouvelot, S. 2022. The microbiota of the grapevine holobiont: A key component of plant health. J. Adv. Res. 40: 1-15.

Bouyahia, H., Boscia, D., Savino, V., La Notte, P., Pirolo, C., Castellano, M. A., Minafra, A., and Martelli, G. P. 2005. *Grapevine rupestris stem pitting-associated virus* is linked with grapevine vein necrosis. Vitis 44:133-137.

Brundrett, M., Bougher, N., Dell, B., Grove, T., and Malajczuk, N. 1996. Working with mycorrhizas in forestry and agriculture. https://www.aciar.gov.au/publication/working-mycorrhizas-forestry-and-agriculture

Brundrett, M. C., and Tedersoo, L. 2018. Evolutionary history of mycorrhizal symbioses and global host plant diversity. New Phytol. 220:1108-1115.

Čarija, M., Černi, S., Stupin-Polančec, D., Radić, T., Gaši, E., and Hančević, K. 2022a. Grapevine leafroll-associated virus 3 replication in grapevine hosts changes through the dormancy stage. Plants 11:3250.

Čarija, M., Radić, T., Černi, S., Mucalo, A., Zdunić, G., Vončina, D., Jagunić, M., and Hančević, K. 2022b. Prevalence of virus infections and GLRaV-3 genetic diversity in selected clones of Croatian indigenous grapevine cultivar Plavac Mali. Pathogens 11:176.

Chen, X. W., Wu, L., Luo, N., Mo, C. H., Wong, M. H., and Li, H. 2019. Arbuscular mycorrhizal fungi and the associated bacterial community influence the uptake of cadmium in rice. Geoderma 337:749-757.

Chiu, C. H., Roszak, P., Orvošová, M., and Paszkowski, U. 2022. Arbuscular mycorrhizal fungi induce lateral root development in angiosperms via a conserved set of MAMP receptors. Curr. Biol. 32:4428-4437.e3.

Crespo-Martínez, S., Ramírez-Lacunza, A., Miranda, C., Urrestarazu, J., and Santesteban, L. G. 2023. Dynamics of GFLV, GFkV, GLRaV-1, and GLRaV-3 grapevine viruses transport toward developing tissues. Eur. J. Plant Pathol. 167:197-205.

Daft, M. J., and Okusanya, B. O. 1973. Effect of *Endogone* mycorrhiza on plant growth V. Influence of infection on the multiplication of viruses in tomato, petunia and strawberry. New Phytol. 72:975-983.

Deja-Sikora, E., Kowalczyk, A., Trejgell, A., Szmidt-Jaworska, A., Baum, C., Mercy, L., and Hrynkiewicz, K. 2020. Arbuscular mycorrhiza changes the impact of Potato virus Y on growth and stress tolerance of *Solanum tuberosum* L. *in vitro*. Front. Microbiol. 10:2971.

Deja-Sikora, E., Mercy, L., Baum, C., and Hrynkiewicz, K. 2019. The contribution of endomycorrhiza to the performance of *Potato virus Y*-infected solanaceous plants: Disease alleviation or exacerbation? Front. Microbiol. 10:516.

Deja-Sikora, E., Werner, K., and Hrynkiewicz, K. 2023. AMF species do matter: *Rhizophagus irregularis* and *Funneliformis mosseae* affect healthy and PVY-infected *Solanum tuberosum* L. in a different way. Front. Microbiol. 14:1127278.

Ebrahimi, S., Eini, O., and Koolivand, D. 2020. Arbuscular mycorrhizal symbiosis enhances virus accumulation and attenuates resistance-related gene expression in tomato plants infected with *Beet curly top Iran virus*. J. Plant Dis. Prot. 127:341-348.

Eichmeier, A., Peňázová, E., and Muljukina, N. 2018. Survey of *Grapevine Pinot gris virus* in certified grapevine stocks in Ukraine. Eur. J. Plant Pathol. 152:555-560.

Elsharkawy, M. M., Shimizu, M., Takahashi, H., and Hyakumachi, M. 2012. The plant growth-promoting fungus *Fusarium equiseti* and the arbuscular mycorrhizal fungus *Glomus mosseae* induce systemic resistance against *Cucumber mosaic virus* in cucumber plants. Plant Soil 361:397-409.

Fuchs, M. 2020. Grapevine viruses: A multitude of diverse species with simple but overall poorly adopted management solutions in the vineyard. J. Plant Pathol. 102:643-653.

Gambino, G. 2015. Multiplex RT-PCR method for the simultaneous detection of nine grapevine viruses. Pages 39-47 in: Plant Virology Protocols: New Approaches to Detect Viruses and Host Responses. Methods in Molecular Biology, vol. 1236. I. Uyeda and C. Masuta, eds. Humana Press, New York, NY, U.S.A.

Gambino, G., Cuozzo, D., Fasoli, M., Pagliarani, C., Vitali, M., Boccacci, P., Pezzotti, M., and Mannini, F. 2012. Co-evolution between *Grapevine rupestris stem pitting-associated virus* and *Vitis vinifera* L. leads to decreased defence responses and increased transcription of genes related to photosynthesis. J. Exp. Bot. 63:5919-5933.

Gambino, G., Minuto, M., Boccacci, P., Perrone, I., Vallania, R., and Gribaudo, I. 2011. Characterization of expression dynamics of WOX homeodomain transcription factors during somatic embryogenesis in *Vitis vinifera*. J. Exp. Bot. 62:1089-1101.

Gaši, E., Radić, T., Čarija, M., Gambino, G., Balestrini, R., and Hančević, K. 2023. Arbuscular mycorrhizal fungi induce changes of photosynthesis-related parameters in virus infected grapevine. Plants 12:1783.

Gasparro, M., Milella, R. A., Alba, V., Giannandrea, M. A., and Caputo, A. R. 2019. Seasonal dynamics and spatial distribution of main *Grapevine viruses* in field-grown grapevine cultivars. Eur. J. Plant Pathol. 155:193-205.

Goddard, M.-L., Belval, L., Martin, I. R., Roth, L., Laloue, H., Deglène-Benbrahim, L., Valat, L., Bertsch, C., and Chong, J. 2021. Arbuscular mycorrhizal symbiosis triggers major changes in primary metabolism together with modification of defense responses and signaling in both roots and leaves of *Vitis vinifera*. Front. Plant Sci. 12:721614.

Hančević, K., Saldarelli, P., Čarija, M., Černi, S., Zdunić, G., Mucalo, A., and Radić, T. 2021. Predominance and diversity of GLRaV-3 in native vines of Mediterranean Croatia. Plants 10:17.

Hao, L., Zhang, Z., Hao, B., Diao, F., Zhang, J., Bao, Z., and Guo, W. 2021. Arbuscular mycorrhizal fungi alter microbiome structure of rhizosphere soil to enhance maize tolerance to La. Ecotoxicol. Environ. Saf. 212:111996.

Hao, Z., Xie, W., and Chen, B. 2019. Arbuscular mycorrhizal symbiosis affects plant immunity to viral infection and accumulation. Viruses 11:534.

Hoagland, D. R., and Arnon, D. I. 1950. The Water-Culture Method for Growing Plants Without Soil. College of Agriculture, University of California, Berkeley, CA, U.S.A.

Kaur, S., and Suseela, V. 2020. Unraveling arbuscular mycorrhiza-induced changes in plant primary and secondary metabolome. Metabolites 10:335.

Khoshkhatti, N., Eini, O., Koolivand, D., Pogiatzis, A., Klironomos, J. N., and Pakpour, S. 2020. Differential response of mycorrhizal plants to *Tomato bushy stunt virus* and *Tomato mosaic virus* infection. Microorganisms 8:2038.

Krishna, H., Singh, S. K., Sharma, R. R., Khawale, R. N., Grover, M., and Patel, V. B. 2005. Biochemical changes in micropropagated grape (*Vitis vinifera* L.) plantlets due to arbuscular-mycorrhizal fungi (AMF) inoculation during ex vitro acclimatization. Sci. Hortic. 106:554-567.

Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25:402-408.

Maffei, G., Miozzi, L., Fiorilli, V., Novero, M., Lanfranco, L., and Accotto, G. P. 2014. The arbuscular mycorrhizal symbiosis attenuates symptom severity and reduces virus concentration in tomato infected by *Tomato yellow leaf curl Sardinia virus* (TYLCSV). Mycorrhiza 24:179-186.

Maillet, F., Poinsot, V., André, O., Puech-Pagès, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E. A., Driguez,

H., Bécard, G., and Dénarié, J. 2011. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469:58-63.
Maliogka, V. I., Martelli, G. P., Fuchs, M., and Katis, N. I. 2015. Control of viruses infecting grapevine. Pages 175-227 in: Advances in Virus Research, Control of Plant Virus Diseases. G. Loebenstein and N. I. Katis, eds. Academic Press, Cambridge, MA, U.S.A.
McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L., and Swan, J. A. 1990. A new method which gives an objective measure of colonization of roots by vesicular—arbuscular mycorrhizal fungi. New Phytol. 115:495-501.
Meng, B., and Gonsalves, D. 2007. *Grapevine rupestris stem pitting-associated virus*: A decade of research and future perspectives. Plant Viruses 1:52-62.
Meng, B., and Rowhani, A. 2017. *Grapevine rupestris stem pitting-associated virus*. Pages 257-287 in: Grapevine Viruses: Molecular Biology, Diagnostics and Management. B. Meng, G. P. Martelli, D. A. Golino, and M. Fuchs, eds. Springer International Publishing, Cham, Switzerland.
Metwally, R. A., Taha, M. A., Abd El-Moaty, N. M., and Abdelhameed, R. E. 2024. Attenuation of *Zucchini mosaic virus* disease in cucumber plants by mycorrhizal symbiosis. Plant Cell Rep. 43:54.
Miozzi, L., Catoni, M., Fiorilli, V., Mullineaux, P. M., Accotto, G. P., and Lanfranco, L. 2011. Arbuscular mycorrhizal symbiosis limits foliar transcriptional responses to viral infection and favors long-term virus accumulation. Mol. Plant-Microbe Interact. 24:1562-1572.
Miozzi, L., Vaira, A. M., Brilli, F., Casarin, V., Berti, M., Ferrandino, A., Nerva, L., Accotto, G. P., and Lanfranco, L. 2020. Arbuscular mycorrhizal symbiosis primes tolerance to Cucumber mosaic virus in tomato. Viruses 12:675.
Miozzi, L., Vaira, A. M., Catoni, M., Fiorilli, V., Accotto, G. P., and Lanfranco, L. 2019. Arbuscular mycorrhizal symbiosis: Plant friend or foe in the fight against viruses? Front. Microbiol. 10:1238.
Montero, R., El aou ouad, H., Pacifico, D., Marzachì, C., Castillo, N., García, E., Del Saz, N. F., Florez-Sarasa, I., Flexas, J., and Bota, J. 2017. Effects of Grapevine leafroll-associated virus 3 on the physiology in asymptomatic plants of *Vitis vinifera*. Ann. Appl. Biol. 171:155-171.
Moreno, A. B., and López-Moya, J. J. 2020. When viruses play team sports: Mixed infections in plants. Phytopathology 110:29-48.
Nuzzo, F., Moine, A., Nerva, L., Pagliarani, C., Perrone, I., Boccacci, P., Gribaudo, I., Chitarra, W., and Gambino, G. 2022. Grapevine virome and production of healthy plants by somatic embryogenesis. Microb. Biotechnol. 15:1357-1373.
Osman, F., Golino, D., Hodzic, E., and Rowhani, A. 2018. Virus distribution and seasonal changes of grapevine leafroll-associated viruses. Am. J. Enol. Vitic. 69:70-76.
Perrone, I., Chitarra, W., Boccacci, P., and Gambino, G. 2017. Grapevine–virus–environment interactions: An intriguing puzzle to solve. New Phytol. 213:983-987.
Possingham, J. V., and Obbink, J. G. 1971. Endotrophic mycorrhiza and the nutrition of grape vines. Vitis 10:120-130.
Rivadeneira, M., Galván, M. Z., Abán, M., Semke, R. E., Rivadeneira, J., Lanza Volpe, M., and Gomez Talquenca, S. 2022. Survey for major grapevine viruses in commercial vineyards of northwestern Argentina. Plants 11:1720.
Rowhani, A., Daubert, S., Arnold, K., Al Rwahnih, M., Klaassen, V., Golino, D., and Uyemoto, J. K. 2018. Synergy between grapevine vitiviruses and grapevine leafroll viruses. Eur. J. Plant Pathol. 151:919-925.
Rúa, M. A., Umbanhowar, J., Hu, S., Burkey, K. O., and Mitchell, C. E. 2013. Elevated CO_2 spurs reciprocal positive effects between a plant virus and an arbuscular mycorrhizal fungus. New Phytol. 199:541-549.
Schreiner, R. P. 2007. Effects of native and nonnative arbuscular mycorrhizal fungi on growth and nutrient uptake of 'Pinot noir' (*Vitis vinifera* L.) in two soils with contrasting levels of phosphorus. Appl. Soil Ecol. 36:205-215.
Schubert, A., and Cravero, M. C. 1985. Occurrence and infectivity of vesicular-arbuscular mycorrhizal fungi in north-western Italy vineyards. Vitis 24:129-138.
Shabanian, M., Xiao, H., and Meng, B. 2020. Seasonal dynamics and tissue distribution of two major viruses associated with grapevine leafroll under cool climate condition. Eur. J. Plant Pathol. 158:1017-1031.
Singhal, P., Nabi, S. U., Yadav, M. K., and Dubey, A. 2021. Mixed infection of plant viruses: Diagnostics, interactions and impact on host. J. Plant Dis. Prot. 128:353-368.
Syller, J., and Grupa, A. 2016. Antagonistic within-host interactions between plant viruses: Molecular basis and impact on viral and host fitness. Mol. Plant Pathol. 17:769-782.
Trouvelot, S., Bonneau, L., Redecker, D., van Tuinen, D., Adrian, M., and Wipf, D. 2015. Arbuscular mycorrhiza symbiosis in viticulture: A review. Agron. Sustain. Dev. 35:1449-1467.
Tsai, C. W., Daugherty, M. P., and Almeida, R. P. P. 2012. Seasonal dynamics and virus translocation of *Grapevine leafroll-associated virus* 3 in grapevine cultivars. Plant Pathol. 61:977-985.
Weng, W., Yan, J., Zhou, M., Yao, X., Gao, A., Ma, C., Cheng, J., and Ruan, J. 2022. Roles of *arbuscular mycorrhizal* fungi as a biocontrol agent in the control of plant diseases. Microorganisms 10:1266.
Xiao, H., Shabanian, M., Moore, C., Li, C., and Meng, B. 2018. Survey for major viruses in commercial *Vitis vinifera* wine grapes in Ontario. Virol. J. 15:127.

3.3. **Publication III**. : "*Tripartite interactions between grapevine, viruses, and arbuscular mycorrhizal fungi provide insights into modulation of oxidative stress responses*"

Journal of Plant Physiology 303 (2024) 154372

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Tripartite interactions between grapevine, viruses, and arbuscular mycorrhizal fungi provide insights into modulation of oxidative stress responses

Tomislav Radić [a], Rosemary Vuković [b], Emanuel Gaši [a,*], Daniel Kujundžić [b], Mate Čarija [a], Raffaella Balestrini [c], Fabiano Sillo [d], Giorgio Gambino [d], Katarina Hančević [a]

[a] Institute for Adriatic Crops and Karst Reclamation, Put Duilova 11, 21 000, Split, Croatia
[b] Josip Juraj Strossmayer University of Osijek, Department of Biology, Ulica cara Hadrijana 8/A, 31000, Osijek, Croatia
[c] Institute of Biosciences and Bioresources, National research Council (IBBR-CNR), via Amendola 165/A, 70126, Bari, Italy
[d] Institute for Sustainable Plant Protection, National Research Council (IPSP-CNR), Strada delle Cacce 73, 10135, Torino, Italy

ARTICLE INFO

Keywords:
Virus infection
Beneficial symbionts
Ascorbate
Superoxide dismutase
Guaiacol peroxidase
Vitis vinifera L

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) can be beneficial for plants exposed to abiotic and biotic stressors. Although widely present in agroecosystems, AMF influence on crop responses to virus infection is underexplored, particularly in woody plant species such as grapevine. Here, a two-year greenhouse experiment was set up to test the hypothesis that AMF alleviate virus-induced oxidative stress in grapevine. The 'Merlot' cultivar was infected with three grapevine-associated viruses and subsequently colonized with two AMF inocula, containing one or three species, respectively. Five and fifteen months after AMF inoculation, lipid peroxidation - LPO as an indicator of oxidative stress and indicators of antioxidative response (proline, ascorbate - AsA, superoxide dismutase - SOD, ascorbate- APX and guaiacol peroxidases - GPOD, polyphenol oxidase - PPO, glutathione reductase - GR) were analysed. Expression of genes coding for a stilbene synthase (*STS1*), an enhanced disease susceptibility (*EDS1*) and a lipoxygenase (*LOX*) were determined in the second harvesting. AMF induced reduction of AsA and SOD over both years, which, combined with not AMF-triggered APX and GR, suggests decreased activation of the ascorbate-glutathione cycle. In the mature phase of the AM symbiosis establishment GPOD emerged as an important mechanism for scavenging H_2O_2 accumulation. These results, together with reduction in *STS1* and increase in *EDS1* gene expression, suggest more efficient reactive oxygen species scavenging in plants inoculated with AMF. Composition of AMF inocula was important for proline accumulation. Overall, our study improves the knowledge on ubiquitous grapevine-virus-AMF systems in the field, highlighting that established functional AM symbiosis could reduce virus-induced stress.

1. Introduction

Numerous studies have provided evidences of beneficial impact of arbuscular mycorrhizal fungi (AMF) on plant response under biotic stresses (Dowarah et al., 2022). AMF are able to improve host tolerance against bacterial, fungal and viral phytopathogens, nematodes and herbivores (Dowarah et al., 2022). Among them, a few studies have been so far dedicated to plant-virus-AMF relationship in a woody fruit crop such as grapevine. AMF benefits for the plant hosts can be seen through the enhanced vigour attained by improved nutrient and water uptake as well as by the induction of "mycorrhiza-induced resistance" (MIR; Cameron et al., 2013). The physiological/molecular modulations in the host plants during symbiosis also lead to a primed status for a more efficient activation of defence mechanisms (Pozo & Azcón-Aguilar, 2007; Jung et al., 2012; Alagna et al., 2020).

Insights in the bioprotection efficiency of AMF against plant viruses are so far inconsistent, depending on the plant species, AMF species, viruses and environmental factors (Hao et al., 2019). Clearly protective influence of AMF in tomato against viral infections (Maffei et al., 2014; Aseel et al., 2019; Miozzi et al., 2020), reduced virus titre in tobacco

* Corresponding author.
E-mail addresses: tomislav.radic@krs.hr (T. Radić), rvukovic@biologija.unios.hr (R. Vuković), emanuel.gasi@krs.hr (E. Gaši), daniel.kujundzic@biologija.unios.hr (D. Kujundžić), mate.carija@krs.hr (M. Čarija), raffaella.balestrini@ipsp.cnr.it (R. Balestrini), fabiano.sillo@ipsp.cnr.it (F. Sillo), giorgio.gambino@ipsp.cnr.it (G. Gambino), katarina.hancevic@krs.hr (K. Hančević).

https://doi.org/10.1016/j.jplph.2024.154372
Received 17 June 2024; Received in revised form 11 October 2024; Accepted 11 October 2024
Available online 11 October 2024
0176-1617/© 2024 Published by Elsevier GmbH.

(Stolyarchuk et al., 2009), and alleviated oxidative stress in virus-infected potato (Deja-Sikora et al., 2023) were already reported. Conversely, AMF reduced growth of potato plant infected with Potato virus Y (Thiem et al., 2014) as well as increased viral accumulation in mycorrhizal *versus* non-mycorrhizal plants in several plant species, suggesting a "mycorrhiza-induced susceptibility" (Miozzi et al., 2019). The majority of available data refer to herbaceous crop species, while data on this tripartite interaction in perennial fruit crops are scarce. Significantly reduced virus impact in sour orange colonized by AMF (Nemec and Myhre, 1984), local and systemic mycorrhiza-induced protection in grapevine against the ectoparasitic nematode *Xiphinema index*, a vector of grapevine fanleaf virus (Hao et al., 2012), and enhanced photosynthesis performance in mycorrhizal and virus infected grapevine (Gaši et al., 2023) have been reported.

Grapevine (*Vitis* spp.) is increasingly considered as a model fruit plant and new high-throughput technologies are introduced to the study of grapevine-environment interactions, providing a growing number of publications on all aspects of grapevine biology and biotechnology (Jaillon, 2007; Perrone et al., 2017). Infection with viruses induces a variety of physiological responses and can lead to reduced growth and development (Fuchs, 2020). One of the first defence reactions triggered in grapevine upon virus infection is an antioxidative response (Sgherri et al., 2013; Hančević et al., 2023). Reactive oxygen species (ROS) are normally generated by the metabolic activity of the plants and can act as signalling molecules for activating plant metabolic pathways. The generation of ROS increases upon environmental stress conditions and their high accumulation through oxidative stress can lead to damage of the cell membranes and biomolecules (Hasanuzzaman et al., 2020). To counteract the effect of increased ROS accumulation, plants are equipped with ROS scavenging mechanisms: i) enzymes such as superoxide dismutase (SOD), catalase (CAT), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and glutathione peroxidase (GPX) and (ii) non-enzymatic antioxidant molecules like ascorbate, α-tocopherols, glutathione, proline, flavonoids and carotenoids (Hernandez et al., 2016). Also, modulated expression of some genes have relevant role in plant antioxidative defence. The gene *EDS1* codes for a lipase-like protein known to be involved in regulating plant defence responses through salicylic acid signaling and ROS production, particularly against biotrophic pathogens (Lapin et al., 2020). The *LOX* gene family encodes lipoxygenases involved in the catalyse the oxygenation of polyunsaturated fatty acids for the synthesis of oxylipins, which have regulatory roles in defence towards plant pathogens (Bannenberg et al., 2009). The *STS1* gene codes for a stilbene synthase, pivotal for the biosynthesis of stilbenes, just as important in counteracting oxidative stress as the production of ROS (Ahn et al., 2014).

Grapevine roots are naturally colonized by AMF (Balestrini et al., 2010) and the biodiversity of AMF in vineyards has been investigated to identify the factors influencing root colonization by these beneficial organisms, and different studies reported that soil characteristics, environmental factors, and agronomic practices impact the intensity of AMF colonization in grapevine roots (Balestrini et al., 2010; Likar et al., 2013). In the last years, several papers have also started to highlight the mechanisms at the basis of the symbiosis between grapevine and AMF as well as the role of these fungi in enhancing the plant response to biotic stresses (Torres et al., 2018; Nerva et al., 2022).

Considering the worldwide spread of grapevine virus infections and their potential severe consequences (Fuchs, 2020), the presence of grapevine-virus-AMF interplay in almost all agroecosystems, the potential AMF benefits (Schreiner, 2005; Trouvelot et al., 2015; Singh and Giri, 2017), as well as the importance of antioxidative defence mechanisms in grapevine coping with virus infection, the relationships among grapevine, viruses and AMF need to be elucidated. This fits into an already recognized challenge for studying the grapevine–virus interactions not only as a binary relationship (host-pathogen), but by considering the plant and its endophytic microorganisms as a unique microecosystem (Perrone et al., 2017). Beneficial endophytes, such as AMF, are an integral part of ROS homeostasis and may be a way of reducing oxidative damage in grapevine (Spagnoletti et al., 2016; Sahu et al., 2022).

Starting from the hypothesis that AM symbiosis might enhance the grapevine's capability of coping with virus-induced oxidative stress, we set up a two-year greenhouse experiment to discern the changes occurring in grapevine antioxidative response in plants concurrently exposed to both virus infection and AMF colonization, in comparison to an AMF-free system.

2. Materials and methods

2.1. Experimental set-up

Merlot cv (*Vitis vinifera* L.). scions were grafted on Kober 5BB rootstock (*Vitis berlandieri* Planch. × *Vitis riparia* Michx.) and rooted in a two-years greenhouse experiment (see for details in Gaši et al., 2023). After determination of initial phytosanitary status by detecting the presence/absence of the ten most dangerous and the most widespread grapevine viruses, the suitable plants were subsequently infected by the targeted viruses and colonized by two types of AMF inoculum. This resulted in five groups of treatments based on virus status: i) plants absent from any of the ten tested viruses, ii) plants infected with GRSPaV only, iii) plants infected with a combination of GRSPaV and GLRaV-3, iv) plants infected with a combination of GRSPaV and GPGV and v) plants infected with all three of these viruses. Subsequently, plants in each of these five treatments were inoculated with one of AMF inoculums: i) only *Rhizophagus irregularis* (Symplanta LLC, Darmstadt, GE), ii) *R. irregularis*, *Funneliformis mosseae* and *F. caledonium* (Inoq LLC, Schnega, Germany), iii) sterilized AMF inoculum. In total, 15 treatments were set up (Supporting Table 1). For assessing oxidative marker and antioxidant content, as well as root colonization, five samples of each treatment were collected at five and 15 months after AMF inoculation, while for the analysis of expression of the three selected genes, three biological replicates for each treatment were sampled 15 months after AMF inoculation. The four or five fully developed mature leaves, between 3rd and 7th or 8th nodes, were collected for the oxidative markers, antioxidative status and genes' expression and immediately put in the dry ice and storaged at −80 °C till the analysis. Young roots were collected for the assessment of the root colonization by AMF, they were washed and stained and microscopy slides were kept at 4 °C till the analysis.

2.2. Preliminary virus detection

To ensure the plant material of desired virus status for the above described experimental set-up, we tested successfully grafted plants for the initial presence of the ten economically most important grapevine viruses. After selecting only plants with no-viruses or with GRSPaV, we tested the success of virus transmission after "chip budding". For these analyses, wood scrapings were sampled for RNA isolation by a CTAB based method (Gambino, 2015). Quantitative PCR (qPCR) detection of grapevine leafroll-associated virus 1 and 2 (GLRaV-1, GLRaV-2), GLRaV-3, grapevine fleck virus (GFkV), arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), GRSPaV, grapevine virus A (GVA), grapevine virus B (GVB) and GPGV was performed for each biological sample to ensure the desired sanitary status (Supporting Table 2). Protocol of reverse transcription to cDNA and qPCR reaction mix and cycling conditions are described in details in Gaši et al., (2023). No virus symptoms typical for GLRaV-3 or GPGV were developed.

2.3. AMF colonization

AMF colonization was checked by microscopic examination of the roots (light microscope, 200 × magnification) and the plants with

Table 1
AMF colonization of the grapevine roots by treatments; total colonization and abundance of AM structures are shown, separately for two measurements in two consecutive years. Main effects of the AMF are shown, as obtained by two-way ANOVA. Different letters indicate a significant difference between treatments ($p < 0.05$, Duncan post-hoc test).

Treatment	Total colonization (%)		Arbuscules (%)		Vesicles (%)		Hyphae only (%)	
	2022	2023	2022	2023	2022	2023	2022	2023
T1	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
T2	78.6 ± 8.4	93.4 ± 7.6	66.1 ± 13.2	83.1 ± 18.6	44.3 ± 24.1	83.3 ± 13.1	12.5 ± 4.8	5.6 ± 6.2
T3	92.4 ± 4.7	97.8 ± 2.6	75.6 ± 15.6	90.6 ± 7	14.8 ± 6.1	54 ± 19.8	15.8 ± 9.9	7.3 ± 5
T4	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
T5	94.3 ± 6.2	95.3 ± 6.8	88.7 ± 12.4	94.3 ± 8.6	76.8 ± 19.9	87.2 ± 12.1	5.7 ± 6.2	1 ± 1.8
T6	81.4 ± 9	87.8 ± 6.1	55.1 ± 10.8	79.8 ± 10	9.7 ± 4.9	31 ± 15.1	26 ± 7.4	8 ± 3.9
T7	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
T8	97.5 ± 1.1	84.1 ± 4.9	93.7 ± 4.7	76.2 ± 8.2	82.4 ± 9.7	69.5 ± 11.7	3.1 ± 3.1	7.9 ± 3.3
T9	87.6 ± 10.7	92.8 ± 7.3	68.8 ± 17.5	87.5 ± 6	18.2 ± 7.1	46 ± 5.3	18.2 ± 9.5	5.3 ± 2.2
T10	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
T11	90.6 ± 8.2	85.6 ± 12.4	86.8 ± 10.1	82.5 ± 11	65.4 ± 15.7	73.9 ± 7.4	3.9 ± 3.7	3.1 ± 4.4
T12	96 ± 2.7	91.5 ± 6.6	85 ± 7.9	86.5 ± 8	28.3 ± 12.1	55.7 ± 12	10.7 ± 6.4	5 ± 1.4
T13	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
T14	94.3 ± 5	92.4 ± 7.7	86.1 ± 11.8	90.9 ± 8.7	71.5 ± 11.7	83.6 ± 7.3	5.8 ± 3.7	1.5 ± 1.4
T15	93.4 ± 8.2	87.2 ± 7.2	85.8 ± 8.6	84.3 ± 9.8	34.1 ± 14.4	50.9 ± 12.9	7.4 ± 1.9	3 ± 2.6
Main effects (AMF):								
No AMF	0^a	0^a	0^a	0^a	0^a	0^a	0^a	0^a
R. irregularis	92.1 ± 8^c	90.4 ± 8.4^b	85.5 ± 13^c	86.3 ± 11.1^b	69.1 ± 19.9^c	80 ± 10.7^c	5.9 ± 4.9^b	3.3 ± 3.8^b
AMF Mix	87.2 ± 11^b	91.4 ± 6.6^b	70.1 ± 18^b	85.7 ± 7.9^b	20.9 ± 13.8^b	47.5 ± 14.9^b	16.7 ± 10^c	5.7 ± 3.3^b

Table 2
P-values obtained by two-way ANOVA (Duncan post-hoc test, $p < 0.05$) with main effects of virus, AMF and their interaction for measured parameters.

PARAMETERS	VIRUS		AMF		VIRUS × AMF	
	2022	2023	2022	2023	2022	2023
LPO	n.s.	0.004	n.s.	<0.001	n.s.	<0.001
AsA	n.s.	n.s.	<0.001	<0.001	n.s.	0.024
Pro	n.s.	<0.001	0.001	<0.001	n.s.	0.045
SOD	n.s.	<0.001	0.022	n.s.	n.s.	n.s.
GPOD	0.038	<0.001	0.015	<0.001	n.s.	<0.001
PPO	0.015	<0.001	n.s.	0.003	0.024	<0.001
APX	<0.001	0.012	0.004	n.s.	n.s.	<0.001
GR	n.s.	n.s.	n.s.	0.030	n.s.	0.040
EDS1	Ø	0.001	Ø	0.014	Ø	0.018
LOX	Ø	0.003	Ø	<0.001	Ø	0.003
*STS1*_leaf	Ø	0.004	Ø	<0.001	Ø	0.002
*STS1*_root	Ø	n.s.	Ø	n.s.	Ø	0.012

LPO - lipid peroxidation, AsA - Ascorbate, Pro - free proline, SOD - superoxide dismutase, GPOD - guaiacol peroxidase, PPO - polyphenol oxidase, APX - ascorbate peroxidase, GR - glutathione reductase, *EDS1* - Enhanced Disease Susceptibility I, *LOX* - lipoxygenase, *STS1* - Stilbene synthase 1, n.s. - non-significant, Ø - not measured.

satisfactory status were used in the final experiment set up (Table 1). Fine roots were collected and washed and stained with Trypan blue overnight (Brundrett et al., 1996). Finally, roots were rinsed with water and kept in 50% glycerol until mounting on slides for microscope observations. Total root colonization, abundance of arbuscules, vesicles and hyphae percentages were estimated by examination of ~150 transects per slide, according to the magnified intersections method (McGonigle et al., 1990).

2.4. Analysis of lipid peroxidation (LPO)

The level of LPO was determined by measuring the content of thiobarbituric acid reactive substances (TBARS) formed as a result of the reaction of thiobarbituric acid (TBA) and LPO products at pH 3.5 (Verma and Dubey, 2003). An aliquot of powdered tissue was homogenised with 0.1% (w:v) trichloroacetic acid (TCA) solution (1:5, w:v), kept on ice for 15' and centrifuged for 15 min at 16,000 g/4 °C. An aliquot of the supernatant (0.5 mL) was mixed with 1 mL of reagent (0.5% (w:v) TBA in 20% (w:v) TCA), followed by 30 min/95 °C incubation in a thermoshaker TS-100 (Biosan, Riga, Latvia). The absorbance of the reaction mixture was measured at 532 and 600 nm using a UV–Vis spectrophotometer LAMBDA 25 equipped with the software package UV WinLab v6.0.4, (PerkinElmer, Waltham, Massachusetts, USA). The results were expressed as nmol of TBARS per g of fresh weight (nmol/g FW).

2.5. Analysis of free proline

Proline content was determined according to the protocol described by Carillo et al. (2008). Fine leaf tissue powder was homogenised with 40% (v:v) ethanol (1:10, w:v), incubated overnight at 4 °C and centrifuged for 5 min (14,000 g/4 °C). The reagent for colorimetric determination of proline content consisted of 1% (w:v) ninhydrin prepared in 60% (v:v) acetic acid and 20% (v:v) ethanol. Ninhidrine reagent (0.1 mL) was mixed with 50 µL of the supernatant extract, and the reaction mixture was incubated for 20 min at 95 °C on a TS-100 Thermo-Shaker (Biosan, Riga, Latvia). Reaction mixture (0.1 mL) was placed in a 96-well plate, and the absorbance was measured at 532 nm using a Spark multimode microplate reader with SparkControl software (Tecan, Männedorf, Switzerland). As a standard, proline in a concentration range of 0.04–1 mM was used, and the results were expressed as in nmol of proline per gram of fresh weight (nmol/g FW).

2.6. Analysis of ascorbate (AsA)

AsA concentration was measured according to the method described by Kampfenkel et al. (1995) and adapted for a semi-high-throughput 96-well assay format by Murshed et al. (2008). Grounded tissue was homogenised with 5% (w:v) 5-sulfosalicylic acid (1:10, w:v), kept on ice for 10 min and centrifuged for 15 min (22,000 g/4 °C). The reagent used for AsA determination was freshly prepared of 42% (v:v) orthophosphoric acid, 10% (w:v) trichloroacetic acid, 4% (w:v) 2.2-bipyridyl dissolved in 70 % ethanol, and 3% (w:v) FeCl3 mixed in 2.5:2:2:1 ratio, respectively. For the reaction, 5 µL of extract, 35 µL of 0.2 mM potassium phosphate buffer, pH 7.4, and 0.15 mL of AsA reagent were mixed, and incubated for 40 min at 42 °C. The absorbance was measured at 525 nm. As a standard, L-ascorbic acid in a concentration range of 5–70 µg/mL was used, and the results were expressed as µg of AsA per g of fresh weight (µg/g FW).

2.7. Proteins extraction and determination of total protein concentration

Previously frozen in liquid nitrogen, grapevine leaves were ground with a bead mill (TissueLyser, Qiagen) for 1 min at 30 Hz speed. An aliquot of tissue powder (0.4 g) was homogenised with 3 mL of freshly prepared extraction buffer (0.5 M 3-(N-morpholino) propanesulfonic acid, pH 7.5, protease inhibitor cocktail tablet (cOmplete, Roche), 3% (w/v) polyethylene glycol 4000). After 15 min of extraction on ice, the homogenate was centrifuged for 15 min at 19,000 g, and 4 °C. Aliquots of the obtained crude protein extracts were stored at −80 °C for the measurement of the GR, APX, PPO and GPOD activity. For the SOD activity estimation, an aliquot of crude protein extract was purified by PD MidiTrap G-25 columns (Cytiva) using a gravity protocol according to the manufacturer's instructions.

Total soluble protein concentrations in crude and purified extracts were estimated (Bradford, 1976), adapted for 96-well plates assay format. Briefly, an aliquot (5 μL) of the protein extract was incubated with 0.25 mL of Bradford's reagent (Sigma-Aldrich, St. Louis, Missouri, USA) for 5 min at room temperature (RT). Bovine serum albumin in the range of 0.125–1.4 mg/mL was used for a standard curve and absorbance was measured at 595 nm on a Spark multimode microplate reader (Tecan, Männedorf, Switzerland).

2.8. Determination of the antioxidant enzyme activities

All enzyme activities were determined by kinetic semi-high-throughput spectrophotometric methods using a Spark multimode microplate reader with SparkControl software (Tecan, Männedorf, Switzerland).

Glutathione reductase (GR, EC 1.6.4.2) activity was determined by the method of Racker (1955), modified for a semi-high-throughput 96-well assay format by Murshed et al. (2008). The reaction mixture for GR activity estimation consisted of 50 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 8.0), 0.5 mM EDTA and 0.25 mM NADPH. The aliquot (10 μL) of the crude protein extract was equilibrated with 0.18 mL of the reaction mixture, and the reaction started by the addition of 0.5 mM GSSG. The decrease in absorbance was recorded at 340 nm every 15 s for 5 min. The specific activity of GR was expressed in units of enzyme activity per gram of protein (U/g protein).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined with the modified method by Nakano and Asada (1981). The reaction mixture for APX activity determination consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA and 0.7 mM L-ascorbic acid. The aliquot (10 μL) of the crude protein extract was equilibrated with 0.18 mL of the reaction mixture for 3 min and 5 mM of H_2O_2 was added. The decrease in absorbance was recorded at 290 nm every 15 s for 5 min. The specific activity of APX was expressed in units of enzyme activity per gram of protein (U/g protein).

Guaiacol peroxidase (GPOD, EC 1.11.1.7) activity estimation was modified according to the method described by Siegel and Galston (1967). The reaction mixture consisted of 18 mM guaiacol and 5 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) and 0.19 mL was mixed with 10 μL of crude protein extract. The absorbance was monitored at 470 nm every 15 s for 3 min. The specific activity of GPOD was expressed in units of enzyme activity per gram of protein (U/g protein).

Polyphenol oxidase (PPO, EC 1.14.18.1) activity was estimated with method that is based on the coupling reaction of the benzoquinone derivative and L-ascorbic acid (Dawson and Magee, 1955; Marumo and Waite, 1986) and modified as described in Matić et al. (2022). The reaction mixture for the PPO activity determination consisted of 50 mM potassium phosphate buffer (pH 6.5), 0.17 mM L-3,4 dihydroxyphenylalanine, 0.07 mM L-ascorbic acid, 0.002 mM EDTA and the aliquot of the crude protein extract (10 μL) in a final volume of 0.3 mL. Absorbance was recorded at 265 nm every 15 s for 3 min. The specific activity of PPO was expressed in units of enzyme activity per gram of protein (U/g protein).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured according to the method of Beauchamp and Fridovich (1971), modified and adapted for a semi-high-throughput 96-well assay. The reaction mixture consisted of 0.05 mM cytochrome C (prepared in 50 mM potassium phosphate buffer, pH 7.8, with 0.1 mM EDTA) and 1 mM xanthine mixed in a 10:1 ratio. The aliquot of the purified protein extract (10 μL) was mixed and equilibrated with 0.24 mL of the reaction mixture for 15 min, and the reaction was initiated by adding 0.0004 U of xanthine oxidase. The increase in absorbance was recorded at 550 nm every 15 s for 3 min at room temperature. SOD activity was calculated using the degree of inhibition of cytochrome C reduction and was expressed in units of SOD activity per mg protein (U/mg protein).

2.9. Gene expression analysis

Relative expression for three genes putatively coding for Enhanced Disease Susceptibility I (*EDS1*), lipoxygenase (*LOX*) and stilbene synthase 1 (*STS1*) was determined. *STS1* has been evaluated in leaves and roots, while the other two genes only in leaves. RNA extraction was performed according to a CTAB-based method (Gambino, 2015). DNA contamination was removed with TURBO DNA-free Kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer instructions. RNA concentration and purity were assessed on Nanodrop One (Thermo Fisher Scientific, Waltham, MA, USA) at 230, 260 and 280 nm. RNA extracts were purified from remaining DNA with the TURBO DNA-free™ Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Synthesis of cDNA was performed with SuperScript II Reverse Transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA) using random primers and following manufacturer's instructions. cDNA was used as template for quantification of the relative expression of the genes. Two endogenous genes, ubiquitin (*UBI*) and elongation factor (*EF*), known to have stable expression across treatments, were used as housekeeping genes (Balestrini et al., 2017). Relative quantification was performed with CFX96 Touch Real-Time PCR (Bio-Rad, Hercules, CA, USA) on three biological replicates, each represented with three technical replicates. Reaction Mix (10 μl) contained 5 μl of iTaq Universal SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA), 0.2 μM primer pairs (Supporting Table 1) and cDNA (1 μl/1:5). qPCR reactions were performed in CFX96 (Bio-rad, United States) instrument, with the following cycling conditions: denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. Melt curve analysis was made (65–95 °C) with a rate of 0.5 °C every 5 s. Transcripts were normalized to the geometric mean of housekeeping genes and the gene expression was evaluated as the mean ΔCt for each treatment.

2.10. Statistical analysis

Prior to statistical analysis we checked whether the data follow a normal distribution (Shapiro-Wilk test). Separately for each of two years, heatmaps were generated to present the overall grouping of used parameters and corresponding clustering. Two-way ANOVA was performed to estimate significance of the AMF/virus influence on the data dynamics, with virus and AMF status as independent variables. For statistical analysis Statistica 14.0.1. software (Tibco, Arlington, VA, USA) and R software (v4.3.2) were used.

3. Results

3.1. AMF root colonization

High level of AMF root colonization was observed in treated grapevines five months after AMF application and persisted a year later without significant differences between the measurements at the two time points (*t*-test, $p > 0.05$). Two-way ANOVA revealed that five combinations of virus status did not cause any distinctive difference in

the occurrence of AMF typical structures. On the other hand, application of mono-species and multispecies AMF inocula resulted in a greater abundance of arbuscules and vesicles in treatments with *R. irregularis* alone (on average 85.5 ± 13 % and 69.1 ± 19.9 % respectively) than in mixed AMF (AMF Mix) inoculum (on average 70.1 ± 18% and 20.9 ± 13.8 %, respectively) in the first sampling point, as already observed in Gaši et al. (2023). One year later, at the second sampling point, the distinct influence of the two types of AMF inocula persisted exclusively in the case of vesicles, regardless of the virus infection (80.7 ± 10.7 % for *R. irregularis* vs. 47.5 ± 14.9 % for AMF Mix) (Table 1).

3.2. Estimation of oxidative and antioxidative status

Samplings and measurements were made in two consecutive years. The data were analysed separately for each year since samplings were not taken in the same grapevine phenophase: in 2022 the samples were

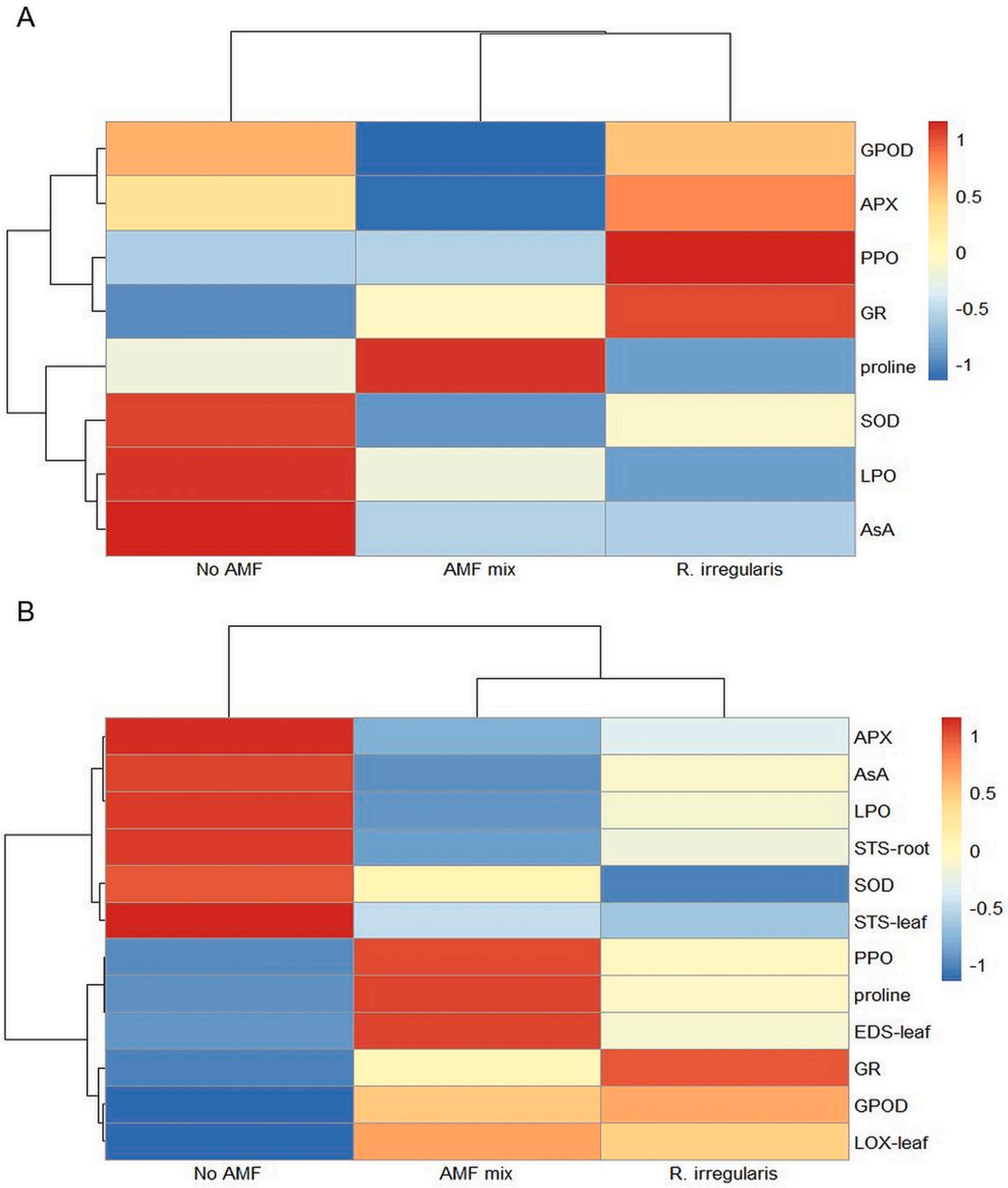


Fig. 1. Heatmap and cluster analysis summarizing the response of oxidative markers to mycorrhizal status of experimental plants. Non mycorrhizal grapevines (No AMF) and vines inoculated with *Rhizophagus irregularis* (R. irregularis) and *R. irregularis, Funneliformis mosseae* and *F. caledonium* (AMF Mix) are displayed with eight in 2022 (a), and twelve oxidative and antioxidative markers in 2023 (b): lipid peroxidation (LPO), ascorbate (AsA), proline, superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidases (GPOD), polyphenol oxidase (PPO), glutathione reductase (GR), stilbene synthase in leaves (STS1-leaf) and roots (STS1-root), enhanced disease susceptibility (EDS1-leaf) and lipoxygenase genes (LOX-leaf). Color scale indicates range between maximum (red, 1) and minimum values (blue, −1). Distance was determined by Euclidean method and clustering was performed using Ward method. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

harvested in the phenophase BBCH 81 and in 2023 in BBCH 89 (Lorenz et al., 1995).

3.2.1. Heatmap and cluster analysis

Strong clustering of parameters was visible on heatmaps for both years as well as their general relation to AMF (Fig. 1). In both years, AMF inoculated treatments clustered separately from the AMF-free controls indicating a relevant relation with the AMF status. General AMF influence was visible for majority of parameters with SOD, AsA and LPO that showed higher values in non-inoculated plants with respect to the AMF-inoculated ones, while PPO, GR and GPOD were higher in AMF-inoculated plants with respect to the not-inoculated control plants. Considering AMF as the only discriminant factor, the relationship between APX and AMF inoculation showed variability over the two considered sampling times, while the influence of AMF Mix in increasing proline concentration was consistent in the two years.

3.2.2. Two-way ANOVA

Two-way ANOVA test was used to verify the differences between treatments, with AMF and virus status as independent variables. Early virus infection and AMF colonization induced significant difference between treatments for all analysed parameters except for GR and LPO, while a year later significant influence of AMF and/or virus inoculum was obtained for all of eight analysed parameters (Table 2). Some (anti) oxidative indicators responded significantly only to AMF (AsA and GR), while others displayed significant changes both due to AMF and virus addition (Figs. 2 and 3). AsA concentrations, as well as SOD activity, displayed the same pattern in both years, significantly decreasing in treatments with AMF (30–67% in treatments with significant difference), with no differences between the two types of AMF inoculum. LPO activities were related to AMF/virus inoculums only in the second year, with variability being triggered by both AMF and virus infection. LPO clear dependence on AMF is visible through significantly decreased values in mycorrhizal treatments (40–60%). Proline concentrations also depended strongly on AMF status of the plants, being the only parameter analysed that showed a difference between the two types of AMF inoculum. Mix-AMF inoculum mainly induced higher proline values compared to the *R. irregularis* alone or to non-AMF controls. Concerning GR, relation to AMF was evident only in the second year in treatments infected with only GRSPaV, with significantly higher concentrations in AMF-inoculated plants with respect to the not-inoculated ones. Similarly to GR, GPOD and APX values were also much more related to the AMF

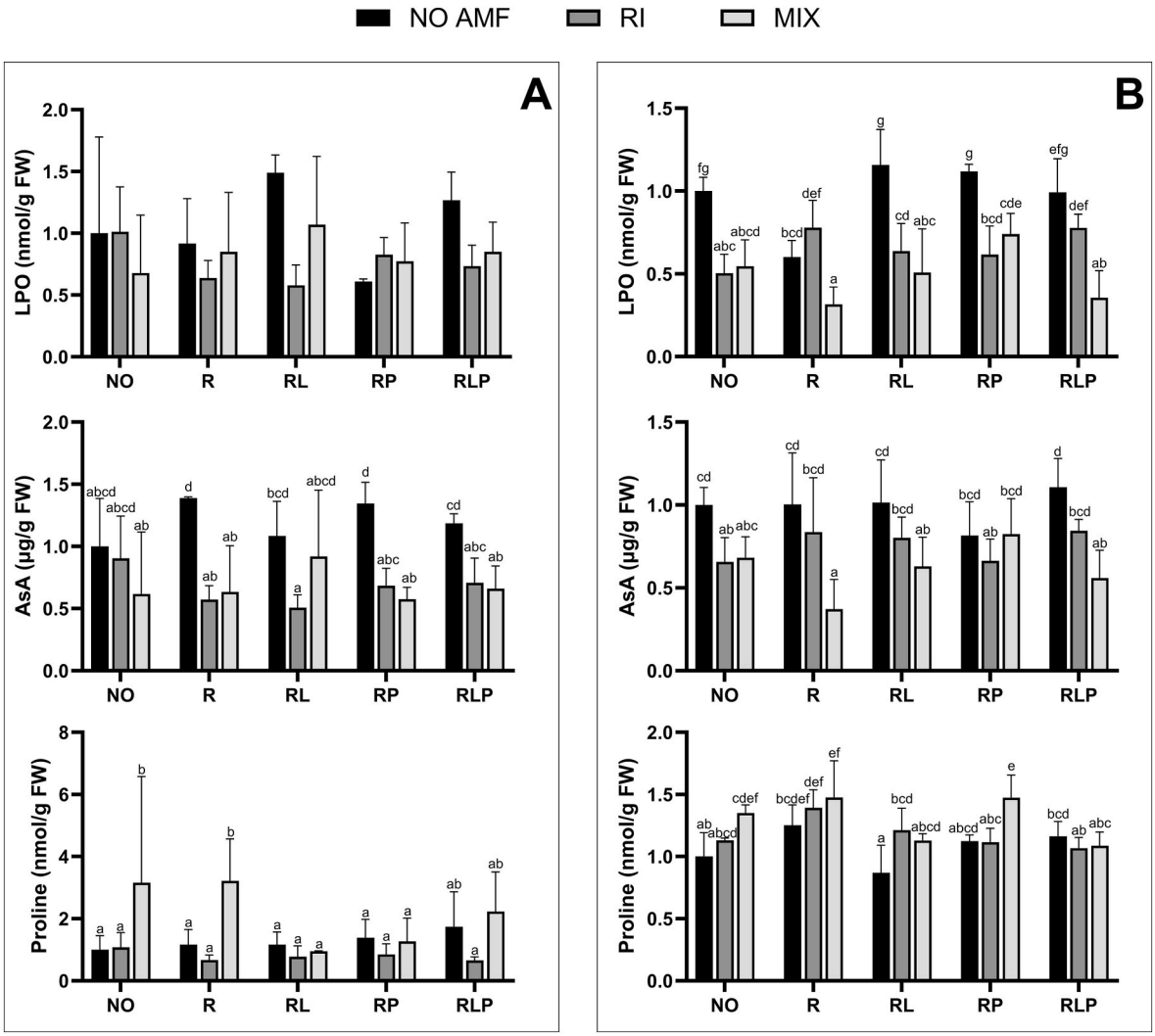


Fig. 2. Non-enzymatic (anti)oxidative parameters in 2022 (A) and 2023 (B): lipid peroxidation (LPO), ascorbate (AsA) and proline. Data were normalized to control treatment that contained no virus and no AMF (T1), for each year separately. Statistical analysis was carried out using two-way ANOVA. Different letters indicate a significant difference between treatments ($p < 0.05$, Duncan post-hoc test). NO – treatments without virus, R – treatments with GRSPaV, RL – GRSPaV + GRLaV3, RP – GRSPaV + GPGV, RLP - GRSPaV + GRLaV3 + GPGV. NO AMF – treatments without AMF, RI – *Rhizophagus irregularis*, MIX – *R. irregularis* + *Funneliformis mosseae*, *F. caledonium*.

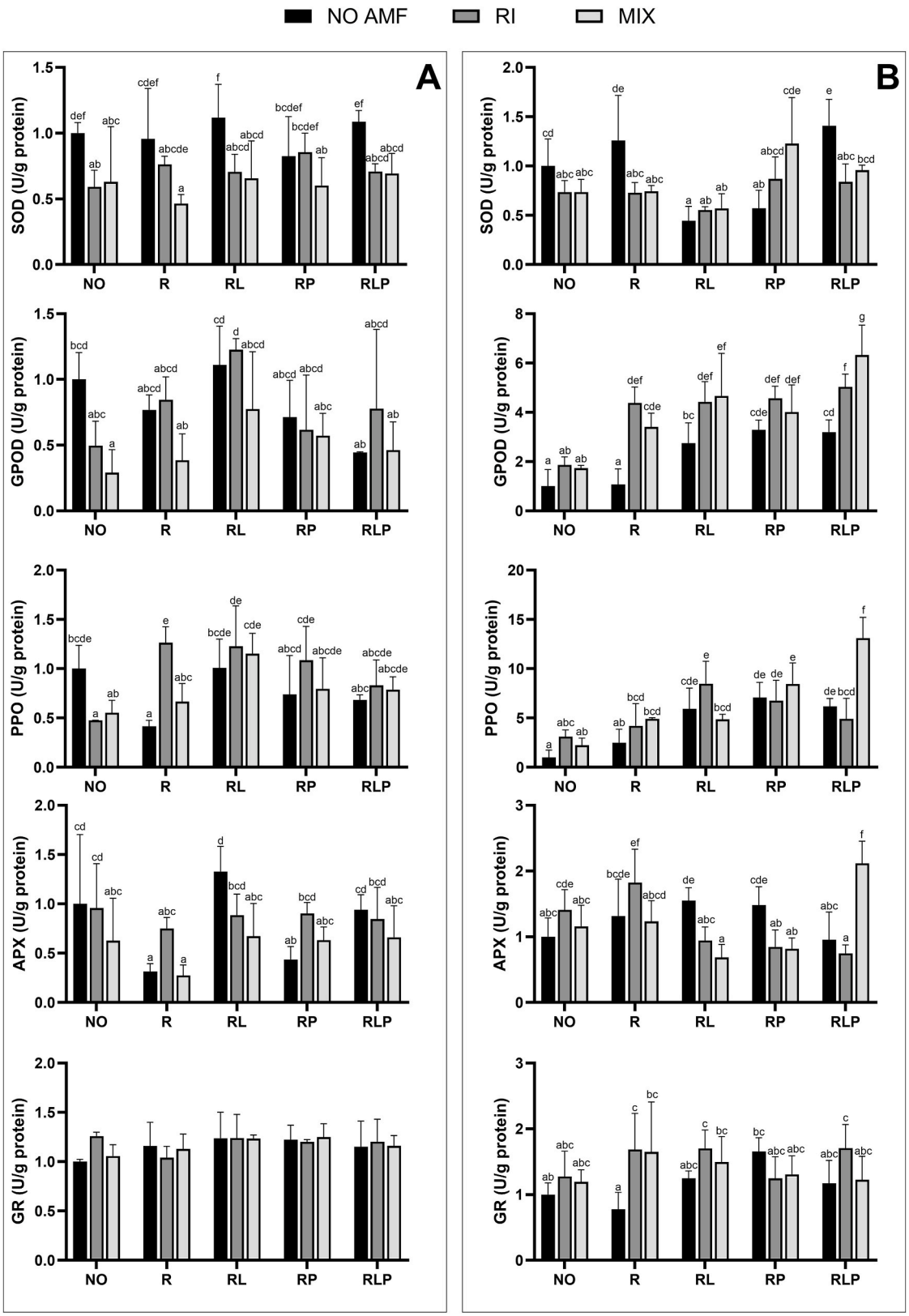


Fig. 3. Antioxidative enzymes in 2022 (A) and 2023 (B): superoxide dismutase (SOD), guaiacol peroxidase (GPOD), polyphenol oxidase (PPO), ascorbate peroxidase (APX) and glutathione reductase (GR). Data were normalized to control treatment that contained no virus and no AMF (T1) for each year separately. Statistical analysis was carried out using two-way ANOVA. Different letters indicate a significant difference between treatments ($p < 0.05$, Duncan post-hoc test). NO – treatments without virus, R – treatments with GRSPaV, RL – GRSPaV + GRLaV3, RP – GRSPaV + GPGV, RLP - GRSPaV + GRLaV3 + GPGV. NO AMF – treatments without AMF, RI – *Rhizophagus irregularis*, MIX – *R. irregularis* + *Funneliformis mosseae*, *F. caledonium*.

status in the second year. Particularly, GPOD revealed strong positive relation to AMF inoculation, with significant increase of activity in AMF-inoculated plants (up to 78 % in GR treatment). For APX, AMF inoculum had weaker influence than virus infection, for both years. AMF induced high APX activity mainly in AMF-free control plants during the second year, but this was not consistent. Although two-way ANOVA showed significant PPO relation to both AMF and viruses, no clear pattern was observed.

3.3. Gene expression

The expression of *EDS1*, *LOX* and *STS1* genes was affected by AMF presence, virus status and their interaction (Table 2, Fig. 4). *EDS1* gene expression responded to both virus infection and AMF colonization. It was the least expressed in treatments infected with all three viruses and the most expressed in GRSPaV-infected grapevines. Nevertheless, in AMF inoculated plants its expression was increased in the presence of GRSPaV and GPGV (64 %) as well as in the combination of GRSPaV, GLRaV-3 and GPGV (85 %).

A similar pattern of dependence on AMF/viral infection was observed also for the *LOX* gene. Higher expression values in AMF treatments compared to non AMF ones were observed mainly where GRSPaV was present in combination with GPGV and with both GPGV and GLRaV-3 (up to 85 % increase). Regarding viral influence, *LOX* was also the least expressed in treatments with all three viruses present and most expressed in treatments with GRSPaV only.

STS1 response to AMF/virus inocula in mature leaves revealed elevated expression in AMF-free controls for the treatments where GRSPaV was in combination with GLRaV-3 or with GPGV and GLRaV-3 (~90 %). Considering roots, while less influenced by AMF colonization or virus status, *STS1* was highly expressed specifically in non-AMF-inoculated plants harbouring the GRSPaV + GLRaV-3 virus combination (higher up to 60 %).

4. Discussion

In this study, the underexplored tripartite plant-virus-AMF interaction using grapevine as a model woody plant system has been addressed. Our findings suggested that the AMF inocula have a substantial potential to affect antioxidative processes in grapevines infected with viruses.

One of the first lines of grapevine, and generally plant defence, against virus attack is the production of ROS through an oxidative burst (Sgherri et al., 2013; Hernandez et al., 2016; Hančević et al., 2023). On the other hand, the formation of arbuscular mycorrhizal symbiosis plays a role in the reinforcement of the antioxidant defence system of the plant for the prevention of oxidative damage (Kapoor and Singh, 2017). Here, a clear difference in antioxidative defence system between AMF-inoculated and non-inoculated grapevine plants in combating viral stressor was revealed. The significantly lower levels of AsA and SOD in AMF-inoculated plants, compared to non-inoculated ones, indicate a reduced production of ROS species. Consequently, this potentially have led to reduced oxidative stress and may have contributed to the enhanced ability of plants to combat viruses when colonized by AMF. SOD are one of the most effective components of the antioxidant defense system in plant cells against ROS toxicity (Berwal and Ram, 2018). These metalloenzymes are significantly impacted by grapevine virus infection, whether only in some phases of infection (Sgherri et al., 2013), or regardless of the length of the infection, virus isolate, and grape cultivar. For this reason, these enzymes have been proposed as indicators of viral infection in grapevine (Hančević et al., 2023). For AsA,

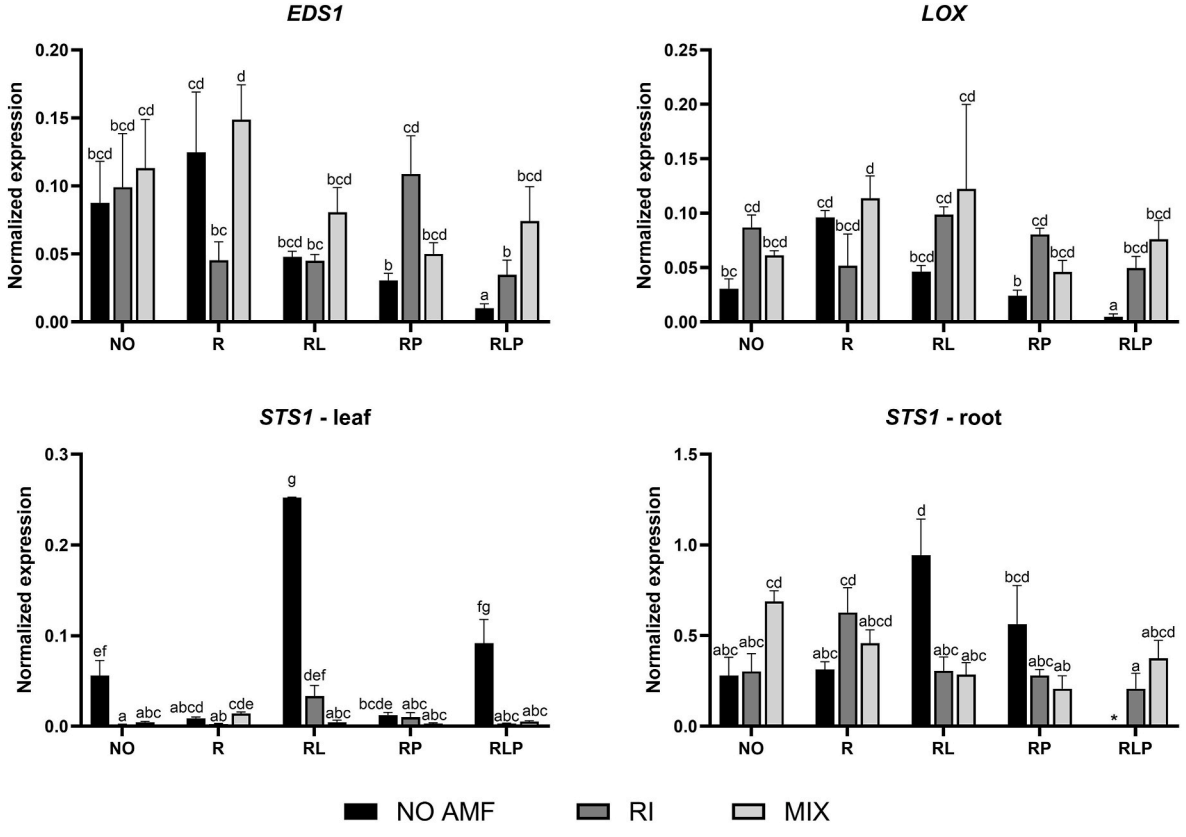


Fig. 4. Relative expression of genes: Enhanced disease susceptibility 1 (*EDS1*), Lipoxygenase (*LOX*), Stilbene synthase 1 in leaf (*STS*-leaf) and Stilbene synthase 1 in root (*STS*-root). Statistical analysis was carried out using two-way ANOVA. Different letters indicate a significant difference between treatments (Duncan post-hoc test, $p < 0.05$). NO – treatments without virus, R – treatments with GRSPaV, RL – GRSPaV + GRLaV3, RP – GRSPaV + GPGV, RLP - GRSPaV + GRLaV3 + GPGV. NO AMF – treatments without AMF, RI – *Rhizophagus irregularis*, MIX – *R. irregularis* + *Funneliformis mosseae*, *F. caledonium*.

as a key antioxidant for scavenging excessive ROS, available data in literature describe its accumulation in virus stressed plants and also in plants colonized by AMF. Sgherri et al. (2013) reported increased synthesis of AsA in vine infected by grapevine fanleaf virus (GFLV), making plants more tolerant to this virus (Carvalho et al., 2015). A similar antioxidant accumulation, linked to a defence response to viruses, has been reported in other crops as well, such as in *Brassica rapa* against turnip mosaic virus (Fujiwara et al., 2016). It is noteworthy that, depending on the type and degree of stress, the use of AMF inocula in other plants species such as tobacco (Begum et al., 2020), rice (Ruiz-Sánchez et al., 2010), maize (Barzana et al., 2015), and tomato (Liu et al., 2016), can increase the production of AsA.

The fact that in this study SOD activity was reduced in mycorrhizal plants compared to non-inoculated plants suggests lower conversion of superoxides into H_2O_2, and consequently lower accumulation of H_2O_2. This was accompanied with poor activation of the next step of H_2O_2 metabolism - ascorbate-glutathione cycle, in which AsA is one of key antioxidants and APX and GR are the main antioxidant enzymes (Pang and Wang, 2010). The reduced activation of this cycle in AMF-inoculated plants was manifested in lower accessibility of AsA as APX substrate, and inconsistent APX activity of H_2O_2 reduction and GR activity, although their levels are expected to increase in the presence of AMF (Ma et al., 2022; Ye et al., 2023). Reduced SOD activity and AsA concentrations, together with a weakly activated ascorbate-glutathione cycle, could potentially imply their reduced capability to deal with oxygen radicals. However, considering these results together with the previously observed enhanced growth performance in AMF-inoculated plants compared to those only infected with viruses, these findings suggest that AMF-plants exhibited an improved vigour and tolerance to viral stress. Namely, AMF-inoculated grapevines exhibited improved growth (shoot length) already five months after inoculation and also a year later, accompanied with the intensified photosynthesis and pigment concentration, increased leaf water potential and dry matter content (Gaši et al., 2023, Gaši et al. unpublished data). In both considered time points, underlying processes resulted in a decrease in ROS generation without the requirement to activate the ascorbate-glutathione cycle. Similarly, in experiment on potato plants, an AMF-induced reduction of H_2O_2 content was observed, suggesting a protective role by AMF against potato virus Y-induced oxidative stress (Deja-Sikora et al., 2023). In the second year, however, AMF-inoculated plants demonstrated an increased degradation of H_2O_2, highlighted by elevated level of GPOD, an enzyme that can be promoted by AMF presence (Mayer et al., 2017; Domokos et al., 2018). This coincides with the GLRaV-3 virus titer increase, depending on tissues, virus combination and AMF inoculum used (Gaši et al., 2024). Increased virus titer could induce increased ROS production, ultimately increasing activity of antioxidative systems to reduce virus induced cell damage (Cui et al., 2016). Thus, we observed evidence of reduced ascorbate-glutathione cycle activation and decreased H_2O_2 synthesis in the early stages of AM symbiosis, whereas accumulation seemed to occur in the later stages of AM establishment. At this phase, even after a year, ROS scavenging activation through ascorbate-glutathione cycle was still reduced. However, AMF significantly boosted the catalytic activity of GPOD on H_2O_2.

In the grapevine-virus-AMF interplay, a significant AMF influence on proline concentrations has been observed. Proline plays diverse roles under different stresses, and amongst others it acts as a general ROS scavenger (Raza et al., 2023). Despite it is known that proline biosynthesis can be activated by plant-pathogen interactions (Fabro et al., 2004), and its high production was reported in leaves of grapevine infected with red blotch-associated virus (GRBaV) (Wallis & Sudarshana, 2016) and partially in grapevine infected with virus mix (Hančević et al., 2023), our study did not find any significant relation to virus infection. On the other hand, the influence of AMF on proline concentration was significant. It is well known that AMF can significantly influence plant proline production: in some cases, AMF reduces proline levels in plants experiencing heat or water stress (Zou et al., 2013; Hazzoumi et al., 2015; Wu et al., 2017), while AMF can also lead to increased proline production in other cases (Chen et al., 2014; Liu et al., 2022). In our study, co-occurrence of multiple AMF species in inoculum was associated with higher proline production, compared to non-inoculated and plants inoculated with a single AMF species. This is in agreement with previous results showing that AMF species identity can differently influence proline accumulation (Chen et al., 2014; Pasquini et al., 2023).

While mentioned antioxidative markers responded to AMF application at both harvesting time points, LPO displayed significant relation to AMF only in the second year. The extent of LPO is a reliable indicator of ROS production leading to oxidative stress (Chakraborty et al., 2024). Decrease in LPO prevents cell membrane injury and maintains integrity and stability of the plasma membrane in AMF-inoculated plants (Evelin et al., 2012; Garg and Chandel, 2015). LPO is expected to increase in virus infected plants (Madhusudhan et al., 2009) and to decrease in mycorrhizal plants (Kapoor and Singh, 2017; Chandrasekaran, 2022). This is confirmed in our study in the second year, where such a pattern was found for all combinations of viruses and AMF, especially in plants infected by more than one virus. Different LPO patterns in two sampling points may be caused by the length of period from AMF inoculation to the harvesting, but also by the different grapevine developmental stages at the two points when the measurements were made. Developmental stage may be an important factor influencing variations of antioxidative responses of grapevine under abiotic stress (Majer and Hideg, 2012). Although LPO was reduced in AMF-inoculated plants in the second year, the gene *LOX* was upregulated in specific treatments with AMF and multiple viruses. The *LOX* genes in plants are known to be activated during the pathogen attack (Rosahl, 1996) and its induction in mycorrhizal plants under stress conditions has been previously reported (Chitarra et al., 2016; Kumar Maurya et al., 2018; Begum et al., 2021). Plants may harbour different *LOX* genes/isoforms that might be involved in processes other than lipid peroxidation, such as growth regulation and senescence (Porta and Rocha-Sosa, 2002). Our results suggest that while the *LOX* gene is upregulated and potentially active, its effect on the lipid peroxidation profile could be moderated by the effects mediated by presence of AMF, or alternatively, this specific *LOX* gene may be involved in other biological processes (*e.g.*, jasmonic acid pathway). This inconsistency should be explored into more details, to highlight the role of LOX pathway in grapevine-virus-AMF interplay.

Expression of *STS1* and *EDS1* revealed a significant relation to AMF but only in specific combinations of virus infection. Stilbenes are an additional class of chemical compounds playing an important role in plant defence against phytopathogens (Valletta et al., 2021) having protective and reversing roles against induced oxidative stress in grapevine (Medrano-Padial et al., 2021). Bruisson et al. (2016) showed that mycorrhization of several grapevine varieties with *R. irregularis* triggered a higher expression of genes involved in stilbene biosynthesis. However, our results match those obtained for SOD and AsA since stilbene synthesis is stimulated in virus infected grapevine without AMF, implying lower level of oxidative stress in mycorrhizal plants. Interestingly, this effect is the most pronounced in grapevine plants co-infected with GLRaV-3 and it is more evident in the leaves than in the roots. *EDS1* relative expressions displayed an opposite pattern – increase in AMF-inoculated plants and only in treatments where GPGV was present in combination with GRSPaV. Expression of *EDS1* has been linked with indirect promotion of scavenging H_2O_2 and thus control of ROS homeostasis in rice under heat stress (Liao et al., 2023). It is also one of responsive differentially expressed defence genes during powdery and downy mildew infections in *V. vinifera* (Goyal et al., 2021). In this line, we can recognize a partial role for *EDS1* in alleviating oxidative stress, exclusively in mycorrhizal grapevines infected with multiple viruses.

The observed modulations in antioxidative response induced by AMF were documented during the early stages of grapevine development, specifically within the first 15 months of their life cycle. These changes were accompanied by a significant enhancement in growth performance

(Gaši et al., 2023, Gaši et al. unpublished data), suggesting an improved ability to cope with viral stress. However, the interaction between grapevines, AMF, and viruses is complex. A detailed analysis of relative virus concentrations in this study indicated that virus accumulation is influenced by the type of tissue analysed, the timing of sample collection, and the specific AMF and virus compositions involved. For GRSPaV, initial accumulation was observed in the roots and decrease in young leaves of AMF-inoculated grapevines, with diminishing influence of AMF over time. Conversely, AMF induced GLRaV-3 increased concentrations in mature leaves even one year post-inoculation, raising concerns about a potential increase in grapevine susceptibility to this significant virus. Simultaneously, the relative concentration of GPGV was found to be reduced in the foliage of AMF-inoculated plants (Gaši et al., 2024). The overall impact of AMF on virus-infected grapevines, particularly as they mature, requires further investigation. The young age of the grapevines studied did not allow for an assessment of AMF's impact on yield and fruit quality, as no fruit set occurred during the observed period. Similarly, symptom development was not evident in such a young infection. A long-term study would provide valuable insights into the overall influence of AMF on the agronomic performance of virus-infected grapevines and show potential impact on the symptoms development.

5. Conclusions

Our work presents a first insight in significant influence of AMF on specific antioxidative indicators in grapevine combating virus stress and confirm AMF to be integral part of ROS homeostasis. The preliminary concept concerning the observed processes indicates that the production of ROS is reduced in grapevines colonized by arbuscular mycorrhizal fungi (AMF). This reduction is evident due to a non-activated ascorbate-glutathione cycle and GPOD activity during the early stages of AMF symbiosis. However, in the later stages, AMF significantly boosts the efficient removal of ROS through the catalytic action of GPOD. Together with reduction in *STS1* and increase in *EDS1* gene expression, our results demonstrate that subsequent mycorrhiza establishment in previously virus-infected grapevine may modulate plant defence responses. Observed effects may be general for some (anti)oxidative markers or dependent on infection time/season, AMF inoculum composition and virus species for other markers. Such AMF influence on certain oxidative and antioxidative markers is important fact to keep in mind since in vineyards grapevine are infected with wide range of virus species but at the same time they are commonly colonized by AMF. AMF role in alleviating virus-induced oxidative stress in grapevine builds on the previous knowledge on AMF beneficial effects in grapevine coping with other abiotic and biotic stress factors and emphasizes the importance of AMF in sustainable viticulture. In future, focused transcriptomic experimental work on tripartite grapevine-virus-AMF interactions is needed to clarify more precise underlying mechanisms leading to the results obtained herein.

CRediT authorship contribution statement

Tomislav Radić: Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Rosemary Vuković:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Emanuel Gaši:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Daniel Kujundžić:** Writing – review & editing, Formal analysis. **Mate Čarija:** Writing – review & editing, Visualization, Investigation. **Raffaella Balestrini:** Writing – review & editing, Supervision, Methodology. **Fabiano Sillo:** Writing – review & editing, Methodology. **Giorgio Gambino:** Writing – review & editing, Supervision, Methodology. **Katarina Hančević:** Writing – review & editing, Investigation, Conceptualization.

Funding information

This study was financed by the Croatian Science Foundation (IP-2020-02-8397).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The work was carried out in the frame of the project "Arbuscular mycorrhiza potential to modify grapevine defence against viruses, MYCO GRAPE" financed by the Croatian Science Foundation (IP-2020-02-8397).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2024.154372.

Data availability

Data will be made available on request.

References

Ahn, S.Y., Kim, S.A., Cho, K.S., Yun, H.K., 2014. Expression of genes related to flavonoid and stilbene synthesis as affected by signaling chemicals and *Botrytis cinerea* in grapevines. Biol. Plant. (Prague) 58 (4), 758–767. https://doi.org/10.1007/s10535-014-0437-2.

Alagna, F., Balestrini, R., Chitarra, W., Marsico, A.D., Nerva, L., 2020. Getting ready with the priming: innovative weapons against biotic and abiotic crop enemies in a global changing scenario. In: Hossain, M.A., Liu, F., Burritt, D.J., Fujita, M., Huang, B. (Eds.). Elsevier eBooks. https://doi.org/10.1016/b978-0-12-817892-8.00003-9.

Aseel, D.G., Rashad, Y.M., Hammad, S.M., 2019. Arbuscular mycorrhizal fungi trigger transcriptional expression of flavonoid and chlorogenic acid biosynthetic pathways genes in tomato against tomato mosaic virus. Sci. Rep. 9, 9692. https://doi.org/10.1038/s41598-019-46281-x.

Balestrini, R., Magurno, F., Walker, C., Lumini, E., Bianciotto, V., 2010. Cohorts of arbuscular mycorrhizal fungi (AMF) in *Vitis vinifera*, a typical Mediterranean fruit crop. Environ. Microbiol. Rep. 2, 594–604. https://doi.org/10.1111/j.1758-2229.2010.00160.x.

Balestrini, R., Salvioli, A., Dal Molin, A., Novero, M., Gabelli, G., Paparelli, E., Marroni, F., Bonfante, P., 2017. Impact of an arbuscular mycorrhizal fungus versus a mixed microbial inoculum on the transcriptome reprogramming of grapevine roots. Mycorrhiza 27 (5), 417–430. https://doi.org/10.1007/s00572-016-0754-8.

Bannenberg, G., Martínez, M., Hamberg, M., Castresana, C., 2009. Diversity of the enzymatic activity in the lipoxygenase gene family of *Arabidopsis thaliana*. Lipids 44, 85–95. https://doi.org/10.1007/s11745-008-3245-7.

Barzana, G., Aroca, R., Ruiz-Lozano, M., 2015. Localized and non-localized effects of arbuscular mycorrhizal symbiosis on accumulation of osmolytes and aquaporins and on antioxidant systems in maize plants subjected to total or partial root drying. Plant Cell Environ. 38, 1613–1627. https://doi.org/10.1111/pce.12507.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44 (1), 276–287. https://doi.org/10.1016/0003-2697(71)90370-8.

Begum, N., Ahanger, M.A., Zhang, L., 2020. AMF inoculation and phosphorus supplementation alleviates drought induced growth and photosynthetic decline in *Nicotiana tabacum* by up-regulating antioxidant metabolism and osmolyte accumulation. Environ. Exp. Bot. 176, 104088. https://doi.org/10.1016/j.envexpbot.2020.104088.

Begum, N., Akhtar, K., Ahanger, M.A., Iqbal, M., Wang, P., Mustafa, N.S., Zhang, L., 2021. Arbuscular mycorrhizal fungi improve growth, essential oil, secondary metabolism, and yield of tobacco (*Nicotiana tabacum* L.) under drought stress conditions. Environ. Sci. Pollut. Res. 28, 45276–45295. https://doi.org/10.1007/s11356-021-13755-3.

Berwal, M.K., Ram, C., 2018. Superoxide dismutase: a stable biochemical marker for abiotic stress tolerance in higher plants. In: de Oliveira, A.B. (Ed.), Abiotic and Biotic Stress in Plants. IntechOpen, pp. 1–11. https://doi.org/10.5772/intechopen.82079.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Bruisson, S., Maillot, P., Schellenbaum, P., Walter, B., Gindro, K., Deglène-Benbrahim, L., 2016. Arbuscular mycorrhizal symbiosis stimulates key genes of the

phenylpropanoid biosynthesis and stilbenoid production in grapevine leaves in response to downy mildew and grey mould infection. Phytochemistry 131, 92–99. https://doi.org/10.1016/j.phytochem.2016.09.002.
Brundrett, M., Bougher, N., Dell, B., Grove, T., Malajczuk, N., 1996. Working with Mycorrhizas in Forestry and Agriculture. ACIA, Canberra. https://doi.org/10.13140/2.1.4880.5444.
Cameron, D.D., Neal, A.L., van Wees, S.C.M., Ton, J., 2013. Mycorrhiza-induced resistance: more than the sum of its parts? Trends Plant Sci. 18 (10), 539–545. https://doi.org/10.1016/j.tplants.2013.06.004.
Carillo, P., Mastrolonardo, G., Nacca, F., Parisi, D., Verlotta, A., Fuggi, A., 2008. Nitrogen metabolism in durum wheat under salinity: accumulation of proline and glycine betaine. Funct. Plant Biol. 35, 412–426. https://doi.org/10.1071/FP08108.
Carvalho, L.C., Vidigal, P., Amâncio, S., 2015. Oxidative stress homeostasis in grapevine (*Vitis vinifera* L.). Front. Environ. Sci. 3, 20. https://doi.org/10.3389/fenvs.2015.00020.
Chakraborty, N., Mitra, R., Dasgupta, D., Ganguly, R., Acharya, K., Minkina, T., Popova, V., Churyukina, E., Keswani, C., 2024. Unraveling lipid peroxidation-mediated regulation of redox homeostasis for sustaining plant health. Plant Physiol. Biochem. 206, 108272. https://doi.org/10.1016/j.plaphy.2023.108272.
Chandrasekaran, M., 2022. Arbuscular mycorrhizal fungi mediated alleviation of drought stress via non-enzymatic antioxidants: a meta-analysis. Plants 11 (19), 2448. https://doi.org/10.3390/plants11192448.
Chen, X., Song, F., Liu, F., Tian, C., Liu, S., Xu, H., Zhu, X., 2014. Effect of different arbuscular mycorrhizal fungi on growth and physiology of maize at ambient and low temperature regimes. Sci. World J. 956141, 1–7. https://doi.org/10.1155/2014/956141.
Chitarra, W., Pagliarani, C., Maserti, B., Lumini, E., Siciliano, I., Cascone, P., Schubert, A., Gambino, G., Balestrini, R., Guerrieri, E., 2016. Insights on the impact of arbuscular mycorrhizal symbiosis on tomato tolerance to water stress. Plant Physiol. 171 (2), 1009–1023. https://doi.org/10.1104/pp.16.00307.
Cui, Z.H., Bi, W.L., Hao, X.Y., Xu, Y., Li, P.M., Walker, A., Wang, Q.C., 2016. Responses of in vitro-grown plantlets (Vitis vinifera) to grapevine leafroll-associated virus-3 and PEG-induced drought stress. Front. Physiol. 7. https://doi.org/10.3389/fphys.2016.00203.
Dawson, C.R., Magee, R.J., 1955. Plant tyrosinase (polyphenol oxidase). Methods Enzymol. 2, 817–827. https://doi.org/10.1016/S0076-6879(55)02309-4.
Deja-Sikora, E., Werner, K., Hrynkiewicz, K., 2023. AMF species do matter: *Rhizophagus irregularis* and *Funneliformis mosseae* affect healthy and PVY-infected *Solanum tuberosum* L. in a different way. Front. Microbiol. 14, 1127278. https://doi.org/10.3389/fmicb.2023.1127278.
Domokos, E., Jakab-Farkas, L., Darkó, B., Bíró-Janka, B., Mara, G., Albert, C., Balog, A., 2018. Increase in *Artemisia annua* plant biomass, artemisinin content and guaiacol peroxidase activity using the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. Front. Plant Sci. 9, 478. https://doi.org/10.3389/fpls.2018.00478.
Dowarah, B., Singh Gill, S., Agarwala, N., 2022. Arbuscular mycorrhizal fungi in conferring tolerance to biotic stresses in plants. J. Plant Growth Regul. 41, 1429–1444. https://doi.org/10.1007/s00344-021-10392-5.
Evelin, H., Giri, B., Kapoor, R., 2012. Contribution of *Glomus intraradices* inoculation to nutrient acquisition and mitigation of ionic imbalance in NaCl-stressed *Trigonella foenum-graecum*. Mycorrhiza 22 (3), 203–217. https://doi.org/10.1007/s00572-011-0392-0.
Fabro, F., Kovács, I., Pavet, V., Szabados, L., Alvarez, M.E., 2004. Proline accumulation and AtP5CS2 gene activation are induced by plant-pathogen incompatible interactions in Arabidopsis. Mol. Plant Microbe Interact. 17 (4), 343–350. https://doi.org/10.1094/MPMI.2004.17.4.343.
Fuchs, M., 2020. Grapevine viruses: a multitude of diverse species with simple but overall poorly adopted management solutions in the vineyard. J. Plant Pathol. 102, 643–653. https://doi.org/10.1007/s42161-020-00579-2.
Fujiwara, A., Togawa, S., Hikawa, T., Matsuura, H., Masuta, C., Inukai, T., 2016. Ascorbic acid accumulates as a defense response to Turnip mosaic virus in resistant Brassica rapa cultivars. J. Exp. Bot. 67 (14), 4391–4402. https://doi.org/10.1093/jxb/erw223.
Gambino, G., 2015. Multiplex RT-PCR method for the simultaneous detection of nine grapevine viruses. In: Uyeda, I., Masuta, C. (Eds.), Plant Virology Protocols. Methods in Molecular Biology, vol. 1236. Humana Press, New York. https://doi.org/10.1007/978-1-4939-1743-3_4.
Garg, N., Chandel, S., 2015. Role of arbuscular mycorrhiza in arresting reactive oxygen species (ROS) and strengthening antioxidant defense in *Cajanus cajan* (L.) Millsp. nodules under salinity (NaCl) and cadmium (Cd) stress. Plant Growth Regul. 75, 521–534. https://doi.org/10.1007/s10725-014-0016-8.
Gaši, E., Radić, T., Čarija, M., Gambino, G., Balestrini, R.M., Hančević, K., 2023. Arbuscular mycorrhizal fungi induce changes of photosynthesis-related parameters in virus infected grapevine. Plants 12, 1783. https://doi.org/10.3390/plants12091783.
Gaši, E., Radić, T., Gambino, G., Čarija, M., Matić, F., Balestrini, R., Hančević, K., 2024. Arbuscular mycorrhizal fungi modify temporal virus accumulation and distribution in different grapevine tissues. Phytobiomes J. https://doi.org/10.1094/PBIOMES-06-24-0066-R.
Goyal, N., Bhatia, G., Garewal, N., Upadhyay, A., Singh, K., 2021. Identification of defense related gene families and their response against powdery and downy mildew infections in *Vitis vinifera*. BMC Genom. 22, 776. https://doi.org/10.1186/s12864-021-08081-4.
Hančević, K., Čarija, M., Radić Brkanac, S., Gaši, E., Likar, M., Zdunić, G., Regvar, M., Radić, T., 2023. Grapevine leafroll-associated virus 3 in single and mixed infections triggers changes in the oxidative balance of four grapevine varieties. Int. J. Mol. Sci. 24 (1), 8. https://doi.org/10.3390/ijms24010008.
Hao, Z., Fayolle, L., van Tuinen, D., Chatagnier, O., Li, X., Gianinazzi, S., Gianinazzi-Pearson, V., 2012. Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode *Xiphinema index* involves priming of defence gene responses in grapevine. J. Exp. Bot. 63, 3657–3672. https://doi.org/10.1093/jxb/ers046.
Hao, Z., Xie, W., Chen, B., 2019. Arbuscular mycorrhizal symbiosis affects plant immunity to viral infection and accumulation. Viruses 11 (6), 534. https://doi.org/10.3390/v11060534.
Hasanuzzaman, M., Bhuyan, M.H.M.B., Zulfiqar, F., Raza, A., Mohsin, S.M., Mahmud, J. A., Fujita, M., Fotopoulos, V., 2020. Reactive oxygen species and antioxidant defense in plants under abiotic stress: revisiting the crucial role of a universal defense regulator. Antioxidants 9, 681. https://doi.org/10.3390/antiox9080681.
Hazzoumi, Z., Moustakime, Y., Elharchli, E.H., Joutei, K.A., 2015. Effect of arbuscular mycorrhizal fungi (AMF) and water stress on growth, phenolic compounds, glandular hairs, and yield of essential oil in basil (*Ocimum gratissimum* L). Chem. Biol. Technol. Agric. 2, 10. https://doi.org/10.1186/s40538-015-0035-3.
Hernandez, J.A., Gullner, G., Clemente-Moreno, M.J., Künstler, A., Juhasz, C., Díaz-Vivancos, P., Kiraly, L., 2016. Oxidative stress and antioxidative responses in plant virus interactions. Physiol. Mol. Plant Pathol. 94, 134–148. https://doi.org/10.1016/j.pmpp.2015.09.001.
Jaillon, O., et al., 2007. (The French–Italian public consortium for grapevine genome characterization). The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449 (7161), 463–467. https://doi.org/10.1038/nature06148.
Jung, S.C., Martinez-Medina, A., Lopez-Raez, J.A., Pozo, M.J., 2012. Mycorrhiza-Induced Resistance and priming of plant defences. J. Chem. Ecol. 38, 651–664. https://doi.org/10.1007/s10886-012-0134-6.
Kampfenkel, K., Van Montagu, M., Inze, D., 1995. Extraction and determination of ascorbate and dehydroascorbate from plant tissue. Anal. Biochem. 225, 165–167. https://doi.org/10.1006/abio.1995.1127.
Kapoor, R., Singh, N., 2017. Arbuscular mycorrhiza and reactive oxygen species. In: Wu, Q.S. (Ed.), Arbuscular Mycorrhizas and Stress Tolerance of Plants. Springer, Singapore. https://doi.org/10.1007/978-981-10-4115-0_10.
Kumar Maurya, A., Kelly, M.P., Mahaney, S.M., Gomez, S.K., 2018. Arbuscular mycorrhizal symbiosis alters plant gene expression and aphid weight in a tripartite interaction. J. Plant Interact. 13, 294–305. https://doi.org/10.1080/17429145.2018.1475020.
Lapin, D., Bhandari, D.D., Parker, J.E., 2020. Origins and immunity networking functions of EDS1 family proteins. Annu. Rev. Phytopathol. 58, 253–276. https://doi.org/10.1146/annurev-phyto-010820-012840.
Liao, M., Ma, Z., Kang, Y., Zhang, B., Gao, X., Yu, F., Yang, P., Ke, Y., 2023. ENHANCED DISEASE SUSCEPTIBILITY 1 promotes hydrogen peroxide scavenging to enhance rice thermotolerance. Plant Physiol. 192, 3106–3119. https://doi.org/10.1093/plphys/kiad257.
Likar, M., Hančević, K., Radić, T., Regvar, M., 2013. Distribution and diversity of arbuscular mycorrhizal fungi in grapevines from production vineyards along the eastern Adriatic coast. Mycorrhiza 23, 209–219. https://doi.org/10.1007/s00572-012-0463-x.
Liu, A., Chen, S., Wang, M., Liu, D., Chang, R., Wang, Z., Lin, X., Bai, B., Ahammed, J., 2016. Arbuscular mycorrhizal fungus alleviates chilling stress by boosting redox poise and antioxidant potential of tomato seedlings. J. Plant Growth Regul. 35, 109–120. https://doi.org/10.1007/s00344-015-9511-z.
Liu, Z., Bi, S., Meng, J., Liu, T., Li, P., Yu, C., Peng, X., 2022. Arbuscular mycorrhizal fungi enhanced rice proline metabolism under low temperature with nitric oxide involvement. Front. Plant Sci. 13, 962460. https://doi.org/10.3389/fpls.2022.962460.
Lorenz, D.H., Eichhorn, K.W., Bleiholder, H., Klose, R., Meier, U., Weber, E., 1995. Growth stages of the grapevine: phenological growth stages of the grapevine (*Vitis vinifera* L. ssp. vinifera)—codes and descriptions according to the extended BBCH scale. Aust. J. Grape Wine Res. 1 (2), 100–103. https://doi.org/10.1111/j.1755-0238.1995.tb00085.x.
Ma, W.Y., Qin, Q.Y., Zou, Y.N., Kuča, K., Giri, B., Wu, Q.S., Hashem, A., Al-Arjani, A.B.F., Almutairi, K.F., Abd_Allah, E.F., Xu, Y.J., 2022. Arbuscular mycorrhiza induces low oxidative burst in drought-stressed walnut through activating antioxidant defense systems and heat shock transcription factor expression. Front. Plant Sci. 13, 1089420. https://doi.org/10.3389/fpls.2022.1089420.
Madhusudhan, K.N., Srikanta, B.M., Shylaja, M.D., Prakash, H.S., Shetty, H.S., 2009. Changes in antioxidant enzymes, hydrogen peroxide, salicylic acid and oxidative stress in compatible and incompatible host-tobamovirus interaction. J. Plant Interact. 4 (3), 157–166. https://doi.org/10.1080/17429140802419516.
Maffei, G., Miozzi, L., Fiorilli, V., Novero, M., Lanfranco, L., Accotto, G.P., 2014. The arbuscular mycorrhizal symbiosis attenuates symptom severity and reduces virus concentration in tomato infected by Tomato yellow leaf curl Sardinia virus (TYLCSV). Mycorrhiza 24, 179–186. https://doi.org/10.1007/s00572-013-0527-6.
Majer, P., Hideg, E., 2012. Developmental stage is an important factor that determines the antioxidant responses of young and old grapevine leaves under UV irradiation in a green-house. Plant Physiol. Biochem. 50, 15–23. https://doi.org/10.1016/j.plaphy.2011.09.018.
Marumo, K., Waite, J.H., 1986. Optimization of hydroxylation of tyrosine and tyrosine-containing peptides by mushroom tyrosinase. BBA-Protein Struc. M. 872 (1–2), 98–103. https://doi.org/10.1016/0167-4838(86)90152-4.
Matić, M., Vuković, R., Vrandečić, K., Štolfa Čamagajevac, I., Ćosić, J., Vuković, A., Dvojković, K., Novoselović, D., 2022. Defense response to Fusarium infection in winter wheat varieties, varying in FHB susceptibility, grown under different nitrogen levels. Agronomy 12, 1746. https://doi.org/10.3390/agronomy12081746.

Mayer, Z., Duc, N.H., Sasvári, Z., Posta, K., 2017. How arbuscular mycorrhizal fungi influence the defense system of sunflower during different abiotic stresses. Acta Biol. Hung. 68 (4), 376–387. https://doi.org/10.1556/018.68.2017.4.4.

McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L., Swan, J.A., 1990. A new method which gives and objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. 115, 495–501. https://doi.org/10.1111/j.1469-8137.1990.tb00476.x.

Medrano-Padial, C., Puerto, M., Richard, T., Cantos-Villar, E., Pichardo, S., 2021. Protection and reversion role of a pure stilbene extract from grapevine shoot and its major compounds against an induced oxidative stress. J. Funct.Foods 79, 104393. https://doi.org/10.1016/j.jff.2021.104393.

Miozzi, L., Vaira, A.M., Catoni, M., Fiorilli, V., Accotto, G.P., Lanfranco, L., 2019. Arbuscular mycorrhizal symbiosis: plant friend or foe in the fight against viruses? Front. Microbiol. 10, 1238. https://doi.org/10.3389/fmicb.2019.01238.

Miozzi, L., Vaira, A.M., Brilli, F., Casarin, V., Berti, M., Ferrandino, A., Nerva, L., Accotto, G.P., Lanfranco, L., 2020. Arbuscular mycorrhizal symbiosis primes tolerance to cucumber mosaic virus in tomato. Viruses 12, 675. https://doi.org/10.3390/v12060675.

Murshed, R., Lopez-Lauri, F., Keller, C., Monnet, F., Sallanon, H., 2008. Acclimation to drought stress enhances oxidative stress tolerance in *Solanum lycopersicum* L. fruits. Plant Stress 2, 145–151.

Nakano, Y., Asada, K., 1981. Hydrogen Peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880. https://doi.org/10.1093/oxfordjournals.pcp.a076232.

Nemec, S., Myhre, D., 1984. Virus-*Glomus etunicatum* interactions in Citrus rootstocks. Plant Dis. 68, 311–314. https://doi.org/10.1094/PD-68-311.

Nerva, L., Giudice, G., Quiroga, G., et al., 2022. Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs. Biol. Fertil. Soils 58, 17–34. https://doi.org/10.1007/s00374-021-01607-8.

Pang, C.H., Wang, B.S., 2010. Role of ascorbate peroxidase and glutathione reductase in ascorbate–glutathione cycle and stress tolerance in plants. In: Anjum, N., Chan, M.T., Umar, S. (Eds.), Ascorbate-Glutathione Pathway and Stress Tolerance in Plants. Springer, Dordrecht. https://doi.org/10.1007/978-90-481-9404-9_3.

Pasquini, D., Zampieri, E., Ioannou, A., Spanos, A., Sillo, F., Giovannini, L., Fotopoulos, V., Brunetti, C., Lumini, E., Balestrini, R., 2023. Impact of the arbuscular mycorrhizal fungal inoculation on growth and biochemical parameters in *Rosmarinus officinalis* and *Lavandula angustifolia*. Symbiosis 91, 107–117. https://doi.org/10.1007/s13199-023-00946-4.

Perrone, I., Chitarra, W., Boccacci, P., Gambino, G., 2017. Grapevine–virus–environment interactions: an intriguing puzzle to solve. New Phytol. 213 (3), 983–987. https://doi.org/10.1111/nph.14271.

Porta, H., Rocha-Sosa, M., 2002. Plant lipoxygenases. Physiological and molecular features. Plant Physiol. 130 (1), 15–21. https://doi.org/10.1104/pp.010787.

Raza, A., Charagh, S., Abbas, S., Hassan, M.U., Saeed, F., Haider, S., Sharif, R., Anand, A., Corpas, F.J., Jin, W., Varshne, R.K., 2023. Assessment of proline function in higher plants under extreme temperatures. Plant Biol. 25, 379–395. https://doi.org/10.1111/plb.13510.

Pozo, M.J., Azcón-Aguilar, C., 2007. Unraveling mycorrhiza-induced resistance. Curr. Opin. Plant Biol. 10, 393–398. https://doi.org/10.1016/j.pbi.2007.05.004.

Racker, E., 1955. Glutathione reductase from bakers' yeast and beef liver. J. Biol. Chem. 217, 855–865. https://doi.org/10.1016/S0021-9258(18)65950-2.

Rosahl, S., 1996. Lipoxygenases in plants–their role in development and stress response. Z. Naturforsch. C Biosci. 51 (3–4), 123–138. https://doi.org/10.1515/znc-1996-3-401.

Ruiz-Sánchez, M., Aroca, R., Muñoz, Y., Polón, R., Ruiz-Lozano, J.M., 2010. The arbuscular mycorrhizal symbiosis enhances the photosynthetic efficiency and the antioxidative response of rice plants subjected to drought stress. J. Plant Physiol. 167 (11), 862–869. https://doi.org/10.1016/j.jplph.2010.01.018.

Sahu, P.K., Jayalakshmi, K., Tilgam, J., Gupta, A., Nagaraju, Y., Kumar, A., Hamid, S., Vardhan Singh, H., Minkina, T., Rajput, V.D., Singh Rajawat, M.V., 2022. ROS generated from biotic stress: effects on plants and alleviation by endophytic microbes. Front. Microbiol. 13, 1042936. https://doi.org/10.3389/fpls.2022.1042936.

Schreiner, R.P., 2005. Mycorrhizas and mineral acquisition in grapevines. In: Christensen, L.P., Smart, D.R., Am, Enol, J., Viticult (Eds.), Proceedings of the Soil Environment and Vine Mineral Nutrition Symposium, pp. 49–60.

Sgherri, C., Ranieri, A., Quartacci, M.F., 2013. Antioxidative responses in *Vitis vinifera* infected by grapevine fanleaf virus. J. Plant Physiol. 170 (2), 121–128. https://doi.org/10.1016/j.jplph.2012.09.016.

Siegel, B.Z., Galston, A.W., 1967. The isoperoxidases of Pisum sativum. Plant Physiol. 42 (2), 221–226. https://doi.org/10.1104/pp.42.2.221.

Singh, I., Giri, B., 2017. Arbuscular mycorrhiza mediated control of plant pathogens. In: Mycorrhiza - Nutrient Uptake. Biocontrol, Ecorestoration – Springer Cham, pp. 131–160. https://doi.org/10.1007/978-3-319-68867-1_7.

Spagnoletti, F.N., Balestrasse, K., Lavado, R.S., Giacometti, R., 2016. Arbuscular mycorrhiza detoxifying response against arsenic and pathogenic fungus in soybean. Ecotoxicol. Environ. Saf. 133, 47–56. https://doi.org/10.1016/j.ecoenv.2016.06.012.

Stolyarchuk, I.M., Shevchenko, T.P., Polischuk, V.P., Kripka, A.V., 2009. Virus infection course in different plant species under influence of arbuscular mycorrhiza. Microb. Biotechnol. 6, 70–75. https://doi.org/10.18524/2307-4663.2009.3(7).103120.

Thiem, D., Szmidt-Jaworska, A., Baum, C., Muders, K., Niefojadlo, K., Hrynkiewicz, K., 2014. Interactive physiological response of potato (*Solanum tuberosum* L.) plants to fungal colonization and Potato virus Y (PVY) infection. Acta Mycol. 49, 291–303. https://doi.org/10.5586/am.2014.015.

Torres, N., Antolín, M.C., Garmendia, I., Goicoechea, N., 2018. Nutritional properties of Tempranillo grapevine leaves are affected by clonal diversity, mycorrhizal symbiosis and air temperature regime. Plant Physiol. Biochem. 130, 542–554. https://doi.org/10.1016/j.plaphy.2018.08.004.

Trouvelot, S., Bonneau, L., Redecker, D., van Tuinen, D., Adrian, M., Wipf, D., 2015. Arbuscular mycorrhiza symbiosis in viticulture: a review. Agron. Sustain. Dev. 35, 1449–1467. https://doi.org/10.1007/s13593-015-0329-7.

Valletta, A., Iozia, L.M., Leonelli, F., 2021. Impact of environmental factors on stilbene biosynthesis. Plants 10 (1), 90. https://doi.org/10.3390/plants10010090.

Verma, S., Dubey, R.S., 2003. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci. 164, 645–655. https://doi.org/10.1016/S0168-9452(03)00022-0.

Wallis, C.M., Sudarshana, M.R., 2016. Effects of Grapevine red blotch-associated virus (GRBaV) infection on foliar metabolism of grapevines. C. J. Plant Pathol. 38 (3), 358–366. https://doi.org/10.1080/07060661.2016.1227374.

Wu, H.H., Zou, Y.N., Rahman, M., Ni, Q.D., Wu, Q.S., 2017. Mycorrhizas alter sucrose and proline metabolism in trifoliate orange exposed to drought stress. Sci. Rep. 7, 42389. https://doi.org/10.1038/srep42389.

Ye, Q., Wang, H., Li, H., 2023. Arbuscular mycorrhizal fungi enhance drought stress tolerance by regulating osmotic balance, the antioxidant system, and the expression of drought-responsive genes in *Vitis vinifera* L. Aust. J. Grape Wine Res., 208341 https://doi.org/10.1155/2023/7208341.

Zou, Y.N., Wu, Q.S., Huang, Y.M., Ni, Q.D., He, X.H., 2013. Mycorrhizal-mediated lower proline accumulation in poncirus trifoliata under water deficit derives from the integration of inhibition of proline synthesis with increase of proline degradation. PLoS One 8 (11), e80568. https://doi.org/10.1371/journal.pone.0080568.

3.4. **Publication IV**. : “*Hormonal changes associated with arbuscular mycorrhizal fungi indicate defense-like alterations in virus-stressed grapevine*”

Received: 13 November 2024 | Revised: 3 February 2025 | Accepted: 6 February 2025

DOI: 10.1111/ppl.70136

ORIGINAL RESEARCH

Physiologia Plantarum

Hormonal changes associated with arbuscular mycorrhizal fungi indicate defense-like alterations in virus-stressed grapevine

Emanuel Gaši[1] | Matevž Likar[2] | Vicent Arbona[3] | Miguel González-Guzmán[3] | Katarina Hančević[1] | Raffaella Balestrini[4] | Mate Čarija[1] | Marjana Regvar[2] | Giorgio Gambino[5] | Fabiano Sillo[5] | Tomislav Radić[1]

[1]Institute for Adriatic Crops and Karst Reclamation, Dept. Plant Sciences, Split, Croatia

[2]Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

[3]Dept. Biologia, Bioquímica I Ciències Naturals, Universitat Jaume I, Castelló, de La Plana, Spain

[4]Institute of Biosciences and Bioresources, National Research Council (IBBR-CNR), Bari, Italy

[5]Institute for Sustainable Plant Protection, National Research Council (IPSP-CNR), Torino, Italy

Correspondence
Tomislav Radić,
Email: tomislav.radic@krs.hr

Funding information
Hrvatska Zaklada za Znanost, Grant/Award Numbers: DOK-2021-02-2381, IP-2020-02-8397

Edited by F. Perrine-Walker

Abstract

Grapevine is an economically important crop, affected by major production losses due to high virus prevalence. Arbuscular mycorrhizal fungi (AMF) can reduce the impact of plant biotic stresses. However, hormonal response to the simultaneous presence of viruses and AMF remains largely unknown. In this study, we explored the potential of AMF to modify the grapevine's defense response to compatible virus infections. We used GRSPaV, GLRaV-3, and GPGV as infectious viral agents, separately or in different combinations. Two AMF inoculums were tested for their bioprotective abilities, RHIZ (*Rhizophagus irregularis)* and MIX (*R. irregularis*, *Funneliformis mosseae*, *F. caledonium*). Generally, MIX induced stronger physiological responses than RHIZ inoculum, especially during the earlier phase of symbiosis. The main findings were connected to the hormonal profile of the grapevine infected by all three viruses and inoculated with MIX. In particular, salicylic acid (SA) and abscisic acid (ABA) concentrations were induced five and fifteen months post AMF inoculation, respectively. Expressions of *VvNCED1* and *VvBG1* were up-regulated in uninoculated grapevines, indicating slower induction of stress response mechanisms. Parameters related to plant vigour and growth were induced in grapevine at both time points, regardless of the virus combination. In conclusion, the defense-like response induced by AMF in grapevines infected with multiple viruses is characterized by the induction of ABA and SA, accompanied by a consistent enhancement of vigor parameters. This study confirms AMF symbiosis as a potentially promising additional tool for combating viral diseases in vineyards.

1 | INTRODUCTION

Grapevine (*Vitis vinifera* L.) is a plant of great value, monetary and culturally, incentivizing farmers across vine-growing regions of the world. However, challenges such as pronounced weather extremes and pathogens impact grapevine health, inducing plant stress with unfavorable physiological changes (Aguilera et al., 2022). A major problem in grapevine production stems from virus-induced diseases. Grapevine serves as a host to over a hundred viruses discovered to date (Fuchs, 2024). Few known viruses are causal agents of grapevine

 © 2025 Scandinavian Plant Physiology Society.

diseases, resulting in a drastic reduction of productivity or grape quality (Fuchs, 2020). Additionally, grapevine infected with viruses can have a minimal negative impact during optimal growing conditions. Still, the burden of virus disease during stressful conditions (e.g., drought, saline stress, herbivory, etc.) can have detrimental effects (El Aou-ouad et al., 2016; Sadras et al., 2024). Therefore, mitigating the negative virus impact on the grapevine host in an environmentally conscious way is very important. Microorganisms can have great potential to influence crop production by potentially managing the negative effects of abiotic and biotic stress on plant health and vigor (González Guzmán et al., 2022). Additionally, arbuscular mycorrhizal fungi (AMF) have been used as mutualistic microorganisms with promising effects on plants facing different stresses. Stress induced by salinity, drought, and heat, but also by biotic factors such as fungal pathogens and nematodes, has been reported to be mitigated by AMF (Belval et al., 2024; Hao et al., 2018; Liu et al., 2023; Nogales et al., 2020; Zhang et al., 2024).

Interactions of AMF with plant viruses have been investigated in a few herbaceous plant species, such as tomato (Miozzi et al., 2020), potato (Deja-Sikora et al., 2023), and cucumber (Metwally et al., 2024), but very rarely have woody perennials been studied. Defining the AMF-induced viral defense through reduced viral titer and reduced symptom development brought some clarity and resulted in binary concepts of susceptibility and resistance as scenarios of the host response to AMF-virus interaction (Deja-Sikora et al., 2019; Hao et al., 2019; Miozzi et al., 2019). So far, the results of multitrophic interactions between the endophyte and the virus have been variable, underpinning the influence of different factors that shape the outcomes of the interactions studied. Additionally, AMF shows high functional variability that varies between and within species, based on intraspecific variations, making AMF impact on plant health and vigor species-specific (Koch et al., 2017; Munkvold et al., 2004). In accordance, *Rhizophagus irregularis* and *Funneliformis mosseae* both showed beneficial responses in virus-stressed potatoes, but *R. irregularis* is more successful in colonizing the roots, inducing better growth response, and mitigating oxidative stress than *F. mosseae* (Deja-Sikora et al., 2023). However, low specificity and high affinity of AMF symbiosis leads to co-occurrence of multiple AMF within the same hosts, with multiple-species inoculation frequently being reported as more advantageous than a single-species counterpart (Frew, 2021; Jansa et al., 2008). The proposal of a 'health triangle' representing a more inclusive approach that takes into consideration the simultaneous existence of pathogenic and beneficial relationships defining plant health is a more holistic approach to understanding plant health (Leveau, 2024). This shift in paradigm represents an important step in understanding the nuances defining the pathogen-host-beneficial microbe interaction.

Since the majority of vineyards worldwide are affected by various viruses (Fuchs, 2020) and at the same time, grapevine is very prone to form arbuscular mycorrhizae spontaneously and may have considerable benefits from it (Trouvelot et al. 2015), grapevine-virus-AMF interaction deserves essential attention. However, pre-invasive defense strategies of stomatal closure or cell wall fortification that would be effective against some fungal and bacterial pathogens tend to be ineffective for viruses since the grapevine virus invasion strategy implies direct delivery to the phloem sap (Armijo et al., 2016; Ton et al., 2009). In grapevine, indirect virus-AMF interaction was studied through the nematode vector *Xiphinema index* (Hao et al., 2018). We have recently studied tripartite interactions among viruses-AMF-grapevine, showing that AMF symbiosis modulates oxidative stress responses in virus-infected plants (Radić et al., 2024). However, there is a need to provide a deeper understanding of this tripartite interaction. Exploring the hormonal profile of grapevine is an essential segment for unravelling the virus-induced defense and disentangling the outcome of multitrophic interactions on grapevine health. For example, abscisic acid (ABA) is involved in plant protection against pathogens. Still, its influence on plant defense is dependent on the type of pathogen and mode of pathogen entry into the host (Ton et al., 2009). Hormonal activity is crucial for signaling a timely defense response to the pathogen attack but also for facilitating and sustaining the mycorrhizal symbiosis (Foo et al., 2013; Islam et al., 2019; Ludwig-Müller, 2010; Zhao and Li, 2021). Since grapevine is very prone to virus diseases, the process of 'priming' of the grapevine host by AMF to convey a pre-invasion protective effect is hardly possible in already established vineyards. Simultaneously, roguing the entire vineyard is laborious and costly, making it an undesirable practice for small growers. Whether subsequent AMF inoculation of already virus-infected grapevine may have a beneficial impact on grapevine and its potential use in the vineyards remains an open question.

The high virus prevalence combined with grapevine strong tendency to form arbuscular mycorrhiza and their widespread interactions in real agroecosystems make such systems highly valuable for studying multitrophic interactions and their effects on host physiology. The primary focus of this study was to investigate whether the subsequent addition of AMF to grapevines already infected with viruses could yield measurable benefits for grapevine hormonal balance and growth. If successful, this approach could provide an additional tool to mitigate virus-induced damage in viticulture. We examined the responsiveness of virus-infected grapevines to AMF addition, gaining insights into specific changes in hormonal profiles and growth at two different time points. For this purpose, we used the grapevine rupestris stem-pitting associated virus (GRSPaV) either alone or in combination with the grapevine leafroll associated virus 3 (GLRaV-3) and the grapevine Pinot Gris virus (GPGV) to induce stress. AMF inoculum, consisting of either single or multiple mycorrhizal species, was used to assess their beneficial effects on grapevine physiology. We hypothesized that the hormonal profile of virus-infected grapevine will change with the addition of mycorrhizal inoculum, reflecting defense-like responses. Further, we expected that inoculum containing multiple species, whether viral or AMF, would exert a more significant influence on grapevine physiology compared to single-species inoculum. Additionally, AM symbiosis is expected to significantly improve grapevine fitness and growth parameters regardless of virus infection.

2 | MATERIALS AND METHODS

2.1 | Experimental setup

The experimental setup was described in the previous publications in detail (Gaši et al., 2023; Radić et al., 2024) and a detailed overview is given in Figure 1. Briefly, scions of Merlot (*Vitis vinifera* L., clone 0343) and rootstocks of Kober 5BB (*Vitis berlandieri* Planch. × *Vitis riparia* Michx., clone 0259; Vitipep's, Sarrians) were grafted and planted in 6 L pots (L15 cm - W15 cm - H25 cm) containing sterilized soil, peat, perlite and quartz sand (1:1:1:1/3) and kept in a greenhouse at the Institute of Adriatic Crops and Karst Reclamation, Split, Croatia. Grapevine wood scrapings were subjected to preliminary virus screening of the most common grapevine viruses (Table S1). For the total RNA extraction, the CTAB method was used (Gambino, 2015), following cDNA synthesis with M-MLV Reverse Transcriptase (Thermo Fisher Scientific). Virus screening details and RT-qPCR cycling conditions were described in Gambino et al., (2011). Only virus-free and GRSPaV-infected grapevine plants were used further in the experimental setup (Gaši et al., 2023; Radić et al., 2024). For the final virus setup, grapevine was infected with GLRaV-3 and/or GPGV through chip-budding with plant material of known sanitary status (Hančević et al., 2021) and the infection was confirmed by RT-qPCR screening of Merlot shoots following the protocol described in Gambino et al., (2011). The last step of setting up the treatments was the introduction of AMF into the substrate as described in detail in prior work (Table S2; Gaši et al., 2023). Two types of AMF inoculum used were: *Rhizophagus irregularis* (RHIZ) DAOM197198 (Symplanta LLC) and a mixture of *R. irregularis*, *Funneliformis mosseae* and *F. caledonium* (MIX; Inoq LLC). Controls were mock inoculated with autoclaved AMF inoculum. Overall, fifteen treatments were designed, each represented with six biological replicates, and placed in a random block design. The sampling was done based on previous established work considering the virus and AMF treated grapevine plants used as treatments (Table S2; Radić et al., 2024). Mycorrhizal colonization was performed by a microscopic investigation of 'Trypan blue' - treated roots (Brundrett et al., 1996). Plants with AMF-colonized roots, confirmed two to five months after the inoculation, were used further in the study, as described in previous works (Gaši et al., 2023; Radić et al., 2024). Samplings were carried out five- and fifteen- months post AMF inoculation, in the years 2022 and 2023, respectively. For most analyses, fully developed mature leaves between the 3rd and 7th nodes were sampled. Additionally, the fine roots were sampled for the expression analysis of root-related genes (see at the 2.5 section). Samples were analysed in triplicates, regardless of tissue type.

2.2 | Plant hormone profiling of grapevine leaves

Plant hormone profiling of salicylic acid (SA), indole-3-acetic acid (IAA), isopentenyl adenine (iP), isopentenyl adenosine (iPR), abscisic acid (ABA), jasmonic acid (JA), jasmonil isoleucine (JA-Ile) and phaseic acid (PA) was carried out by LC/MS as described in De Ollas et al. (2021). Three biological replicates per treatment were prepared from freeze dried plant material (c.a. 15 mg per extraction). Before extraction, plant samples were spiked with specific amounts of $^{2}H_{6}$-ABA, ^{13}C-SA, $^{2}H_{2}$-iPR, $^{2}H_{5}$-IAA, and dihydro jasmonic acid as internal standards to correct for analyte loss. Extraction was carried out in 1 mL ultrapure water for 10 min in a ball mill at room temperature using 2 mm glass beads. After the extraction, homogenates were centrifuged at 4700 × *g* for 10 min at 4°C and the supernatants were recovered. The resulting solutions were partitioned twice against an equal volume of di-ethyl ether after adjusting pH to 3.0 with a 30% acetic acid solution. The combined organic layers were evaporated under vacuum in a centrifuge concentrator (Jouan) and the dry residues were reconstituted in 0.5 mL of a

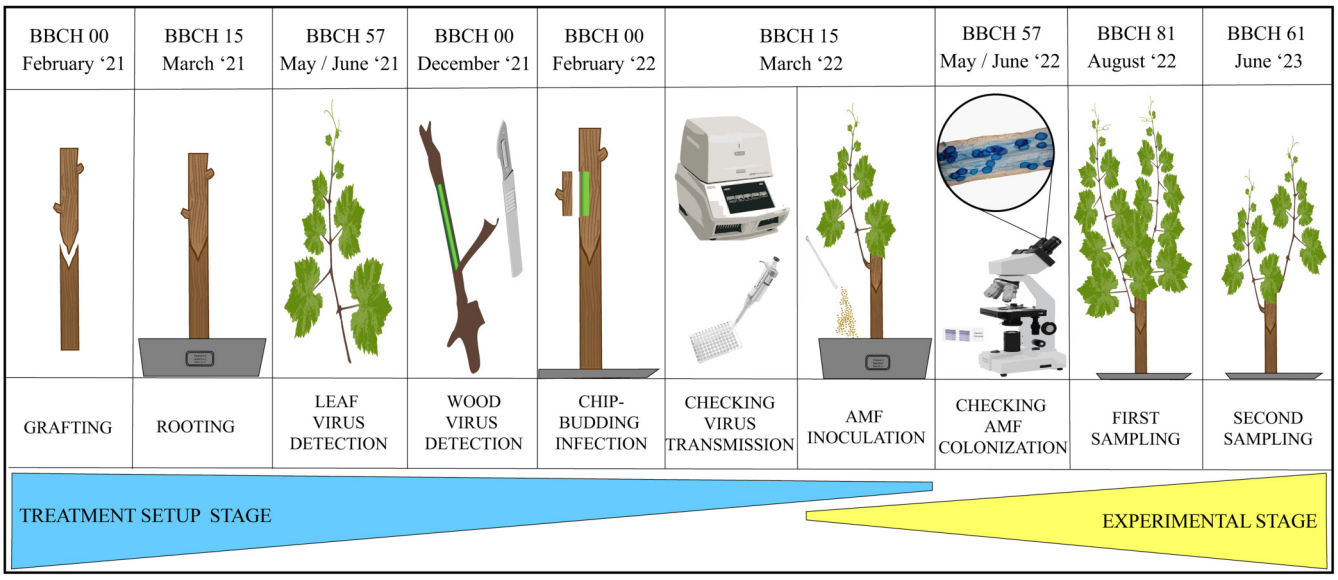


FIGURE 1 The detailed schematic overview of the experimental setup and sampling of grapevine tissues. Each phase is described with a phenological stage of the grapevine, the date, a graphical and textual description of the main task performed.

10% aqueous methanol solution. Prior to injection, extracts were filtered through a 0.20 µm PTFE syringe membrane and the filtrates were recovered in chromatography amber glass vials. The samples were analysed by tandem LC/MS in an Acquity SDS UPLC system (Waters Corp.) coupled to a triple quadrupole mass spectrometer (Xevo TQ-S, Micromass Ltd.) through an electrospray ionization source. Separations were carried out on a C18 column (Luna Omega Polar C18, 50 × 2.1 mm, 1.6 µm particle size, Phenomenex) using a linear gradient of ultrapure acetonitrile and water, both supplemented with formic acid to a 0.1% (v/v) concentration, at a constant flow rate of 0.3 mL min^{-1}. During analyses, the column temperature was maintained at 40°C and the samples were maintained at 10°C to slow down degradation. Plant hormones were detected in negative electrospray mode following their specific precursor-to-product ion transitions and quantitated using an external calibration curve with standard samples of known amount.

2.3 | Element and pigment analysis of grapevine leaves

For the analysis of the elements in the grapevine tissue, fully developed leaves were lyophilized and 500 mg of sample was burned at 550°C for 5 h in the muffle furnace. The ash samples were dissolved in 2 mL of HCl and diluted with distilled water for the final volume of 50 mL. Concentration of phosphorus was quantified following the method by Olsen and Sommers (1982). For quantification of the potassium concentration, the flame photometer (Model 410, Sherwood) was used. Concentrations of all other elements (Zn, Mn, Cu, Fe, Mg, Ca) were measured using an atomic absorption spectrometer (Spectraa 220, Varian). Chlorophyll *a*, chlorophyll *b* and total carotenoids were quantified spectrophotometrically using the method by Lichtenthaler (1987). Briefly, pigments were extracted overnight from 10 mg of lyophilized and pulverized leaf material using 95% ethanol and extracts were used to measure absorbance at 470 nm, 647 nm and 663 nm.

2.4 | Grapevine ecophysiological parameters

Measurements of leaf gas exchanges were performed in the period between 09:00 and 12:00 h on fully developed leaves between the 5th and 8th shoot from the base. Measurements of photosynthetic parameters were done using an open gas exchange system Li-6400 (Li-Cor. Inc.). Li-Cor parameters used in this study were net photosynthesis rate (A_N), leaf transpiration (E), intercellular CO_2 concentration (Ci) and conductance to water (gs). The measurement was performed with device parameters set up as described in Gaši et al. (2023). The measurement of leaf water potential (ψ leaf) was quantified using the pressure chamber model 670 (PMS Instrument Company) and values were expressed in bars. Finally, differences in grapevine growth were quantified by measuring stem length (DS), node number (IS) and average internode length (DI) calculated as the ratio of the stem length divided by the number of nodes.

2.5 | RNA extraction and gene expression analysis

For gene expression analysis, fully developed leaves and fine grapevine roots were sampled. Total RNA was extracted from 100 mg per sample using the CTAB protocol (Gambino, 2015). Purity and concentrations of the extracts were assessed on a Nanodrop One (Thermo Fisher Scientific). DNA contamination was removed using the TURBO DNA-free Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Absence of genomic DNA was checked by quantitative real time PCR (qPCR) before the cDNA synthesis, using *VvUBI* primers (Gambino et al., 2011). First-strand cDNA synthesis was done by using the SuperScript II Reverse Transcriptase kit (Thermo Fisher Scientific) and 250 ng of purified RNA extracts. Relative quantification was performed with CFX96 Touch Real-Time PCR (Bio-Rad) by using specific primers for the targeted genes (Table S3). Specifications of the PCR mix and the cycling conditions for the RT-qPCR are explained in detail in Gaši et al. (2024). Specific annealing of the primers was controlled on dissociation kinetics performed at the end of each PCR run. Ubiquitin (*VvUBI*) and actin (*VvACT*) were used as reference genes (Gambino et al., 2011) and the expression of transcripts was quantified after normalization to the geometric mean of the two reference genes. For statistical analysis of the differences in relative quantification of the genes of interest, ΔCt was calculated by subtracting the Ct value from the gene of interest from the geometric mean of the two reference genes e.g. ΔCt = GEOMEAN [Ct (*VvACT*) + Ct (*VvUBI*)] − Ct (gene of interest). Expressions of 9-cis-epoxy carotenoid dioxygenase 1 (*VvNCED1*), beta-glucosidase 1 (*VvBG1*), ABA 8′-hydroxylase (*VvABA8OH1*), callose synthase (*VvCAS2*), Abscisate Beta-Glucosyltransferase-like (*VvGT*), Chitinase III (*VvChitIII*), Phosphate transporter 1–3 (*VvPT1-3*) and Sugar transporter 13 (*VvSTP13*) were measured (Table S3).

2.6 | Statistical analysis

For statistical analysis, R v4.2.0, https://www.Rproject.org/ (R Foundation for Statistical Computing) was used. The non-metric multidimensional scaling (NMDS) was performed using the *vegan* (v2.6–6; Oksanen et al., 2024) library. The differences between the grouping variables were evaluated with a permutational multivariate analysis of variance based on 999 permutations (perMANOVA, adonis2 function in the vegan library). Two-way ANOVA was performed to test for treatment factors (virus, AMF and interaction) and the differences between means were considered significant if $p < 0.05$. The significant differences in treatments were examined based on the Tukey's post-hoc test. The correlation matrices were created using the *corrplot* (v0.92; Wei & Simko, 2024) library. Data visualization of the ordination methods, correlation matrices and boxplots with summary statistics were done using the *ggplot2* (v3.5.1; Wickham et al., 2019), *ggpubr* (v.0.6.0; Kassambara, 2023) and *multicompview* (v0.1–10; Graves et al., 2024) libraries.

3 | RESULTS

Based on the NMDS analysis, distinct differences in treatment effects were observed at two sampling points, with older AMF symbiosis showing more pronounced changes (Figure 2). Two parts of the treatment setup, AMF inoculum and virus composition, were both contributing factors in the visual separation of the parameters, with the hormonal profile being an important factor differing between the sampling years.

3.1 | Hormonal changes and ABA homeostasis in AMF-inoculated, virus-infected grapevine

Comparing all the treatments, we observed pronounced hormonal changes in treatments inoculated with MIX AMF, and the strongest response was in the three-virus coinfection (RLP, Figure 3). After the first five months from AMF inoculation, a transient SA increase was measured in the MIX AMF treatments, followed by a statistically insignificant but noticeable ABA decrease (Figure S1A, B). Additionally, grapevine containing GPGV, showed reduced iP and increased IAA concentrations following MIX AMF inoculation (Figure S1C, D). Fifteen months post AMF inoculation, we observed different hormonal changes than in the early, five-month old AMF symbiosis. Once again, MIX AMF and mix virus (RLP) had the most pronounced changes led by a powerful induction of ABA and PA (Figure 2A, B) and a strong positive correlation between the two (Figure S2). Since ABA response was drastically induced in some mycorrhizal grapevine, the expression of genes involved in different parts of ABA biosynthesis and catabolism was investigated. The Vv*NCED1* gene was measured in both grapevine leaves and roots, and was heightened in all virus treatments and in both tissue types. Generally, we observed that genes involved in ABA biosynthesis were up regulated in uninoculated grapevine (Figure 3C, D), but showed low overall concentrations of active ABA and PA in the leaves. However, ABA and PA concentrations were higher in AMF-inoculated grapevine, particularly in treatments harbouring GPGV (Figure 3A, B). Additionally, β- glucosidase homolog 1 (Vv*BG1*) and glucosyltransferase (Vv*GT*) expressions were significantly higher in uninoculated than in AMF inoculated grapevine carrying GPGV (Figure 3E, F). Other phytohormones did not show meaningful changes, but SA was induced in MIX grapevine infected with RL, RP and RLP as opposed to unifected grapevine (Figure S3).

3.2 | Vigour parameters and physiological changes of AMF - virus interplay in grapevine host

All analyses were repeated in both samplings, five and fifteen months after AMF inoculation, with additional gene expressions for investigating the ABA homeostasis. Fifteen months post AMF inoculation, the NMDS analysis revealed clear AMF inoculum-based differences but also showed virus-based data separation (Figure S4). The parameters that had an influence on the overall differences in treatments were also connected to the plant vigour parameters. Mycorrhizal symbiosis drastically induced heightened photosynthesis rates, increased stem length, and leaf phosporous content (Figure 4A-C). Similarly, only five

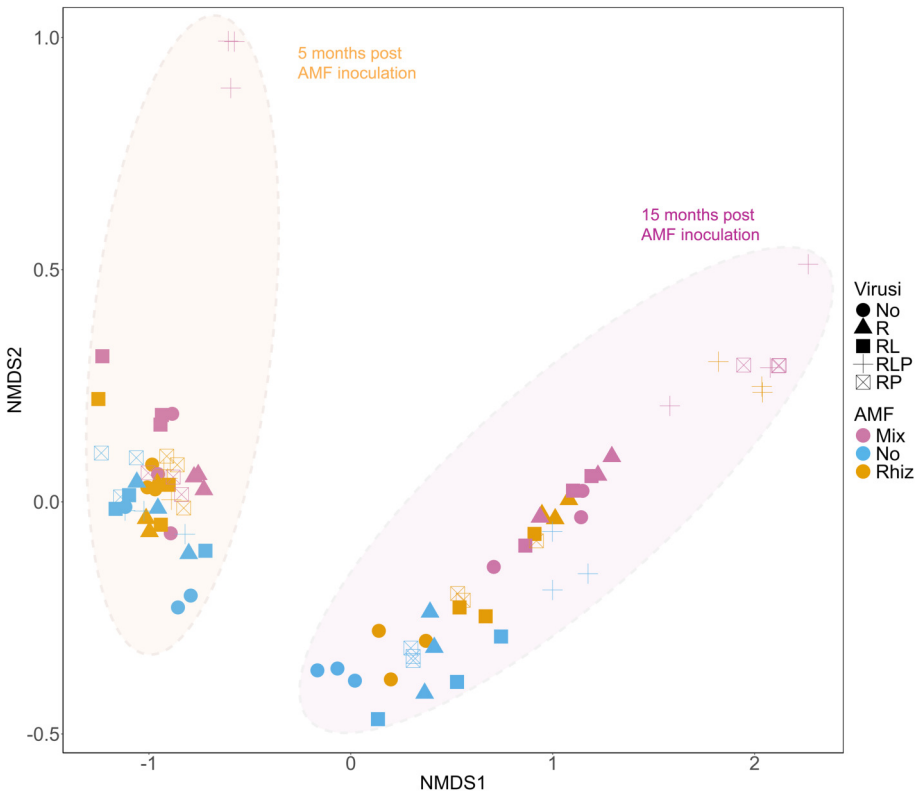


FIGURE 2 The non-metric multidimensional scaling (NMDS) analysis of parameters measured in the two-year experimental period based on Euclidean distance matrix. The differences between the grouping variables were evaluated with perMANOVA ($p < 0.001$) based on 999 permutations, $n = 3$. Abbreviations: NO, no virus; NO-AMF, plants without mycorrhizal symbiosis; MIX, plants inoculated with *R. irregularis*, *Funneliformis mosseae* and *F. caledonium*; R, GRSPaV; RHIZ, plants inoculated with *Rhizophagus irregularis*; RL, GRSPaV and GRLaV-3; RLP, GRSPaV, GRLaV-3 and GPGV; RP, GRSPaV and GPGV.

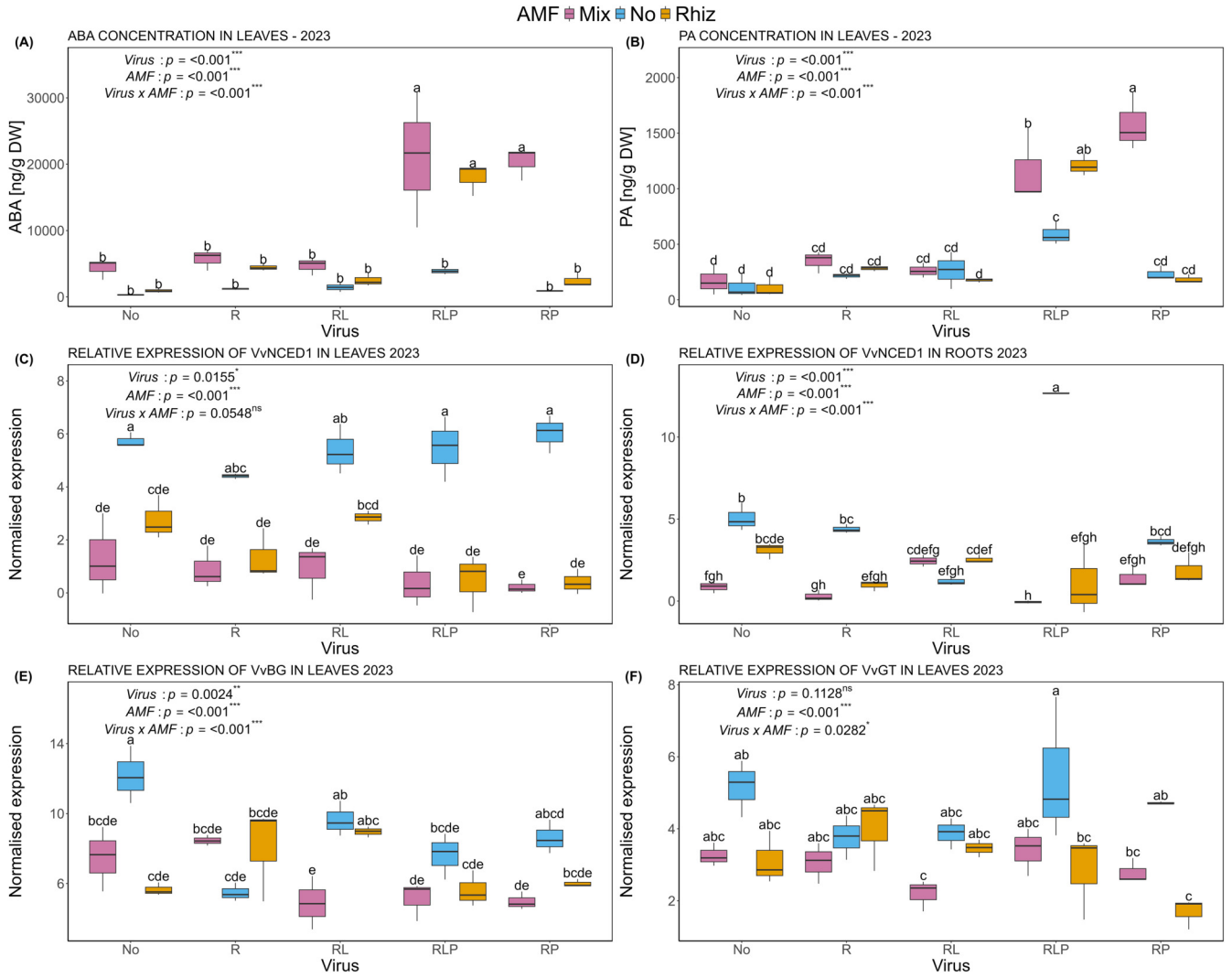


FIGURE 3 Boxplots of ABA related parameters that showed significant differences between treatments in the second sampling in 2023. Two-way ANOVA and Tukey post-hoc test were used to calculate the significance ($p < 0.05$) and the differences between treatments are represented with different lowercase letters. Parameters presented here are as follows: (A) concentration of ABA, Abscisic acid, (B) concentration of PA, Phaseic acid, (C) expression of NCED1, 9-cis-epoxy carotenoid dioxygenase gene in the leaves, D) expression of NCED1, 9-cis-epoxy carotenoid dioxygenase gene in the roots, E) expression of BG1, β-glucosidase homolog 1gene in the leaves, F) expression of GT, glycosyltransferase gene in the leaves. $n = 3$. Abbreviations: NO, no virus; NO-AMF, plants without mycorrhizal symbiosis; MIX, plants inoculated with *R. irregularis*, *Funneliformis mosseae* and *F. caledonium*; R, GRSPaV; RHIZ, plants inoculated with *Rhizophagus irregularis*; RL, GRSPaV and GRLaV-3; RLP, GRSPaV, GRLaV-3 and GPGV; RP, GRSPaV and GPGV.

months after AMF inoculation, we already observed a beneficial effect of AMF symbiosis reflected in improved vigour and growth parameters, as described after fifteen months. Additionally, MIX AMF improved the measured plant ecophysiological parameters in more cases than a single species (RHIZ) inoculation (Figure S5).

4 | DISCUSSION

The focus of this study was to explore the mycorrhizal symbiosis capability to modify grapevines response to virus infections. Physiological parameters were used to gain insights into the biotic stress response of AMF inoculated grapevine. AMF has been noted to have a beneficial influence on plants in the form of increased nutrition and water uptake, the priming of plants defense responses and overall an increase in plant vigour and health (Darriaut et al., 2022; Zhu et al., 2022). However, plant viruses can have negative impacts on their host physiology, e.g. GLRaV-3, perturbed sugar metabolism, changes of the hormonal and antioxidative profile and overall health and homeostasis disturbance were frequently reported (Hančević et al., 2023; Mishra et al., 2020; Naidu et al., 2015). In this study, we showed differential grapevine responses to multiple virus infections, five and fifteen months after AMF inoculation.

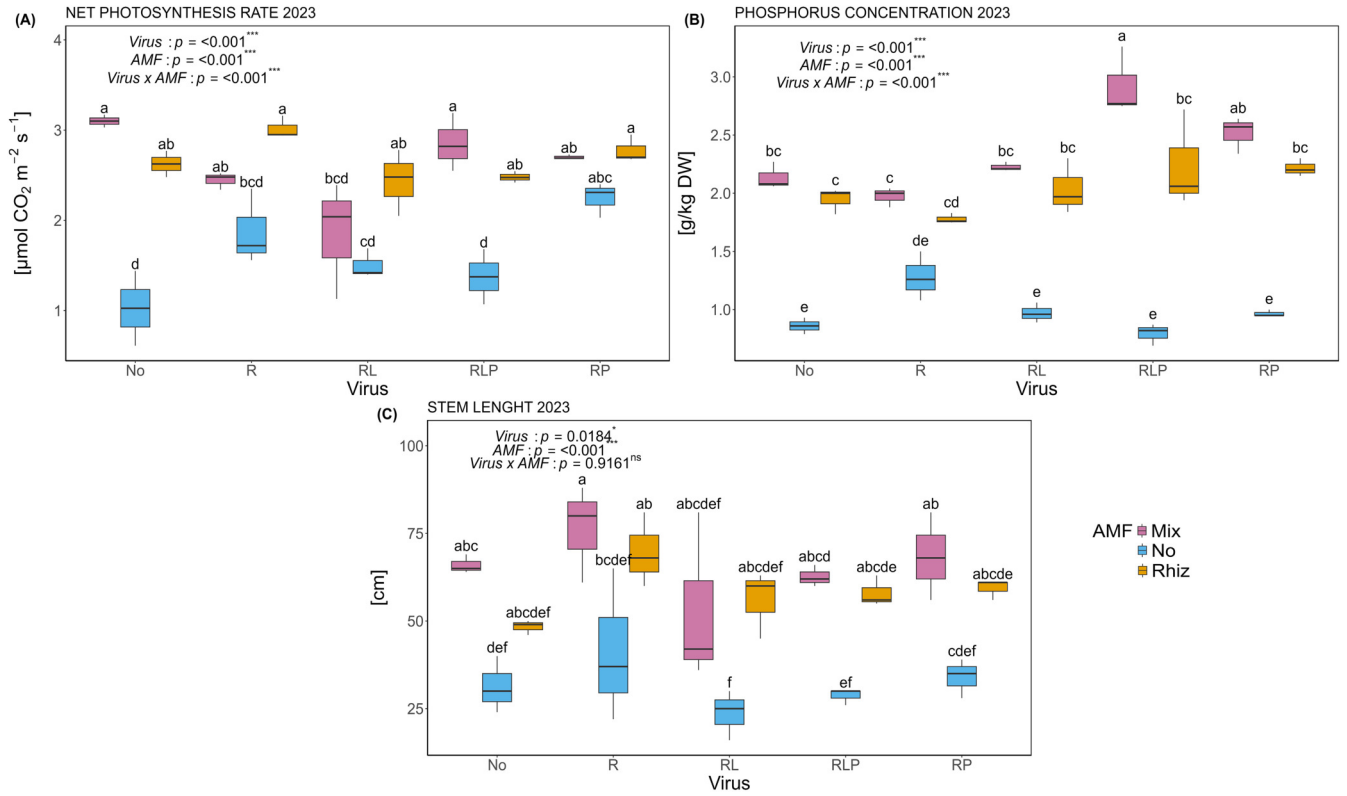


FIGURE 4 Boxplots of selected physiological and growth parameters measured in the second sampling year. Two-way ANOVA and Tukey post-hoc test was used to calculate the significance ($p < 0.05$) and the differences between the treatments are represented with different lowercase letters. Parameters presented here are as follows: (A) Net photosynthesis rate, (B) phosphorus leaf concentration and (C) stem length. $n = 3$. Abbreviations: NO, no virus; NO-AMF, plants without mycorrhizal symbiosis; MIX, plants inoculated with *R. irregularis*, *Funneliformis mosseae* and *F. caledonium*; R, GRSPaV; RHIZ, plants inoculated with *Rhizophagus irregularis*; RL, GRSPaV and GRLaV-3; RLP, GRSPaV, GRLaV-3 and GPGV; RP, GRSPaV and GPGV.

4.1 | SA and ABA are crucial in AMF - virus crosstalk after five and fifteen months, respectively

Five months post-AMF inoculation, grapevines infected with all three viruses and treated with MIX inoculum showed a defense response marked by elevated SA levels. SA is known for having a pivotal role in plants combating biotic stresses through its influence on systemic responses and pathogen identification (Klessig et al., 2018; Murphy et al., 2020; Zhang & Li, 2019). However, SA accumulation was not always connected to decreased virus titer or the reduction of symptom development (Murphy et al., 2020; Nie, 2006). Insights from prior work indicate that GRSPaV was present in similar relative concentrations in the mature grapevine leaves throughout the duration of the experiment (Gaši et al., 2024). Accumulation of SA in three-virus combinations could be seen as a mechanism of virus self-regulation processes to minimize damage in a susceptible host (Carr et al., 2019; Murphy et al., 2020). Interestingly, ABA concentration was reduced in grapevine infected with all three viruses and inoculated with MIX AMF, possibly indicating SA-ABA crosstalk in treatments with multiple viruses and AMF species (Figure S1A, B). ABA interference with the plant immunity has been shown through antagonistic interactions with SA (Pérez-Clemente et al., 2019). For MIX AMF, auxins and cytokinins showed different responses in grapevine harbouring GPGV. Auxins, regulating plant growth and development, can have a disrupted signalling by the rice dwarf virus, resulting in stunted growth (Jin et al., 2016). However, our MIX grapevine plants had induced growth regardless of the virus infection. Increased IAA concentration was also connected to an increased susceptibility to biotrophic infections through the antagonistic interaction between SA and IAA (Robert-Seilaniantz et al., 2011). However, the effect of antagonistic interactions between SA and IAA was not evident, possibly due to combined effects of both viruses and mycorrhizal inoculum.

Fifteen months after the AMF inoculation, the hormonal profiling shows the induction of ABA concentrations in RHIZ and MIX grapevine infected with all three viruses (Figure 3A). Increased ABA concentration in leaves can be connected to an early drought response (Lehr et al., 2022). Additionally, the role of ABA has been shown in biotic stress alleviation as well (Alazem & Lin, 2017; Asselbergh et al., 2008; Xie et al., 2018). Strong positive correlation between ABA and PA was expected, since PA is a catabolite of the ABA hydroxylation process (Nambara & Marion-Poll, 2005). Increased ABA and PA concentrations of virus stressed plants, when inoculated with AMF, are indicative of a physiologically responsive grapevine, possibly leading to a more resilient phenotype when exposed to biotic stress. In prior work, Radić et al., (2024) have determined a strong antioxidative enzyme activity connected to ABA increase. Specifically, guaiacol

peroxidase, polyphenol oxidase and ascorbate peroxidase activities were induced in this specific treatment (Radić et al., 2024). ABA is known to induce H_2O_2 production through RBOHD induction (Arve et al., 2014). An active antioxidative system is required for efficient ROS elimination, a process that is crucial for minimizing cell damage in plants facing virus induced stress.

Expression of genes connected to ABA biosynthesis and metabolism were used to further investigate the ABA accumulation in the grapevine leaves and roots (Figure 3C-F). Interestingly, *VvNCED1* expression, that is generally related to de novo ABA synthesis (Sah et al. 2016) in leaves and roots was higher in NOAM treatments, regardless of the virus combination used, as opposed to RHIZ or MIX inoculums. Since *VvNCED1* had low expressions in the treatments that are high in ABA and PA concentrations, the hormonal change was possibly a transient and sensitive response to environmental cues that could be a result of the AMF - virus interplay. Another mode of increase in ABA concentration is through the hydrolyzation of inactive glucosyl ester form, with β-glucosidases (*VvBG1*) having a crucial role in this process (Xu et al., 2012). The *VvBG1* gene was highly expressed in NO- and RLP- virus combinations of NOAM grapevine (Figure 3E). In these plants, ABA concentrations were low, but possibly originating more from the pool of inactive ABA. Glucosyltransferases are involved in glucosylation of ABA into ABA-GE, an inactive form of ABA (Dong et al., 2014). *VvGT* gene expression was significantly higher in NOAM than in RHIZ inoculated plants when infected with GPGV and had low concentrations of active ABA in the leaves (Figure 3F). The NOAM plants in GPGV-infected grapevine actively synthesized ABA but also effectively eliminated it by converting it into inactive ABA-GE, as indicated by low overall ABA and PA concentrations in the leaves. Few studies have pointed out the possible positive influence of ABA on the immunity of virus-infected plants (Alazem & Lin, 2017; He et al., 2023). Taken together, in this study, the role of ABA in combating virus stress was possibly through its contribution to oxidative cell homeostasis. Although ABAs role in plant defense is ambiguous, the general effect on plant immunity is stimulatory in the beginning phases of pathogen invasion (pre-invasive immunity), but an inhibitory effect is noted in the later stages of pathogen invasion, specifically for fungal pathogens and oomycetes (Cao et al., 2011; Ton et al., 2009). However, there is very limited knowledge about ABA involvement during plant virus infections (Alazem & Lin, 2017). He et al. (2023) have noted the accumulation of ABA in response to apple necrotic mosaic virus infection of *Nicotiana benthamiana*. They demonstrated a mechanism of action where virus infection triggers ABA accumulation, promoting the ABA-insensitive 5 transcription factor that represses the transcription of ATP synthase and leads to host susceptibility to virus infection.

4.2 | Grapevine physiology changes differ with duration of mycorrhizal symbiosis and virus infection

In both samplings, regardless of hormonal changes, the beneficial influence of AMF on grapevine has been noted. In particular, higher photosynthetic activity, growth parameters and higher concentrations of phosphorous in the leaf dry matter (Figure 4; Figure S5). AMF have been known to have beneficial influence on grapevine growth and vigour (Darriaut et al., 2022; Gaši et al., 2023; Trouvelot et al., 2015). However, each sampling was presented with a unique set of changes in measured parameters. Differences in response to the treatment between the years may have been due to different phenological phases of the plants for each sampling (August and June), but also due to the difference in duration of the interactions between grapevine and the microorganisms used. The differences caused by mycorrhizal inoculums were variable, but more pronounced in the five-month-old symbiosis, when the RHIZ inoculum seemed to be less effective than the MIX inoculum. Depending on the virus combination, the RHIZ inoculum failed to induce higher photosynthesis rates or phosphorus concentrations as opposed to the MIX inoculum. The differential response of used inoculums could have been due to functional differences of species being present in the inoculums. For example, the single-species AMF inoculum was shown to induce growth of *Lolium perenne* on contaminated soil, but the mixture of three AMF species did not produce the same response (Malicka et al., 2021). Balestrini et al. (2017) showed that one AMF species inoculum and mixed bacterial and fungal inoculum lead to significant transcriptional reprograming in grapevine, with unique responses between inoculums. *Glomus claroideum* and *Glomus intraradices* also induced higher phosphorus content in leek after two-month colonization with both fungi together compared to single species inoculums (Jansa et al., 2008). Additionally, virus infection can reduce phosphorus content in specific grapevine cultivars (Čarija et al., 2022), while AMF can compensate for this reduction. This further highlights the importance of AMF in mitigating the effects of viral infections in grapevines. Taking into consideration the differences in identity and composition of the mycorrhizal inoculum used, the MIX inoculum shows earlier and stronger influences on the hormonal profile of grapevine. The MIX inoculum differed from the RHIZ inoculum in the presence of two *Funneliformis* species. It has been suggested that *F. mosseae* is a less competitive species, that forms mycorrhizal symbiosis with the grapevine roots early on, without maintaining a long-lived symbiosis (Noceto et al., 2023). Further advanced analyses of the grapevine roots need to be employed to confirm the presence and duration of symbiosis with each individual species in the MIX inoculum, in order to discuss the contribution of each AMF species. However, RHIZ and MIX had similar effects on virus infected grapevines after fifteen months, indicating that the MIX inoculum might induce beneficial effects more rapidly in grapevine.

5 | CONCLUSIONS

Here we investigated grapevine subjected to virus stress and the AMF beneficial influence in a two-years greenhouse experiment. The hormone profiles were the most perturbed in grapevine harbouring GPGV or when it was accompanied with GLRaV-3 and GRSPaV viruses. Of the two AMF inoculums used, the MIX inoculum consisting of *R. irregularis*, *F. mosseae* and *F. caledonium* had a more pronounced impact on virus-stressed grapevine. In the five-month interaction

period, grapevine inoculated with mycorrhizal fungi showed defense-like responses based on the measured induction of SA. Fifteen months post AMF inoculation, virus infected grapevine showed an induction of defense through the accumulation of ABA, regardless of AMF inoculum type. Mycorrhizal inoculum has generally been shown to benefit grapevine through a heightened nutritional status and photosynthetic processes in both five- and fifteen-months post AMF inoculation. Overall, AMF symbiosis established after the virus infection could be beneficial in dealing with biotic stress through induced hormonal changes, while maintaining better growth and nutrition, making this approach a valuable alternative to time consuming and cost-heavy rouging of infected vineyards.

AUTHOR CONTRIBUTIONS

T.R. developed the concept of the study. The methodology was developed by G.G., R.B., V.A., M.G.G. Validation was performed by M.L., T.R., K.H., M.Č., G.G., M.R., R.B., V.A., F.S. and M.G.G. The formal analysis was conducted by E.G., T.R., V.A., M.G.G. and M.L. The investigation and funding was acquired by T.R. Data curation was handled by E.G., and M.L. The original draft preparation was completed by E.G., M.L., T.R. and K.H. and visualization was carried out by E.G. and M.L. Manuscript was reviewed and edited by E.G., M.L., T.R., K.H., M.Č., G.G., M.R., R.B., V.A., F.S. and M.G.G. Supervision by T.R.

ACKNOWLEDGMENTS

This research was funded by Croatian Science Foundation/Research project [IP-2020-02-8397 and DOK-2021-02-2381].

FUNDING INFORMATION

This research was funded by Croatian Science Foundation/Research project [IP-2020-02-8397 and DOK-2021-02-2381].

DATA AVAILABILITY STATEMENT

Data will be made available upon request.

ORCID

Emanuel Gaši https://orcid.org/0000-0002-2554-3162
Matevž Likar https://orcid.org/0000-0002-3086-6433
Vicent Arbona https://orcid.org/0000-0003-2232-106X
Miguel González-Guzmán https://orcid.org/0000-0003-1000-9255
Katarina Hančević https://orcid.org/0000-0001-7993-7450
Raffaella Balestrini https://orcid.org/0000-0001-7958-7681
Mate Čarija https://orcid.org/0000-0003-4004-7105
Marjana Regvar https://orcid.org/0000-0002-2623-1099
Giorgio Gambino https://orcid.org/0000-0003-1590-4478
Fabiano Sillo https://orcid.org/0000-0002-1218-9985
Tomislav Radić https://orcid.org/0000-0002-1633-6516

REFERENCES

Aguilera, P., Ortiz, N., Becerra, N., Turrini, A., Gaínza-Cortés, F., Silva-Flores, P., Aguilar-Paredes, A., Romero, J.K., Jorquera-Fontena, E., Mora, M. de L.L, & Borie, F. (2022) Application of Arbuscular Mycorrhizal Fungi in Vineyards: Water and Biotic Stress Under a Climate Change Scenario: New Challenge for Chilean Grapevine Crop, *Frontiers in Microbiology*, 13, 826571

Alazem, M. & Lin, N.-S. (2017) Antiviral Roles of Abscisic Acid in Plants. *Frontiers in Plant Science*, 8, 1760

Armijo, G., Schlechter, R., Agurto, M., Muñoz, D., Nuñez, C. & Arce-Johnson, P. (2016) Grapevine Pathogenic Microorganisms: Understanding Infection Strategies and Host Response Scenarios. *Frontiers in Plant Science*, 7, 382

Arve, L.E., Carvalho, D.R.A., Olsen, J.E. & Torre, S. (2014) ABA induces H_2O_2 production in guard cells, but does not close the stomata on *Vicia faba* leaves developed at high air humidity. *Plant Signaling & Behavior*, 9, e29192

Asselbergh, B., De Vleesschauwer, D. & Höfte, M. (2008) Global Switches and Fine-Tuning - ABA Modulates Plant Pathogen Defense. *Molecular Plant-Microbe Interactions*, 21, 709–719

Balestrini, R., Salvioli, A., Dal Molin, A., Novero, M., Gabelli, G., Paparelli, E., Marroni, F. & Bonfante, P. (2017) Impact of an arbuscular mycorrhizal fungus versus a mixed microbial inoculum on the transcriptome reprogramming of grapevine roots. *Mycorrhiza*, 27, 417–430

Belval, L., Roth, L., Martin, I.R., Laloue, H., Deglene-Benbrahim, L., Valat, L., Goddard, M.-L. & Chong, J. (2024) Effect of arbuscular mycorrhizal symbiosis on grapevine response to *Neofusicoccum parvum*, a major trunk disease fungus. *Plant Stress*, 14, 100582

Brundrett, M., Bougher, N., Dell, B., Grove, T. & Malajczuk, N. (1996) Working with Mycorrhizas in Forestry and Agriculture. 10.13140/2.1.4880.5444

Cao, F.Y., Yoshioka, K. & Desveaux, D. (2011) The roles of ABA in plant–pathogen interactions. *Journal of Plant Research*, 124, 489–499

Čarija, M., Černi, S., Stupin-Polančec, D., Radić, T., Gaši, E. & Hančević, K. (2022) Grapevine Leafroll-Associated Virus 3 Replication in Grapevine Hosts Changes through the Dormancy Stage. *Plants*, 11, 3250

Carr, J.P., Murphy, A.M., Tungadi T., Yoon, J.-Y. (2019) Plant defense signals: Players and pawns in plant-virus-vector interactions. *Plant Science*, 279, 87–95

Darriaut, R., Lailheugue, V., Masneuf-Pomarède, I., Marguerit, E., Martins, G., Compant, S., Ballestra, P., Upton, S., Ollat, N. & Lauvergeat, V. (2022) Grapevine rootstock and soil microbiome interactions: Keys for a resilient viticulture. *Horticulture Research*, 9, uhac019

De Ollas, C., González-Guzmán, M., Pitarch, Z., Matus, J.T., Candela, H., Rambla, J.L., Granell, A., Gómez-Cadenas, A. & Arbona, V. (2021) Identification of ABA-Mediated Genetic and Metabolic Responses to Soil Flooding in Tomato (*Solanum lycopersicum* L. Mill). *Frontiers in Plant Science*, 12, 613059

Deja-Sikora, E., Mercy, L., Baum, C. & Hrynkiewicz, K. (2019) The Contribution of Endomycorrhiza to the Performance of Potato Virus Y-Infected Solanaceous Plants: Disease Alleviation or Exacerbation? *Frontiers in Microbiology*, 10, 516

Deja-Sikora, E., Werner, K. & Hrynkiewicz, K. (2023) AMF species do matter: *Rhizophagus irregularis* and *Funneliformis mosseae* affect healthy and PVY-infected *Solanum tuberosum* L. in a different way. *Frontiers in Microbiology*, 14, 1127278

Dong, T., Xu, Z.-Y., Park, Y., Kim, D.H., Lee, Y. & Hwang, I. (2014) Abscisic Acid Uridine Diphosphate Glucosyltransferases Play a Crucial Role in Abscisic Acid Homeostasis in Arabidopsis. *Plant Physiology*, 165, 277–289

El Aou-ouad, H., Montero, R., Medrano, H. & Bota, J. (2016) Interactive effects of grapevine leafroll-associated virus 3 (GLRaV-3) and water stress on the physiology of *Vitis vinifera* L. cv. Malvasia de Banyalbufar and Giro-Ros. *Journal of Plant Physiology*, 196–197, 106–115

Foo, E., Ross, J.J., Jones, W.T. & Reid, J.B. (2013) Plant hormones in arbuscular mycorrhizal symbioses: an emerging role for gibberellins. *Annals of Botany*, 111, 769–779

Frew, A. (2021) Contrasting effects of commercial and native arbuscular mycorrhizal fungal inoculants on plant biomass allocation, nutrients, and phenolics. *Plants People Planet*, 3, 536–540

Fuchs, M. (2024) Grapevine viruses: Did you say more than a hundred? *Journal of Plant Pathology*, https://doi.org/10.1007/s42161-024-01819-5

Fuchs, M. (2020) Grapevine viruses: a multitude of diverse species with simple but overall poorly adopted management solutions in the vineyard. *Journal of Plant Pathology*, 102, 643–653

Gambino, G. (2015) Multiplex RT-PCR method for the simultaneous detection of nine grapevine viruses. In: Uyeda, I., Masuta, C. (eds) Plant Virology Protocols. *Methods in Molecular Biology, Humana Press*, 1236, 39–47

Gambino, G., Minuto, M., Boccacci, P., Perrone, I., Vallania, R. & Gribaudo, I. (2011) Characterization of expression dynamics of WOX homeodomain transcription factors during somatic embryogenesis in *Vitis vinifera*. *Journal of Experimental Botany*, 62, 1089–1101

Gaši, E., Radić, T., Čarija, M., Gambino, G., Balestrini, R. & Hančević, K. (2023) Arbuscular Mycorrhizal Fungi Induce Changes of Photosynthesis-Related Parameters in Virus Infected Grapevine. *Plants* 12, 1783

Gaši, E., Radić, T., Gambino, G., Čarija, M., Matić, F., Balestrini, R. & Hančević, K. (2024) Arbuscular mycorrhizal fungi modify temporal virus accumulation and distribution in different grapevine tissues. *Phytobiomes Journal*, https://doi.org/10.1094/PBIOMES-06-24-0066-R

González Guzmán, M., Cellini, F., Fotopoulos, V., Balestrini, R. & Arbona, V. (2022) New approaches to improve crop tolerance to biotic and abiotic stresses. *Physiologia Plantarum*, 174, e13547

Graves, S., Piepho, H. & Dorai-Raj, S. (2024) multcompView: Visualizations of Paired Comparisons. R package version 0.1–10

Hančević, K., Čarija, M., Radić Brkanac, S., Gaši, E., Likar, M., Zdunić, G., Regvar, M. & Radić, T. (2023) Grapevine Leafroll-Associated Virus 3 in Single and Mixed Infections Triggers Changes in the Oxidative Balance of Four Grapevine Varieties. *International Journal of Molecular Sciences*, 24, 8

Hančević, K., Saldarelli, P., Čarija, M., Černi, S., Zdunić, G., Mucalo, A. & Radić, T. (2021) Predominance and Diversity of GLRaV-3 in Native Vines of Mediterranean Croatia. *Plants* 10, 17

Hao, Z., van Tuinen, D., Fayolle, L., Chatagnier, O., Li, X., Chen, B., Gianinazzi, S. & Gianinazzi-Pearson, V. (2018) Arbuscular mycorrhiza affects grapevine fanleaf virus transmission by the nematode vector *Xiphinema index*. *Applied Soil Ecology*, 129, 107–111

Hao, Z., Xie, W. & Chen, B. (2019) Arbuscular Mycorrhizal Symbiosis Affects Plant Immunity to Viral Infection and Accumulation. *Viruses* 11, 534

He, C., Xing, F., Liang, J., Zhang, Z., Zhan, B., Habili, N., Wang, H. & Li, S. (2023) The ABI5-dependent down-regulation of mitochondrial ATP synthase OSCP subunit facilitates apple necrotic mosaic virus infection. *Journal of Experimental Botany*, 74, 4189–4207

Islam, W., Naveed, H., Zaynab, M., Huang, Z. & Chen, H.Y.H. (2019) Plant defense against virus diseases; growth hormones in highlights. *Plant Signaling & Behavior*, 14, 1596719

Jansa, J., Smith, F.A. & Smith, S.E. (2008) Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? *New Phytologist*, 177, 779–789

Jin, L., Qin, Q., Wang, Y., Pu, Y., Liu, L., Wen, X., Ji, S., Wu, J., Wei, C., Ding, B. & Li, Y. (2016) Rice Dwarf Virus P2 Protein Hijacks Auxin Signaling by Directly Targeting the Rice OsIAA10 Protein, Enhancing Viral Infection and Disease Development. *PLOS Pathogens*, 12, e1005847

Kassambara, A. (2023) ggpubr:'ggplot2' Based Publication Ready Plots. R package version 0.6.0

Koch, A.M., Antunes, P.M., Maherali, H., Hart, M.M. & Klironomos, J.N. (2017) Evolutionary asymmetry in the arbuscular mycorrhizal symbiosis: conservatism in fungal morphology does not predict host plant growth. *New Phytologist* 214, 1330–1337

Lehr, P. P., Hernández-Montes, E., Ludwig-Müller, J., Keller, M. & Zörb, C. (2022) Abscisic acid and proline are not equivalent markers for heat, drought and combined stress in grapevines. *Australian Journal of Grape and Wine Research*, 28, 119–130.

Leveau, J.H.J. (2024) Re-Envisioning the Plant Disease Triangle: Full Integration of the Host Microbiota and a Focal Pivot to Health Outcomes. *Annual Review of Phytopathology*, 62, 31–47

Lichtenthaler, H.K. (1987) Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes, in: Methods in Enzymology, Plant Cell Membranes. *Academic Press*, 148, 350–382

Liu, M.-Y., Li, Q.-S., Ding, W.-Y., Dong, L.-W., Deng, M., Chen, J.-H., Tian, X., Hashem, A., Al-Arjani, A.-B.F., Alenazi, M.M., Abd-Allah, E.F. & Wu, Q.-S. (2023) Arbuscular mycorrhizal fungi inoculation impacts expression of aquaporins and salt overly sensitive genes and enhances tolerance of salt stress in tomato. *Chemical and Biological Technologies in Agriculture*, 10, 5

Ludwig-Müller, J. (2010) Hormonal Responses in Host Plants Triggered by Arbuscular Mycorrhizal Fungi, in: Koltai, H., Kapulnik, Y. (Eds.), *Arbuscular Mycorrhizas: Physiology and Function*. Springer, Netherlands, 169–190

Malicka, M., Magurno, F., Posta, K., Chmura, D. & Piotrowska-Seget, Z. (2021) Differences in the effects of single and mixed species of AMF on the growth and oxidative stress defense in *Lolium perenne* exposed to hydrocarbons. *Ecotoxicology and Environmental Safety*, 217, 112252

Metwally, R.A., Taha, M.A., Abd El-Moaty, N.M. and Abdelhameed, R.E. (2024) Attenuation of Zucchini mosaic virus disease in cucumber plants by mycorrhizal symbiosis. *Plant Cell Reports*, 43, 54

Miozzi, L., Vaira, A.M., Brilli, F., Casarin, V., Berti, M., Ferrandino, A., Nerva, L., Accotto, G.P. & Lanfranco, L. (2020) Arbuscular Mycorrhizal Symbiosis Primes Tolerance to Cucumber Mosaic Virus in Tomato. *Viruses*, 12, 675

Miozzi, L., Vaira, A.M., Catoni, M., Fiorilli, V., Accotto, G.P. & Lanfranco, L. (2019) Arbuscular Mycorrhizal Symbiosis: Plant Friend or Foe in the Fight Against Viruses? *Frontiers in Microbiology*, 10, 1238

Mishra, J., Srivastava, R., Trivedi, P.K. & Verma, P.C. (2020) Effect of virus infection on the secondary metabolite production and phytohormone biosynthesis in plants. *3 Biotech*, 10, 547

Munkvold, L., Kjøller, R., Vestberg, M., Rosendahl, S. & Jakobsen, I. (2004) High functional diversity within species of arbuscular mycorrhizal fungi. *New Phytologist* 164, 357–364

Murphy, A.M., Zhou, T. & Carr, J.P. (2020) An update on salicylic acid biosynthesis, its induction and potential exploitation by plant viruses. *Current Opinion in Virology*, 42, 8–17.

Naidu, R.A., Maree, H.J. & Burger, J.T. (2015) Grapevine Leafroll Disease and Associated Viruses: A Unique Pathosystem. *Annual Reviews in Phytopathology*, 53, 613–634

Nambara, E. & Marion-Poll, A. (2005) Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology*, 56, 165–185

Nie, X. (2006) Salicylic Acid Suppresses Potato virus Y Isolate N:O-Induced Symptoms in Tobacco Plants. *Phytopathology*, 96, 255–263

Noceto, P.A., Durney, C., van Tuinen, D., de Sousa, J., Wipf, D. & Courty, P.E. (2023) Arbuscular mycorrhizal fungal communities differ in neighboring vineyards of different ages. *Mycorrhiza* 33, 241–248

Nogales, A., Ribeiro, H., Nogales-Bueno, J., Hansen, L.D., Gonçalves, E.F., Coito, J.L., Rato, A.E., Peixe, A., Viegas, W. & Cardoso, H. (2020) Response of Mycorrhizal'Touriga Nacional'Variety Grapevines to High Temperatures Measured by Calorespirometry and Near-Infrared Spectroscopy. *Plants* 9, 1499

Olsen, S. R. & Sommers, L. E. (1982) Phosphorus, in: *Methods of Soil Analysis*. John Wiley & Sons, 403–430

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M., Lahti, L., McGlinn, D.,

Ouellette, M., Ribeiro Cunha, E., Smith, T., Stier, A., Ter Braak, C & Weedon, J. (2024) vegan: Community Ecology Package. R package version 2, 6–6

Pérez-Clemente, R.M., Montoliu, A., Vives-Peris, V., Arbona, V. & Gómez-Cadenas, A. (2019) Hormonal and metabolic responses of Mexican lime plants to CTV infection. *Journal of Plant Physiology*, 238, 40–52

Radić, T., Vuković, R., Gaši, E., Kujundžić, D., Čarija, M., Balestrini, R., Sillo, F., Gambino, G. & Hančević, K. (2024) Tripartite interactions between grapevine, viruses, and arbuscular mycorrhizal fungi provide insights into modulation of oxidative stress responses. *Journal of Plant Physiology*, 303, 154372

Robert-Seilaniantz, A., MacLean, D., Jikumaru, Y., Hill, L., Yamaguchi, S., Kamiya, Y. & Jones, J.D.G. (2011) The microRNA miR393 re-directs secondary metabolite biosynthesis away from camalexin and towards glucosinolates. *Plant Journal*, 67, 218–231.

Sadras, V., Guirao, M., Moreno, A. & Fereres, A. (2024) Inter-virus relationships in mixed infections and virus-drought relationships in plants: a quantitative review. *Plant Journal*, 117, 1786–1799

Sah, S.K., Reddy, K.R. & Li, J. (2016) Abscisic Acid and Abiotic Stress Tolerance in Crop Plants. *Frontiers in Plant Science*, 7, 571

Ton, J., Flors, V. & Mauch-Mani, B. (2009) The multifaceted role of ABA in disease resistance. *Trends in Plant Science*, 14, 310–317

Trouvelot, S., Bonneau, L., Redecker, D., van Tuinen, D., Adrian, M. & Wipf, D. (2015) Arbuscular mycorrhiza symbiosis in viticulture: a review. *Agronomy for Sustainable Development*, 35, 1449–1467

Wei, T. & Simko, V. (2024). R package'corrplot': Visualization of a Correlation Matrix

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L.D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T.L., Miller, E., Bache, S.M., Müller, K., Ooms, J., Robinson, D., Seidel, D.P., Spinu, V., Takahashi, K., Vaughan, D., Wilke, C., Woo, K. & Yutani, H. (2019) Welcome to the tidyverse. *Journal of Open Source Software*, 4, 1686

Xie, K., Li, L., Zhang, H., Wang, R., Tan, X., He, Y., Hong, G., Li, J., Ming, F., Yao, X., Yan, F., Sun, Z. & Chen, J. (2018) Abscisic acid negatively modulates plant defence against rice black-streaked dwarf virus infection by suppressing the jasmonate pathway and regulating reactive oxygen species levels in rice. *Plant Cell & Environment*, 41, 2504–2514

Xu, Z.-Y., Lee, K.H., Dong, T., Jeong, J.C., Jin, J.B., Kanno, Y., Kim, D.H., Kim, S.Y., Seo, M., Bressan, R.A., Yun, D.-J. & Hwang, I. (2012) A Vacuolar β-Glucosidase Homolog That Possesses Glucose-Conjugated Abscisic Acid Hydrolyzing Activity Plays an Important Role in Osmotic Stress Responses in Arabidopsis. *Plant Cell*, 24, 2184–2199.

Zhang, W., Xia, K., Feng, Z., Qin, Y., Zhou, Y., Feng, G., Zhu, H. & Yao, Q. (2024) Tomato plant growth promotion and drought tolerance conferred by three arbuscular mycorrhizal fungi is mediated by lipid metabolism. *Plant Physiology and Biochemistry*, 208, 108478

Zhang, Y., Li, X. (2019) Salicylic acid: biosynthesis, perception, and contributions to plant immunity. *Current Opinion in Plant Biology*, 50, 29–36

Zhao, S. & Li, Y. (2021) Current understanding of the interplays between host hormones and plant viral infections. *PLoS Pathogen*, 17, e1009242

Zhu, B., Gao, T., Zhang, D., Ding, K., Li, C. & Ma, F. (2022) Functions of arbuscular mycorrhizal fungi in horticultural crops. *Scientia Horticulturae*, 303, 111219

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Gaši, E., Likar, M., Arbona, V., González-Guzmán, M., Hančević, K., Balestrini, R. et al. (2025) Hormonal changes associated with arbuscular mycorrhizal fungi indicate defense-like alterations in virus-stressed grapevine. *Physiologia Plantarum*, 177(1), e70136. Available from: https://doi.org/10.1111/ppl.70136

4. **DISCUSSION**

Arbuscular mycorrhizal symbiosis is one of the most well studied type of symbiosis between plants and fungi. Its beneficial effects have been investigated in a wide range of different environmental conditions, under both abiotic and biotic stressors. However, less is known about AMF interaction with woody plants species, such as the grapevine, and their pathogens. Grapevine industry has been subjected to plethora of pathogen-induced biotic stresses. Among the more prevalent pathogens, viruses are one of the most important biotic stressors in the grapevine. Complex interaction of AMF with different stressors and the implication of AMF symbiosis on grapevine physiology is increasingly being investigated. In the papers presented as a part of this thesis, the impact of AMF symbiosis on physiology of established virus-grapevine interaction has been investigated. During two years post-AMF inoculation, different analyses were conducted to investigate the possible bioprotective effects of AMF symbiosis on grapevine infected with viruses.

4.1. **Virus concentration changes in different grapevine tissues based on AMF presence in the grapevine roots**

After AMF inoculation of grapevine plants, successful colonisation was assessed after five and fifteen months. Two inoculums used in the experiment were *R. irregularis* inoculum alone ('RHIZ') and a mixture of three AMF species ('MIX AMF'). MIX AMF consisted of *R. irregularis*, *Funneliformis mosseae* and *F. caledonium*. Light microscopy of 'Trypan blue' coloured roots revealed that both inoculums were highly effective in colonising grapevine roots. However, slight difference was observed in the frequency of arbuscular and vesicular structures in RHIZ, compared to MIX, five months after inoculation. The differences in mycorrhizal inoculum performance were also noted in analysis of physiological parameters of the grapevine and is discussed in the second part of the discussion. Additionally, in the second sampling, fifteen months post-AMF inoculation, while both RHIZ and MIX had high root colonisation percentages, RHIZ had more frequent vesicular structures than MIX AMF. Although some virus-AMF interactions were described to have negative impact on the root fresh weight (Maffei *et al.*, 2014) or arbuscule formation (Deja-Sikora *et al.*, 2023), the different virus treatment did not affect AMF root colonisation in this experiment. On the

other hand, relative concentration of analysed viruses was modified in different grapevine tissues, depending on the mycorrhizal inoculum used.

Grapevine was infected with three distinct viruses, GRSPaV alone or in coinfection with GLRaV-3 and GPGV. GRSPaV quantification was achieved by RNA extraction, cDNA synthesis and measured with quantitative reverse transcription polymerase chain reaction (qRT-PCR). GRSPaV relative concentration was assessed in four distinct tissues (roots, petioles, young leaves and mature leaves) and in three time points. Even though distribution of viruses is highly variable across different plant tissues, seasons of the year and different grapevine-virus systems (Montero *et al.*, 2017; Osman *et al.*, 2018; Gasparro *et al.*, 2019; Shabanian *et al.*, 2020; Nuzzo *et al.*, 2022), attempt was made to describe relative virus quantification of AMF inoculated grapevine. In AMF inoculated grapevine, relative concentration of GRSPaV in the roots of infected grapevine increased drastically in the first sampling, accompanied by the decreased GRSPaV concentration in the young leaves. Virus concentration has been variably reported as both either increased or decreased in different plant-virus-AMF tripartite systems (Deja-Sikora *et al.*, 2019; Hao *et al.*, 2019; Miozzi *et al.*, 2019). Under a 'long-term' infection scenario, increased tomato spotted wilt virus concentration was noted in tomato host (Miozzi *et al.*, 2011). Here, this effect was observed in the young leaves of AMF-inoculated grapevine, where GRSPaV concentration steadily increased in the course of the duration of the experiment. Finally, in the last sampling there was no difference in the GRSPaV concentration between young leaves of AMF inoculated and uninoculated grapevine. Similarly, AMF reduced cucumber mosaic virus titre in the cucumber was noted in the early stages of interaction, but effect was lost in the later sampling points (Elsharkawy *et al.*, 2012). The MIX AMF inoculum showed more pronounced effect on GRSPaV accumulation and its relative concentration as opposed to RHIZ inoculum. The more pronounced effects of MIX AMF have been noted in other parameters analysed in this thesis and will be mentioned throughout the discussion. Nevertheless, grapevine virus concentration increased in the AMF inoculated roots during the early interaction period, subsequently decreasing to the levels of non-mycorrhizal grapevine at the later sampling point. The opposite effect was noted in the young leaf grapevine tissue. Since, establishing a functional AMF symbiosis results in a more developed root systems (Krishna *et al.*, 2005), it is possible that heightened

root development intensified virus replication in the roots. Nutrient transport to the apical portion of the plant could result in the increase of virus concentration in the young leaves, at the later stages of systemic infection. Additionally, under the long-term infection scenario, AMF inoculation affected GLRaV-3 and GPGV relative concentrations. GLRaV-3 concentration drastically increased in all tissues in the AMF inoculated grapevine after one year, while GPGV relative concentrations were high in the roots of AMF inoculated grapevine, but not in the foliage. Similar differential response was noted in the tomato infected with tomato bushy stunt or tomato mosaic viruses with opposing effect of AMF on virus accumulation in the leaves (Khoshkhatti *et al.*, 2020). This differential influence of AMF on a specific virus does not take into the account the virus-virus synergistic or antagonistic interaction and the specificity of a virus strain (Syller and Grupa, 2016; Perrone *et al.*, 2017). The complex interplay between virus-AMF and plants is reflected in different observations of the AMF influence on a relative concentration of a virus (Hao *et al.*, 2019; Miozzi *et al.*, 2019; Deja-Sikora *et al.*, 2023). However, symptomatology or latency of the virus-induced damage cannot be described purely based on the virus quantification, especially since young grapevines kept in the greenhouse tend to be symptomless in the first few years (Gilardi *et al.*, 2020).

4.2. **Physiology of virus-infected grapevine points to reduced stress when inoculated with arbuscular mycorrhizal fungi**

One of the first lines of defence in grapevine facing biotic stress induced by virus infection, is the cell Ca^{2+} influx and production of ROS (Aldon *et al.*, 2018; Sahu *et al.*, 2022). Simultaneously, activation of antioxidative systems is crucial for effective elimination of the produced ROS and management of oxidative cell damage (Sharma *et al.*, 2012). In grapevine infected with GRSPaV, GLRaV-3 and/or GPGV, oxidative homeostasis is crucial for minimising the ROS-induced damage. Since all mentioned viruses are compatible with the grapevine host, massive ROS production is lacking and infection cannot be localised, because the oxidative burst is absent (Glazebrook, 2005). However, oxidative stress can still be present in compatible plan-virus interactions. In virus-infected grapevine, lipid peroxidation (LPO) is expected to increase, while the opposite is observed for mycorrhizal plants (Madhusudhan *et al.*,

2009; Chandrasekaran and Paramasivan, 2022). The noted LPO reduction in AMF-inoculated grapevine is indicative of reduced oxidative damage of lipid molecules. This was found to be the case especially in the second sampling, fifteen months after AMF inoculation. At that time point, different virus coinfections resulted in increased, while AMF inoculation, especially MIX AMF, resulted in decreased LPO concentration. Difference between two samplings may be explained by the duration of period of AMF and virus interaction or grapevine developmental stage differences. Additionally, formation of AMF symbiosis also triggers antioxidative defence system (Kapoor and Singh, 2017). Ascorbate (AsA) and superoxide dismutase (SOD) activity were decreased in AMF-inoculated grapevine, pointing to the reduced production of ROS after AMF inoculation. The reduced SOD activity is suggestive of lower conversion of superoxide ion and lower accumulation of H_2O_2. The AsA is involved in the metabolism of H_2O_2 through ascorbate-glutathione cycle (Pang and Wang, 2010). In potato plants, AMF-induced reduction of H_2O_2 is highlighted as bioprotective role against virus-induced oxidative stress (Deja-Sikora *et al.*, 2023). Nevertheless, after fifteen months of interaction, AMF inoculated grapevine showed increase in GPOD-mediated degradation of H_2O_2 indicating heightened oxidative stress. Changes in oxidative homeostasis coincides with the increased GLRaV-3 and GPGV accumulation in AMF inoculated grapevines. Increased virus load has a profound effect on oxidative stress, as the antioxidative systems are activated to avoid excessive cell damage (Cui *et al.*, 2016). Additionally, genes encoding for proteins involved in oxidative homeostasis and defence response, particularly stilbene synthase (*STS1*) and enhanced disease susceptibility (*EDS1*), were upregulated in grapevine infected with a specific virus combinations. *STS1* is a key enzyme connected to the synthesis of resveratrol and stilbene, both important in defence response (Vannozzi *et al.*, 2012) and was upregulated in non-mycorrhizal grapevine, possibly indicating lack of defence response or unstressed conditions in mycorrhizal grapevine. On the other hand, *EDS1* is upregulated in AMF-inoculated grapevine, infected with all three viruses. The role of *EDS1* is in promotion of oxidative balance through H_2O_2 scavenging (Liao *et al.*, 2023). Taken together, both oxidative parameters and genes involved in oxidative homeostasis are pointing to either a reduced stress or otherwise favourable conditions in grapevine inoculated with AMF, even under higher virus load, confirming AMF to be an integral part of ROS homeostasis. These findings position AMF to be an integral part of ROS homeostasis and suggest tolerance to viral stress and beneficial influence

on grapevine physiology, particularly in the context of improved growth, photosynthesis and pigment concentration.

Grapevine's vigour was assessed through measurement of photosynthesis-related parameters, leaf pigments, growth, and leaf elements concentration. Beneficial AMF influence has been noted in all photosynthesis-related measurements. During the first five months of AMF-virus interaction, RHIZ inoculum was more effective in inducing higher levels of photosynthesis, but after the fifteen-months of symbiosis MIX AMF was more beneficial in this regard. However, MIX AMF caused higher concentrations of leaf pigments and more vigorous growth of virus-infected grapevine than RHIZ inoculum. These discrepancies may be due to differences in symbiosis establishment between the two inoculums and possible competing scenario in multiple AMF species inoculum (Roger *et al.*, 2013). In the first sampling, net photosynthesis rate, transpiration rate, intercellular CO_2 concentration and conductance of water did not vary significantly with different virus confections, but changed drastically with the establishment of the AMF symbiosis. The net photosynthetic rate induction of mycorrhizal plants has been well documented in numerous hosts, including grapevine (Ye *et al.*, 2022; Sandrini *et al.*, 2024). Additionally, chlorophyll and carotenoids concentrations, and dry matter content were increased in AMF-treated plants. Nevertheless, plants infected with GLRaV-3 had a significant reduction in chlorophyll *a* and total carotenoid concentration, as opposed to plants which were not infected with GLRaV-3. The chlorophyll *a* reduction was noted in leafroll-infected grapevine leaves, and was due to chlorophyllase activity (Bertamini *et al.*, 2004). Further, concentrations of various elements were changed in mycorrhizal grapevine, at both sampling times, five and fifteen months after the inoculation. In particular, out of all analysed elements, P concentration increased the most, especially in the leaves of AMF-inoculated grapevine, regardless of virus infection. AMF are known to induce P uptake in plants, which can explain this observation (Etesami *et al.*, 2021). For grapevine, it was shown that AMF communities significantly shape the plant growth and physiological responses, and indirectly influence the chlorophyll content by increasing the uptake of various elements from the soil (Moukarzel *et al.*, 2023). Taken together, AMF-dependent stimulation of growth, nutrition, photosynthesis related parameters, and pigments shows that AMF makes grapevine more vigorous during virus infection. Additionally, as seen from induced defence response, AMF-inoculated grapevines

seem to be overall more tolerant to virus infection, even though the virus relative concentrations might not reflect that fact or indicate any meaningful resistance. However, in depth 'omics' studies focused on pathogenesis related genes and segments of gene silencing mechanism could highlight the existence of AMF-induced virus resistance in the grapevine.

Early interaction, five months post AMF inoculation, also induced hormonal physiological changes. In particular, SA was induced in mycorrhizal grapevine infected with all three viruses. This induction in SA can be viewed as a defence response to virus infection, but also as a mechanism of self-regulation by the virus itself (Murphy *et al.*, 2020). In grapevine, SA induction has been reported to reduce both biotic (Nutricati *et al.*, 2023) and abiotic (Li *et al.*, 2022b) stress. In tobacco plants, both endogenous and exogenous SA are crucial for the resistance to tomato mosaic virus, possibly by limiting virus spread through plasmodesmata (Jiang *et al.*, 2021). Additionally, at this time ABA has been downregulated in grapevine inoculated with MIX AMF and multiple viruses, possibly indicating SA-ABA crosstalk. However, after fifteen months, hormonal profile showed unique changes, particularly in ABA, PA and JA-Ile concentrations. This perturbed hormonal change was noted for grapevines harbouring GPGV in coinfection with only GRSPaV, or both GRSPaV and GLRaV-3. Interestingly, ABA concentrations increased drastically in both MIX and RHIZ inoculated grapevine. ABA has been shown to alleviate virus-induced stress (Alazem and Lin, 2017; Zhao and Li, 2021), thus increase in ABA concertation might be due to increased oxidative stress (Karimi *et al.*, 2021). ABA is known to modify ROS homeostasis and antioxidative activity under different stress scenarios (Li *et al.*, 2022a). In particular, activity of guaiacol peroxidase (GPOD), polyphenol oxidase (PPO) and ascorbate peroxidase (APX) was increased along with concentration of ABA in the leaves, pointing to increased tolerance to virus-induced stress in AMF-inoculated grapevine. In summary, from eight different phytohormones, analysed in the grapevine leaves, SA and ABA show most changes in their concentrations, five and fifteen months after AMF inoculation, respectively.

Overall, the differences made by both RHIZ and MIX inoculums in the context of physiological changes of virus-infected grapevine, provides an insight into the beneficial role of AMF-mediated defence response and stress alleviation. The overall more advantageous nature of MIX inoculum, consisting of three mycorrhizal species,

could be due to different factors not accounted for in this thesis. However, this pronounced effect of MIX inoculum as opposed to one-species AMF inoculum has been previously reported in different contexts. For example, grapevine leaf number or leaf surface is more increased in mixed inoculums than in one-species inoculums (Krishna *et al.*, 2005). Additionally, multiple stressful factors such as nutrient deficiencies and heavy metal pollution are mitigated more by mixture of different AMF species (Crossay *et al.*, 2019). Indeed, generally AMF effect is dependent on the inoculum composition, and mixed mycorrhizal inoculums are thought to be more advantageous for plant growth and development (Jansa et al., 2008; Trouvelot *et al.*, 2015), although not always the case (Malicka *et al.*, 2021). However, without further analyses, conclusions about AMF species-specific contribution to grapevine defence alleviation cannot be drawn.

5. CONCLUSION

The main focus of this thesis was to investigate the bioprotective potential of AMF in grapevine under different virus infection conditions. The focus was on AMF-virus shared impact on grapevine physiology. Thus, obtained findings imply significant modification of grapevine response to virus infection, highlighting the beneficial influence of AMF under virus-induced biotic stress.

For the purpose of studying AMF-grapevine-virus interaction, two mycorrhizal inoculums, RHIZ inoculum containing *R. irregularis* and MIX inoculum containing R. *irregularis*, *F. mosseae*, and *F. caledonium*, were used in experimental design. Both AMF inoculums successfully colonised grapevine to a high degree, with development of significantly larger number of vesicular structures for RHIZ inoculum, both five and fifteen months after AMF inoculation. Compared to grapevine without established AMF symbiosis, both inoculums were successful in inducing beneficial physiological changes, namely increased photosynthesis rate amount of leaf pigments, nutrients concentrations and plant growth. However, MIX inoculum was more successful in inducing stronger beneficial response in multiple virus coinfections than RHIZ, corroborating the hypothesis that mixed inoculums exert a stronger influence on grapevine's physiological response than one-species AMF inoculums. Nevertheless, regardless of AMF inoculum used, mycorrhized grapevine showed better physiological performance than non-mycorrhized grapevine.

Tissue-specific modifications of GRSPaV, GLRaV-3 and GPGV relative concentrations were related to mycorrhizal symbiosis presence. In particular, early interaction of MIX AMF with the GRSPaV increased its concentration in the roots and decreased it in the young grapevine leaves. GRSPaV was more evenly distributed across different tissues at later time points. One year after AMF inoculation, AMF-virus interaction resulted in the increased GLRaV-3 and decreased GPGV concentrations in the grapevine leaves. These results confirm the hypothesis that AMF symbiosis alters virus concentration across grapevine tissues, without clear, species-specific pattern.

Main findings of physiological changes resulting from the AMF-virus interaction align with the hypothesis that AMF symbiosis reduces virus-induced stress in the grapevine, making it more tolerant to virus infection. The mycorrhizal grapevine subjected to virus stress showed reduced lipid peroxidation and modifications of antioxidative systems, particularly ascorbate-glutathione cycle and antioxidative

enzyme activity, in the five- and fifteen-months post AMF inoculation, respectively. Additionally, hormonal profile of virus-infected grapevine changed with the establishment of AMF symbiosis. Salicylic acid was the main hormonal response during the first five months, and abscisic acid concentration increase was crucial in the later phase of the interaction, fifteen months after the AMF inoculation. The growth of the plant and nutrient acquisition were stimulated in the presence of AMF. Additionally, photosynthetic rate was increased in MIX and RHIZ inoculated plants, in both analysed time points. All these changes reduced oxidative stress and enhanced stress tolerance in AMF-inoculated plants.

In summary, this thesis highlights the potential of AMF to modify grapevine defence response against virus infections. The unequivocal, direct resistance to virus infection was not shown, however AMF symbiosis did induce heightened tolerance of grapevine to virus infection through improved physiological performance and stress responses.

6. ACADEMIC ACKNOWLEDGEMENTS

I greatly thank Tomislav Radić for mentoring and guiding me throughout the PhD journey, for being the best supporter in every aspect of collaboration, for giving me advices, help and creating amazing work environment. Additionally, thank You to Tomislav for being involved in any and all aspects of this PhD, including experimental setup, laboratory work, data and manuscript preparation, help in carrying out the mobility programme and much more. Without Tomislav this PhD would not exist.

Thank You to Katarina Hančević and Mate Čarija for being involved in MycoGrape project and for giving me advice and assistance in all aspects of laboratory work, glasshouse work, data analysis, manuscript preparation and all other shared activities.

Thank You to Giorgio Gambino and Amedeo Moine for guiding me through virus relative quantification. Especially thank You to Giorgio for giving me the opportunity to work with him in his laboratory and for providing guidance in treatment setup, manuscript preparation and data interpretation.

Thank You to Rafaella Balestrini and Fabiano Sillo for the opportunity to work side-by-side with them in their *Institute for Sustainable Plant Protection*. In particular, thank You for the guidance and advice on gene expression analysis, data interpretation and manuscript preparation.

Thank You to Rosemary Vuković for providing the analysis of oxidative stress parameters and alongside Tomislav Radić, creating comprehensive overview of grapevine biotic stress response. Additionally, thank You to Daniel Kujundžić, for their involvement in oxidative parameter analysis.

Thank You to Marjana Regvar and Matevž Likar for giving me the opportunity to visit their *Biotechnical Faculty, University of Ljubljana*. Thank You for giving me valuable guidance in data analysis and manuscript preparation.

Thank You to Miguel González-Guzmán and Vicent Arbona for providing the metabolic analysis of the grapevine hormonal changes and for their help in data interpretation and in manuscript preparation.

Thank You to Frano Matić for helping with the interpretation of the data and for explain complex statistical concepts in a friendly and understandable way.

Thank You to Silvija Milišić for giving me the opportunity to work on sample preparation. Particularly thank You for carrying out the elemental analysis of grapevine leaves.

Thank You to Radojka Plećaš for advising and helping us in the maintenance of the greenhouse experiment and for sample preparation.

7. REFFERENCES

Abdelaal, K., Alaskar, A. and Hafez, Y. (2024) 'Effect of arbuscular mycorrhizal fungi on physiological, bio-chemical and yield characters of wheat plants (*Triticum aestivum* L.) under drought stress conditions', *BMC Plant Biology*, 24(1), p. 1119. https://doi.org/10.1186/s12870-024-05824-9.

Aerts, N., Pereira Mendes, M. and Van Wees, S.C.M. (2021) 'Multiple levels of crosstalk in hormone networks regulating plant defense', *The Plant Journal*, 105(2), pp. 489–504. https://doi.org/10.1111/tpj.15124.

Akiyama, K., Matsuzaki, K. and Hayashi, H. (2005) 'Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi', *Nature*, 435(7043), pp. 824–827. https://doi.org/10.1038/nature03608.

Alazem, M. and Lin, N.-S. (2015) 'Roles of plant hormones in the regulation of host–virus interactions', *Molecular Plant Pathology*, 16(5), pp. 529–540. https://doi.org/10.1111/mpp.12204.

Alazem, M. and Lin, N.-S. (2017) 'Antiviral Roles of Abscisic Acid in Plants', *Frontiers in Plant Science*, 8, p. 1760. 10.3389/fpls.2017.01760.

Aldon, D. *et al.* (2018) 'Calcium Signalling in Plant Biotic Interactions', *International Journal of Molecular Sciences*, 19(3), p. 665. https://doi.org/10.3390/ijms19030665.

Allen, J.W. and Shachar-Hill, Y. (2009) 'Sulfur Transfer through an Arbuscular Mycorrhiza', *Plant Physiology*, 149(1), pp. 549–560. https://doi.org/10.1104/pp.108.129866.

Amaro, R. *et al.* (2023) 'Hormone Changes in Tolerant and Susceptible Grapevine Leaves Under Powdery Mildew Infection', *Journal of Plant Growth Regulation*, 42(6), pp. 3606–3614. https://doi.org/10.1007/s00344-022-10823-x.

Anikina, I. *et al.* (2023) 'Plant protection from virus: a review of different approaches', *Frontiers in Plant Science*, 14, p. 1163270 https://doi.org/10.3389/fpls.2023.1163270.

Armijo, G. *et al.* (2016) 'Grapevine Pathogenic Microorganisms: Understanding Infection Strategies and Host Response Scenarios', *Frontiers in Plant Science*, 7, p. 382. https://doi.org/10.3389/fpls.2016.00382.

Asselbergh, B., De Vleesschauwer, D. and Höfte, M. (2008) 'Global Switches and Fine-Tuning—ABA Modulates Plant Pathogen Defense', *Molecular Plant-Microbe Interactions*, 21(6), pp. 709–719. https://doi.org/10.1094/MPMI-21-6-0709.

Balestrini, R. *et al.* (2010) 'Cohorts of arbuscular mycorrhizal fungi (AMF) in *Vitis vinifera*, a typical Mediterranean fruit crop', *Environmental Microbiology Reports*, 2(4), pp. 594–604. https://doi.org/10.1111/j.1758-2229.2010.00160.x.

Balestrini, R., Berta, G. and Bonfante, P. (1992) 'The plant nucleus in mycorrhizal roots: positional and structural modifications', *Biology of the Cell*, 75(3), pp. 235–243. https://doi.org/10.1016/0248-4900(92)90145-Q.

Balestrini, R. and Bonfante, P. (2005) 'The interface compartment in arbuscular mycorrhizae: A special type of plant cell wall?', *Plant Biosystems*, 139(1), pp. 8–15. https://doi.org/10.1080/11263500500056799.

Bari, R. and Jones, J.D.G. (2009) 'Role of plant hormones in plant defense responses', *Plant Molecular Biology*, 69(4), pp. 473–488. https://doi.org/10.1007/s11103-008-9435-0.

Bedini, A. *et al.* (2018) 'Unraveling the Initial Plant Hormone Signaling, Metabolic Mechanisms and Plant Defense Triggering the Endomycorrhizal Symbiosis Behavior', *Frontiers in Plant Science*, 9, p. 1800. https://doi.org/10.3389/fpls.2018.01800.

Belkina, D. *et al.* (2023) 'Grapevine Virome of the Don Ampelographic Collection in Russia Has Concealed Five Novel Viruses', *Viruses*, 15(12), p. 2429. https://doi.org/10.3390/v15122429.

Berens, M.L. *et al.* (2017) 'Evolution of Hormone Signaling Networks in Plant Defense', *Annual Review of Phytopathology*, 55, pp. 401–425. https://doi.org/10.1146/annurev-phyto-080516-035544.

Bertamini, M., Muthuchelian, K. and Nedunchezhian, N. (2004) 'Effect of Grapevine Leafroll on the Photosynthesis of Field Grown Grapevine Plants (*Vitis vinifera* L. cv. Lagrein)', *Journal of Phytopathology*, 152(3), pp. 145–152. https://doi.org/10.1111/j.1439-0434.2004.00815.x.

Bertazzon, N. *et al.* (2020) 'Two New Putative Plant Viruses from Wood Metagenomics Analysis of an Esca Diseased Vineyard', *Plants*, 9(7), p. 835. https://doi.org/10.3390/plants9070835.

Besserer, A. *et al.* (2006) 'Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria', *PLoS biology*, 4(7), p. e226. https://doi.org/10.1371/journal.pbio.0040226.

Brundrett, M.C. and Tedersoo, L. (2018) 'Evolutionary history of mycorrhizal symbioses and global host plant diversity', *New Phytologist*, 220(4), pp. 1108–1115. https://doi.org/10.1111/nph.14976.

Burger, J.T. *et al.* (2017) 'Grapevine leafroll-associated virus3', in B. Meng et al. (eds) *Grapevine Viruses: Molecular Biology, Diagnostics and Management*. Cham: Springer International Publishing, pp. 167–195. https://doi.org/10.1007/978-3-319-57706-7_8.

Cameron, D.D. *et al.* (2013) 'Mycorrhiza-induced resistance: more than the sum of its parts?', *Trends in plant science*, 18(10), pp. 539–545. https://doi.org/10.1016/j.tplants.2013.06.004.

Campos, L. *et al.* (2014) 'Salicylic acid and gentisic acid induce RNA silencing-related genes and plant resistance to RNA pathogens', *Plant Physiology and Biochemistry*, 77, pp. 35–43. https://doi.org/10.1016/j.plaphy.2014.01.016.

Cangahuala-Inocente, G.C. *et al.* (2011) 'Arbuscular mycorrhizal symbiosis elicits proteome responses opposite of P-starvation in SO4 grapevine rootstock upon root colonisation with two *Glomus* species', *Mycorrhiza*, 21(6), pp. 473–493. https://doi.org/10.1007/s00572-010-0352-0.

Carvalho, L.C., Vidigal, P. and Amâncio, S. (2015) 'Oxidative stress homeostasis in grapevine (*Vitis vinifera* L.)', *Frontiers in Environmental Science*, 3, p. 20. https://doi.org/10.3389/fenvs.2015.00020.

Cavagnaro, T.R. *et al.* (2006) 'Arbuscular Mycorrhizas, Microbial Communities, Nutrient Availability, and Soil Aggregates in Organic Tomato Production', *Plant and Soil*, 282(1–2), pp. 209–225. https://doi.org/10.1007/s11104-005-5847-7.

Chandrasekaran, M. and Paramasivan, M. (2022) 'Arbuscular mycorrhizal fungi and antioxidant enzymes in ameliorating drought stress: a meta-analysis', *Plant and Soil*, 480(1), pp. 295–303. https://doi.org/10.1007/s11104-022-05582-3.

Choi, J., Summers, W. and Paszkowski, U. (2018) 'Mechanisms Underlying Establishment of Arbuscular Mycorrhizal Symbioses', *Annual Review of Phytopathology*, 56, pp. 135–160. https://doi.org/10.1146/annurev-phyto-080516-035521.

Coelho, J. *et al.* (2019) 'The study of hormonal metabolism of Trincadeira and Syrah cultivars indicates new roles of salicylic acid, jasmonates, ABA and IAA during grape ripening and upon infection with *Botrytis cinerea*', *Plant Science*, 283, pp. 266–277. https://doi.org/10.1016/j.plantsci.2019.01.024.

Collum, T.D. and Culver, J.N. (2016) 'The impact of phytohormones on virus infection and disease', *Current Opinion in Virology*, 17, pp. 25–31. https://doi.org/10.1016/j.coviro.2015.11.003.

Comby, M. *et al.* (2017) 'Arbuscular Mycorrhizal Fungi as Potential Bioprotectants Against Aerial Phytopathogens and Pests', in Q.-S. Wu (ed.) *Arbuscular Mycorrhizas and Stress Tolerance of Plants*. Singapore: Springer, pp. 195–223. https://doi.org/10.1007/978-981-10-4115-0_9.

Crossay, T. *et al.* (2019) 'Is a mixture of arbuscular mycorrhizal fungi better for plant growth than single-species inoculants?', *Mycorrhiza*, 29(4), pp. 325–339. https://doi.org/10.1007/s00572-019-00898-y.

Cui, Z.-H. *et al.* (2016) 'Responses of In vitro-Grown Plantlets (*Vitis vinifera*) to Grapevine leafroll-Associated Virus-3 and PEG-Induced Drought Stress', *Frontiers in Physiology*, 7. https://doi.org/10.3389/fphys.2016.00203.

Culver, J.N. and Padmanabhan, M.S. (2007) 'Virus-induced disease: altering host physiology one interaction at a time', *Annual Review of Phytopathology*, 45, pp. 221–243. https://doi.org/10.1146/annurev.phyto.45.062806.094422.

Dahan, J. *et al.* (2023) 'Grapevine Endophyte Endornavirus and Two New Endornaviruses Found Associated with Grapevines (*Vitis vinifera* L.) in Idaho, USA', *Viruses*, 15(6), p. 1347. https://doi.org/10.3390/v15061347.

Dai, H.-Y. *et al.* (2024) 'Improvement of *Panax notoginseng* saponin accumulation triggered by methyl jasmonate under arbuscular mycorrhizal fungi', *Frontiers in Plant Science*, 15, p. 1360919. https://doi.org/10.3389/fpls.2024.1360919.

Dangl, J.L. and Jones, J.D.G. (2001) 'Plant pathogens and integrated defense responses to infection', *Nature*, 411(6839), pp. 826–833. https://doi.org/10.1038/35081161.

Das, D. and Gutjahr, C. (2020) 'Role of phytohormones in arbuscular mycorrhiza development', in *The Model Legume Medicago truncatula*. John Wiley & Sons, Ltd, pp. 485–500. https://doi.org/10.1002/9781119409144.ch61.

Das, S. and Sarkar, S. (2024) 'Arbuscular mycorrhizal fungal contribution towards plant resilience to drought conditions', *Frontiers in Fungal Biology*, 5, p. 1355999. https://doi.org/10.3389/ffunb.2024.1355999.

Deja-Sikora, E. *et al.* (2019) 'The Contribution of Endomycorrhiza to the Performance of Potato Virus Y-Infected Solanaceous Plants: Disease Alleviation or Exacerbation?', *Frontiers in Microbiology*, 10, p. 516. https://doi.org/10.3389/fmicb.2019.00516.

Deja-Sikora, E. *et al.* (2020) 'Arbuscular Mycorrhiza Changes the Impact of Potato Virus Y on Growth and Stress Tolerance of *Solanum tuberosum* L. in vitro', *Frontiers in Microbiology*, 10, p. 2971. https://doi.org/10.3389/fmicb.2019.02971.

Deja-Sikora, E., Gołębiewski, M. and Hrynkiewicz, K. (2024) 'Transcriptomic responses of *Solanum tuberosum* cv. Pirol to arbuscular mycorrhiza and potato virus Y (PVY) infection', *Plant Molecular Biology*, 114(6), p. 123. https://doi.org/10.1007/s11103-024-01519-9.

Deja-Sikora, E., Werner, K. and Hrynkiewicz, K. (2023) 'AMF species do matter: *Rhizophagus irregularis* and *Funneliformis mosseae* affect healthy and PVY-infected *Solanum tuberosum* L. in a different way', *Frontiers in Microbiology*, 14, p. 1127278. https://doi.org/10.3389/fmicb.2023.1127278.

Elsharkawy, M.M. *et al.* (2012) 'The plant growth-promoting fungus *Fusarium equiseti* and the arbuscular mycorrhizal fungus *Glomus mosseae* induce systemic

resistance against Cucumber mosaic virus in cucumber plants', *Plant and Soil*, 361(1), pp. 397–409. https://doi.org/10.1007/s11104-012-1255-y.

Ertunç, F. (2020) 'Physiology of virus-infected plants', in L.P. Awasthi (ed.) *Applied Plant Virology*. Academic Press, pp. 199–205. https://doi.org/10.1016/B978-0-12-818654-1.00015-3.

Espinoza, C., Vega, A., *et al.* (2007a) 'Gene expression associated with compatible viral diseases in grapevine cultivars', *Functional & Integrative Genomics*, 7(2), pp. 95–110. https://doi.org/10.1007/s10142-006-0031-6.

Espinoza, C., Medina, C., *et al.* (2007b) 'Senescence-associated genes induced during compatible viral interactions with grapevine and Arabidopsis', *Journal of Experimental Botany*, 58(12), pp. 3197–3212. https://doi.org/10.1093/jxb/erm165.

Etesami, H., Jeong, B.R. and Glick, B.R. (2021) 'Contribution of Arbuscular Mycorrhizal Fungi, Phosphate–Solubilizing Bacteria, and Silicon to P Uptake by Plant', *Frontiers in Plant Science*, 12. https://doi.org/10.3389/fpls.2021.699618.

Faoro, F. *et al.* (1992) 'Cytopathology of grapevine leafroll-associated virus III (GLRaV-III)', *Rivista di Patologia Vegetale*, 2(2), pp. 67–83.

Faoro, F. and Carzaniga, R. (1995) 'Cyotchemistry and immunocytochemistry of the inclusion bodies induced by grapevine leafroll-associated closteroviruses GLRaV-1 and GLRaV-3', *Rivista di Patologia Vegetale*, 5(3), pp. 85–94.

Ferrandino, A. and Lovisolo, C. (2014) 'Abiotic stress effects on grapevine (*Vitis vinifera* L.): Focus on abscisic acid-mediated consequences on secondary metabolism and berry quality', *Environmental and Experimental Botany*, 103: pp. 138–147. https://doi.org/10.1016/j.envexpbot.2013.10.012.

Fiorilli, V. *et al.* (2024) 'Plant Immunity Modulation in Arbuscular Mycorrhizal Symbiosis and Its Impact on Pathogens and Pests', *Annual Review of Phytopathology*, 62(1), pp. 127–156. https://doi.org/10.1146/annurev-phyto-121423-042014.

Fonouni-Farde, C., Diet, A. and Frugier, F. (2016) 'Root Development and Endosymbioses: DELLAs Lead the Orchestra', *Trends in Plant Science*, 21(11), pp. 898–900. https://doi.org/10.1016/j.tplants.2016.08.012.

Foo, E. *et al.* (2016) 'Interactions between ethylene, gibberellins, and brassinosteroids in the development of rhizobial and mycorrhizal symbioses of pea', *Journal of Experimental Botany*, 67(8), pp. 2413–2424. https://doi.org/10.1093/jxb/erw047.

Foyer, C.H. and Noctor, G. (2003) 'Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria', *Physiologia Plantarum*, 119(3), pp. 355–364. https://doi.org/10.1034/j.1399-3054.2003.00223.x.

Foyer, C.H., Noctor, G. and Hodges, M. (2011) 'Respiration and nitrogen assimilation: targeting mitochondria-associated metabolism as a means to enhance nitrogen use efficiency', *Journal of Experimental Botany*, 62(4), pp. 1467–1482. https://doi.org/10.1093/jxb/erq453.

Fracetto, G.G.M., Peres, L.E.P. and Lambais, M.R. (2017) 'Gene expression analyses in tomato near isogenic lines provide evidence for ethylene and abscisic acid biosynthesis fine-tuning during arbuscular mycorrhiza development', *Archives of Microbiology*, 199(5), pp. 787–798. https://doi.org/10.1007/s00203-017-1354-5.

Fuchs, M. (2020) 'Grapevine viruses: a multitude of diverse species with simple but overall poorly adopted management solutions in the vineyard', *Journal of Plant Pathology*, 102(3), pp. 643–653. https://doi.org/10.1007/s42161-020-00579-2.

Fuchs, M. (2025) 'Grapevine viruses: Did you say more than a hundred?', *Journal of Plant Pathology*, 107, pp. 217–227. https://doi.org/10.1007/s42161-024-01819-5.

Gambino, G. *et al.* (2012) 'Co-evolution between Grapevine rupestris stem pitting-associated virus and *Vitis vinifera* L. leads to decreased defense responses and increased transcription of genes related to photosynthesis', *Journal of Experimental Botany*, 63(16), pp. 5919–5933. https://doi.org/10.1093/jxb/ers244.

Garcia, K. and Zimmermann, S.D. (2014) 'The role of mycorrhizal associations in plant potassium nutrition', *Frontiers in Plant Science*, 5, p. 337. https://doi.org/10.3389/fpls.2014.00337.

Gasparro, M. *et al.* (2019) 'Seasonal dynamics and spatial distribution of main Grapevine viruses in field-grown grapevine cultivars', *European Journal of Plant Pathology*, 155(1), pp. 193–205. https://doi.org/10.1007/s10658-019-01761-8.

Giampetruzzi, A. *et al.* (2012) 'A new grapevine virus discovered by deep sequencing of virus- and viroid-derived small RNAs in Cv Pinot gris', *Virus Research*, 163(1), pp. 262–268. https://doi.org/10.1016/j.virusres.2011.10.010.

Gilardi, G. *et al.* (2020) 'Biological and molecular interplay between two viruses and powdery and downy mildews in two grapevine cultivars', *Horticulture Research*, 7(1), pp. 1–14. https://doi.org/10.1038/s41438-020-00413-x.

Glazebrook, J. (2005) 'Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens', *Annual Review of Phytopathology*, 43, pp. 205–227. https://doi.org/10.1146/annurev.phyto.43.040204.135923.

Govindarajulu, M. *et al.* (2005) 'Nitrogen transfer in the arbuscular mycorrhizal symbiosis', *Nature*, 435(7043), pp. 819–823. https://doi.org/10.1038/nature03610.

Graham, R.D. and Webb, M.J. (1991) 'Micronutrients and Disease Resistance and Tolerance in Plants', in *Micronutrients in Agriculture*. John Wiley & Sons, Ltd, pp. 329–370. https://doi.org/10.2136/sssabookser4.2ed.c10.

Gutha, L.R. *et al.* (2010) 'Modulation of flavonoid biosynthetic pathway genes and anthocyanins due to virus infection in grapevine (*Vitis vinifera* L.) leaves', *BMC plant biology*, 10, p. 187. https://doi.org/10.1186/1471-2229-10-187.

Gutjahr, C. (2014) 'Phytohormone signaling in arbuscular mycorhiza development', *Current Opinion in Plant Biology*, 20, pp. 26–34. https://doi.org/10.1016/j.pbi.2014.04.003.

Halldorson, M.M. and Keller, M. (2018) 'Grapevine leafroll disease alters leaf physiology but has little effect on plant cold hardiness', *Planta*, 248(5), pp. 1201–1211. https://doi.org/10.1007/s00425-018-2967-x.

Hamiduzzaman, M.Md. *et al.* (2005) 'β-Aminobutyric Acid-Induced Resistance Against Downy Mildew in Grapevine Acts Through the Potentiation of Callose Formation and Jasmonic Acid Signaling', *Molecular Plant-Microbe Interactions*, 18(8), pp. 819–829. https://doi.org/10.1094/MPMI-18-0819.

Hančević, K. *et al.* (2023) 'Grapevine Leafroll-Associated Virus 3 in Single and Mixed Infections Triggers Changes in the Oxidative Balance of Four Grapevine Varieties', *International Journal of Molecular Sciences*, 24(1), p. 8. https://doi.org/10.3390/ijms24010008.

Hao, Z. *et al.* (2018) 'Arbuscular mycorrhiza affects grapevine fanleaf virus transmission by the nematode vector *Xiphinema index*', *Applied Soil Ecology*, 129, pp. 107–111. https://doi.org/10.1016/j.apsoil.2018.05.007.

Hao, Z., Xie, W. and Chen, B. (2019) 'Arbuscular Mycorrhizal Symbiosis Affects Plant Immunity to Viral Infection and Accumulation', *Viruses*, 11(6), p. 534. https://doi.org/10.3390/v11060534.

Harrison, M.J. *et al.* (2010) 'Phosphate Transporters in Arbuscular Mycorrhizal Symbiosis', in H. Koltai and Y. Kapulnik (eds) *Arbuscular Mycorrhizas: Physiology and Function*. Dordrecht: Springer Netherlands, pp. 117–135. https://doi.org/10.1007/978-90-481-9489-6_6.

He, Q. *et al.* (2020) 'All Roads Lead to Susceptibility: The Many Modes of Action of Fungal and Oomycete Intracellular Effectors', *Plant Communications*, 1(4), p. 100050. https://doi.org/10.1016/j.xplc.2020.100050.

Heinlein, M. (2015) 'Plant virus replication and movement', *Virology*, 479–480, pp. 657–671. https://doi.org/10.1016/j.virol.2015.01.025.

Hernández, J.A. *et al.* (2016) 'Oxidative stress and antioxidative responses in plant–virus interactions', *Physiological and Molecular Plant Pathology*, 94, pp. 134–148. https://doi.org/10.1016/j.pmpp.2015.09.001.

Herrera-Medina, M.J. *et al.* (2007) 'Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza', *New Phytologist*, 175(3), pp. 554–564. https://doi.org/10.1111/j.1469-8137.2007.02107.x.

Hodge, A., Campbell, C.D. and Fitter, A.H. (2001) 'An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material', *Nature*, 413(6853), pp. 297–299. https://doi.org/10.1038/35095041.

Huang, S. *et al.* (2023) 'Uncovering the impact of AM fungi on wheat nutrient uptake, ion homeostasis, oxidative stress, and antioxidant defense under salinity stress', *Scientific Reports*, 13(1), p. 8249. https://doi.org/10.1038/s41598-023-35148-x.

Hull, R. (2014) 'Chapter 11 - Virus–Plant Interactions in Non-Permissive and Permissive Hosts', in R. Hull (ed.) *Plant Virology (Fifth Edition)*. Boston: Academic Press, pp. 605–668. https://doi.org/10.1016/B978-0-12-384871-0.00011-X.

Islam, W. *et al.* (2019) 'Plant defense against virus diseases; growth hormones in highlights', *Plant Signaling & Behavior*, 14(6), p. 1596719. https://doi.org/10.1080/15592324.2019.1596719.

Jahromi, F. *et al.* (2008) 'Influence of Salinity on the In Vitro Development of Glomus intraradices and on the In Vivo Physiological and Molecular Responses of Mycorrhizal Lettuce Plants', *Microbial Ecology*, 55(1), pp. 45–53. https://doi.org/10.1007/s00248-007-9249-7.

Jansa, J., Smith, F.A. and Smith, S.E. (2008) 'Are there benefits of simultaneous root colonisation by different arbuscular mycorrhizal fungi?', *New Phytologist*, 177(3), pp. 779–789. https://doi.org/10.1111/j.1469-8137.2007.02294.x.

Jiang, Y. *et al.* (2021) 'NbWRKY40 Positively Regulates the Response of *Nicotiana benthamiana* to Tomato Mosaic Virus via Salicylic Acid Signaling', *Frontiers in Plant Science*, 11, p. 603518. https://doi.org/10.3389/fpls.2020.603518.

Jones, J.D.G. and Dangl, J.L. (2006) 'The plant immune system', *Nature*, 444(7117), pp. 323–329. https://doi.org/10.1038/nature05286.

Jung, S.C. *et al.* (2012) 'Mycorrhiza-Induced Resistance and Priming of Plant Defenses', *Journal of Chemical Ecology*, 38(6), pp. 651–664. https://doi.org/10.1007/s10886-012-0134-6.

Kapoor, R. and Singh, N. (2017) 'Arbuscular Mycorrhiza and Reactive Oxygen Species', in Q.-S. Wu (ed.) *Arbuscular Mycorrhizas and Stress Tolerance of Plants*. Singapore: Springer, pp. 225–243. https://doi.org/10.1007/978-981-10-4115-0_10.

Karimi, R., Ebrahimi, M. and Amerian, M. (2021) 'Abscisic acid mitigates NaCl toxicity in grapevine by influencing phytochemical compounds and mineral nutrients in leaves', *Scientia Horticulturae*, 288, p. 110336. https://doi.org/10.1016/j.scienta.2021.110336.

Kaur, K. *et al.* (2023) 'The genetic variability of grapevine Pinot gris virus (GPGV) in Australia', *Virology Journal*, 20(1), p. 211. https://doi.org/10.1186/s12985-023-02171-3.

Keller, M. (2015) 'Chapter 8 - Living with Other Organisms', in M. Keller (ed.) *The Science of Grapevines (Second Edition)*. San Diego: Academic Press, pp. 343–367. https://doi.org/10.1016/B978-0-12-419987-3.00008-X.

Khoshkhatti, N. *et al.* (2020) 'Differential Response of Mycorrhizal Plants to Tomato bushy stunt virus and Tomato mosaic virus Infection', *Microorganisms*, 8(12), p. 2038. https://doi.org/10.3390/microorganisms8122038.

Kim, K.S., Gonsalves, D., Teliz, D., Lee, K.W. (1989) 'Ultrastructure and Mitochondrial Vesiculation Associated with Closteroviruslike Particles in Leafroll-Diseased Grapevines', *Phytopathology,* 79, pp. 357–360. 10.1094/Phyto-79-357.

Kloppholz, S., Kuhn, H. and Requena, N. (2011) 'A Secreted Fungal Effector of *Glomus intraradices* Promotes Symbiotic Biotrophy', *Current Biology*, 21(14), pp. 1204–1209. https://doi.org/10.1016/j.cub.2011.06.044.

Koch, A.M. *et al.* (2017) 'Evolutionary asymmetry in the arbuscular mycorrhizal symbiosis: conservatism in fungal morphology does not predict host plant growth', *New Phytologist*, 214(3), pp. 1330–1337. https://doi.org/10.1111/nph.14465.

Kretzschmar, T. *et al.* (2012) 'A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching', *Nature*, 483(7389), pp. 341–344. https://doi.org/10.1038/nature10873.

Krishna, H. *et al.* (2005) 'Biochemical changes in micropropagated grape (*Vitis vinifera* L.) plantlets due to arbuscular-mycorrhizal fungi (AMF) inoculation during ex vitro acclimatization', *Scientia Horticulturae*, 106(4), pp. 554–567. https://doi.org/10.1016/j.scienta.2005.05.009.

Leveau, J.H.J. (2024) 'Re-Envisioning the Plant Disease Triangle: Full Integration of the Host Microbiota and a Focal Pivot to Health Outcomes', *Annual Review of Phytopathology*, 62(Volume 62, 2024), pp. 31–47. https://doi.org/10.1146/annurev-phyto-121423-042021.

Li, S. *et al.* (2022a) 'The interaction of ABA and ROS in plant growth and stress resistances', *Frontiers in Plant Science*, 13. https://doi.org/10.3389/fpls.2022.1050132.

Li, Z. *et al.* (2022b) 'Salicylic acid alleviates selenium stress and promotes selenium uptake of grapevine', *Physiology and Molecular Biology of Plants*, 28(3), pp. 625–635. https://doi.org/10.1007/s12298-022-01169-5.

Liao, M. *et al.* (2023) 'ENHANCED DISEASE SUSCEPTIBILITY 1 promotes hydrogen peroxide scavenging to enhance rice thermotolerance', *Plant Physiology*, 192(4), pp. 3106–3119. https://doi.org/10.1093/plphys/kiad257.

Linderman, R.G. and Davis, E.A. (2001) 'Comparative Response of Selected Grapevine Rootstocks and Cultivars to Inoculation with Different Mycorrhizal Fungi', *American Journal of Enology and Viticulture*, 52(1), pp. 8–11. https://doi.org/10.5344/ajev.2001.52.1.8.

Lou, X. *et al.* (2021) 'The Synergy of Arbuscular Mycorrhizal Fungi and Exogenous Abscisic Acid Benefits *Robinia pseudoacacia* L. Growth through Altering the Distribution of Zn and Endogenous Abscisic Acid', *Journal of Fungi (Basel, Switzerland)*, 7(8), p. 671. https://doi.org/10.3390/jof7080671.

Lough, T.J. and Lucas, W.J. (2006) 'Integrative Plant Biology: Role of Phloem Long-Distance Macromolecular Trafficking', *Annual Review of Plant Biology*, 57, pp. 203–232. https://doi.org/10.1146/annurev.arplant.56.032604.144145.

Ludwig-Müller, J. *et al.* (2002) 'Reduced arbuscular mycorrhizal root colonisation in *Tropaeolum majus* and *Carica papaya* after jasmonic acid application can not

be attributed to increased glucosinolate levels', *Journal of Plant Physiology*, 159(5), pp. 517–523. https://doi.org/10.1078/0176-1617-00731.

Ludwig-Müller, J. (2010) 'Hormonal Responses in Host Plants Triggered by Arbuscular Mycorrhizal Fungi', in H. Koltai and Y. Kapulnik (eds) *Arbuscular Mycorrhizas: Physiology and Function*. Dordrecht: Springer Netherlands, pp. 169–190. https://doi.org/10.1007/978-90-481-9489-6_8.

Madhusudhan, K.N. *et al.* (2009) 'Changes in antioxidant enzymes, hydrogen peroxide, salicylic acid and oxidative stress in compatible and incompatible host-tobamovirus interaction', *Journal of Plant Interactions*, 4(3), pp. 157–166. https://doi.org/10.1080/17429140802419516.

Maffei, G. *et al.* (2014) 'The arbuscular mycorrhizal symbiosis attenuates symptom severity and reduces virus concentration in tomato infected by Tomato yellow leaf curl Sardinia virus (TYLCSV)', *Mycorrhiza*, 24(3), pp. 179–186. https://doi.org/10.1007/s00572-013-0527-6.

Malicka, M. *et al.* (2021) 'Differences in the effects of single and mixed species of AMF on the growth and oxidative stress defense in *Lolium perenne* exposed to hydrocarbons', *Ecotoxicology and Environmental Safety*, 217, p. 112252. https://doi.org/10.1016/j.ecoenv.2021.112252.

Malinowski, R. *et al.* (2024) 'Vascular tissue – boon or bane? How pathogens usurp long-distance transport in plants and the defense mechanisms deployed to counteract them', *New Phytologist*, 243(6), pp. 2075–2092. https://doi.org/10.1111/nph.20030.

Maliogka, V.I. *et al.* (2015) 'Chapter Six - Control of Viruses Infecting Grapevine', in G. Loebenstein and N.I. Katis (eds) *Advances in Virus Research*. Academic Press (Control of Plant Virus Diseases), pp. 175–227. https://doi.org/10.1016/bs.aivir.2014.11.002.

Maree, H.J. *et al.* (2013) 'Grapevine leafroll-associated virus 3', *Frontiers in Microbiology*, 4, p. 82. https://doi.org/10.3389/fmicb.2013.00082.

Martelli, G.P. (2017) 'An Overview on Grapevine Viruses, Viroids, and the Diseases They Cause', in B. Meng et al. (eds) *Grapevine Viruses: Molecular Biology,*

Diagnostics and Management. Cham: Springer International Publishing, pp. 31–46. https://doi.org/10.1007/978-3-319-57706-7_2.

Martín-Rodríguez, J.A. *et al.* (2016) 'Gibberellin–Abscisic Acid Balances during Arbuscular Mycorrhiza Formation in Tomato', *Frontiers in Plant Science*, 7, p. 1273. https://doi.org/10.3389/fpls.2016.01273.

Meixner, C. *et al.* (2005) 'Lack of mycorrhizal autoregulation and phytohormonal changes in the supernodulating soybean mutant nts1007', *Planta*, 222(4), pp. 709–715. https://doi.org/10.1007/s00425-005-0003-4.

Meng, B. and Rowhani, A. (2017) 'Grapevine rupestris stem pitting-associated virus', in Baozhong Meng et al. (eds) *Grapevine Viruses: Molecular Biology, Diagnostics and Management*. Cham: Springer International Publishing, pp. 257–287. https://doi.org/10.1007/978-3-319-57706-7_12.

Metwally, R.A. *et al.* (2024) 'Attenuation of Zucchini mosaic virus disease in cucumber plants by mycorrhizal symbiosis', *PLANT CELL REPORTS*, 43(2), p. 54. https://doi.org/10.1007/s00299-023-03138-y.

Miozzi, L. *et al.* (2011) 'Arbuscular Mycorrhizal Symbiosis Limits Foliar Transcriptional Responses to Viral Infection and Favors Long-Term Virus Accumulation', *Molecular Plant-Microbe Interactions*, 24(12), pp. 1562–1572. https://doi.org/10.1094/MPMI-05-11-0116.

Miozzi, L. *et al.* (2019) 'Arbuscular Mycorrhizal Symbiosis: Plant Friend or Foe in the Fight Against Viruses?', *Frontiers in Microbiology*, 10, p. 1238. https://doi.org/10.3389/fmicb.2019.01238.

Miozzi, L. *et al.* (2020) 'Arbuscular Mycorrhizal Symbiosis Primes Tolerance to Cucumber Mosaic Virus in Tomato', *Viruses*, 12(6), p. 675. https://doi.org/10.3390/v12060675.

Mishra, S. *et al.* (2024) 'Salicylic acid (SA)-mediated plant immunity against biotic stresses: An insight on molecular components and signaling mechanism', *Plant Stress*, 11, p. 100427. https://doi.org/10.1016/j.stress.2024.100427.

Mittler, R. *et al.* (2022) 'Reactive oxygen species signalling in plant stress responses', *Nature Reviews Molecular Cell Biology*, 23(10), pp. 663–679. https://doi.org/10.1038/s41580-022-00499-2.

Mongès, A. *et al.* (2023) 'Are Histidine Kinases of Arbuscular Mycorrhizal Fungi Involved in the Response to Ethylene and Cytokinins?', *Molecular Plant-Microbe Interactions*, 36(10), pp. 656–665. https://doi.org/10.1094/MPMI-05-23-0056-R.

Montero, R. *et al.* (2017) 'Effects of Grapevine leafroll-associated virus 3 on the physiology in asymptomatic plants of *Vitis vinifera*', *Annals of Applied Biology*, 171(2), pp. 155–171. https://doi.org/10.1111/aab.12356.

Moukarzel, R. *et al.* (2023) 'Soil Arbuscular Mycorrhizal Fungal Communities Differentially Affect Growth and Nutrient Uptake by Grapevine Rootstocks', *Microbial Ecology*, 86(2), pp. 1035–1049. https://doi.org/10.1007/s00248-022-02160-z.

Murphy, A.M., Zhou, T. and Carr, J.P. (2020) 'An update on salicylic acid biosynthesis, its induction and potential exploitation by plant viruses', *Current Opinion in Virology*, 42, pp. 8–17. https://doi.org/10.1016/j.coviro.2020.02.008.

Naidu, R. *et al.* (2014) 'Grapevine Leafroll: A Complex Viral Disease Affecting a High-Value Fruit Crop', *Plant disease*, 98(9). https://doi.org/10.1094/PDIS-08-13-0880-FE.

Naidu, R.A., Maree, H.J. and Burger, J.T. (2015) 'Grapevine Leafroll Disease and Associated Viruses: A Unique Pathosystem', *Annual Review of Phytopathology*, 53, pp. 613–634. https://doi.org/10.1146/annurev-phyto-102313-045946.

Nemec, S. and Myhre, D. (1984) 'Virus-*Glomus etunicatum* Interactions in Citrus Rootstocks', *Plant Disease*, 68, pp. 311-314. https://doi.org/10.1094/PD-68-311.

Neumann, E. and George, E. (2010) 'Nutrient Uptake: The Arbuscular Mycorrhiza Fungal Symbiosis as a Plant Nutrient Acquisition Strategy', in H. Koltai and Y. Kapulnik (eds) *Arbuscular Mycorrhizas: Physiology and Function*. Dordrecht: Springer Netherlands, pp. 137–167. https://doi.org/10.1007/978-90-481-9489-6_7.

Ngou, B.P.M., Ding, P. and Jones, J.D.G. (2022) 'Thirty years of resistance: Zig-zag through the plant immune system', *The Plant Cell*, 34(5), pp. 1447–1478. https://doi.org/10.1093/plcell/koac041.

Ngwene, B., Gabriel, E. and George, E. (2013) 'Influence of different mineral nitrogen sources (NO_3^--N vs. NH_4^+-N) on arbuscular mycorrhiza development and N transfer in a *Glomus intraradices*–cowpea symbiosis', *Mycorrhiza*, 23(2), pp. 107–117. https://doi.org/10.1007/s00572-012-0453-z.

Nobori, T. and Tsuda, K. (2019) 'The plant immune system in heterogeneous environments', *Current Opinion in Plant Biology*, 50, pp. 58–66. https://doi.org/10.1016/j.pbi.2019.02.003.

Nogales, A. *et al.* (2021) 'The effects of field inoculation of arbuscular mycorrhizal fungi through rye donor plants on grapevine performance and soil properties', *Agriculture, Ecosystems & Environment*, 313, p. 107369. https://doi.org/10.1016/j.agee.2021.107369.

Nutricati, E. *et al.* (2023) 'Signaling Cross-Talk between Salicylic and Gentisic Acid in the "*Candidatus Phytoplasma Solani*" Interaction with Sangiovese Vines', *Plants*, 12(14), p. 2695. https://doi.org/10.3390/plants12142695.

Nuzzo, F. *et al.* (2022) 'Grapevine virome and production of healthy plants by somatic embryogenesis', *Microbial Biotechnology*, 15(5), pp. 1357–1373. https://doi.org/10.1111/1751-7915.14011.

Oldroyd, G.E.D. (2013) 'Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants', *Nature Reviews Microbiology*, 11(4), pp. 252–263. https://doi.org/10.1038/nrmicro2990.

Osman, F. *et al.* (2018) 'Virus Distribution and Seasonal Changes of Grapevine Leafroll-Associated Viruses', *American Journal of Enology and Viticulture*, 69(1), pp. 70–76. https://doi.org/10.5344/ajev.2017.17032.

Ozdemir, G. *et al.* (2010) 'Effect of Inoculation with Mycorrhizal Fungi on Growth and Nutrient Uptake of Grapevine Genotypes (*Vitis* spp.)', *European Journal of Horticultural Science*, 75, pp. 103–110.

Padmanabhan, M.S., Shiferaw, H. and Culver, J.N. (2006) 'The Tobacco mosaic virus Replicase Protein Disrupts the Localization and Function of Interacting Aux/IAA Proteins', *Molecular Plant-Microbe Interactions*, 19(8), pp. 864–873. https://doi.org/10.1094/MPMI-19-0864.

Pang, C.-H. and Wang, B.-S. (2010) 'Role of Ascorbate Peroxidase and Glutathione Reductase in Ascorbate–Glutathione Cycle and Stress Tolerance in Plants', in N.A. Anjum, M.-T. Chan, and S. Umar (eds) *Ascorbate-Glutathione Pathway and Stress Tolerance in Plants*. Dordrecht: Springer Netherlands, pp. 91–113. https://doi.org/10.1007/978-90-481-9404-9_3.

Parniske, M. (2008) 'Arbuscular mycorrhiza: the mother of plant root endosymbioses', *Nature Reviews. Microbiology*, 6(10), pp. 763–775. https://doi.org/10.1038/nrmicro1987.

Pel, M.J.C. and Pieterse, C.M.J. (2013) 'Microbial recognition and evasion of host immunity', *Journal of Experimental Botany*, 64(5), pp. 1237–1248. https://doi.org/10.1093/jxb/ers262.

Perrone, I. *et al.* (2017) 'Grapevine–virus–environment interactions: an intriguing puzzle to solve', *New Phytologist*, 213(3), pp. 983–987. https://doi.org/10.1111/nph.14271.

Pieterse, C.M.J. *et al.* (2009) 'Networking by small-molecule hormones in plant immunity', *Nature Chemical Biology*, 5(5), pp. 308–316. https://doi.org/10.1038/nchembio.164.

Pieterse, C.M.J. *et al.* (2012) 'Hormonal Modulation of Plant Immunity', *Annual Review of Cell and Developmental Biology*, 28(1), pp. 489–521. https://doi.org/10.1146/annurev-cellbio-092910-154055.

Pieterse, C.M.J. *et al.* (2014) 'Induced systemic resistance by beneficial microbes', *Annual Review of Phytopathology*, 52, pp. 347–375. https://doi.org/10.1146/annurev-phyto-082712-102340.

Pietersen, G., Bell, V.A. and Krüger, K. (2017) 'Management of Grapevine Leafroll Disease and Associated Vectors in Vineyards', in B. Meng et al. (eds) *Grapevine Viruses: Molecular Biology, Diagnostics and Management*. Cham:

Springer International Publishing, pp. 531–560. https://doi.org/10.1007/978-3-319-57706-7_26.

Pons, S. *et al.* (2020) 'Phytohormone production by the arbuscular mycorrhizal fungus *Rhizophagus irregularis*', *PLOS ONE*, 15(10), p. e0240886. https://doi.org/10.1371/journal.pone.0240886.

Pozo, M.J. and Azcón-Aguilar, C. (2007) 'Unraveling mycorrhiza-induced resistance', *Current Opinion in Plant Biology*, 10(4), pp. 393–398. https://doi.org/10.1016/j.pbi.2007.05.004.

Pruitt, R.N., Gust, A.A. and Nürnberger, T. (2021) 'Plant immunity unified', *Nature Plants*, 7(4), pp. 382–383. https://doi.org/10.1038/s41477-021-00903-3.

Pumplin, N. and Harrison, M.J. (2009) 'Live-Cell Imaging Reveals Periarbuscular Membrane Domains and Organelle Location in *Medicago truncatula* Roots during Arbuscular Mycorrhizal Symbiosis', *Plant Physiology*, 151(2), pp. 809–819. https://doi.org/10.1104/pp.109.141879.

Qi, S. *et al.* (2022) 'Arbuscular Mycorrhizal Fungi Contribute to Phosphorous Uptake and Allocation Strategies of *Solidago canadensis* in a Phosphorous-Deficient Environment', *Frontiers in Plant Science*, 13, p. 831654. https://doi.org/10.3389/fpls.2022.831654.

Qu, L., Wang, M. and Biere, A. (2021) 'Interactive Effects of Mycorrhizae, Soil Phosphorus, and Light on Growth and Induction and Priming of Defense in *Plantago lanceolata*', *Frontiers in Plant Science*, 12, p. 647372. https://doi.org/10.3389/fpls.2021.647372.

Radić, T. *et al.* (2014) 'Occurrence of root endophytic fungi in organic *versus* conventional vineyards on the Croatian coast', *Agriculture, Ecosystems & Environment*, 192, pp. 115–121. https://doi.org/10.1016/j.agee.2014.04.008.

Radić, T. *et al.* (2021) 'Root-associated community composition and co-occurrence patterns of fungi in wild grapevine', *Fungal Ecology*, 50, p. 101034. https://doi.org/10.1016/j.funeco.2020.101034.

Regvar, M., Gogala, N. and Zalar, P. (1996) 'Effects of jasmonic acid on mycorrhizal *Allium sativum*', *New Phytologist*, 134(4), pp. 703–707. https://doi.org/10.1111/j.1469-8137.1996.tb04936.x.

Reynolds, A.G. (2017) 'The Grapevine, Viticulture, and Winemaking: A Brief Introduction', in B. Meng et al. (eds) *Grapevine Viruses: Molecular Biology, Diagnostics and Management*. Cham: Springer International Publishing, pp. 3–29. https://doi.org/10.1007/978-3-319-57706-7_1.

Ridgway, H.J., Kandula, J. and Stewart, A. (2006) 'Optimising the medium for producing arbuscular mycorrhizal spores and the effect of inoculation on grapevine growth', *New Zealand Plant Protection*, 59, pp. 338–342. https://doi.org/10.30843/nzpp.2006.59.4591.

Riedle-Bauer, M. (2000) 'Role of Reactive Oxygen Species and Antioxidant Enzymes in Systemic Virus Infections of Plants', *Journal of Phytopathology*, 148(5), pp. 297–302. https://doi.org/10.1046/j.1439-0434.2000.00503.x.

Roger, A. *et al.* (2013) 'Relatedness among arbuscular mycorrhizal fungi drives plant growth and intraspecific fungal coexistence', *The ISME Journal*, 7(11), pp. 2137–2146. https://doi.org/10.1038/ismej.2013.112.

Rúa, M.A. *et al.* (2013) 'Elevated CO2 spurs reciprocal positive effects between a plant virus and an arbuscular mycorrhizal fungus', *New Phytologist*, 199(2), pp. 541–549. https://doi.org/10.1111/nph.12273.

Sahu, P.K. *et al.* (2022) 'ROS generated from biotic stress: Effects on plants and alleviation by endophytic microbes', *Frontiers in Plant Science*, 13. https://doi.org/10.3389/fpls.2022.1042936.

Saldarelli, P. *et al.* (2017) 'Grapevine Pinot gris virus', in B. Meng et al. (eds) *Grapevine Viruses: Molecular Biology, Diagnostics and Management*. Cham: Springer International Publishing, pp. 351–363. https://doi.org/10.1007/978-3-319-57706-7_17.

Sandrini, M. *et al.* (2024) 'Microbial consortia inoculants differently shape ecophysiological and systemic defense responses of field-grown grapevine cuttings', *Plant Stress*, 14, p. 100686. https://doi.org/10.1016/j.stress.2024.100686.

Schmitz, A.M. and Harrison, M.J. (2014) 'Signaling events during initiation of arbuscular mycorrhizal symbiosis', *Journal of Integrative Plant Biology*, 56(3), pp. 250–261. https://doi.org/10.1111/jipb.12155.

Schreiner, R.P. (2005) 'Mycorrhizas and Mineral Acquisition in Grapevines', in *Proceedings of the Soil Environment and Vine Mineral Nutrition Symposium.* American Society for Enology and Viticulture, pp. 49–60.

Schreiner, R.P. (2007) 'Effects of native and nonnative arbuscular mycorrhizal fungi on growth and nutrient uptake of "Pinot noir" (*Vitis vinifera* L.) in two soils with contrasting levels of phosphorus', *Applied Soil Ecology*, 36(2), pp. 205–215. https://doi.org/10.1016/j.apsoil.2007.03.002.

Sgherri, C., Ranieri, A. and Quartacci, M.F. (2013) 'Antioxidative responses in *Vitis vinifera* infected by grapevine fanleaf virus', *Journal of Plant Physiology*, 170(2), pp. 121–128. https://doi.org/10.1016/j.jplph.2012.09.016.

Shabanian, M., Xiao, H. and Meng, B. (2020) 'Seasonal dynamics and tissue distribution of two major viruses associated with grapevine Leafroll under cool climate condition', *European Journal of Plant Pathology*, 158(4), pp. 1017–1031. https://doi.org/10.1007/s10658-020-02137-z.

Sharma, P. *et al.* (2012) 'Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions', *Journal of Botany*, 2012(1), p. 217037. https://doi.org/10.1155/2012/217037.

Shvets, D. *et al.* (2022) 'Metagenomic Analysis of Ampelographic Collections of Dagestan Revealed the Presence of Two Novel Grapevine Viruses', *Viruses*, 14(12), p. 2623. https://doi.org/10.3390/v14122623.

Singh, M. *et al.* (2024) 'Unveiling arbuscular mycorrhizal fungi: the hidden heroes of soil to control the plant pathogens', *Archives of Phytopathology and Plant Protection*, 57(6), pp. 427–457. https://doi.org/10.1080/03235408.2024.2368112.

Sipahioglu, M.H. *et al.* (2009) 'Biological Relationship of Potato virus Y and Arbuscular Mycorrhizal Fungus *Glomus intraradices* in Potato', *Pest Tehnology*, 3(1), pp. 63-66.

Smith, S.E. and Read, D. (2008a) '1 - The symbionts forming arbuscular mycorrhizas', in S.E. Smith and D. Read (eds) *Mycorrhizal Symbiosis (Third Edition)*. London: Academic Press, pp. 13–41. https://doi.org/10.1016/B978-012370526-6.50003-9.

Smith, S.E. and Read, D. (2008b) '3 - Genetic, cellular and molecular interactions in the establishment of arbuscular mycorrhizas', in S.E. Smith and D. Read (eds) *Mycorrhizal Symbiosis (Third Edition)*. London: Academic Press, pp. 91–116. https://doi.org/10.1016/B978-012370526-6.50005-2.

Smith, S.E. and Read, D. (2008c) '4 - Growth and carbon economy of arbuscular mycorrhizal symbionts', in S.E. Smith and D. Read (eds) *Mycorrhizal Symbiosis (Third Edition)*. London: Academic Press, pp. 117–144. https://doi.org/10.1016/B978-012370526-6.50006-4.

Smith, S.E. and Read, D. (2008d) '5 - Mineral nutrition, toxic element accumulation and water relations of arbuscular mycorrhizal plants', in S.E. Smith and D. Read (eds) *Mycorrhizal Symbiosis (Third Edition)*. London: Academic Press, pp. 145–VI. https://doi.org/10.1016/B978-012370526-6.50007-6.

Smith, S.E. and Smith, F.A. (2011) 'Roles of Arbuscular Mycorrhizas in Plant Nutrition and Growth: New Paradigms from Cellular to Ecosystem Scales', *Annual Review of Plant Biology*, 62, pp. 227–250. https://doi.org/10.1146/annurev-arplant-042110-103846.

Song, F. *et al.* (2011) 'Regulatory mechanisms of host plant defense responses to arbuscular mycorrhiza', *Acta Ecologica Sinica*, 31(6), pp. 322–327. https://doi.org/10.1016/j.chnaes.2011.09.001.

Stumpe, M. *et al.* (2005) 'Lipid metabolism in arbuscular mycorrhizal roots of *Medicago truncatula*', *Phytochemistry*, 66(7), pp. 781–791. https://doi.org/10.1016/j.phytochem.2005.01.020.

Syller, J. and Grupa, A. (2016) 'Antagonistic within-host interactions between plant viruses: molecular basis and impact on viral and host fitness', *Molecular Plant Pathology*, 17(5), pp. 769–782. https://doi.org/10.1111/mpp.12322.

Tanaka, Y. and Yano, K. (2005) 'Nitrogen delivery to maize via mycorrhizal hyphae depends on the form of N supplied', *Plant, Cell & Environment*, 28(10), pp. 1247–1254. https://doi.org/10.1111/j.1365-3040.2005.01360.x.

Tarquini, G. *et al.* (2023) 'Traditional Approaches and Emerging Biotechnologies in Grapevine Virology', *Viruses*, 15(4), p. 826. https://doi.org/10.3390/v15040826.

Thiem, D. *et al.* (2014) 'Interactive physiological response of potato (*Solanum tuberosum* L.) plants to fungal colonisation and Potato virus Y (PVY) infection', *Acta Mycologica*, 49(2), pp. 291–303. https://doi.org/10.5586/am.2014.015.

Ton, J., Flors, V. and Mauch-Mani, B. (2009) 'The multifaceted role of ABA in disease resistance', *Trends in Plant Science*, 14(6), pp. 310–317. https://doi.org/10.1016/j.tplants.2009.03.006.

Torres, N., Goicoechea, N., Morales, F., Antolín, M.C. (2016) 'Berry quality and antioxidant properties in *Vitis vinifera* cv. Tempranillo as affected by clonal variability, mycorrhizal inoculation and temperature', *Crop and Pasture Science,* 67(9), pp. 961–977. https://doi.org/10.1071/CP16038.

Torres, N., Yu, R., Kurtural, S.K. (2021) 'Arbuscular Mycrorrhizal Fungi Inoculation and Applied Water Amounts Modulate the Response of Young Grapevines to Mild Water Stress in a Hyper-Arid Season', *Frontiers in Plant Science*, 11:622209. 10.3389/fpls.2020.622209

Tripathi, D. *et al.* (2018) 'Extracellular ATP Acts on Jasmonate Signaling to Reinforce Plant Defense', *Plant Physiology*, 176(1), pp. 511–523. https://doi.org/10.1104/pp.17.01477.

Tripathi, R. *et al.* (2022) 'Plant mineral nutrition and disease resistance: A significant linkage for sustainable crop protection', *Frontiers in Plant Science*, 13, p. 883970. https://doi.org/10.3389/fpls.2022.883970.

Trouvelot, S. *et al.* (2015) 'Arbuscular mycorrhiza symbiosis in viticulture: a review', *Agronomy for Sustainable Development*, 35(4), pp. 1449–1467. https://doi.org/10.1007/s13593-015-0329-7.

Vannozzi, A. *et al.* (2012) 'Genome-wide analysis of the grapevine stilbene synthase multigenic family: genomic organization and expression profiles upon biotic and

abiotic stresses', *BMC Plant Biology*, 12(1), p. 130. https://doi.org/10.1186/1471-2229-12-130.

Wang, Y. *et al.* (2021) 'Auxin is involved in arbuscular mycorrhizal fungi-promoted tomato growth and NADP-malic enzymes expression in continuous cropping substrates', *BMC Plant Biology*, 21, p. 48. https://doi.org/10.1186/s12870-020-02817-2.

Waszczak, C. *et al.* (2015) 'Oxidative post-translational modifications of cysteine residues in plant signal transduction', *Journal of Experimental Botany*, 66(10), pp. 2923–2934. https://doi.org/10.1093/jxb/erv084.

Whitham, S.A., Yang, C. and Goodin, M.M. (2006) 'Global Impact: Elucidating Plant Responses to Viral Infection', *Molecular Plant-Microbe Interactions*, 19(11), pp. 1207–1215. https://doi.org/10.1094/MPMI-19-1207.

Wijayawardene, N.N. *et al.* (2024) 'Classes and phyla of the kingdom Fungi', *Fungal Diversity*, 128(1), pp. 1–165. https://doi.org/10.1007/s13225-024-00540-z.

Yang, S.-Y. *et al.* (2012) 'Nonredundant regulation of rice arbuscular mycorrhizal symbiosis by two members of the phosphate transporter1 gene family', *The Plant Cell*, 24(10), pp. 4236–4251. https://doi.org/10.1105/tpc.112.104901.

Ye, Q., Wang, H. and Li, H. (2022) 'Arbuscular Mycorrhizal Fungi Improve Growth, Photosynthetic Activity, and Chlorophyll Fluorescence of *Vitis vinifera* L. cv. Ecolly under Drought Stress', *Agronomy*, 12(7), p. 1563. https://doi.org/10.3390/agronomy12071563.

Yu, Y. *et al.* (2022) 'Induced Systemic Resistance for Improving Plant Immunity by Beneficial Microbes', *Plants*, 11(3), p. 386. https://doi.org/10.3390/plants11030386.

Zamioudis, C. and Pieterse, C.M.J. (2012) 'Modulation of Host Immunity by Beneficial Microbes', *Molecular Plant-Microbe Interactions*, 25(2), pp. 139–150. https://doi.org/10.1094/MPMI-06-11-0179.

Zhang, C. *et al.* (2022) 'GLRaV-2 protein p24 suppresses host defenses by interaction with a RAV transcription factor from grapevine', *Plant Physiology*, 189(3), pp. 1848–1865. https://doi.org/10.1093/plphys/kiac181.

Zhang, H. *et al.* (2023) ‘Different viral effectors suppress hormone-mediated antiviral immunity of rice coordinated by OsNPR1’, *Nature Communications*, 14(1), p. 3011. https://doi.org/10.1038/s41467-023-38805-x.

Zhang, R.-Q. *et al.* (2013) ‘Arbuscular mycorrhizal fungal inoculation increases phenolic synthesis in clover roots via hydrogen peroxide, salicylic acid and nitric oxide signaling pathways’, *Journal of Plant Physiology*, 170(1), pp. 74–79. https://doi.org/10.1016/j.jplph.2012.08.022.

Zhao, H. *et al.* (2023) ‘Species diversity, updated classification and divergence times of the phylum *Mucoromycota’*, *Fungal Diversity*, 123(1), pp. 49–157. https://doi.org/10.1007/s13225-023-00525-4.

Zhao, S. and Li, Y. (2021) ‘Current understanding of the interplays between host hormones and plant viral infections’, *PLoS Pathogens*, 17(2), p. 1009242. https://doi.org/10.1371/journal.ppat.1009242.

8. *CURRICULUM VITAE*

Emanuel Gaši, mag. oecol., was born on 20 December 1996 in Zagreb, Croatia. In 2018, he graduated with a Bachelor's degree in Environmental Science at the Faculty of Science, University of Zagreb. The same year he enrolled in a Master's degree programme in Environmental Science at the Faculty of Science, University of Zagreb. During his Master's degree programme, Emanuel finished one semester at the University of Vienna, through Erasmus+ mobility programme. He gained the current academic degree in 2021. Shortly after graduating, he started working as a research assistant at the Institute for Adriatic Crops and Karst Reclamation, Split, and enrolled in a PhD programme in Biology at the Faculty of Science, University of Zagreb. His PhD research was carried out under the Croatian Science Foundation project, "Arbuscular mycorrhiza potential to modify grapevine defense against viruses - MycoGrape", under the supervision of Tomislav Radić, PhD. Apart from research project connected to his thesis, Emanuel has been involved in Croatian-Slovenian bilateral project, two science-popular projects and two regional projects. He is the first author of three and a co-author of four scientific publications indexed in the SCI-expanded database. During his PhD programme, he participated in two short-term mobilities and five international congresses, with a contribution of eight poster or oral presentations and a long-term mobility training funded by the Croatian Science Foundation at the Institute for Sustainable Plant Protection, Turin, Italy.

9. PROŠIRENI SAŽETAK

Arbuskularne mikorizne gljive (AMF) su mikroorganizmi koji ostvaruju mutualističku interakciju s korijenskim sustavima širokog spektra biljaka, osiguravajući različite pogodnosti biljci domaćinu. Glavna korist koju AMF pružaju domaćinu je povećano opskrbljivanje nutrijentima čime potiču snažniji rast i razvoj biljke. Također, AMF potiču povećanu toleranciju biljaka na različite stresore poput kontaminacije tla teškim metalima, klimatskih promjena te patogenih organizama i štetnika. U interakciji s patogenim mikroorganizmima, AMF uzrokuju otpornost putem fiziološke indukcije obrambenog odgovora i smanjenja simptoma bolesti. Međutim, virusi predstavljaju patogene od značajnog ekonomskog interesa, čija interakcija s biljkama u simbiozi sa arbuskularnim mikoriznim gljivama još nije u potpunosti razjašnjena. Štoviše, kod dosad istraživanih biljnih vrsta je osim određenih korisnih utjecaja AMF na tijek virusne bolesti, zabilježena i veća podložnost infekciji, vidljiva kroz povećanu koncentraciju virusa i izraženije simptome bolesti.

Vinova loza (*Vitis vinifera* L.) jedna je od višegodišnjih voćnih kultura s iznimno važnim ekonomskim i kulturnim značajem. Mnogi čimbenici koji mogu uzrokovati smanjenje prinosa i ekonomske gubitke detaljno se istražuju. Između ostalog, vinova loza je jedna od poljoprivrednih kultura koja je domaćin najvećem broju opisanih virusa – više od 100 ih koristi vinovu lozu kao primarnog domaćina, pri čemu trećina tih virusa uzrokuje bolesti sa značajnim utjecajem na vinogradarstvo.

Utjecaj AMF-a na biljke zaražene virusima slabo je istražen kod višegodišnjih domaćina poput vinove loze. Zbog nedostatka obrambenih odgovora vinove loze na virusne infekcije, AMF predstavljaju potencijalni alternativni pristup održivom vinogradarstvu i smanjenju ekonomskih gubitaka uzrokovanih virusnim infekcijama. Stoga je cilj ove doktorske disertacije istražiti interakciju AMF–vinova loza–virusi kroz promjene u fiziološkim parametrima domaćina. Utjecaj ove interakcije promatran je kroz tri formirane hipoteze koje ispituju učinak mikoriznih gljiva na odabrane fiziološke parametre vinove loze zaražene virusima, kao i na titar virusa u različitim tkivima mikorizirane vinove loze. Posljednja hipoteza odnosi se na ispitivanje učinkovitosti različitih virusnih i AMF inokuluma u indukciji snažnijeg fiziološkog odgovora vinove loze.

U svrhu ispitivanja hipoteza postavljen je eksperiment na vinovoj lozi u stakleničkim uvjetima. Plemke sorte Merlot nakalemljene su na podloge Kober 5B i

ukorijenjene u sterilnom supstratu, nakon čega je utvrđen njihov fitosanitarni status i prisutnost deset najčešćih virusa vinove loze putem lančane reakcije polimerazom (PCR). Potom je provedena čip-inokulacija kojom su loze zaražene virusom jamičavosti drva vinove loze rupestris (GRSPaV) zasebno ili u kombinaciji s virusom uvijenosti lista vinove loze 3 (GLRaV-3) i/ili virusom pinota sivog (GPGV). Nakon uspješne infekcije biljke su inokulirane jednom ili više vrsta AMF, a kolonizacija korijena provjerena je mikroskopski. Uzorkovanja su provedena u dvije vremenske točke – pet i petnaest mjeseci nakon inokulacije s AMF-om – a analize provedene u sklopu istraživanja obuhvatile su fotosintetske parametre i vodni potencijal, mjerenje koncentracije reaktivnih kisikovih vrsta i aktivnosti antioksidativnih enzima, profile fitohormona, relativne koncentracije virusa, ekspresija odabranih gena u korijenju i listovima te sadržaj pojedinih elemenata u listovima vinove loze.

Utjecaj mikoriznog inokuluma na titar virusa GRSPaV mjeren je u tri vremenske točke tijekom godine dana. Na početku je mikoriza uzrokovala višu relativnu koncentraciju virusa u korijenju, a nižu u mladim listovima, posebice kod inokuluma s više vrsta AMF. Međutim, s vremenom su se razlike između tkiva smanjivale; koncentracija virusa u korijenu je opadala, dok je u mladim listovima rasla. Također, stari listovi i peteljke pokazali su razmjerno stabilne koncentracije virusa tijekom trajanja eksperimenta. U posljednjem uzorkovanju, godinu dana nakon infekcije, dodatno su ispitane relativne koncentracije virusa GLRaV-3 i GPGV. Za GLRaV-3 ustanovljena je visoka koncentracija virusa u listovima mikoriziranih loza, dok je GPGV bio najviše koncentriran u korijenju, što ukazuje na potencijalno stimulativan učinak AMF-a na titar različitih virusa vinove loze, ovisno o tkivu i fenološkoj fazi.

U kontekstu fizioloških promjena, interakcija AMF-a s vinovom lozom uzrokovala je brojne korisne promjene na domaćinu već nakon pet mjeseci, uključujući značajno intenzivniji rast, veću koncentraciju fosfora u listovima i veću stopu fotosinteze u odnosu na nemikorizirane biljke. Iako nije bilo razlike u postotku kolonizacije korijena između različitih AMF inokuluma, inokulum s više vrsta AMF općenito je inducirao snažnije odgovore vinove loze od inokuluma s jednom vrstom AMF. Također, u prvom uzorkovanju uočena je viša akumulacija salicilne kiseline, hormona važnog za obranu.

U drugom uzorkovanju, petnaest mjeseci nakon inokulacije s AMF-om, zabilježene su značajne promjene u fiziologiji vinove loze zbog interakcije virusa i AMF-a. Promjene u lipidnoj peroksidaciji, aktivnosti enzima askorbat peroksidaze, superoksid dismutaze i gvajakol peroksidaze ukazivale su na smanjen stres kod zaražene vinove loze u simbiozi s AMF-om. Od fitohormona, koncentracija abscizinske kiseline značajno se povećala u drugom mjerenju kao odgovor na interakciju AMF-a i virusa. Korisne promjene zabilježene u prvom mjerenju također su bile prisutne u drugom mjerenju, gdje se inokulum s više vrsta AMF pokazao uspješnijim u induciranju pozitivnih promjena kod zaraženog domaćina.

U proučavanju utjecaja interakcije virusa i mikoriznih gljiva na obrambeni status vinove loze uočene su brojne promjene u fiziologiji vinove loze. Općenito, infekcija kombinacijom virusa uzrokovala je veći stres, dok je kombinacija mikoriznih gljiva inducirala snažnije pozitivne fiziološke promjene. Ispitani parametri i učinak mikoriznih gljiva na relativnu koncentraciju virusa ukazali su na pozitivan učinak AMF-a u obrani vinove loze od virusa, povećavajući toleranciju na stres uzrokovan virusnim infekcijama.

10. APPENDIX

Appendix 1. Overview of the treatments designed for this study

Treatment	***Mycorrhizal inoculum***	***Virus composition***
NO	NO	No virus
	RHIZ (RI)	
	MIX	
R (GR)	NO	Grapevine rupestris stem-pitting associated virus (GRSPaV)
	RHIZ (RI)	
	MIX	
RL (GRGL)	NO	Grapevine rupestris stem-pitting associated virus (GRSPaV) Grapevine leafroll-associated virus 3 (GLRaV-3)
	RHIZ (RI)	
	MIX	
RP (GRGP)	NO	Grapevine rupestris stem-pitting associated virus (GRSPaV) Grapevine Pinot Gris virus (GPGV)
	RHIZ (RI)	
	MIX	
RLP (GRGLGP)	NO	Grapevine rupestris stem-pitting associated virus (GRSPaV) Grapevine leafroll-associated virus 3 (GLRaV-3) Grapevine Pinot Gris virus (GPGV)
	RHIZ (RI)	
	MIX	

Mycorrhizal inoculum abbreviations: NO – autoclaved AMF used as a mock inoculum, RHIZ – *Rhizophagus irregularis*, MIX - *R. irregularis*, *Funneliformis mosseae* and *F. caledonium*.

Appendix 2. List of specific primers used in this study

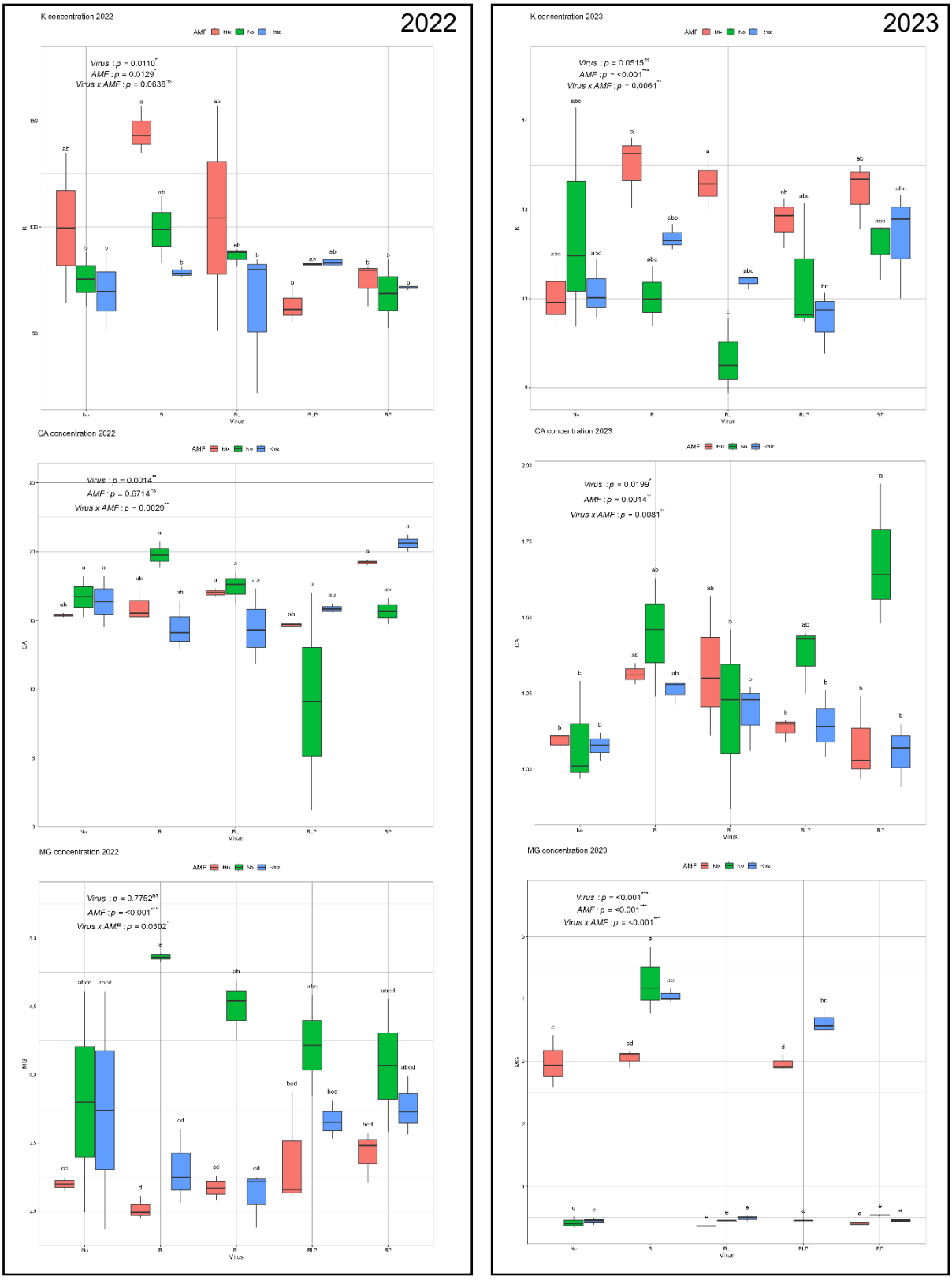
Target (abbreviation)	***Primer sequences 5'-3' (F- forward, R- reverse)***	***Accession no. / gene ID***	***Reference***
PRIMERS FOR 'VIRUS DETECTION'			
Grapevine virus B (GVB)	F GTGCTAAGAACGTCTTCACAGC R ATCAGCAAACACGCTTGAACCG	NC_003602	Minafra and Hadidi, 1994
Arabis mosaic virus (ArMV)	F TGACAACATGGTATGAAGCACA R TATAGGGCCTTTCATCACGAAT	NC_006057	Gambino and Gribaudo, 2006
Grapevine virus A(GVA)	F GAGGTAGATATAGTAGGACCTA R TCGAACATAACCTGTGGCTC	NC_003604	Goszczynski and Jooste, 2003
Grapevine leafroll-associated virus 1 (GLRaV-1)	F TCTTTACCAACCCCGAGATGAA R GTGTCTGGTGACGTGCTAAACG	XM_002282480 AF195822	Gambino et al., 2011 Gambino and Gribaudo, 2006
Grapevine leafroll-associated virus 2 (GLRaV-2)	F TGAAGTTCAAACCGGCAACA R TCGAGCGCAAACAATGTATCA	VIT_16s0098g01190 NC_007448.1	Gambino et al., 2012
Grapevine leafroll-associated virus 3 (GLRaV-3)	F TTGGTGGATGAGGTGCACAT R GTTGCGAAGACGCCTAGTTGT	AF195822 NC_004667.1	Nuzzo et al., 2022
Grapevine fleck virus (GFkV)	F CCTCCATTCCTGAACCTTTCAT R TGCGCATGCACGTGAGA	NC_007448.1 NC_003347.1	Gambino and Gribaudo, 2006 Nuzzo et al., 2022
Grapevine Pinot Gris virus (GPGV)	F GAATCGCTTGCTTTTTCATG R CTACATACTAAATGCACTCTCC	NC_004667.1 FR877530	Bianchi et al., 2015
Grapevine fanleaf virus (GFLV)	F TGGCACAGTCTGTCATGCAA R CAAACTTGGCCATCTGCAACT	FR877530 MN889891	Nuzzo et al., 2022
PRIMERS FOR 'REFERENCE GENES'			

Continuation from the previous page:

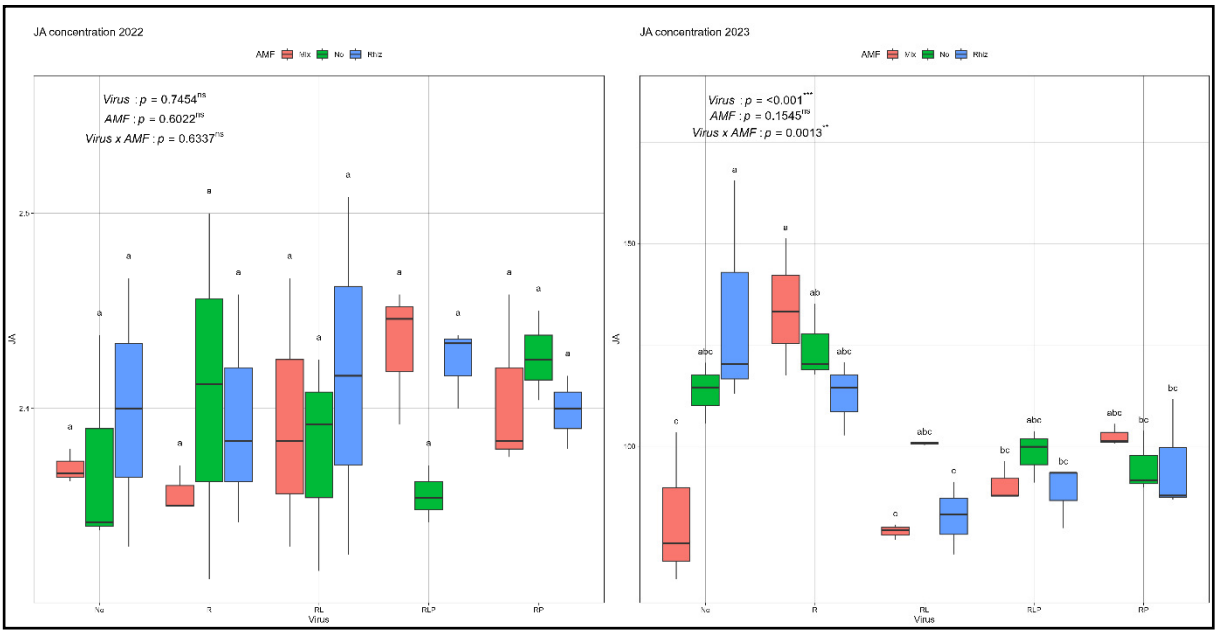
Ubiquitin (UBI)	F	TCTGAGGTTCGTGGTGGTA	VIT_16s0098g00580	Chitarra et al., 2017
	R	AGGCGTGCATAACATTTGCG		
Elongation factor 1-alpha (EF)	F	GAACTGGGTGCTTGATAGGC	VIT_06s0004g03240	Balestrini et al., 2017
	R	AACCAAAATATCCGGAGTAAAAGA		
Actin (ACT)	F	GCCCCTCGTCTGTGACAATG	VIT_04s0044g00580	Chitarra et al., 2017
	R	CCTTGGCCGACCCACAATA		
PRIMERS FOR 'GENES OF INTEREST'				
Chitinase III (CHIT)	F	TGCCAAAATCGAGGCACTAAGG	VIT 16s0050g02210	Balestrini et al., 2017
	R	TGGCCGAGACGATGATTTTC		
Phosphate transporter 1-3 (PT1-3)	F	GCACAAATCGAGAAATGGT	VIT 16s0050g02370	Balestrini et al., 2017
	R	GCGAGCACAGAATTAATACGAC		
ABA 8' hydroxylase-1 (ABA8OH1)	F	ATGGACTTCCAGCCAGATTG	VIT_18s0001g10500	Nerva et al., 2022
	R	GGACATCTCTCCAACCCAGA		
Beta Glucosidase 1 (BG1)	F	TGATGGAACCGGGAAAATAA	VIT_01s0011g00760	Nerva et al., 2022
	R	CCTGTCACCAAACTGCTGAA		
Callose synthase (CAS2)	F	TTCACCCCAGTTGCATTTCT	VIT_06s0004g01270	Chitarra et al., 2018
	R	CCGATCCTTCCTATGACCAC		
Abscisate Beta-Glucosyltransferase-like (GT)	F	CAAATGGGGAAGAAGGCGTG	VIT_17s0000g07200	Nerva et al., 2022
	R	CAGGCCTGCTCATCAATGGA		
9-cis-epoxycarotenoid dioxygenase (NCED1)	F	GGTGGTGAGCCTCTGTTCCT	VIT_19s0093g00550	Ferrero et al., 2018
	R	CTGTAAATTCGTGGCGTTCACT		
Sugar transporter 13 (STP13)	F	GGGTACGGCAATGGATTCG	VIT_07s0151g00110	Chitarra et al., 2017
	R	CCCTCCCCATACACCACTAATCT		

Continuation from the previous page:

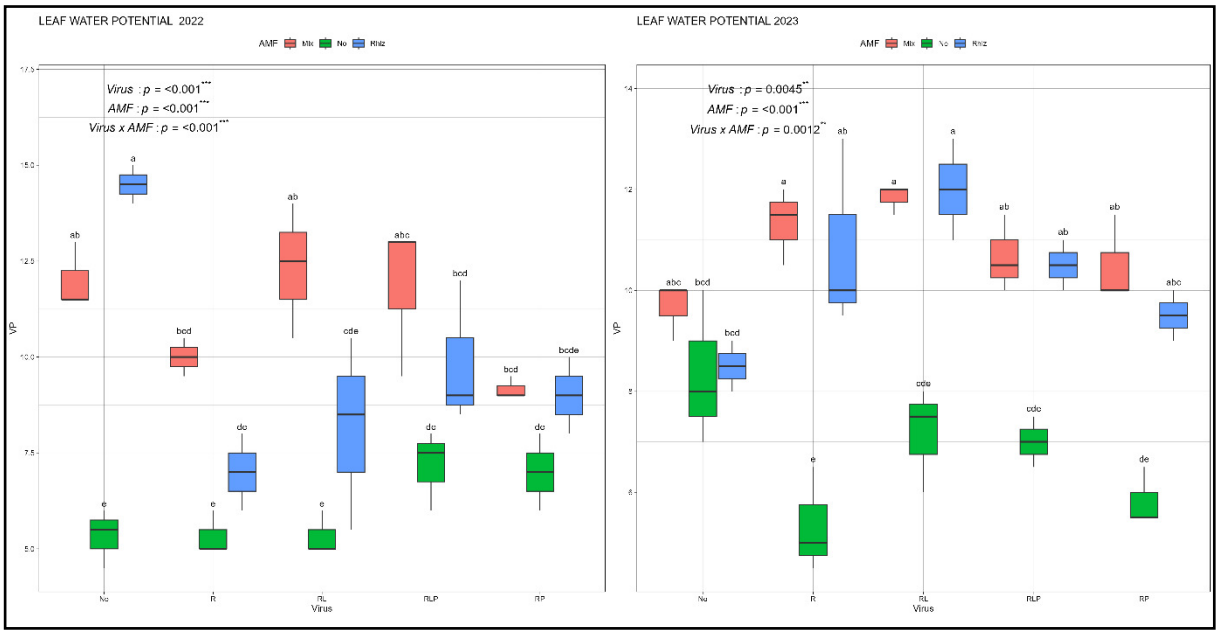
Enhanced Disease Susceptibility 1 (EDS)	F	GGCTACTCGTCGGGTGCTCC	VIT_17s0000g07400	Nerva et al. 2022
	R	GGGTGGGCTCTGATTGGGCT		
Stilbene synthase 1 (STS)	F	TGGCCCTGCAATTCTTGATG	VIT_16s0100g01030	Balestrini et al., 2017
	R	TTAGCACATGCCTCGTTGCTTC		
Lipoxygenase (LOX)	F	TAAAGCCCATCGCAATCGAG	VIT 09s0002g01080	Balestrini et al., 2017
	R	TGGAGCAGACATGAGCTTTTGC		



Appendix 3. Concentration of K, Ca and Mg in grapevine leaves in 2022 (left) and 2023 (right). Different lowercase letters show statistically significant difference between treatments, calculated with two-way ANOVA and Tukey post-hoc test ($p < 0.05$, $n = 3$).



Appendix 4. Concentration of JA in the grapevine leaves in 2022 (left) and 2023 (right). Different lowercase letters show statistically significant difference between treatments, calculated with two-way ANOVA and Tukey post-hoc test ($p < 0.05$, n = 3) .



Appendix 5. Grapevine leaf water potential (VP) measured in 2022 (left) and 2023 (right). Different lowercase letters show statistically significant difference between treatments, calculated with two-way ANOVA and Tukey post-hoc test ($p < 0.05$, n = 3).