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New plant breeding techniques and priming as a multiple level strategy for the control of downy mildew infection in grapevine

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thesis presented by

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Summary

The present thesis relates on three complementary approaches for a more sustainable control of *Plasmopara viticola*: cisgenesis, RNAi and plant defence priming. A brief general introduction is presented in the first chapter, touching the main aspects relative to viticulture in Europe, characteristics of the disease, new biotechnological strategies and priming of plant defence. The second chapter consists of a review article describing with detail the most recent biotechnological approaches for crop protection, including cisgenesis, genome editing, RNAi and epigenetics. In the third chapter the activities concerning cisgenesis for grapevine downy mildew resistance are reported, the study initially focuses on the induction of somatic embryogenesis from elite germplasm, optimising the cultivation of floral tissues for the generation of embryogenic calli. The resistance genes *TNL2a* and *TNL2b* belonging to the RPV3-1 locus, which confers resistance to *Plasmopara viticola,* were then selected for the development of cisgenic varieties, with the construction of a cisgenic vector harbouring those two genes. Finally, the chapter reports on the *Agrobacterium tumefaciens* transformation of embryogenic calli that are currently cultivated on selective medium, and on the future activities for the regeneration of transformed cisgenic plants.

In the fourth and fifth chapters, two papers addressing different aspects related to the exploitation of plant immune system are presented: the first study aimed at clarifying the effects of arbuscular mycorrhiza priming on the grapevine growth-defence trade-off while the second study was focused on the use of alternative protection protocols for the control of downy mildew in a commercial vineyard. Particularly, in the fourth chapter "Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs", the potential benefits of an inoculum formed by two arbuscular mycorrhiza fungal species, with or without a monosaccharide addition, were evaluated on young grapevine cuttings grafted onto 1103P and SO4 rootstocks. The influence of the different treatments was assessed by combining the analysis of agronomic features with biochemical and molecular techniques. The results showed that despite the opposite behaviour of the two selected rootstocks, in mycorrhized samples the whole root microbiome is actively involved in the growth-defence trade off balance.

Finally in the fifth chapter the submitted paper "Novel sustainable strategies to control *Plasmopara viticola* in grapevine unveil new insights on priming responses and arthropods ecology" is presented. The study addresses the reduction of fungicide consumption in viticulture and its associated risks by the exploitation of alternative protocols for the control of downy mildew infection in grapevine, compared to a standard winery protection protocol. In the first protocol, only resistance inducers were used, while the second and third protocols

followed the standard protocol but substituting phosphonates with phosphorus pentoxide and *Ecklonia maxima* extract. The results showed that, at véraison, downy mildew incidence and severity were significantly reduced on both canopy and bunches in the plants treated with all tested protocols compared to non-treated controls. The study also revealed interesting insights about the direct effect of protocols for phosphite substitution on the crosstalk between salicylic and jasmonic acid signalling pathways. Interestingly, by priming plant defences, the resistance inducers caused a short delay in bunch ripening, involving changes in carbohydrate metabolism, regulation of defence related genes, systemic acquired resistance and reactive oxygen species detoxification.

In the thesis conclusion, the main findings are then summarised for each chapter, by examining the most critical aspects and including a brief discussion on the preliminary activities that were conducted to exploit the RNAi technique for silencing two essential genes of *Plasmopara viticola*.

Riassunto

La presente tesi riguarda tre approcci complementari per un controllo più sostenibile del patogeno *Plasmopara viticola*: cisgenesi, RNAi e priming di difesa delle piante. Nel primo capitolo viene presentata una breve introduzione generale, toccando i principali aspetti relativi alla viticoltura in Europa, alle caratteristiche della malattia, alle nuove strategie biotecnologiche e al priming nella difesa delle piante. Nel secondo capitolo viene presentata una review che descrive in dettaglio i più recenti approcci biotecnologici per la protezione delle colture, tra cui la cisgenesi, l'editing del genoma, l'RNAi e l'epigenetica. Nel terzo capitolo sono riportate le attività relative alla cisgenesi per l'introduzione della resistenza alla peronospora nella vite, lo studio si è concentrato inizialmente sull'induzione dell'embriogenesi somatica in germoplasma d'élite, ottimizzando la coltivazione dei tessuti floreali per la generazione di calli embriogenici. I geni di resistenza TNL2a e TNL2b appartenenti al locus RPV3-1, che conferiscono resistenza a *Plasmopara viticola*, sono stati quindi selezionati per lo sviluppo di varietà cisgeniche, con la costruzione di un vettore cisgenico che ospita questi due geni. Viene quindi descritta la trasformazione dei calli embriogenici con i ceppi ingegnerizzati di *Agrobacterium tumefaciens* e le future attività per la rigenerazione di piante cisgeniche trasformate.

Nel quarto e nel quinto capitolo vengono presentati due articoli che affrontano diversi aspetti legati allo sfruttamento del sistema immunitario delle piante: il primo studio mira a chiarire gli effetti del priming indotto da micorrize sul bilancio tra crescita e difesa nella vite mentre il

secondo studio si concentra sull'utilizzo di protocolli di protezione alternativi per il controllo della peronospora in un vigneto commerciale. In particolare, nel quarto capitolo "Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs", sono stati valutati i potenziali benefici di un inoculo formato da due specie di micorrize arbuscolari, con o senza aggiunta di monosaccaridi, su giovani barbatelle innestate sui portainnesti 1103P e SO4. L'influenza dei diversi trattamenti è stata valutata combinando l'analisi delle caratteristiche agronomiche con tecniche biochimiche e molecolari. I risultati hanno mostrato che, nonostante il comportamento opposto dei due portainnesti selezionati, nei campioni trattati con le micorrize l'intero microbioma della radice è attivamente coinvolto nel bilanciamento dei costi/benefici tra crescita e difesa.

Infine, nel quinto capitolo, viene presentato l'articolo "Novel Sustainable Strategy to control *Plasmopara viticola* in grapevine, unveil new insights on priming responses and artropods ecology". Lo studio affronta la riduzione del consumo di fungicidi in viticoltura e dei rischi associati attraverso lo sfruttamento di protocolli alternativi per il controllo della peronospora nella vite confrontandoli con un protocollo di protezione standard adottato da una cantina commerciale. Nel primo protocollo sono stati utilizzati solo induttori di resistenza, mentre il secondo e il terzo protocollo hanno seguito il protocollo standard ma sostituendo i fosfonati con anidride fosforica ed estratto di *Ecklonia maxima*. I risultati hanno mostrato che all'invaiatura l'incidenza e la gravità della peronospora in tutti i protocolli testati erano significativamente ridotte rispetto ai controlli non trattati sia sulla chioma che sui grappoli. Lo studio ha anche mostrato degli spunti interessanti sulla rimodulazione dell'acido salicilico e dell'acido jasmonico nei due protocolli per la sostituzione dei fosfiti. È interessante notare come gli induttori di resistenza attivando le difese della pianta abbiano indotto anche un breve ritardo nella maturazione dei grappoli, agendo, sul metabolismo dei carboidrati, sulla regolazione dei geni di difesa, sulla risposta sistemica acquisita e sulla disintossicazione dalle specie reattive dell'ossigeno. Nella conclusione sono quindi riassunti i principali risultati di ciascun capitolo, esaminandone gli aspetti più critici, inclusa una breve discussione delle attività preliminari che sono state condotte sull'uso dell'RNAi per il silenziamento di due geni essenziali di *Plasmopara viticola*.

CHAPTER 1

General Introduction – A multiple level strategy in the sustainable fight against grapevine downy mildew

CHAPTER 1

1. General Introduction – A multiple level strategy in the sustainable fight against grapevine downy mildew

1.1 Viticulture in the European Union

Grapevine cultivation represents a strategic sector for the European agriculture, being Europe the world's first wine producer with 15.8 bn litres produced in 2019, of which the 19.6% has been exported outside the EU borders, mainly to the United Kingdom and the United States. Italy was the biggest exporter with 1.1 bn litres in 2019, followed by France and Spain, for a total EU export value of 11 bn Euro in 2019/2020 (data source: https://agridata.ec.europa.eu/extensions/DataPortal/wine.html). The European union promotes the development of agriculture and of the wine sector through its Common Agriculture Policy (CAP), established in 1962, and reformed over time in order to fulfil the needs posed by the evolution of the economic scenarios and citizen's needs (Pomarici and Sardone 2020). The last reform, started in 2018, was conceived to increase agriculture competitivity, strengthening the attention on social and environmental aspects following the Green Deal objectives and is going to be implemented from January 2023. Three sustainability dimensions, economic, environmental and social, shape the nine objectives of the new CAP, where the limitation of risks associated to the climate change and protection of the environment stands out for their relevance (Pomarici and Sardone 2020):

- Ensure a fair income to farmers
- Increase competitiveness
- Rebalance the power in the food chain
- Climate change action
- Environmental care
- Preserve landscapes and biodiversity
- Support generational renewal
- Vibrant rural areas
- Protect food quality and health

In the light of this context, it arises the need of researching new tools and strategies to increase viticulture sustainability, particularly considering that, compared to others, this cropping system requires intensive pesticide applications.

1.2 Physiological and economical aspects of the grapevine-downy mildew pathosystem

Estimation of the total costs sustained in the EU or by its member states for fungicide treatments against *Plasmopara viticola*, the causal agent of grapevine downy mildew, is arduous, and even if a lack of studies addressing the economic aspects related to this disease persist, a study performed in the Piemonte region (Italy) estimated the annual cost for controlling downy mildew between 8 and 16 million euros (Salinari et al. 2006).

The disease, caused by the oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, is native from North America where it is endemic in wild *Vitis* species. According to Baldacci's observations, overwintering oospore germination occurs when at least 10mm of rain fell in the previous 48h, temperatures reached 10°C and grapevine shoots reached about 10 cm in length (Gessler et al. 2011). Germination of the oospores leads to the formation of macrosporangia that contain zoospores, these are released in wet conditions and are able to actively reach the host tissues thanks to their flagella or to be transported by wind and rainsplash on the plant canopy (Gessler et al. 2011). The host infection occurs through stomata and, after encystment the colonization of host mesophyll proceeds through the formation of a mycelium with many haustoria. When warm and high humidity are present, sporangiophores emerge through the stomatal cavity, then secondary cycles of infection repeat along the growing season on leaves and berries when growth conditions are met.

Climate change is also expected to affect plant-pathogen interactions, indeed controlled environment experiments showed that heat waves and rise of CO_2 concentration in the atmosphere, due to greenhouse gas emissions, could lead to an increase in downy mildew incidence. Moreover, these conditions will extend the vegetative season giving pathogens more time to evolve, with a potential increase in survival rate (Pugliese et al. 2011; Gullino et al. 2018). These observations, together with the need of reducing the environmental impact of fungicide treatments, make the challenge for the control of downy mildew even harder, making clear that a differentiated set of approaches could represent a great benefit in the fight against this disease.

1.3 Cisgenesis and RNAi, new approaches in the fight against Plasmopara viticola

Despite the many advantages offered by genetically modified (GM) crops, including protection from pests and yield increase with a lower agricultural input (Carzoli et al. 2018),

the adoption of GM crops has been severely hindered by concerns about potential risks for human health and environment. A recent study, however, demonstrated a renewed willingness of the public toward cisgenesis, where one or more genes are transferred from sexually compatible species, even if cisgenic plants are still considered as transgenic organisms by the EU regulation (Delwaide et al. 2015). Cisgenesis shares with traditionally GM approaches the advantage of introducing new traits in an elite genotype without losing many of the positive characteristics previously selected by the breeder, thereby avoiding the linkage drag associated to plant crossings. This approach could sensibly shorten the time span needed to develop new commercial varieties and it also gives the possibility to receive a greater consumer's acceptance thanks to the absence of transgenes. These features make cisgenic plants good candidates to overcome the scepticism toward GM plants, favouring their wide adoption in the optic to increase agriculture sustainability.

Another approach of great interest for the specificity of its mechanism of action and the low environmental impact is based on the RNAi technology, that allows to silence selected genes through the specific targeting of mRNAs by dsRNA molecules (Rosa et al. 2018). This approach has been widely used in reverse genetics and functional genomic studies, however its implementation as disease management strategy in agriculture is lacking, since plants engineered for the production of dsRNA molecules are transgenic and suffer from the same limitations encountered by GM crops, included the high cost for their registration (Rosa et al. 2018; Taning et al. 2021). More recently, a different approach based on the exogenous application of dsRNAs is gaining popularity thanks to the simplicity of its application trough spraying (SIGS) or trunk injection and because plants treated with RNAi products are not GM organisms (Dalakouras et al. 2016; Sang and Kim 2020).

Thanks to the high specificity toward pathogens and the very low environmental impact, both cisgenesis and RNAi represent reliable and effective biotechnological approaches that can greatly help to reduce the input of fungicide treatments. A comprehensive review of these techniques is presented in the second chapter.

1.4 The priming of plant defence in the plant-pathogen interaction

It is possible to imagine the immune system of plants as a structure consisting of several defence lines communicating with each other and evolved under the scenario of a great arms race, in which reciprocal forms of attack and counterattack have been developed (Bürger and

Chory 2019). Pathogens are first recognized by innate immunity receptors (PRRs, Pattern Recognition Receptors), activating the first line of defence, called pathogen-associated molecular pattern (PAMP) triggered immunity (PTI) (Chagas et al. 2018; Bürger and Chory 2019). PTI can induce several responses, including closure of stomata, construction of physical barriers to counter the pathogen diffusion (i.e. callose deposition) or production of defence secondary metabolites, such as phytoalexins, and reactive oxygen species (ROS) (Chagas et al. 2018). Pathogens, however, have evolved strategies to escape PTI or to interfere with the plant signalling system through production of effector molecules (Chagas et al. 2018). Plants in turn have developed a second sensing system, mainly constituted by R-genes (resistance genes, including nucleotide-binding leucine-rich repeat receptors NB-LRR), and referred to as the effector-triggered immunity (ETI), that activates the second line of defence upon detection of pathogen effectors (Bürger and Chory 2019). ETI associated response represents the main strategy against biotrophic pathogens that do not feed on dead tissues (including grapevine downy mildew), and it involves salicylic acid production and activation of localised cell-death at the infection site, better known as hypersensitive response (HR) (Chagas et al. 2018; Bürger and Chory 2019). The need to protect uninfected tissues leads to the formation of long-distance systemic defence mechanisms, the systemic acquired resistance (SAR) and the induced systemic resistance (ISR), activated through signalling of PTI and SAR responses and respectively regulated by salicylic acid and jasmonate hormones (Chagas et al. 2018; Bürger and Chory 2019).

Control of fungal pathogens is usually achieved by the regular use of synthetic or inorganic fungicides over years (during the growing season), posing risks for the environment and human health when toxic effects are associated to the accumulation of residuals (Wightwick et al. 2010; Burdziej et al. 2021), as in case of copper-based products. An alternative to the use of fungicides that has increased its popularity with time, attracting the interest of researchers, is given by the exploitation of the plant immune system. Indeed, it has been demonstrated that plant exposition to certain stresses, but also to resistance inducers and plant growth-promoting microorganisms, can stimulate the plant defences and ability to face future stress expositions (Cameron et al. 2013; Mhlongo et al. 2018; Llorens et al. 2020). This phenomenon, defined as “priming”, is a form of “stress training” characterised by the existence of a lag phase between the first and the second stress exposition in which adaptations take place at molecular, cellular, and physiological level (Llorens et al. 2020). After the first exposition to the stress, plants enter in a physiological state of readiness, called

"primed state", that allows them to respond faster and more robustly to future stress events (Alagna et al. 2020; Llorens et al. 2020; Tajima et al. 2020).

Resistance inducers are chemical molecules that could have natural or synthetic origins and are characterised by significantly lower risks or non-toxic effects for the environment compared to fungicides (Tripathi et al. 2019; Jamiołkowska 2020), these molecules induce the activation of plant innate immunity and systemic signals, that allow plants to restrict pathogens growth and overcome the stress. Also the interaction with mutualistic microorganisms, including plant grow promoting bacteria (PGPB, and rhizobacteria PGPR), mycorrhizae and fungi can induce the priming of plant defences, in a process that starts with the recognition of microbial PRR, induction of PTI response and transient accumulation of SA, in turn leading to the activation of SAR response and systemic priming of SAR-related defences (Cameron et al. 2013; Alagna et al. 2020).

Being exposed to a wide range of pathogens and environmental conditions, plants evolved a great adaptability to survive in such a complex scenario, developing a large array of defence and metabolic responses including PTI, SAR and HR (Huot et al. 2014). Since nutrients availability is usually limited and defence mechanisms imply a metabolic cost, the fitness maximization requires a dynamic balance of nutrients allocation between growth and defence that varies with relation to the ecological and environmental conditions (Karasov et al. 2017). Several studies also indicated that growth and defence are tightly coregulated at transcriptional, post-transcriptional and biochemical level, with numerous crosstalk between growth hormones signalling pathways (Huot et al. 2014; Karasov et al. 2017 and references therein).

Based on the above, it is therefore fundamental for the development of sustainable agronomic strategies to achieve a comprehensive understanding of the biological mechanisms underpinning the priming of plant defences.

1.5 Aims and objectives

The aim of the thesis is to provide a comprehensive view of alternative strategies to answer the need of increasing the sustainability of the grapevine defence against *Plasmopara viticola*.

To fulfil this goal the work has been organized to addressing two main objectives:

1. The development of a cisgenic approach for the introduction of downy mildew resistance genes into elite grape varieties.
2. Evaluate the effect of defence priming on grapevine disease response:
    - analysing the effects of mycorrhiza induced resistance (MIR)
    - using alternative protection protocols for the pathogen control in the vineyard

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CHAPTER 2

Novel and emerging biotechnological crop protection approaches

Review article

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CHAPTER 2

2. Novel and emerging biotechnological crop protection approaches

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2.1 Abstract

Traditional breeding or Genetically Modified Organisms (GMOs) have for a long time been the sole approaches to effectively cope with biotic and abiotic stresses and implement the quality traits of crops. However, emerging diseases as well as unpredictable climate changes affecting agriculture over the entire globe forces scientists to find alternative solutions required to quickly overcome seasonal crises. In this review, we first focus on cisgenesis and genome editing as challenging biotechnological approaches for breeding crops more tolerant to biotic and abiotic stresses. In addition, we take into consideration a toolbox of new techniques based on applications of RNA interference and epigenome modifications, which can be adopted for improving plant resilience. Recent advances in these biotechnological applications are mainly reported for non-model plants, and woody crops in particular. Indeed, the characterization of RNAi machinery in plants is fundamental to transform available information into biologically or biotechnologically applicable knowledge. Finally, here we discuss how these innovative and environmentally friendly techniques combined with traditional breeding, can sustain a modern agriculture and be of potential contribution to climate change mitigation.

Keywords

New plant breeding techniques, Cisgenesis, Genome editing, RNA interference, Disease resilience, Abiotic stress, DNA methylation, Epigenetics

2.2 Introduction

Increasing plant resilience against biotic or abiotic stress and improvement of quality traits to make crops more productive as well as nutritious are focal targets in plant breeding programs. Opposing pressure comes from the increasing virulence of a large number of pests and diseases, caused by insects, fungi, bacteria, viruses and nematodes (Gimenez et al., 2018), and legislation limiting the use of agrochemicals (Directive 335 2009/128/EC and Regulation (EC) No 1107/2009 of the European Parliament and of the Council). On the other hand, climate changes expand abiotic stress conditions forcing plant breeders to select genotypes resistant to water and thermal stresses to cope with the modification of rainfall patterns and rise in temperatures (Porter et al., 2014; Mohanta et al., 2017b). These unfavorable constraints

are leading to insufficient yield and a strong decrease in quality features (Ebi and Loladze, 2019).

The development of genetically improved varieties of crop plants has long been taking advantage of crossings and mutagenesis to obtain plants with better characteristics in terms of yield and quality features, as well as improved stress resilience traits (Dempewolf et al., 2017). Since the 1920s, when introgression of the desired traits from the available germplasm has not been possible, mutagenesis through radiation or chemical agents has been used. Over the last century, genetic engineering and biotechnologies have broadened the toolbox of geneticists and breeders with new instruments and approaches, leading to the creation of genetically modified organisms (GMOs) (Lusser et al., 2012). The potential of this approach to obtain improved disease resistance, abiotic stress resistance and nutritionally improved genetically modified crops have been widely demonstrated and discussed, together with the limitations and the concerns associated with the use of GMOs (Low et al., 2018; Kumar et al., 2020; Van Esse et al., 2020; Sabbadini et al., 2021).

Thanks to these techniques, the gene pool potentially available to plant breeders has considerably increased, allowing the isolation and transferring of genes to crops from sexually incompatible plant species as well as from other organisms (Carrière et al., 2015). Although in 2018 GM crops covered 191.7 million hectares with remarkable benefits (Brookes and Barfoot, 2016; Change, 2018), their use is still associated with strong public concern, which is related to putative risks for human health and environment contamination (Frewer et al., 2011; Carzoli et al., 2018). Insertion in the crop genome of genes isolated from genetically distant and/or unrelated organisms (transgenes), which usually includes selectable markers (e.g. resistance to antibiotics), is one of the most criticized aspects by citizens. Over the years, to overcome GM crop limitations, many techniques have been developed up to the latest New Plant Breeding Techniques (NPBTs, e.g. genome editing).

In the last 15 years, Next Generation Sequencing (NGS) technologies fostered a major advancement in crop genomics and contributed to the public availability of many reference crop genomes (Jaillon et al., 2007; Xu et al., 2011; Sato et al., 2012; Verde et al., 2017; Linsmith et al., 2019). Moreover, high-throughput re-sequencing of hundreds of genotypes allowed researchers to describe the allele diversity of both domesticated and wild plant populations (Morrell et al., 2012). In this context, the increased data availability on genome structures deepened the comprehension of plant domestication history, the identification of genes responsible for traits of agrochemical interest and gene functions, promoting the development of NPBTs for overcoming the major GMO laborious and costly regulatory

evaluation processes and public concerns. Actually, NPBTs allow a single gene to be transferred, mimicking sexually compatible crosses (cisgenesis) and precise modification of specific DNA sequences (genome editing).

In this review, we summarize the main features, advantages and challenges of various biotechnological approaches, providing examples of applications for the amelioration of plant traits to better cope with biotic and abiotic stresses. The common thread is to describe the recent biotechnological advancements which allow crop traits to be precisely modified and overcome the restrictions imposed on genetically modified products. Therefore, we focused our discussion on cisgenesis and genome editing as the more known techniques, but we also addressed our attention on latest innovative crop breeding technologies, such as RNA interference and epigenome editing. Emphasis is given to non-model plants, such as woody crops, for which the application of biotechnological approaches is not as easy as for herbaceous model plants.

2.3 Cisgenesis: approaches and potentials in plant protection

The idea of cisgenesis was first proposed by Shouten in 2006. In its widely accepted definition, the results of cisgenic approaches are crops modified with genes isolated exclusively from sexually compatible plants, including gene introns and regulative regions, such as promoters and terminators, in their sense orientation (Schouten et al., 2006a; Schouten et al., 2006b).

Cisgenic strategies

Cisgenic plants may resemble plants derived from traditional breeding and share the same genetic pool with them, since genes of interest are isolated from a species that could be used for traditional crosses and transferred, preserving its “native” form. One of the main drawbacks of gene introgression in a crop genome by classical crosses is that a large number of undesirable associated genes are transmitted along with the gene(s) of interest to the next generation, often negatively influencing many agronomic traits, related to products quality and yield. This phenomenon, defined as linkage drag, is common in introgression breeding and Marker Assisted Selection (MAS) is often adopted to reduce the amount of undesired genes (Hospital, 2005). The use of MAS-complex schemes slows down new cultivar release, which can require decades in the case of woody plants that have long juvenile phases. Cisgenesis allows the linkage drag issue to be overcome by transferring only the desired gene(s) in a single step, preserving all the quality traits selected in the élite cultivars.

The limit of cisgenesis is its suitability only to monogenic traits, although it could also be applied to oligogenic characters: indeed, the technical complexity of the procedure is directly correlated with the number of genes to be transferred. On the other hand, cisgenic plants display greater public and farmers positive consensus compared to transgenic ones (Delwaide et al., 2015; Rousselière and Rousselière, 2017; De Steur et al., 2019).

Detailed methods and strategies with an interesting success rate for the development of cisgenic plants have been comprehensively reviewed by several authors over the last decade (Schaart et al., 2011; Espinoza et al., 2013; Holme et al., 2013; Cardi, 2016) so these approaches are quite mature for a wide use.

Since its initial application, several strategies have been conceived for cisgenesis (Fig.1), by considering the differences in transformation and regeneration efficiency and length of the breeding cycle, which depend on the selected plant species. The simplest approach consists of the use of vectors where only the gene of interest is cloned in the T-DNA region, transferred to plants through *Agrobacterium*-mediated transformation and then selected by PCR analysis (Fig.1a) (De Vetten et al., 2003; Basso et al., 2020). Another similar strategy exploits minimal gene cassettes, made just by promoter, coding sequence and terminator, which are introduced into the plant genome by biolistic transformation (Fig.1b), thus avoiding partial or complete backbone integrations (Vidal et al., 2006; Low et al., 2018). Nevertheless, these systems require long and expensive PCR screenings, and are suitable only for species with a high transformation efficiency (Vidal et al., 2006; Malnoy et al., 2010; Petri et al., 2011; Low et al., 2018). In species where transformation is recalcitrant, the transformation with cisgenic reporter genes or co-transformation with selectable marker genes could greatly simplify the recovery of transformed plants. For example, Myb transcription factors involved in the regulation of anthocyanin biosynthesis were tested in apple (Krens et al., 2015) and grapevine (Li et al., 2011) as selectable markers for cisgenic plants. The use of exogenous or endogenous reporter genes have been already successfully applied in herbaceous species (Basso et al., 2020). However, the possibility of using such reporters is confined to those cases where tissue coloration does not interfere with selection for other traits of interest. In seed propagated crops (e.g. wheat, barley, rice, tomato, etc…) it is possible to use a co-transformation strategy (Fig.1c), crossing them with the parental or original variety and hence exploiting segregation of the selectable marker in the progeny, obtaining plants with the cisgene but without the selectable marker (Holme et al., 2012a).

For vegetative propagated species with poor transformation efficiencies, a novel developed approach relies on the excision of unwanted DNA sequences after the selection of

transformed plants through recombination systems (Fig.1d). In 1991, Dale and Ow used the bacteriophage P1 Cre/lox recombinase/sites for markers excision in tobacco plants (Dale and Ow, 1991). Since then other alternative systems from *Zygosaccharomyces rouxii (*R/Rs) and *Saccharomyces cerevisiae* (FLP/frt)*,* have been tested (Lyznik et al., 1993; Schaart et al., 2011). In all these systems, the recombinase expression is usually controlled by chemical or heat shock inducible promoters to avoid a premature excision of the selectable markers (Fig.1d) (Schaart et al., 2011; Dalla Costa et al., 2016).

Stress tolerant cisgenic crops

Cisgenic approaches were adopted in potato, apple, grapevine, melon, wheat, barley, poplar, rice and strawberry (Gadaleta et al., 2008a; Benjamin et al., 2009; Han et al., 2011; Dhekney et al., 2011; Holme et al., 2012a; Krens et al., 2015; Haverkort et al., 2016; Tamang, 2018; Maltseva et al., 2018). In most cases the aim was to increase pathogen resistance, although some studies were focused on quality trait improvement.

Haverkort and colleagues pursued a marker free approach to obtain four cisgenic Late Blight (*Phytophthora infestans*) resistant potato varieties, by transferring from one to three resistance genes (Haverkort et al., 2016). In addition, cisgenic apple varieties were developed by introducing the apple scab (*Venturia inaequalis*) resistance gene *Rvi6* in the susceptible cultivar 'Gala' (Schaart et al., 2011). In the same work, the authors achieved the removal of the selectable marker gene by inducing the *recombinase* R with dexamethasone. The obtained cisgenic plants were tested in field conditions for three years and showed a stable resistant phenotype (Krens et al., 2015). Interestingly, the effectiveness of the same recombinase system was recently also tested in banana, inducing the excision of the green fluorescent protein, used as reporter gene (Kleidon et al., 2019).

Several pathogen resistance genes (PR1 variants, *VvTL1*, *VvAlb1*, homologues of *VvAMP1* and *VvAMP2*/defensin, and an orthologue of Snakin-1) have been isolated from species sexually compatible with *Vitis vinifera* and overexpressed in transgenic lines, which are now under evaluation in field conditions (Gray et al., 2014). In grapevine, methods using a heat-shock controlled FLP/frt recombination system for selectable marker excision have also been reported (Dalla Costa et al., 2016; Dalla Costa et al., 2020).

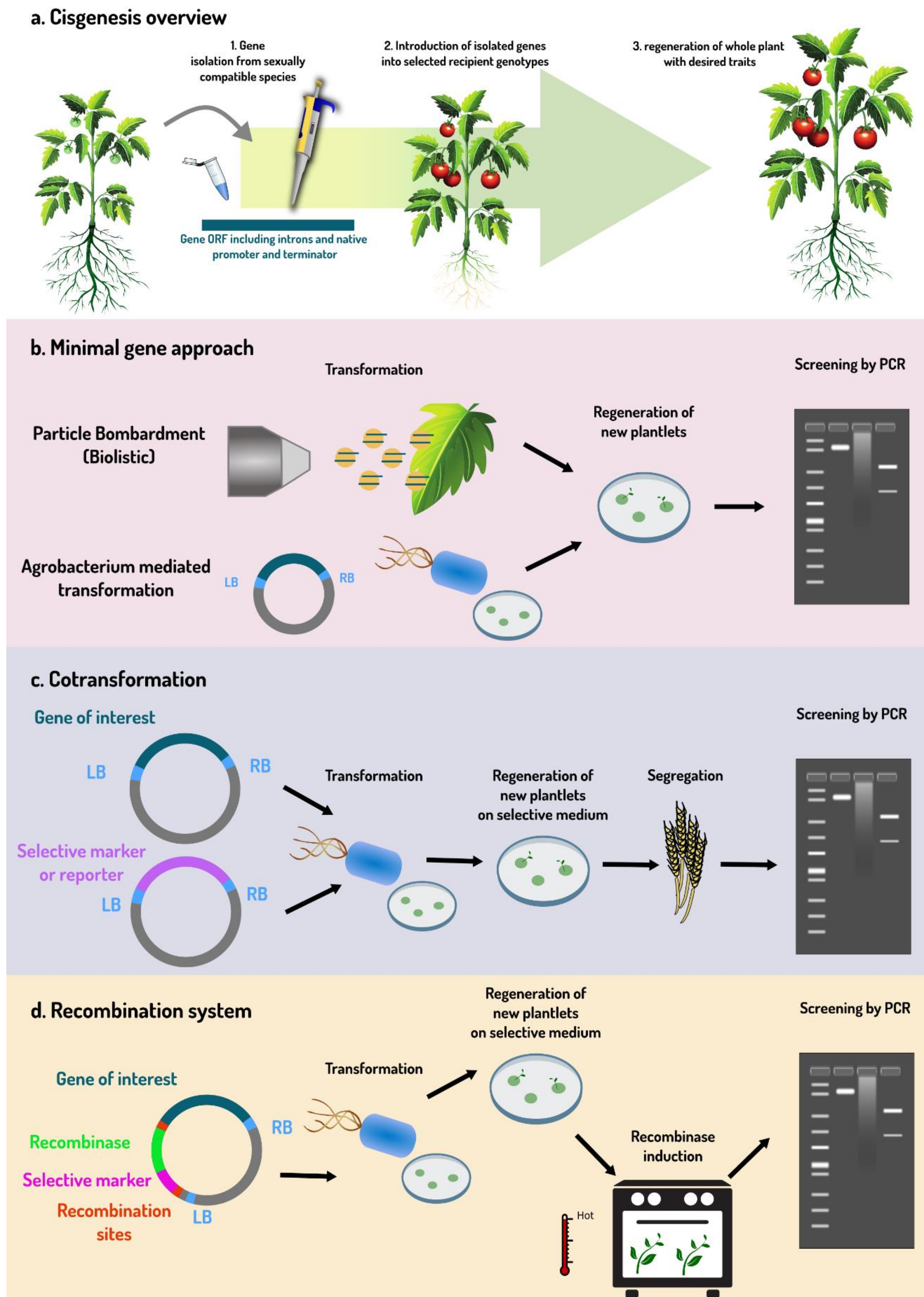

Figure 1. a) Overview of cisgenic strategies from gene selection to plant phenotyping; b) Minimal gene approach, only the gene of interest is cloned in the T-DNA region; c) Co-transformation strategy, the selective marker and gene of interest are introduced by independent transformation events, segregation of the genes allows the selection of cisgenic plants in F1 progeny; d) Excision of unwanted DNA sequences through recombination systems: chemical or physical stimulation induce the excision of DNA fragments flanked by the recombination sites.

Transgenic lines of melon have been developed overexpressing the glyoxylate aminotransferase *At1* and *At2* genes, conferring resistance to *Pseudoperonospora cubensis*, which causes downy mildew in cucurbits (Benjamin et al., 2009). Since the resistance is given by the increased transcription level of these genes, it remains to be assessed whether such an increase can be obtained in cisgenic lines.

In durum wheat, biolistic co-transformation with minimal gene cassettes was used to develop cisgenic lines expressing *1Dy10* HMW glutenin gene, isolated from bread wheat and associated to an improved baking quality. Homozygous cisgenic lines were obtained by segregation at the 4th generation (Gadaleta et al., 2008b; Gadaleta et al., 2008c). Moreover, cisgenic lines of wheat carrying a class I chitinase gene displayed partial resistance to fungal pathogens (Maltseva et al., 2018). Holme et al. (2012b) used a barley phytase gene (*HvPAPhy_a*) and the co-transformation strategy to test cisgenic feasibility in barley, obtaining lines with increased phytase activity (Holme et al., 2012a).

Cisgenesis has also been applied in rice, to overcome one of the most diffuse and devastating pathogens (*Magnaporthe grisea*), by using a co-transformation strategy to introduce rice blast disease resistance gene *Pi9* into elite rice cultivars (Tamang, 2018).

In addition to stress resistance, cisgenesis is also an effective approach for modifying other crop traits as it has been demonstrated in poplar. Genes from *Populus trichocarpa* (*PtGA20ox7*, *PtGA2ox2*, *PtRGL1_2*) involved in gibberellin metabolism were transformed in *Populus tremula* x *alba*, showing that negative gibberellic acid regulators determined a slower growth (*PtGA2ox2*) and longer xylem fibers (*PtRGL1_2*), while the positive regulator determined an increased growth rate (*PtGA20ox7*). However, the poplar plants obtained still contained the positive selectable marker and cannot be considered as cisgenic (Han et al., 2011).

Intragenic plants, as in the case of cisgenesis, possess only genetic material deriving from sexually compatible species, but the inserted gene is the result of a genetic element isolated from different species (e.g. a gene promoter from one species and a coding sequence from another, both sexually compatible) (Holme et al., 2013). An interesting example of this approach comes from the overexpression of cisgenic polygalacturonase inhibitor protein (*FaPGIP*) in strawberry which conferred resistance to grey mold (*Botrytis cinerea*). The overexpression was achieved by cloning the *FaPGIP* coding sequence under the promoter of the strawberry expansin-2 gene and for this reason should be referred to as intragenic (Schaart, 2004).

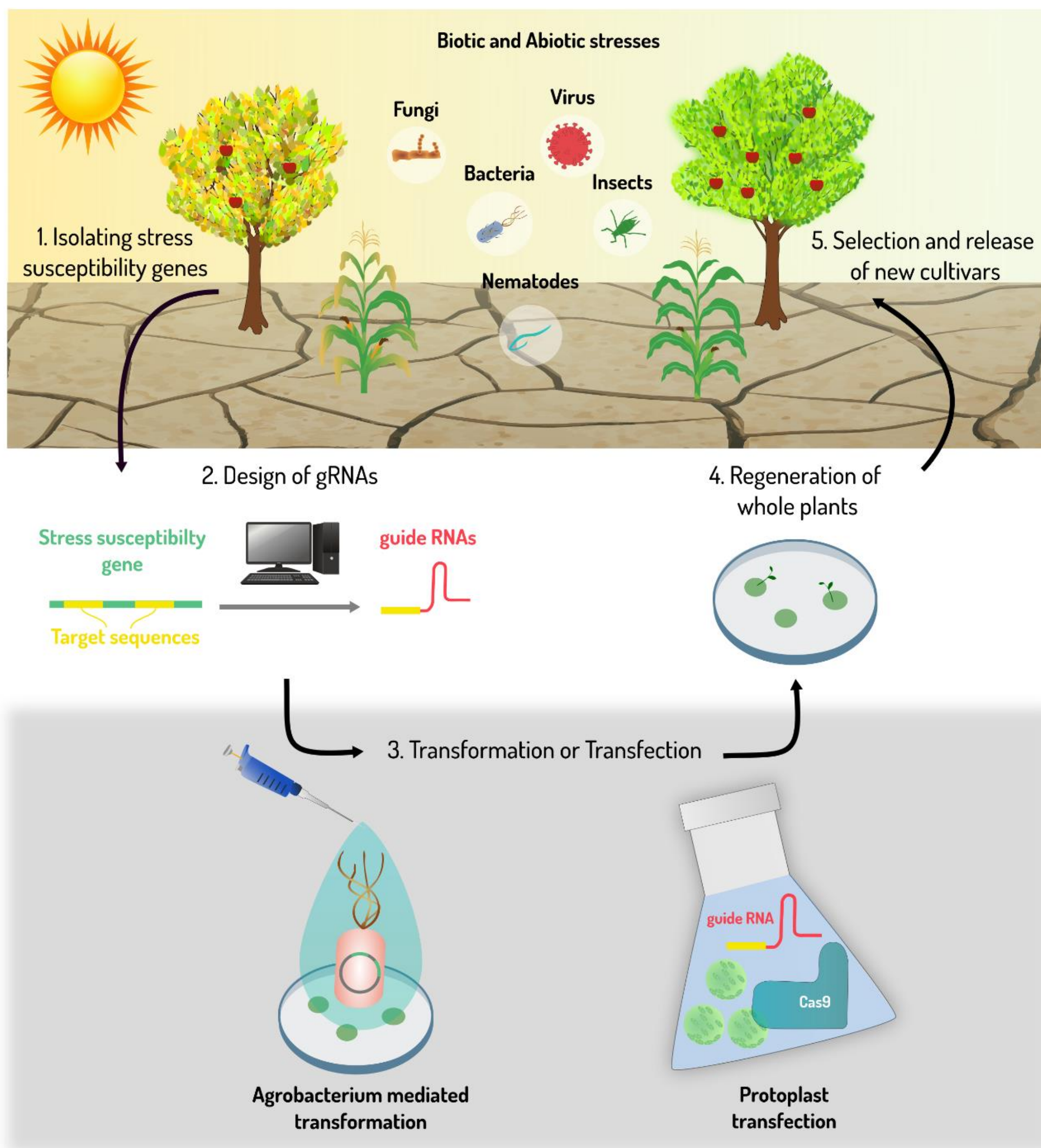

Figure 2. Workflow for the development of Genome edited stress resistant crops: 1) Susceptibility genes are isolated and characterized by genetic and functional genomics studies; 2) Informatics-aided design of gRNAs for increased specificity and off-target minimization; 3) Agrobacterium tumefaciens mediated transformation of plant tissue cultures or ribonucleoprotein protoplast transfection. 4) Regeneration and selection of transformed plants; 5) Testing and selection of transformed lines, release of new varieties.

2.4 Genome Editing

Genome editing introduces changes in specific target DNA sequences without altering other regions (including the target flanking regions) and with the potential to avoid introduction of foreign DNA. The genome editing is performed using endonucleases which are able to recognize specific DNA sequences. Once the target sequence is recognized, the endonuclease introduces a double strand DNA (dsDNA) break (DSB) and induces subsequent activation of the DNA repair pathway (Manghwar et al., 2019). This result can be achieved by exploiting three different classes of enzymes: Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effectors Nucleases (TALENs) and Cas proteins (Zhang et al., 2017). Strong efforts have been made by numerous researchers all over the world to improve the Cas-mediated genome editing technology, which became the most used and efficient tool to edit target genomes (Xie and Yang, 2013). The ability of genome editing techniques to help breeders in improving plant resistance against biotic and abiotic stresses is only in its infancy, but some examples are already available and a concise overview of the steps involved in the development of edited plants is presented in Figure 2.

<u>Focus on CRISPR-Cas: a brief overview</u>

The Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)-Cas systems, discovered as conserved mechanisms against viral invasions in bacteria, require three distinct components: a protein with nuclease activity (e.g. Cas9, Cas12, Cas13 etc.), a single guide RNA (sgRNA) necessary to guide the Cas protein on target sites and a Protospacer Adjacent Motif (PAM), a short sequence upstream of the complementary DNA strand acting as tag of the target site (Fig.3a) (Doudna and Charpentier, 2014). The sgRNA-Cas complex scans the genomic DNA looking for the complementary sequence and, once identified, the Cas protein induces a dsDNA cleavage at a specific position that is determined by the Cas type (Jiang and Doudna, 2017). After DNA cleavage, there are two major pathways of DNA repair in plants: homologous recombination (HR) and nonhomologous end joining (NHEJ), the latter being the most commonly used (Schwartz, 2005; Ran et al., 2013). These two repair mechanisms are the basis for exploiting the Cas in NPBTs.

The CRISPR-Cas system shows very versatile features to produce knock-out mutants, to insert a DNA fragment using a donor vector through the HR system, to base edit a target sequence (e.g. substitutions of C to T and/or A to G etc.), to induce mutation in regulatory sequences, and modify the epigenome (Vats et al., 2019). Nevertheless, if multiple genes that are closely related have to be targeted (e.g. gene family members, multiple alleles of the same

gene) two different strategies are available: i) multiple guide RNAs under the control of a same promoter (polycistronic construct) or multiple guides under the control of their own specific promoter (Xing et al., 2014; Tang et al., 2016; Cermak et al., 2017) and ii) one or a few sgRNAs capable of driving the Cas protein on different genes (Yu et al., 2018).

Initial steps through a wide use of CRISPR/Cas system

The first reported genome-editing application using CRISPR/Cas systems in plant was achieved in 2013 using two model organisms: *Arabidopsis thaliana* and *Nicotiana benthamiana* and easily observable reporter genes (Li et al., 2013; Mao et al., 2013). Over the years, more progress has been made, with several reports in different herbaceous plant species (e.g. tomato, rice, soybean, wheat, etc.) up to the application in woody species (e.g. citrus, apple, grape, etc.) (Ghogare et al., 2020). Furthermore, different laboratories are committed in developing new delivery methods for plant systems. Indeed, classically the DNA sequences encoding for Cas and sgRNA(s) have to be delivered into the host plant genome, and to date, different methods have been tested: *Agrobacterium*-mediated transformation, nanoparticle platforms, biolistic transformation and protoplast transfection (Ahmad and Amiji, 2018; Kalinina et al., 2020). Even though *Agrobacterium*-mediated transformation is widely used in plants, this method requires integration of T-DNA into the host genome together with selectable marker genes (Dalla Costa et al., 2016; Duensing et al., 2018). Actually, the integration of selectable markers is an important legislative issue as it can be stably transferred to sexually compatible species and also to other organisms, without reproduction or human intervention, as a consequence of horizontal gene transfer (HGT) (Keese, 2008; Soda et al., 2017). Conversely, protoplasts transient transformation and regeneration approach allows the direct delivery of ribonucleoproteins (RNPs) in plant tissues without introducing foreign DNA and GM plant creation (Baltes et al., 2015; Cermak et al., 2017; Bruetschy, 2019). Recently the *Agrobacterium*-mediated transformation was compared with the RNPs delivery through PEG-mediated protoplasts transfection approaches in apple and grapevine (Osakabe et al., 2018). Although the biolistic method allows the production of transgene-free plants it displays huge limitations in woody plants (Osakabe et al., 2018) due to restraint in obtaining the embryogenic tissue, which is then able to regenerate the edited plant (Altpeter et al., 2005).

CRISPR technology as a valuable tool to improve crop protection

One of the main tools to enhance plant resistance against fungal and bacterial pathogens relies on targeting susceptible genes (S genes) (Pavan et al., 2009) as proven in *Theobroma cacao* and

several other species (Fister et al., 2018; Langner et al., 2018). Susceptibility gene distinctiveness relies on the fact that they are genes that critically facilitate the compatibility between the plant and the pathogen. They are essential for their interaction, especially in the case of biotrophic pathogens. Therefore, mutation or loss of an S gene can limit the ability of the pathogen to cause disease (van Schie and Takken, 2014). An interesting example was given by Paula et al. (2016), who introduced a mutation in *Solanum lycopersicum DMR6* gene lowering tomato susceptibility not only to downy mildew but also to *Pseudomonas syringae, Phytophthora capsici* and *Xanthomonas* spp. (Paula de Toledo Thomazella et al., 2016). A similar approach was used in apple (*Malus domestica*) to achieve resistance against *Erwinia amylovora* (Pessina et al., 2016). Pompili et al. (2020) used the Cas9 system to produce an *MdDIPM4* knock-out mutant enhancing plant resistance against the fire blight pathogen. A novelty introduced by this approach is an inducible recombination system (FLP/frt) able to remove almost all the T-DNA insertions after confirming the editing event. CRISPR technology was latterly applied to rice in order to obtain bacterial blight resistant varieties: Cas9-mediated genome editing to introduce mutation in one or multiple susceptible genes, belonging to the sugar transporters SWEET family, was successfully achieved in recent works (Oliva et al., 2019; Zeng et al., 2020). Finally, another interesting application of CRISPR to counteract biotic stress was provided in tomato. By targeting a microRNA (miRNA) it was demonstrated the possibility to enhance plant immunity against *Fusarium oxysporum* f. sp. *Lycopersici*, the causal agent of tomato wilt disease, enhancing the basal expression of nucleotide-binding site-leucine rich repeat (NBS-LRR) protein (Gao et al., 2020).

As for fungal and bacterial pathogens, the CRISPR technology can provide a strategy to generate plants with virus resistance. For instance, it is possible to both directly target viral replication, by producing GMO plants expressing constitutive Cas protein and gRNA(s) that target viral sequences (Baltes et al., 2015; Ji et al., 2015) or to generate virus resistant cultivars through modification of plant genes (Kalinina et al., 2020 and references therein).

Beyond biotic stresses, and despite a limited number of papers, abiotic stresses such as water deficit, high temperature and soil salinity can also be tackled by editing plant genes involved in stress response (Nguyen et al., 2018; Zafar et al., 2020; Joshi et al., 2020). An interesting example was reported in a work where the *OST2/AHA1* locus (which regulates stomata response to abscisic acid) was edited to obtain Arabidopsis with increased stomatal responses upon drought and a consequent lower water loss rate (Osakabe and Osakabe, 2017). In parallel, if not directly applied to achieve drought-resistant crops, CRISPR technology can be exploited to study the function of gene(s) along complex regulatory mechanisms. This was the

case of nonexpressor of pathogenesis-related gene 1 (NPR1), a special receptor of salicylic acid (SA), considered as an integral part in systemic acquired resistance (SAR) (Wu et al., 2012). Cas9 was used to obtain NPR1 tomato mutants, which showed reduced drought tolerance, demonstrating that, despite its involvement in biotic stress responses, NPR1 is also involved in abiotic stress resilience (Li et al., 2019). More recently, the CRISPR activation (CRISPRa) system (Brocken et al., 2018) (based on an inactivated version of the nuclease known as dead Cas9 -see next paragraph for more information- fused with a transcription activator) targeting the promoter of ABA-responsive element binding proteins (AREB) was used to study stress-related responses and enhance the drought tolerance in *Arabidopsis* (Roca Paixão et al., 2019).

New frontiers in CRISPR/Cas application

Although genome editing has been widely used for editing specific plant genes, several studies relied on the improvement of its efficiency, versatility and specificity (Gleditzsch et al., 2019). Indeed, despite many theoretical advantages and potential applications, the genome editing techniques still present one major drawback: Cas proteins can recognize PAM sites in non-target sequences and thus induce DSBs in these sequences, leading to undesirable phenotypes. To mitigate the off-target activities, different bioinformatic approaches were developed and used for computational prediction of Cas activity on specific genomes (Bae et al., 2014; Lin and Wong, 2018; Liu et al., 2020a). Moreover, development of Cas variants with improved specificity, such as Cas12a and b (Ming et al., 2020; Schindele and Puchta, 2020), eSpCas9 (Slaymaker et al., 2016), HiFi-Cas9 (Kleinstiver et al., 2016) and HypaCas9 (Ikeda et al., 2019) tried to mitigate the off-target activity and these variants have already been applied in plant genome editing strategies.
Beside the improved Cas variants, different authors have been focusing on the implementation of dead Cas9 (dCas9) (a Cas9 where both the nuclease domains have been inactivated) that could be used for several purposes. The simplest one is the ability to interfere with transcription via steric blockage of polymerase without performing endonuclease activity (Brocken et al., 2018). Furthermore, the dCas9 system can be engineered by linking it to a transcription activator or repressor. These systems can be applied to species that lack a controllable expression system or to study the overexpression or downregulation of target genes, without changing the genome context or introducing a transgene (Mohanta et al., 2017a; Moradpour et al., 2020).

The CRISPR-Cas system has also been engineered to perform base-editing. Base-editing is the ability to directly manipulate DNA sequences enabling the conversion of one base pair to another without performing a DSBs (Anzalone et al., 2019; Yang et al., 2019). A few years ago,

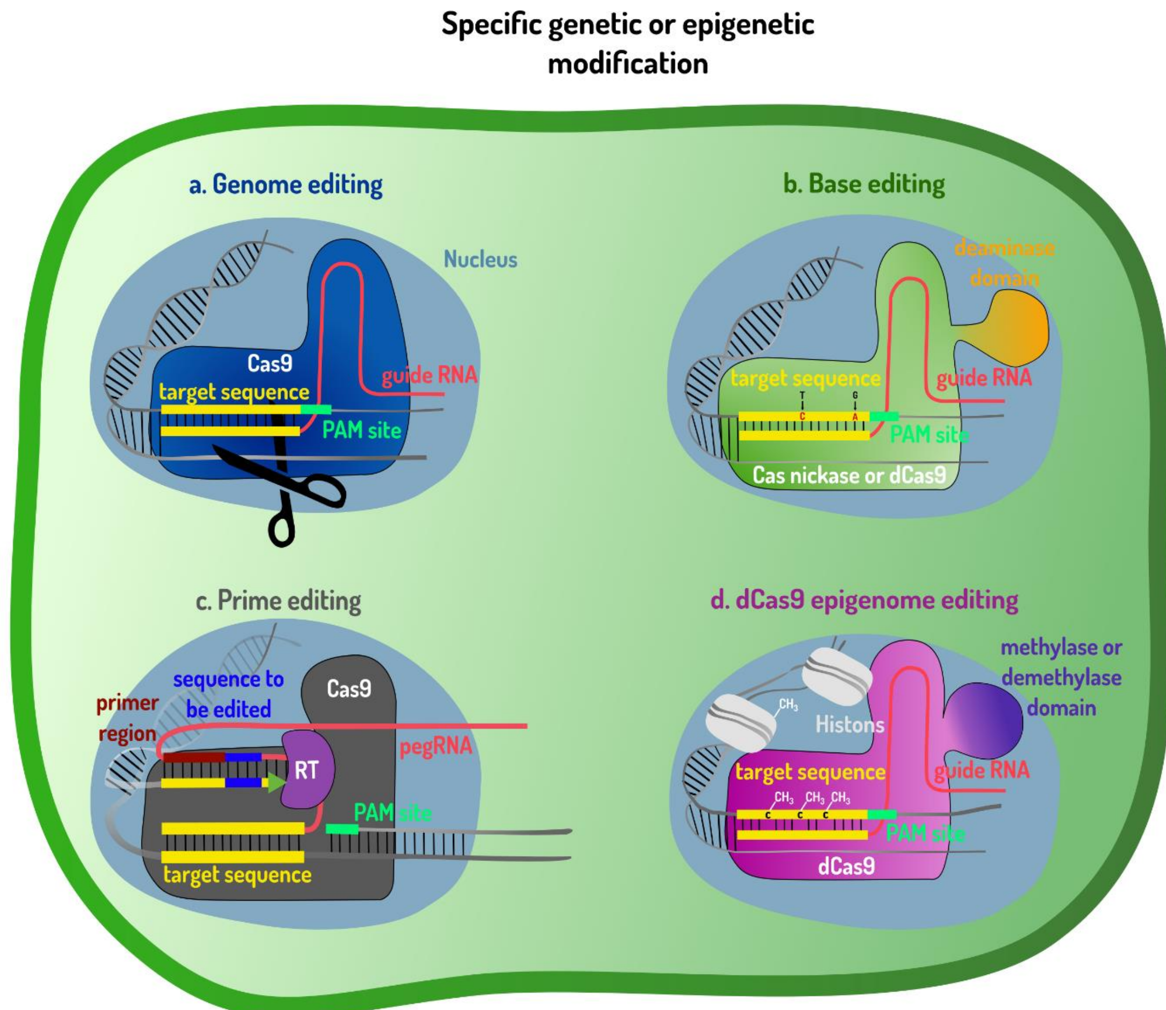

Figure 3. Highly specific genetic and epigenetic modifications by CRISPR-Cas technology: 3a-Genome editing; 3b-Base Editing; 3c-Prime Editing; 3d-Epigenome Editing.

Shimatani and colleagues (2017) used CRISPR-Cas9 fused to *Petromyzon marinus* cytidine deaminase *(PmCDA1)* and gRNAs to introduce point mutations in the acetolactate synthase (ALS) gene of rice and tomato, obtaining herbicide resistance (Shimatani et al., 2017). Recently, base editing has been improved thanks to the development of prime-editing, which is more efficient than the classic base editing (Anzalone et al., 2019; Yang et al., 2019). Differently from the classic dCas9, in prime-editing only one nuclease domain is inactivated, generating a DNA nickase enzyme. The latter, combined with a retrotranscriptase enzyme (RT) and a Prime Editing Guide RNA (called pegRNA), can produce both transition and transversion mutations, extending the possibility of common base editing (Fig.3b-c) (Anzalone

et al., 2019). In a recent article, Plant Prime Editing (PPE) was tested in rice and wheat, giving the first proof of concept in plants. The authors chose six different genes and by evaluating the single base editing efficiencies, confirmed the ability of PPE to produce all kinds of base substitutions (Lin et al., 2020).

Lastly, it is worth noting that a new class of CRISPR-Cas systems specifically targets RNA instead of DNA (Abudayyeh et al., 2017) and has been successfully used in plants to induce interference toward RNA viruses (Lotterhos et al., 2018). Added to this RNA targeting ability of the Cas13, a dCas13 conjugated to a deaminase was also suitable for RNA editing converting A to G and hence obtaining a system that can be used to edit full-length transcripts with pathogenic mutations (Cox et al., 2017). The rapid development of such a powerful and innovative techniques is the basis to achieve increased crop yields, resilient crops to both biotic and abiotic stress and to address consumer's concerns on GMOs approaches as well as nutritional needs (Kumar et al., 2020).

2.5 Towards new GMO-free approaches: exogenous dsRNA application for crop protection

Small RNAs (sRNAs) and RNA interference (RNAi) have emerged as modulators of gene expression in plant immune responses, pathogen virulence, and communications in plant-microbe interactions. Since the RNAi machinery discovery, many efforts have been made to improve its applicability in plant protection (Cagliari et al., 2019; Dalakouras et al., 2020). In plants, RNAi is well known as a conserved regulatory strategy playing key roles in endogenous transcript regulation as well as viral defense, resulting in the post transcriptional downregulation of the target RNA sequence(s). The RNAi machinery is triggered by double stranded RNA (dsRNA) molecules that, once produced in the cell, are processed by RNase III DICER-LIKE endonucleases and cleaved into 21-24 nt short interfering RNAs (siRNAs) (Liu et al., 2020b). After cleavage, one of the two siRNA strands associates to ARGONAUTE (AGOs) proteins to form RNA-induced silencing complexes (RISCs) (Poulsen et al., 2013; Meister, 2013). Consequently, these RISCs specifically interact with transcripts on sequenced-based complementarity, resulting in mRNA cleavage or translational repression, in a process known as Post Transcriptional Gene Silencing (PTGS) (Fig. 4) (Kim, 2008; Mi et al., 2008). Additionally, siRNAs can promote the deposition of repressive chromatin marks in target genomic DNA sequences triggering transcriptional gene silencing (TGS). In plants and invertebrates, siRNAs also have an important function in plant host-pathogen interactions: in the case of viral infections siRNAs are produced in infected cells directly by processing

dsRNA molecules derived from the viral genome itself. Interestingly, there is evidence that siRNAs, once produced in a specific cell, are able to move via plasmodesmata reaching the surrounding

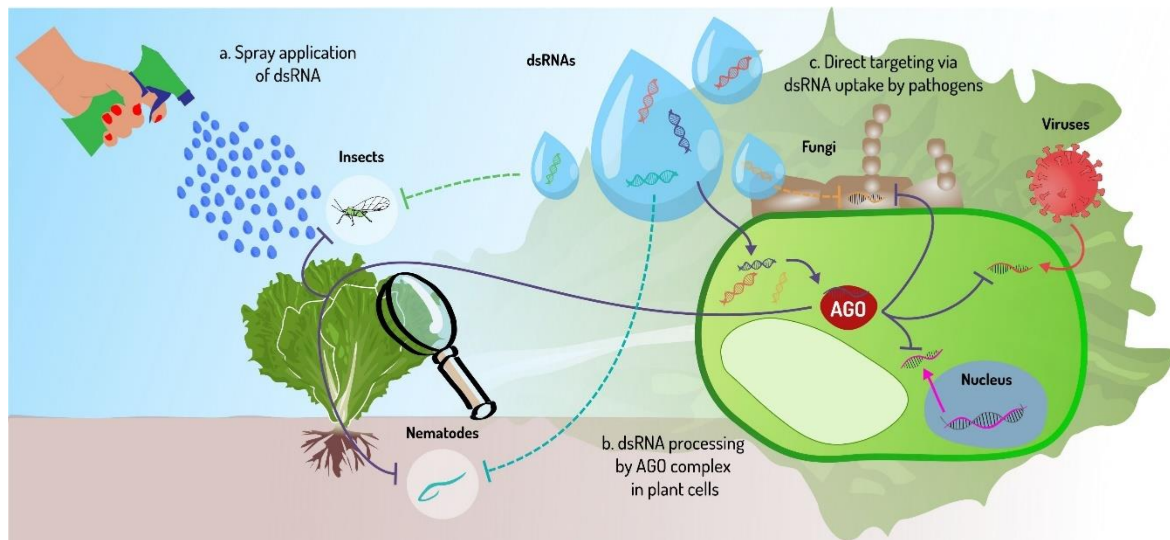

Figure 4. dsRNAs applications in crop protection: a) dsRNA are sprayed on plants in field conditions; b) dsRNAs penetrate the plant cells and after being processed by DICER-like nucleases associated with Argonaute protein (AGO) inducing Post Transcriptional Gene Silencing toward pathogens or endogenous genes, continuous lines (▬); c) dsRNA directly enters pathogen cells silencing one or more essential genes, dotted lines (---).

cells and, through the vascular system, up to distal parts of the plant, inducing the systemic silencing. Both siRNAs short distance and long distance transport mechanisms to the whole plant have been documented and are still under scrutiny (Ham and Lucas, 2017).

Natural cross-kingdom RNAi and its biotechnological application

The RNAi processes are also pivotal in triggering plant immunity against pests and pathogens, modulating their development and virulence. There are lines of evidence supporting the observation that sRNAs can be exchanged bidirectionally among the interacting partners (e.g. plant-fungi) inducing gene silencing in each other and leading to a mechanism named as cross-kingdom RNAi (Wang et al., 2016a; Cai et al., 2018b; Ma et al., 2020). The latter is mediated by exosome-like extracellular vesicles able to deliver sRNAs into the interacting organisms, as recently demonstrated in *Arabidopsis*-*B. cinerea* pathosystem (Cai et al., 2018a). In particular, it was demonstrated that plant delivered sRNAs can downregulate the production of pathogen effectors, whereas *Botrytis* is able to deliver sRNAs, which turn off plant defenses. All this evidence indicates that cross-kingdom RNAi can be utilized to control plant diseases caused

by pathogens, including fungi, viruses, and pests, such as nematodes and insects and foster the application of RNAi strategy to counteract crop pathogens.

Indeed, beside the fascinating mechanisms of siRNA production and translocation in plants, RNAi also represents a promising sustainable and environmentally friendly tool that can be used against crop pests and pathogens and might represent a good alternative to the application of chemicals. So far, in plants, RNAi has been largely used in functional genomic studies or for inducing resistance against insects in transgenic plants (e.g. in maize against *Diabrotica virgifera virgifera* (Fishilevich et al., 2016). *Agrobacterium*-mediated transformation has been applied to express pathogen/pest gene-targeting sRNAs or dsRNA against a selected target. This procedure named as host-induced gene silencing, HIGS, has led to the production of GM crop varieties, not commercialized in Europe (Baulcombe, 2015; Dalakouras et al., 2020 and references therein). Alternatively, a virus-induced gene silencing (VIGS) approach can be applied to express designed pathogen-targeting RNAs in plant tissue and circumvent the generation of GMOs (Lee et al., 2012; Dommes et al., 2019). Indeed, a recent report demonstrated the potentiality of VIGS as a tool for transiently targeting diverse regulatory circuits within a plant and indirectly affecting important agronomic traits, without incorporating transgenic modifications (Torti et al., 2021). However, VIGS relies on the use of virus expression vectors, which are themselves pathogenic to the plant and currently the development of a low or non-pathogenic virus expression vector is a major obstacle to the application of VIGS in crops.

The new frontier of RNAi for crop protection

GMO-free RNAi strategies, based on exogenous dsRNA/siRNA direct applications on plants (Dubrovina and Kiselev, 2019) are among the new approaches developed to overcome plant transformation and its limitations. Some examples of plant endogene modulation by exogenous dsRNAs application are available in the literature. In Arabidopsis, dsRNAs mixed with nanoparticles were adsorbed by plant roots and triggered RNAi against *SHOOT MERISTEMLESS* (*SSTM*) and *WEREWOLF* (*WER*) genes, which are involved in apical meristem and root epidermis regulation (Jiang et al., 2014). In another work, the authors suppressed the expression of a *MYB1* gene using crude bacterial extract containing dsRNAs (Lau et al., 2015). These studies confirmed the activation of RNAi in plants by dsRNAs adsorption through different tissues and by root soaking in a solution of dsRNAs (Li et al., 2015; Dalakouras et al., 2016; Dalakouras et al., 2018). These results also suggest that dsRNAs direct application could represent an effective disease-control strategy against fungal

pathogens in crops. Several articles have indeed reported that the exogenous application *in vitro* or *in vivo* of synthesized long dsRNAs (through bacteria-mediated biosynthesis), hairpin RNAs (hpRNAs) or siRNAs can down-regulate the expression of pest essential genes, thus controlling harmful insects, fungal and viral pathogens. The RNA molecules were successfully applied by using several methods, such as high or low pressure spraying (spray induced gene silencing, SIGS), trunk injection, petiole absorption, soil/root drenching or mechanical inoculation and delivered naked or loaded into carriers (e.g. clay nanosheet, nanoparticles, proteins) to facilitate their uptake and survivability in plant tissues up to 7-8 weeks (Mitter et al., 2017; Dubrovina and Kiselev, 2019; Dalakouras et al., 2020). In the past few years, reports on plant-mediated delivery of dsRNAs against insects demonstrated the lowering of biological activity and/or increased mortality of aphids, whiteflies, mites, and marmorated sting bugs in tomato and bean crops (Gogoi et al., 2017; Ghosh et al., 2018). In addition, dsRNAs microinjection in *Euscelidius variegatus,* a natural vector for phytoplasmas, has recently been reported (Abbà et al., 2019). In this respect, Dalakouras et al. (2018) provided very useful information to improve the plant-mediated dsRNAs efficacy against insects, suggesting the delivery of intact dsRNA, by using specific methods (e.g. petiole adsorption or trunk injection) to avoid the activation of plant RNA processing mechanisms. Indeed, the intact dsRNAs can be translocated by xylem vessels to plant distal tissues, picked up by insects and processed into siRNAs by their own RNAi system, resulting in a more effective response.

Exogenously delivered dsRNAs have been successfully applied in several fungal-plant pathosystems. As for insects, also in fungi, intact dsRNAs are proved to be more efficient in controlling pathogen development. This was first demonstrated by Koch et al. (2016), in which spraying dsRNAs on barley leaves achieved control of *Fusarium graminearum*. In addition, SIGS was effective against several fungal pathogens such as, for example, *Sclerotinia sclerotiorum* in *Brassica napus* (McLoughlin et al., 2018), *Fusarium asiaticum* in wheat coleoptiles (Song et al., 2018b), *Botrytis cinerea* in several plants (Wang et al., 2016a) including grapevine, in both natural and post-harvest condition (Nerva et al., 2020).

The exogenous dsRNAs applications for plant gene regulation still require further investigation and development, especially as concerns the necessity to unveil cell regulatory aspects, which are still largely ignored. In detail, some reports showed that the majority of plant endo-genes display a low RNAi susceptibility, depending on the presence of introns, well known to suppress the RNA silencing processes (Christie et al., 2011). Similarly, it is worth noting that several technological developments are still needed to achieve the wide diffusion of dsRNAs as protective molecules in crops. First of all, formulations with nanoparticles

and/or other synthetic carriers are needed to slow down the rapid dsRNAs degradation, which is a major hurdle in the practical application of SIGS. Secondly, new delivery strategies such as the high-pressure spraying or brush-mediated leaf applications (Dalakouras et al., 2016; Dalakouras et al., 2018) need to be implemented for effective field applications. Finally, a specific science-based risk assessment procedure for exogenous application of dsRNA have to implemented since the actual evaluation of plant protection products (PPP) is not appropriate to establish the environmental fate and the risk associated to the field application of such products (Mezzetti et al., 2020).

Challenges for exogenous dsRNAs application in crop protection

In addition to the above-mentioned formulation issues, it is worth noting that the application of dsRNAs as bio-based pesticides requires a good knowledge of the target organisms. In fact, differences in dsRNAs susceptibility among different organisms and even among genera belonging to the same family have been reported. Specifically, concentrations, length of dsRNA molecules, uptake and recognition pattern by the RNAi machinery can influence the efficacy of the applied treatments.

The total amount of sprayed/supplied dsRNA is one the most variable factors among different reports: effective concentrations from pmol to mg per treated organism were reported (Das and Sherif, 2020 and references therein). This might be one of the most important limiting factors for field applications and implementation, because the amount of dsRNAs /treatment would affect the price per treatment, discouraging their application in case of high costs. Encapsulation methods would probably reduce this problem protecting from degradation and/or facilitating the entrance of dsRNAs into the target tissues (Dalakouras et al., 2020). Together with the concentration, other parameters which show discrepancy in the literature is the optimum length of dsRNAs: lengths from 21bp to more than 1kb were analyzed in several works. In this case all reports highlighted that dsRNAs within a size from 150bp to 500bp are the most efficient in inducing the activation of the RNAi pathway (Das and Sherif, 2020; He et al., 2020; He et al., 2020; Höfle et al., 2020). These results are explained by the nature of RNAi pathway, which requires sequences long enough to be recognized by the molecular machinery but which also need to pass through the cell membrane (and in case of plants and fungi the cell wall) which works as a molecular sieve.

The other important parameters, which represent the most limiting factors at the moment, are the uptake mechanisms of dsRNAs into cells and, once entered, the recognition of specific pattern/sequences by the target RNAi machinery. The dsRNAs uptake mechanism was first

described in *C. elegans*, with the description of Systemic RNAi Defective (SID) proteins, which are involved in the acquisition and transportation of dsRNAs and the derived siRNA along the nematode body (Winston et al., 2002; Winston et al., 2007; Hinas et al., 2012). Several SID-like proteins were described in insects with not uniform results: in some insects these proteins are crucial for the activation of a strong RNAi response, whereas in some other cases they seem to be unnecessary (Wytinck et al., 2020a and references therein). Another mechanism which has been proposed as one of the preferred routs of entry for dsRNAs is the clathrin mediated endocytosis. Both in insects and fungi it has been demonstrated that endocytosis facilitated the uptake of dsRNAs (Wang et al., 2016b; Pinheiro et al., 2018; Wytinck et al., 2020b) but further studies are needed to clarify the mechanism in more details. Information about adsorption and transportation is fundamental also to understand the onset of resistance mechanisms in pest and pathogens, as already reported for *D. virgifera*, which showed a reduced dsRNAs uptake with an increased resistance to the treatment in just 11 generations (Khajuria et al., 2018). Additionally, one of the most important, but poorly understood factor is the recognition of the dsRNAs by the RNAi pathway of the target organism. In this respect, contrasting results have been reported for fungi and insects. In case of fungi, application of dsRNAs to the plant, that will process them into siRNAs, and which are then adsorbed by the fungus results the most effective strategy (Wang et al., 2016b; Song et al., 2018a; Nerva et al., 2020). These results are consistent with the inability of fungi to activate a secondary siRNA amplification mechanism and the exploitation of the plant machinery to enhance the gene silencing treatment effectiveness. In contrast to fungi, insects display a puzzling variety of responses, which are not always linked to evolutive features and show differences among genera of the same family. For example, as recently reviewed (Dalakouras et al., 2020), Coleoptera order are the most susceptible to RNAi, whereas lepidopterans and hemipterans seem recalcitrant to RNAi due to either impaired dsRNAs uptake or to the production of nucleases in their saliva. For this reason GMO approaches relaying on the expression of dsRNAs in chloroplasts, which do not process them into siRNA, displayed a stronger efficacy (Bally et al., 2018). Apart from the preference of siRNAs or intact dsRNA delivery treatments, there is also a lack of information about the recognition of preferred nucleotide residues on the dsRNA for their processing into siRNAs by dicer-like enzymes (DCL). Particularly, DCL sequence evolution characteristics appear to be species-dependent (Guan et al., 2018; Arraes et al., 2020) and can lead to the generation of siRNAs with species-dependent length distribution among different insects (Santos et al., 2019). Taken together these data suggest that for an optimal exploitation of dsRNAs as sustainable plant

protection strategies, data on formulations (intended as dsRNAs size and concentration), uptakes mechanisms and features of RNAi machinery of target pests/pathogens need to be implemented.

2.6 Epigenetic signatures and modifications to improve crop resilience against biotic and abiotic stresses

Both PTGS and TGS are involved in plant immunity and specifically in the control of viral virulence through RNA silencing. However, plants use gene silencing mechanisms, and in particular the RNA-dependent DNA Methylation pathway (RdDM) for regulation of their own gene expression and the transcriptional repression of transposable elements (TEs).

In plants, chromatin can be modified at the level of DNA sequence by DNA methylation at CG, CHG, and CHH (H = A, T or C) contexts through distinct pathways. While METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) are plant enzymes responsible for the maintenance of CG and CHG methylation, respectively, after DNA replication, CHH methylation is established *de novo* through two pathways. Plant RNA-dependent DNA Methylation pathway (RdDM) involves the biogenesis of small interfering RNAs. ARGONAUTE (AGO) family members target 24-nt siRNAs to corresponding genomic loci, which in turn are methylated in CHH and CHG context *via* DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2). DRM2 is responsible for *de novo* DNA methylation of transposons located within euchromatic regions (Yaari et al., 2019). A second pathway requires CHROMOMETHYLASE 2 (CMT2) through interaction with DECREASE IN DNA METHYLATION1 (DDM1) in histone H1-enriched chromatic regions (Zemach et al., 2013). A family of bifunctional methyl-cytosine glycosylases-apurinic/apyrimidinic lyase actively removes DNA methylation, through a base excision repair mechanism (Penterman et al., 2007). DNA methylation may affect gene expression, regulate imprinting and activate transposable elements (TEs) and TE-associated genes, particularly in response to environmental cues (Law and Jacobsen, 2010).

Numerous studies indicate that DNA methylation plays a part in the pathogen-induced immune system and can strongly influence the resistance response in different plant species, as recently reviewed in Tirnaz and Batley (2019). Among these studies, interestingly it has been reported in rice that the epigenetic regulation of PigmS, a gene involved in resistance to rice blast caused by the fungal pathogen *Pyricularia oryzae*, affects plant resistance and indirectly yield. A genome wide methylation analysis demonstrated that the PigmS promoter region contains two tandem miniature transposons MITE1 and MITE2 that are repressed by DNA

methylation. Indeed, CHH methylation levels at MITE1 and MITE2 and in particular RdDM-mediated silencing of the MITE-nested PigmS promoter control PigmS expression and consequently resistance to rice blast (Deng et al., 2017). Intriguingly, this work on rice highlights the need for a thorough characterization of the RdDM epigenetic pathway and DNA methylation pathway in crops. The double aim of studying the involvement of these pathways in plant pathogen interactions can be to clarify how they regulate the expression of resistance genes and what genes are activated in crops, when exogenous double stranded RNAs are introduced in the plant cell. Answering these questions might pave the way for new strategies both for crop protection management and breeding programs for plant resistance, which can incorporate DNA methylation as a new source of variation.

In the plant cell, along with DNA methylation, other chromatin marks can arrange various chromatin states that epigenetically determine specific transcriptional outputs, thus influencing both biotic and abiotic plant stress response (Pecinka et al., 2020). Nucleosome association to DNA is influenced by many kinds of reversible covalent posttranslational modifications (PTMs e.g. acetylation, methylation, phosphorylation, ubiquitination and many others) of the histone tails, in particular of histone H3 and H4 that are enriched in lysine (K) and arginine (R). In addition to PTMs and the positioning of nucleosomes, DNA accessibility is also affected by the incorporation of histone variants (H2A.Z, H2A.X, H3.1, H3.3) which have different specialized properties and can replace canonical core histones in the nucleosome. The histone code hypothesis postulates that deposition, removal, and recognition of each PTM to histones requires specialized enzymes defined as writers, erasers and readers, respectively (Jenuwein, 2001). Although there is some evidence that histone modifiers and chromatin remodelers can affect the expression of genes involved in the plant immune response, this evidence is limited to a few plant species, such as Arabidopsis and rice (Ramirez-Prado et al., 2018). Histone deacetylases (HDACs), acetyltransferases (HATs), methylases, demethylases, ubiquitinases, can act as positive and negative regulators in plant resistance to different stressors. In a recent work, the authors have studied the interactions between the bacterium *Pseudomonas piscium*, from the wheat head microbiome, and the plant pathogenic fungus *Fusarium graminearum*. They have observed that phenazine-1-carboxamide, a compound secreted by the bacteria, influences the activity of a fungal histone acetyltransferase, leading to deregulation of histone acetylation suppression of fungal growth, virulence and mycotoxin biosynthesis. This study highlights a novel mechanism of epigenetic regulation in antagonistic bacterial–fungal interaction that might be potentially useful in crop protection (Chen et al., 2018).

Genome editing tools for epigenome modification

Genome-wide mapping of epigenomic marks and epigenetic target identification are currently two major efforts in many important crops. In the future, it is desirable that these efforts will offer breeders new application to increase and manipulate epigenomic variability, for selecting novel crop varieties more resilient to biotic and abiotic stresses. In recent years, different techniques have been developed to modify the epigenome globally or at target sites. In crops, gene silencing and variation in DNA methylation profiles could be achieved by inducing siRNA expression, because DNA methylation deficient mutants, which would be useful to alter the methylome, have not been identified in all crops, suggesting that they might be lethal (Kawakatsu and Ecker, 2019). At specific genome sites, fusions of epigenome-modifying enzymes to programmable DNA-binding proteins can achieve targeted DNA methylation and diverse histone modifications (Rivenbark et al., 2012; Mendenhall et al., 2013). Particularly, the genome editing tool CRISPR/deadCas9 can be fused to epigenetic-state modifying enzymes and targeted to genes or cis-regulatory elements (CREs) to modulate plant gene expression. A complete set of plant epigenetic editing tools can be generated by fusing CRISPR-dCas9 system to target modifying enzymes for applications in plant breeding for crop protection. The so-called epigenome editing can be used to re-write an epigenetic mark modifying the endogenous gene expression level of one or several genes (Hilton et al., 2015; Miglani et al., 2020) (Fig.3d). An example of such an approach was given in Arabidopsis using a dCas9 linked to the histone acetyltransferase *AtHAT1* to improve the transcription of *AREB1*, a gene involved in abscisic acid (ABA) perception (Roca Paixão et al., 2019; Miglani et al., 2020). The epigenome-edited plant showed enhanced drought resilience and chlorophyll content when compared to controls.

The use of genome editing tools that modify the epigenome at the recombination sites has been proposed as a possible application for manipulating the rate and positions of crossing over (CO), to increase the genetic and epigenetic variation accessible to breeders. In Arabidopsis the disruption of histone 3 di-methylation on lysine 9 (H3K9me2) and non-CG DNA methylation pathways increases meiotic recombination in proximity to the centromeres (Underwood et al., 2018). Although the results obtained in a model species suggest that manipulation of epigenetic marks can allow CO position and frequency to be expanded, further studies are needed to determine the effectiveness of similar approaches in different plant species. Strategies for controlling recombination represent novel potential tools to both

reveal unexplored epigenetic diversity and control its inheritance, since they have the potential to reduce the time for breeding novel more resilient crops.

2.7 Beyond the limits

A main factor limiting the success of NPBTs is plant regeneration after *in vitro* manipulation, particularly for woody plants, being sometimes a cultivar-dependent process. Although the key pathways and molecules have recently been unveiled (Sugimoto et al., 2019), the mechanism of regeneration is not fully understood, and technical issues are still present. Improvements of the regeneration efficiency have been obtained by crop transformation with morphogenic regulators (e.g. *Baby boom* and *Wuschel* genes) which can induce a more efficient meristem differentiation in recalcitrant species (Lowe et al., 2016; Maher et al., 2020; Yavuz et al., 2020). Despite the great potential of such approach, the fact that gene sequences of morphogenic regulators are protected by patents from private companies (Lowe et al., 2016; Maher et al., 2020) might limit the application of this technological innovation. Hence, it is fundamental to achieve higher regeneration efficiency, opening the way to the minimal gene approach even in recalcitrant woody plant species.

Another limiting factor is the low number of available genes involved in the resistance response with an identified function. Indeed, the identification of resistance genes from landraces and wild crop relatives and their functional genetic validation represents the first steps toward the development of new cisgenic varieties. The importance of these steps was recently reported in several herbaceous and woody plants. In wheat, for example, several genes conferring partial resistance to stem rust have been cloned, including *SR35* (Saintenac et al., 2013), *SR33* (Periyannan et al., 2013), *SR50* (Mago et al., 2015), *SR60* (Chen et al., 2020) and *SR55/LR67* (Moore et al., 2015). For woody plants, resistance genes *Rpv1* and *Run1* conferring resistance to *Plasmopara viticola* and *Erysiphe necator* have been identified in the wild grapevine relative *Muscadinia rotundifolia* (Feechan et al., 2013) and are good candidates on which several research groups are working. In spite of this, the number of genes with a known function is still limited. In parallel, more information on promoters, transcriptional terminators and regulatory elements to control the transcription efficiency has to be addressed because of the high impact on gene of interest expression levels and consequently on the final phenotype (Low et al., 2018; Basso et al., 2020).

With respect to the CRISPR/Cas DNA editing, RNA editing using Cas13 has the advantage that it is not stable but reversible. This could enable a delicate temporal control over the editing process when editing RNA, both edited and non-edited transcripts can be present

simultaneously in the cells, which could enable fine-tuning of the edited transcript amount, whereas DNA editing affects all transcripts. Furthermore, in addition to classic gene knock-out mediated by CRISPR/Cas systems, new approaches were developed to target micro RNA genes (MIR) instead of protein coding ones. By fine-tuning specific MIR genes, the up- or downregulation of derived miRNAs and target mRNAs can be achieved, for controlling either crop different biological responses or phenotypes and, consequently, specific agronomic traits (Basso et al., 2020 and references therein). Similarly, an approach called gene editing-induced gene silencing (Kuscu et al., 2017) can be applied to target redundant non-coding RNA sequences that are involved in miRNA/siRNA biogenesis. Once modified, the new RNA molecule will target new sequences, which could be endogenous plant sequences (leading to transcript downregulation) or pathogen vital genes. Contrary to traditional gene editing techniques, gene editing-induced gene silencing could be used to indirectly target pathogenic genes by redirecting the silencing activity of the endogenous RNA interference (RNAi) pathway, supporting a more sustainable crop protection (Zotti et al., 2018).

2.8 Concluding remarks and future prospects

The NPBTs Era displays the potential to revolutionize the agricultural research field (Pandey et al., 2019). Indeed, recent applications and literature data available to date represent only the tip of the iceberg of further discoveries that may change molecular biology. Just as an example, through the combination of DNA and RNA editing systems, the cellular transcriptome can now be manipulated on the transcriptional and posttranscriptional level simultaneously, allowing delicate, and also reversible fine-tuning of gene expression (Schindele et al., 2018).

Taking them singularly, they all still present limitations. Pros and cons can be found both in fine tuning each application as well as their application in a wide range of species. For instance, looking at cisgenic strategies, these have been developed and tested for woody and herbaceous crops, but their application still seems far from fulfilling their potential. The lack of efficient tissue culture and regeneration protocols for many crops hinders the range of possible applications. In addition, the identification of candidate genes involved in abiotic and biotic stresses still represents an important limit. For this reason, all NPBTs could greatly benefit from functional genomics, metabolomic and proteomic studies.

Nevertheless, a wide range of different techniques are becoming mature for substituting GMO approaches and supporting traditional breeding, with a realistic possibility of being largely accepted by the international community. Several NPBTs, making small modifications to plant

own DNA without introducing foreign genes, do not leave any trace of their application in the improved phenotype. Despite the high impact of such techniques, and because the genome modifications introduced by genome editing are indistinguishable from those introduced by spontaneous mutations or conventional breeding (Bortesi and Fischer, 2015), to date the debate about considering organisms obtained by NPBTs as non-GMO is still open (Purnhagen et al., 2018).

Although NPBTs are powerful tools for basic research and more precise crop improvement, further knowledge, such as the comprehension of the genetic bases of important crop traits, have to be produced for efficiently transferring these tools from the lab to the field. Indeed, NPBTs can pave the way for further understanding of plant-pathogen interaction and different facets of climate change adaptation and for exploiting them for improving food security and nutrition quality.

Conflict of interest statement

No conflict of interest declared.

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Vectors attributions

Some elements present in the figures were obtained from www.Vecteezy.com

Authors' contribution statement

GG, LM, LN and WC wrote the introduction. GG wrote the cisgenesis sections. LM and LN wrote the genome editing paragraphs. WC and LN wrote the RNAi strategies sections. SV wrote the epigenetic paragraphs. MFC, CB, GDL and RV commented on the first draft and critically reviewed the final manuscript.

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CHAPTER 3

A cisgenic approach for the introduction of downy mildew resistance into elite grapevine germplasm

Article draft

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3. A cisgenic approach for the introduction of downy mildew resistance into elite grapevine germplasm

3.1 Introduction

In the last decade the EU has shown a growing interest towards a more sustainable production system and an efficient and renewable use of biological resources, that will at the same time reduce greenhouse gas emissions. There is a pressing need to produce more food minimizing environmental and health impact, meeting consumers' needs. To pursuit these intents, the European union has fostered the research and innovation on numerous fields related to the agro-bio industry through Horizon 2020 (https://ec.europa.eu/programmes/horizon2020/en/home, the EU program for the global competitiveness) and its Common Agricultural Policy (https://ec.europa.eu/info/food-farming-fisheries/key-policies/common-agricultural-policy/cap-glance_en).

In this light, the use of the new breeding techniques, and specifically of a cisgenic approach to introduce the already characterized resistance haplotype RPV3-1 (Foria et al. 2019) in some elite *Vitis vinifera* varieties, highly appreciated by the wine industry, will be of great interest.

Wild North American vines have long been used in breeding programs to introgress resistance to *Plasmopara viticola* into the European grape (*Vitis vinifera*) and it has been demonstrated that genes at RPV3 locus, located in chromosome 18 are able to confer resistance to grapevine downy mildew (Foria et al. 2018). The RPV3-1 haplotype induce localised necrosis in grapevine leaves in response to the infection of several *Plasmopara viticola* strains, through the activation of ETI response (Bellin et al. 2009). Specifically two TIR NB-LRR genes TNL2a and TNL2b were mapped to the RPV3 locus and identified as the causal agents of the downy mildew resistance (Foria et al. 2019). A cisgenic approach with these two genes will allow to introduce only the desired trait by using native genes from *Vitis* spp., interfertile with *V. vinifera*, reducing the agrochemicals needs and the risks associated with their use, increasing the profitability of the vineyard and consumers' appreciation.

A fundamental prerequisite of plant genetic transformation is the existence of an efficient regeneration system, that will allow the obtainment of a new individual from few modified cells (Sugimoto et al. 2019). In grapevine, despite many approaches, including direct organogenesis, have been attempted using different tissues as starting material, the most

exploited regeneration system is somatic embryogenesis (Martinelli and Gribaudo 2009; Rama et al. 2009; Sabbadini et al. 2019). This process has been defined as the induction of embryos from somatic cells (Martinelli and Gribaudo 2009), and has also been applied in the obtainment of virus free-plants or as source of genetic variation in plant breeding programs thanks to somaclonal variability (Gribaudo et al. 2004; Martinelli and Gribaudo 2009).

Even if a lot of efforts have been done to standardise the obtainment of somatic embryos in *Vitis* species (Gribaudo et al. 2004), the whole procedure remains long and difficult. Anthers, ovaries and whole flowers, are the most frequently adopted explants and since they must be collected at the optimal developmental stage, inflorescence harvest represents one of the most critical aspects being cultivar dependent and subject to environmental variability, greatly affecting somatic embryogenesis efficiency (Gribaudo et al. 2004; Gambino et al. 2007). Collection of anthers and ovaries from the flower has to be done with the aid of a stereomicroscope in sterile conditions, resulting time consuming and manually intensive, even more considering that thousands of explants are indispensable to achieve a good chance of success. In addition, several months of cultivation on callus induction and differentiation media are required for the development of embryogenic calli (Torregrosa 1998; Gribaudo et al. 2004; Gambino et al. 2007; Martinelli and Gribaudo 2009).

Given these considerations, selection of downy mildew susceptible cultivars has been conducted taking into account commercial interest and regeneration efficiency, selecting "Chardonnay" and "Pinot Noir" for their international diffusion and for the great regeneration efficiency reported in previous studies, while "Sangiovese" and "Glera" were selected for the high commercial interest among the Italian cultivars (Perrin et al. 2001, 2004; Gribaudo et al. 2004, 2017; Gambino et al. 2007; Boso et al. 2014; Maillot et al. 2016; OIV 2017).

The aim of the present work is to demonstrate in grapevine the effectiveness of a cisgenic approach for the development of downy mildew resistant elite varieties.

3.2 Materials and methods

Plant material

Tissue cultures for the obtainment of embryogenic calli started from immature anthers, ovaries or whole flowers of *V.vinifera L.* cultivars "Chardonnay", "Sangiovese", "Pinot Noir" and "Glera". Inflorescences belonging to the selected cultivars were collected from 20 to 10

days before flowering, when the anthers appeared translucent with a light green colour, according to (Gribaudo et al. 2004).

After collection, the inflorescences were surface-sterilised with 33% commercial bleach (3-5% chlorine) for 20', adding few drops of Tween 20 as surfactant, and rinsed 4 times with sterilised water (10' for each rinse). Flower buds were then stored in sterile petri dishes at 4°C for 36h and surface sterilised for a second time using the same disinfection protocol. Explants were then cultivated at 26°C in the dark on callus induction medium (PIV medium: Nitsch and Nitsch mineral salts, Murashige and Skoog vitamins, 6% sucrose, 0.3% gelrite, 4.5 µM 2,4-D, 8.9 µM BAP, pH 5.8)(Franks et al. 1998; Gambino et al. 2007) for 2 months. Developing callus was then selected and transferred on CI medium (Nitsch and Nitsch mineral salts, Murashige and Skoog vitamins, 6% sucrose, 0.5% gelrite, 5 µM 2,4-D and 1 µM Benzylaminopurine (BAP), pH 5.8, Gambino et al. 2005). For the maintenance of embryogenic cultures subcultures were performed monthly on CI medium. Prior transformation embryogenic calli were transferred for 15 days to embryo proliferation medium (GS1CA, Nitsch and Nitsch mineral salts, Murashige and Skoog vitamins, 6% sucrose, 0.4% gelrite, 10 µM naphthoxiacetic acid (NOA), 1 µM BAP, 20 µM Indol Acetic Acid (IAA, filter sterilized and added after autoclaving), 0.25% activated charcoal, pH 5.8, Franks et al. 1998; Gambino et al. 2007).

Plasmid construction

Sequences of the genes TNL2a (5306bp) and TNL2b (5416bp) belonging to RPV3-1 locus were obtained through the collaboration with IGA institute (Parco Scientifico e Tecnologico Luigi Danieli di Udine, Via J. Linussio, 51, 33100 Udine, Italy). Sequences of unique restriction sites were added to the extremities of the gene sequences, specifically XmaJI and MluI were added respectively to the 3' and 5' extremity of TNL2a while MluI and SgsI were added respectively to the 3' and 5' extremities of TNL2b. A 112bp adaptor containg HindIII compatible ends at 3' and 5' extremities and containing XmaJI, MluI and SgsI restriction sites was also designed for cloning the gene fragments into the pNS14 vector. The gene fragments and the adaptor were synthetised in outs ourcing, TNL2a and TNL2b fragments were provided already cloned into distinct amplification vectors, that were used to transform DH5α chemically competent *E.coli* cells. Positive colonies were confirmed by PCR analysis and grown overnight in 4 mL of LB medium supplemented with antibiotic, then subjected to plasmid extraction using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, Irvine, CA, USA). The fragments of the two genes were excised by digestion with the correspondent

restriction enzymes, separated by gel electrophoresis on 1% agarose gel and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer instructions.

For the construction of the cisgenic vector carrying the RPV3 gene sequences, the plasmid pNS14 has been used (Fig.1 a), this binary vector contain in the T-DNA region, an excisable cassette with the 35S:NPTII gene for kanamycin selection in plants and the Cre recobinase controlled by the soybean heat shock promoter 17.5E. Spectinomycin selection gene is instead present in the vector backbone. The vector was first digested with HindIII to remove the multiple cloning site then circularised again after purification obtaining the pNS13 vector. The adapter was then cloned into the pNS13 plasmid previously linearised with HindIII and dephosphorylated with alkaline phosphatase (Thermo Fisher Scientific, MA, USA) to prevent self-ligation (Fig.1 b). Following transformation of the ligation products into DH5α chemically competent cells, positive colonies were confirmed by PCR analysis and selected for the amplification and extraction of the pNS13-AD vector, as previously described. TNL2a fragment was then directionally cloned into the pNS13-AD vector, previously digested with the correspondent restriction enzymes, the obtained vector pNS13-AD-TNL2a was used to transform the chemically competent DH10B *E.coli* cells. Positive colonies were confirmed by PCR screening and used for plasmid production as above mentioned. Finally, the same approach was used for cloning TNL2b into pNS13-AD-TNL2a (Fig.1 c), after digesting the vector with MluI and SgsI restriction enzymes, ligation, transformation of DH10B *E. coli* cells, and plasmid production were performed as above mentioned. In order to confirm the correctness of plasmid sequences the pNS13-AD-TNL2a-TNL2b vector was sequenced with Sanger method at Bio-Fab Research (Roma, Italy). The binary vectors pNS13-AD and pNS13-AD-TNL2a-TNL2b were then used to transform electrocompetent *Agrobacterium tumefaciens* cells of the strains GV3101 and EHA105, that were plated on LB media supplemented with 25 μg/mL of rifampicin and 50 μg/mL of spectinomycin. Positive colonies were screened by PCR and stored at −80 °C for the next activities.

Transformation and selection

Cultures of Agrobacterium were grown overnight in LB medium at 28° C in a shaking incubator. Once the Agrobacterium suspension reached an OD600=0.5, the culture was centrifuged at 4500 rpm for 10' and induced for 3h in the LCM induction medium (Nitsch and Nitsch mineral salts, Murashige and Skoog vitamins, 1% sucrose, pH 5.8) supplemented with 100 μM Acetosyringone. Embryogenic calli growing on GS1CA medium were then

collected in a new petri and incubated for 10' at room temperature with the *Agrobacterium* suspension. The tissues were then collected with the aid of a 100 µm cell strainer and transferred to a new petri dish containing three filter paper sheets soaked with fresh GS1CA medium and co-cultured with *Agrobacterium t.* for 48h at 26°c in the dark. After co-cultivation the calli were rinsed in liquid GS1CA with 600 mg/L cefotaxime and placed on new plates containing GS1CA medium with 450 mg/L cefotaxime to prevent *Agrobacterium* growth. After 1 month the calli were moved to fresh GS1CA medium supplemented with 450mg/L cefotaxime and 100 mg/L kanamycin for selection of transformed tissues and subcultured monthly. After 4 months cefotaxime was removed from the media maintaining the transformed calli on GS1CA supplemented with 100 mg/L kanamycin by monthly subcultures.

Plantlets regeneration

To regenerate the whole plants, transformed embryos differentiating on GS1CA medium supplemented with 100 mg/L kanamycin are transferred on MS ½ medium (Murashige and Skoog salts at half dose, MS vitamins, 30 g/L Sucrose, 0,5% gelrite, pH 5.8) once reaching a dimension of about 5 mm, and it easily possible to distinguish cotyledons and root tips structures. Germination of somatic embryo usually occur in about 30 days, the seedlings are then subculture monthly on MS ½ medium.

3.3 Preliminary results

To obtain the embryogenic calli that will be used for the generation of transformed grapevines through somatic embryogenesis, tissue cultures starting from immature anthers and ovaries (Fig.1) were conducted in 2019, 2020 and 2021 (Table 1). No embryogenic material was obtained in 2019 for the cultivars 'Glera', 'Sangiovese' and 'Pinot Noir', while embryogenic calli developed after 6 months of subculture on CI medium for the cultivar 'Chardonnay'. These results suggest that 'Glera' is a recalcitrant cultivar for the development of embryogenic material, while a greater efficacy was observed for 'Chardonnay'. These data and observations were used to better tailor the 2020 season, where the efforts were focused on the 'Chardonnay' cultivar, with the collection of 10000 between anthers and ovaries, while only whole flowers were collected in case of 'Glera', that confirmed to be a recalcitrant genotype also in 2020. Since previous studies demonstrated that collection of whole flowers is sensibly faster and at least for some cultivars somatic embryogenesis frequencies are similar or even

better than those showed by anthers explants (Gambino et al. 2007), we conducted a preliminary trial on 'Glera' to tackle its low regeneration efficiency, by cultivating 3000 whole flowers. Unluckily no embryogenic calli were obtained despite a total of 15000 anthers and 3000 ovaries. A better result compared to 2019 was obtained for 'Chardonnay' thanks to the higher number of cultivated anthers/ovaries that provided a greater availability of embryogenic calli (Table1). In 2021 we decided to focus in addition to the cultivar 'Chardonnay', for which a total of 5500 anthers/ovaries were collected, on the cultivar 'Pinot noir' for which a total of 14000 anthers/ovaries were collected (Table1). The tissue cultures of the 2021 season already produced calli however more time is required to assess the embryogenic potential of these cultures.

Variety	**2019**	**2020**	**2021**
Chardonnay	1500	10000	5500
Glera	10000	3000 (whole flowers)	
Sangiovese	1000		
Pinot Noir	3000		14000

Table1: Estimates of the collected number of anther/ovaries per year

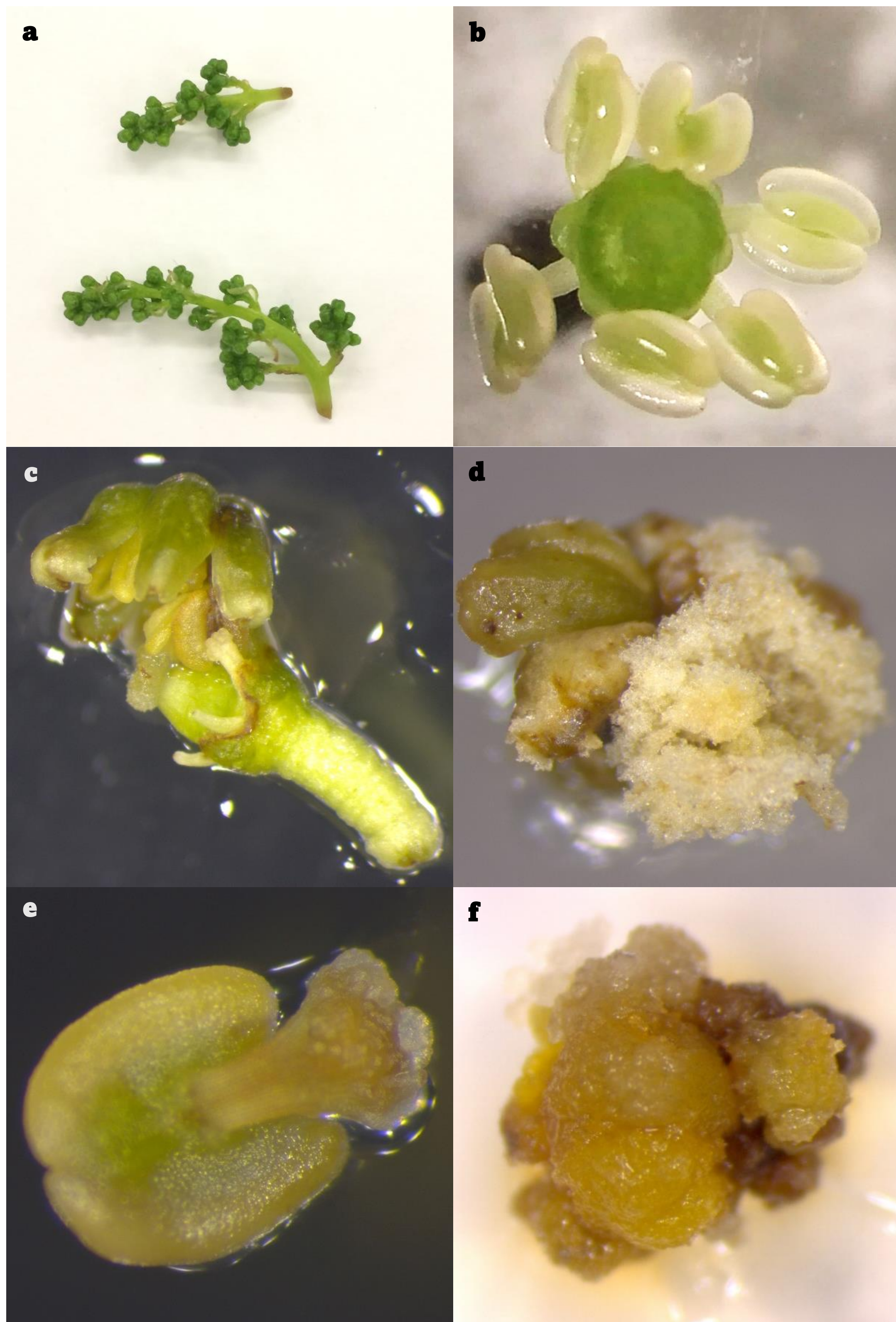

Figure 1. Tissue cultures of grapevine floral tissues for the development of embryogenic calli. a) Collected inflorescences of cultivar 'Chardonnay' prior sterilization. b) Grapevine immature flower after removing the calyptra, note the translucent pale green color of the anthers. c) Whole flower of cultivar 'Glera' on callus induction medium PIV. d) Non embryogenic callus obteined from 'Glera' flowers at 45 days from the harvest. e) Callus formation on the anther filament of cultivar 'Chardonnay' at 14 days from the harvest. f) Callus obtained from cultivar 'Chardonnay' at 90 days from the harvest.

To introduce the RPV3-1 resistance in the cultivar 'Chardonnay', embryogenic calli from 2020 season have been transformed and are currently under kanamycin selection on GS1CA medium, on the plate is possible to observe the selection of transformed tissues along with differentiating embryos emerging from the brown callus mass (Fig.3).

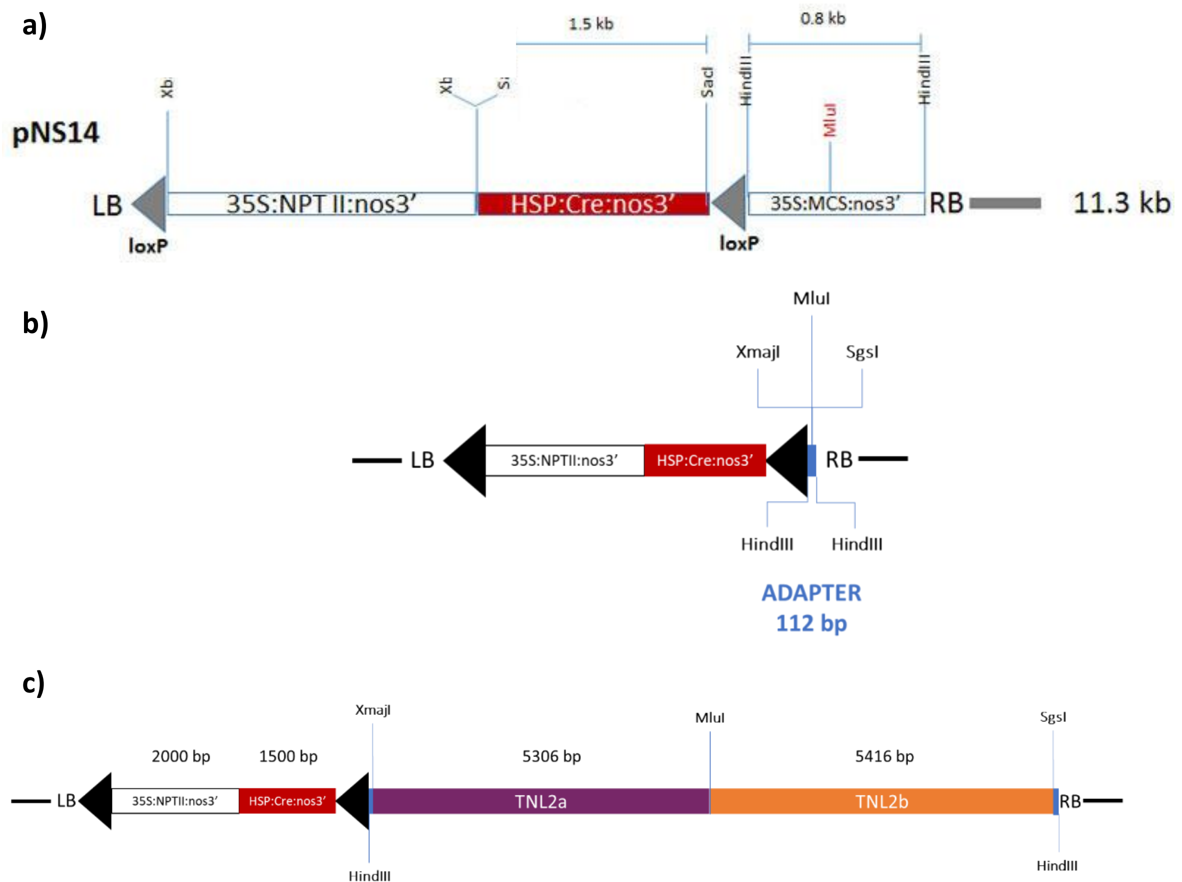

Figure 2. Construction of the RPV3-1 cisgenic vector. a) Representation of the t-DNA region of the pNS14 vector carrying an excisable cassette with NPTII and Cre recombinase between the two loxP sites. b) After removing the MCS, a 112 bp adapter with XmajI, MluI and SgsI restriction sites, that are not present in the genes of interest, was cloned beside the excisable cassette in the HindIII restriction site. c) The restriction sites present in the adapter were used for directional cloning of TNL2a and TNL2b.

Figure 3. Embryogenic calli of the cultivar 'Chardonnay' that have been transformed with the cisgenic vector pNS13-AD-TNL2a-TNL2b under selection on GS1CA medium supplemented with 100 mg/L kanamycin. Differentiating embryos are clearly recognizable by the light green colour.

In the next months the regenerated plantlets will be screened by PCR analysis for the presence of TNL2a and TNL2b genes, selecting the positive seedlings. Positive plants will be assayed by qPCR and Southern blot analysis to establish the number of integration events. Selected plants will then micropropagated on MS ½ medium (Murashige and Skoog salts at half dose, MS vitamins, 30 g/L Sucrose, 0,5% gelrite, pH 5.8) and subjected to heat shock treatment (42°C for 4h) for the excision of the fragment containing the selective marker and the Cre recombinase. After the excision induction, a new PCR screening will be conducted to confirm the remotion of the excisable cassette and the absence of backbone sequences in the plant genome.

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CHAPTER 4

Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs

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CHAPTER 4

4. Mycorrhizal symbiosis balances rootstock-mediated growth-defence tradeoffs

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4.1 Abstract

It is well known that AM symbiosis provides several ecosystem services leading to plant adaptation in different environmental conditions and positively affects physiological and production features. Although beneficial effects from grapevine and AM fungi interactions have been reported, the impact on growth-defence tradeoffs features has still to be elucidated. In this study, the potential benefits of an inoculum formed by two AM fungal species, with or without a monosaccharide addition, were evaluated on young grapevine cuttings grafted onto 1103P and SO4 rootstocks. Inoculated and non-inoculated plants were maintained in potted vineyard substrate under greenhouse conditions for three months. Here, agronomic features were combined with biochemical and molecular techniques to assess the influence of the different treatments. Despite the opposite behaviour of the two selected rootstocks, in AM samples the evaluation of gene expression, agronomic traits and metabolites production, revealed an involvement of the whole root microbiome in the growth-defence tradeoffs

balancing. Noteworthy, we showed that rootstock genotypes and treatments shaped the root-associated microbes, stimulating plant growth and defence pathways. Progresses in this field would open new perspectives, enabling the application of AMF or their inducers to achieve a more sustainable agriculture also in light of the ongoing climate change.

Keywords

AM fungi, trade-off, plant priming, stress tolerance, N, growth-defence balance

4.2 Introduction

Grapevine is one of the most cultivated crop worldwide since its great economic importance resulting from grape and wine production, and commercialization (Chitarra et al. 2017). For this reason, over the years viticulture industry has selected several cultivars showing different traits (*i.e.*, flavour, yields, colour) influenced by geology, soil-scape and climate features, driving some major wine peculiarities (Priori et al. 2019). These components, and their interactions, concur to define the *terroir* of a particular environment (Resolution OIV/VITI 333/2010). Besides scion variety features, rootstocks are able to strongly affect scion performances by means of water transport, biochemical and molecular processes, impacting the whole plant functions and its response to biotic/abiotic stress factors (Chitarra et al. 2017). In the last decade, research on scion/rootstock interactions strongly increased, aiming to develop more sustainable practices against pests and ameliorating plant adaptability to the ongoing climate change (Lovisolo et al. 2016; Warschefsky et al. 2016; Zombardo et al. 2020). Key drivers influencing defence features and adaptive traits are thought to be the microbial communities residing in plant tissues. To date, several studies reported evidence about their influence on physiological performances (*e.g.*, production of flavours, hormones, VOCs) in many plants, including grapevine, where residing microbiota contribute to defining the *microbial terroir* (Gilbert et al. 2014).

According to the Intergovernmental Panel on Climate Change (IPCC 2014), an increase in the global surface temperature is expected over the next years, affecting crop production as a consequence of the predicted occurrence of biotic and abiotic stresses (Mittler and Blumwald 2010). To achieve resilience to stress, numerous efforts have been done over the years, such as the adoption of specific breeding programs and genetic engineering approaches (Cushman and Bohnert 2000). Researchers have been focusing just recently their attention on the exploitation of 'native' plant defence mechanisms (e.g. hormone signalling, plant immunity activation) against biotic and abiotic stressful factors (Feys and Parker 2000; Jones and Dangl 2006;

Hirayama and Shinozaki 2007). The triggering of these responses can occur using chemical treatments (Balestrini et al. 2018), root-associated microorganisms and RNA interference technologies (Alagna et al. 2020), leading plants in a state of alertness - 'Primed state' or 'Priming' – and enabling them to respond more quickly and robustly in case of the exposure to a stress (Beckers and Conrath 2007).

Among soil beneficial microorganisms, arbuscular mycorrhizal fungi (AMF) establish symbioses with the majority of land plants showing an important role in providing nutrients, particularly phosphate and N, but also water and other elements to the host plant (Jacott et al. 2017; Balestrini and Lumini 2018). Mycorrhizal symbiosis is able to influence plant growth and productivity and enhance the tolerance to biotic and abiotic stresses as demonstrated in many crops (Balestrini and Lumini 2018; Balestrini et al. 2018; Alagna et al. 2020). In addition, AM fungi are able to increase aggregation of soil surrounding roots, improving soil matrix stability and physicochemical characteristics (Uroz et al. 2019). Grapevine roots are naturally colonized by native AM fungi with a great impact on growth, yield, quality and development performances (Deal et al. 1972; Karagiannidis et al. 1995; Linderman and Davis 2001; Trouvelot et al. 2015). Thanks to the application of metagenomics approaches to soil and roots, new insights about the AMF living in symbiosis with grapevine have been discovered (Balestrini et al. 2010; Holland et al. 2014; Balestrini and Lumini 2018).

Rootstocks-mediated adaptation to a specific environment is based on the growth-defence trade-offs-mediated mechanisms (Chitarra et al. 2017). Trade-off phenomenon was firstly observed in forestry plants-insect interaction studies and is based on the idea that the limited carbon resources produced by photosynthesis are allocated toward growth or defence processes in order to maximize the adaptation strategies and fitness costs in diverse environments (Huot et al. 2014; Chitarra et al. 2017; Züst and Agrawal 2017). Stresses impair plant growth, redirecting energy and carbon sources toward defence, reducing growth and reproduction performances (Bandau et al. 2015; Züst and Agrawal 2017). Recently, it was suggested that through a meta-analysis, that the increased plant resistance promoted by *Epichloë* fungal endophytes does not compromise plant growth, eliminating the trade-off between growth and defence (Bastías et al. 2021). A role in tradeoffs balance has been demonstrated also for AM symbioses, improving nutrient uptake, disease tolerance and abiotic stress resilience (Jacott et al. 2017).

In this study, we aimed to evaluate if AM fungi and rootstocks can concomitantly contribute to fine-tuning growth-defence tradeoffs features in grapevine, thus enabling plants to trigger earlier and enhanced defence responses against a potential stressor. The use of specific

molecules that can promote the AM fungal colonization have been proposed to improve mycorrhizal inoculum applications under practical field condition (Bedini et al. 2018). In this context, an affordable strategy is the application at low doses of oligosaccharides (*i.e.*, glucose, fructose, and xylose) that have a stimulant effect on AM symbiosis colonization (Lucic and Mercy 2014 - Patent application EP2982241A1). These compounds, initially called as elicitors, in relation to the impact on plant defense, can promote mycorrhizal performances and, for this reason, the term "inducer" was proposed (Bedini et al. 2018). In this work, the impact of an inoculum formed by two AMF species (*Funneliformis mosseae* and *Rhizophagus irregularis*), already reported among the species present in vineyards (Berruti et al. 2018), with or without the addition of a monosaccharide (D-glucose) at low dose (the so called inducer), has been evaluated on young grapevine cuttings *cv.* Glera grafted onto 1103 Paulsen and SO4 rootstocks, well known to trigger an opposite growth-defence behaviour in the scion. The effect of the several treatments on the root-associate microbiota has been also evaluated, to verify the response mediated by the AM and its recruited mycorrhizosphere.

4.3 Materials and methods

Biological materials and experimental set-up

Two hundred one year-old dormant vines of 'Glera' cultivar grafted onto 1103 Paulsen (1103P) and SO4 rootstocks certified as 'virus free' were purchased from an Italian vine nursery (Vivai Cooperativi Rauscedo, Italy; http://www.vivairauscedo.com). Vine roots were washed with tap water and cut to about 4 cm before plantation in 2 L pot containers filled with not sterilized substrate mixture of vineyard soil/*Sphagnum* peat (8:2, v:v) to better simulate the field conditions. The substrate composition was a sandy-loam soil (pH 7.8; available P 10.4 mg kg^{-1}; organic matter 1.80 %; cation exchange capacity 20.11 mew 100 g^{-1}).
Grapevine cuttings were inoculated with AMF mixed inoculum (INOQ GmbH, Germany, 238,5 Million propagule per kg inoculum) at planting time by placing it in the hole and in contact with the roots following the manufacturer's instructions. Mycorrhizal inoculum, a powder based mycorrhizal root fragment (Advantage Grade II, 2016 - INOQ GmbH) contained 50% *Rhizoglomus irregulare* (syn. *Rhizophagus irregularis*; 450 million propagules per Kg) and 50 % *Funneliformis mosseae* (27 million propagules per Kg). The fungal lines were produced *ex vitro*, on *Zea mays* and *Plantago lanceolata* (sand/vermiculite, v/v). Both AMF inoculum and D-glucose at low dose (i.e., the Inducer) were prepared by Louis Mercy (INOQ GmbH; patent EP2982241A1). The containers were prepared according to treatments as follow: i) 25 plants for each rootstock as uninoculated control plants (C); ii) 25 plants for each rootstock

inoculated with 50 mg/L of AMF mixed inoculum (M); iii) 25 plants for each rootstock inoculated with 50 mg/L of AMF mixed inoculum + inducer (M+I); iv) 25 plants for each rootstock amended with 50 mg/L of inducer to stimulate the exploitation of native AMF symbiosis (I). Daily watered grapevine plants were kept under partially climate-controlled greenhouse, under natural light and photoperiod conditions for three months.

After three months, at the end of the experiment, engraftment, growth index and chlorophyll content were recorded. Leaf and root samples for molecular and biochemical analysis were collected from at least three randomly selected plants and immediately stored at -80°C. A part of the root apparatus was used to estimate the level of mycorrhiza formation as described (Balestrini et al. 2017).

Morphological observations in the colonized fragments of thin roots allowed to identify the presence of the typical structures of the symbiosis, regardless of the thesis. However, the patchy level of colonization, and the quality of the root segments after the staining, made morphological quantification difficult, and therefore the AMF presence has been assessed by molecular analyses (see below).

Growth index, engraftment, and chlorophyll content

At the end of the experiment, phenological stages were recorded and classified according to Biologische Bundesanstalt, bundessortenamt und CHemische industrie (BBCH) scale (from 00 to 12, from dormancy to 9 or more leaves unfolded, respectively). BBCH scales have been developed for many crops, including grapevine, and it is based on a decimal code system that identify the growth stage (Lancashire et al. 1991), engraftment % (i.e. rooting %) were visually determined for each plant and treatment. Chlorophyll content was determined using a portable chlorophyll meter SPAD (Konica Minolta 502 Plus). Readings were collected from the second or third leaf from the top on at least three leaves per plant on five randomly selected vines for each experimental condition (Chitarra et al. 2016).

Targeted metabolite analyses

Contents of *trans*-resveratrol, viniferin and abscisic acid (ABA) were quantified on at least three biological replicates per condition according to the protocol previously described (Pagliarani et al. 2019, 2020; Mannino et al. 2020). Leaves and roots from two randomly selected plants were pooled to form a biological replicate, immediately frozen in liquid nitrogen, freeze-dried and stored at -80°C until use. Briefly, about 100 mg of freeze-dried sample (leaf or root) were transferred with 1 mL of methanol:water (1:1 v/v) acidified with 0.1

% (v/v) of formic acid in an ultrasonic bath for 1 h. Samples were centrifuged for 2 min at 4°C and 23.477 *g*, and the supernatant was analysed by high-performance liquid chromatography (HPLC). Original standards of resveratrol (purity ≥ 99 %), viniferin (purity ≥ 95 %) and ABA (purity ≥ 98.5%, Sigma-Aldrich) were used for the identification by comparing retention time and UV spectra. The quantification was made by external calibration method. The HPLC apparatus was an Agilent 1220 Infinity LC system (Agilent R, Waldbronn, Germany) model G4290B equipped with gradient pump, auto-sampler, and column oven set at 30°C. A 170 Diode Array Detector (Gilson, Middleton, WI, United States) set at 265 nm (ABA and IAA) and 280 nm (for stilbenes) was used as detector. A Nucleodur C18 analytical column (250x4.6 mm i.d., 5 µm, Macherey Nagel) was used. The mobile phases consisted in water acidified with formic acid 0.1% (A) and acetonitrile (B), at a flow rate of 0.500 mL min^{-1} in gradient mode, 0-6 min: from 10 to 30 % of B, 6-16 min: from 30 % to 100 % B, 16-21 min: 100% B. Twenty µL was injected for each sample.

Total N, soluble carbohydrate content in leaf and net nitrate uptake in root

The Kjeldahl method was performed according to method 981.10 of the AOAC International (2016), using VELP Scientifica DKL 20 Automatic Kjeldahl Digestion Unit and UDK 159 Automatic Kjeldahl Distillation and Titration System. Approximately 0.2 g of leaf raw material was hydrolyzed with 15 mL concentrated sulfuric acid (H_2SO_4) containing one catalyst tablets (3.47 g K_2SO_4 + 0.003 Se, VELP Scientifica, Italy) in a heat block (DK Heating Digester, VELP Scientifica, Italy) at 300°C for 2 h. After cooling, H_2O was added to the hydrolysates before neutralization with NaOH (30%) and subsequently distilled in a current of steam. The distillate was collected in 25 mL of H_3BO_3 (1%) and titrated with HCl 0.1 M. The amount of total N in the raw materials were calculated.

Leaf soluble carbohydrate content was quantified (Chitarra et al. 2018). At the end of the experiment, white non-lignified roots (0.5 – 1 g) were collected from four randomly selected plants for each treatment and rootstock. Root samples were washed in 0.5 mmol L^{-1} $CaSO_4$ for 15 min, then transferred to a 20 mL aerated uptake solution containing 0.5 mmol L^{-1} $Ca(NO_3)_2$ and 0.5 mmol L-1 $CaSO_4$. Net uptake of NO_3^- was measured removing samples of uptake solution (aliquot of 200 µL) for its determination every 2 min for 10 min(Tomasi et al. 2015). The aliquots were carefully mixed with 800 µL of salicylic acid (5% w/v in concentrated H_2SO_4) and incubated for 20 min at room temperature following the addition of 19 ml of 2 mol L^{-1} NaOH. After cooling, nitrate concentration was measured at the absorbance of 410

nm (Shimadzu UV Visible Spectrophotometer UVmini-1240. Kyoto, Japan) and the net nitrate uptake was expressed as µmol (g FW h^{-1}).

RNA isolation and RT-qPCR

Expression changes of target transcripts were profiled on root and leaf samples (three independent biological replicate for each treatment) by quantitative real-time PCR (RT-qPCR) (Chitarra et al. 2018). Total RNA was isolated from the same lyophilized samples (leaves and roots) used for HPLC-DAD analysis and cDNA synthesis was performed as previously reported (Chitarra et al. 2016). The absence of genomic DNA contamination was checked before cDNA synthesis by qPCR using *VvUBI* specific primers of grapevine. RT-qPCR reactions were carried out in a final volume of 15 µL containing 7.5 µL of Rotor-Gene™ SYBR® Green Master Mix (Qiagen), 1 µL of 3 µM specific primers and 1:10 of diluted cDNA. Reactions were run in the Rotor Gene apparatus (Qiagen) using the following program: 10 min preincubation at 95°C, followed by 40 cycles of 15 s at 95°C, and 30 s at 60°C. Each amplification was followed by melting curve analysis (60–94°C) with a heating rate of 0.5°C every 15 s. All reactions were performed with at least two technical replicates. The comparative threshold cycle method was used to calculate relative expression levels using plant (elongation factors, actin and ubiquitin, *VvEF* and *VvUBI* for root and *VvACT* and *VvEF* for leaf tissue) reference genes. While *R. irregularis* and *F. mosseae* elongation factors (*RiEF1*, *FmEF,* respectively) were used to normalized the expression of the AMF phosphate transporter (*PT*) genes. Oligonucleotide sequences are listed in Supplementary Table 1. Gene expression data were calculated as expression ratio (Relative Quantity, RQ) to Control 1103P plants (C 1103P).

Root DNA isolation and sequencing

Root samples were lyophilized prior to DNA extraction. About 30 to 40 mg of freeze-dried and homogenized material were used to extract total DNA following manufacturer instruction of plant/fungi DNA isolation kit (Norgen Biotech Corp., Thorold, ON, Canada) as previously reported (Nerva et al. 2019). Total DNA was quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific,Waltham, MA, USA), and DNA integrity was inspected running the extracted samples on a 1% agarose electrophoretic gel. Before sending DNA to sequencing a further quantification was performed using a Qubit 4 Fluorometer (Thermo Fisher Scientific,Waltham, MA, USA).

To inhibit plant material amplification, we added a mixture of peptide nucleotide acid (PNA) blockers oligos (Kaneka Eurogentec S.A., Belgium) targeted at plant mitochondrial and chloroplast 16S rRNA genes (mitochondrial and plastidial) and plant 5.8S nuclear rRNA. Mitochondrial sequence was derived from (Lundberg et al. 2013) with a 1bp mismatch, mitochondrial sequence was derived from (Cregger et al. 2018).
PNA was custom-designed for *V. vinifera* (VvpPNA: GGCTCAACCCTGGACAG; Vv-ITS-PNA: CGAGGGCACGCCTGCCTGG; Vv-mPNA: GGCAAGTGTTCTTCGGA). Thermal cycler conditions were maintained as suggested by the Illumina protocol as previously reported (Nerva et al. 2019).
Sequences were deposited in NCBI database under the BioProject PRJNA718015, BioSamples SAMN18520793 to SAMN18520808 and SRR14089924 to SRR14089939.

<u>Rhizoplane metaphylogenomic analyses, taxonomic distributions</u>

A first strict quality control on raw data was performed with PrinSeq v0.20.4 (Schmieder and Edwards 2011) and then processed with Qiime2 (Bolyen et al. 2019). A previously reported and specific pipeline was used for fungal analysis: retained reads were used to identify the start and stop sites for the ITS region using the hidden Markov models (HMMs) (Rivers et al. 2018), created for fungi and 17 other groups of eukaryotes, which enable the selection of ITS-containing sequences. Briefly, the software allows to distinguish true sequences from sequencing errors, filtering out reads with errors or reads without ITS sequences. To distinguish true sequences from those containing errors, sequences have been sorted by abundance and then clustered in a greedy fashion at a threshold percentage of identity (97%). Trimmed sequences were analyzed with DADA2 (Callahan et al. 2016) and sequence variants were taxonomically classified through the UNITE (Abarenkov et al. 2010) database (we selected the reference database built on a dynamic use of clustering thresholds). For graphic representation, only genera with an average relative abundance higher than the settled threshold (1%) were retained.
A 16S specific pipeline was used for bacteria: quality filtering was performed with DADA2 which is able to perform chimera removal, error-correction and sequence variant calling with reads truncated at 260 bp and displaying a quality score above 20. Feature sequences were summarized and annotated using the RDP classifier (Cole et al. 2014) trained to the full length 16S database retrieved from the curated SILVA database (v132) (Quast et al. 2012).
<u>Statistics</u>

Metagenome analyses were performed using R version 3.6.3 (2020-02-29). Fungal and bacterial data were imported and filtered with Phyloseq package (version 1.28.0) (McMurdie and Holmes 2013), keeping only the operational taxonomic units (OTUs) with a relative abundance above 0.01 in at least a single sample. Differential abundance of taxa due to the effects of rootstock-treatment interaction was then tested using DESeq2 (version 1.24.0) (Love et al. 2014) package.

For phenotypic, biochemical and RT-qPCR data, when ANOVA indicated that for either Rootstock (R, 1103P and SO4), Inducer (I, NI) and Myc inoculum (M, Myc and NMyc) factors or their interaction was significant, mean separation was performed according to Tukey's HSD test at a probability level of $P \leq 0.05$. ANOVA and Tukey's HSD test were also used to analyze the treatments effects for each rootstock individually. The standard deviation (SD) or error (SE) of all means were calculated.

4.4 Results

Growth, primary metabolism and N uptake and accumulation

The impact of an AM inoculum, an inducer and a combination of both was evaluated on growth parameters (both rooting % and growth stages coded by BBCH scale) in two grapevine rootstock genotypes (R, 1103P and SO4). Four conditions for each genotype were considered: C, not inoculated plants; I, plants treated with the inducer (I); M, AM-inoculated plants; M+I, AM-inoculated plants + inducer.

Results showed a similar impact of the three treatments on the cutting growth parameters (Fig. 1, Table S2), independently from the genotype. Particularly, in SO4 genotype both the rooting % and the BBCH values were higher in treated plants with respect to the control (Fig. 1a,b). Chlorophyll Content Index (CCI) has been evaluated at the end of the experiment, showing no strong differences among the genotypes and treatments (Fig. 1c), although it was significantly influenced by root colonization (M), the inducer (I) and the M x I interaction in both rootstock genotypes.

Treatments generally led to slightly lower values of carbohydrates content in leaves with the exception of M, and only R and I factors significantly influenced this measurement (Fig. 1d). In detail, for each rootstock I and M+I plants showed significant lower levels of carbohydrates (Fig. 1d).

Net Nitrate uptake (NNU) was evaluated (Fig. 2a Table S2), showing that it was significantly affected by M factors and the interaction M x I with lower values in treated samples for both genotypes, particularly in M SO4 plants with respect to C SO4 ones (Fig. 2a).

As for the CCI, only slight differences in total N content in leaves were evident among genotypes and treatments, although was significantly affected by the M factor and the M x I interactions (Fig. 2b).

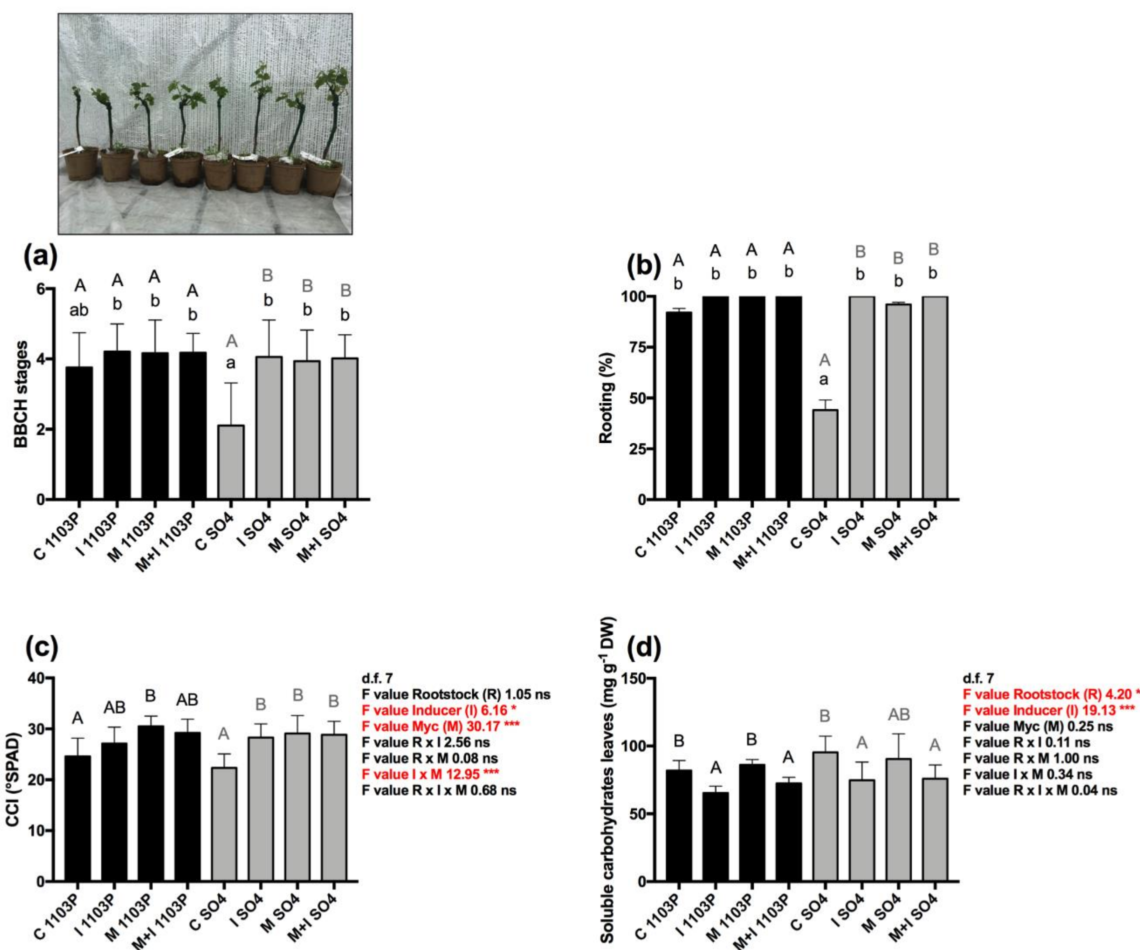

Fig. 1 Growth-related traits and metabolites. a Growth index according to BBCH scale recorded for each treatment at the end of the experiment (n = 25). Upper picture showed an overview of the cuttings' development in response to the treatments at the end of the experiment. b Rooting % of cuttings at the end of the experiment (n = 25). c Chlorophyll Content Index (CCI) measured at the end of the experiment (n = 25). d Quantification of soluble carbohydrates contents in leaves at the end of the experiment (n = 4). All data are expressed as mean SD. ns, *, **, and ***: non-significant or significant at P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test (P ≤ 0.05), considering R x I x M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test (P ≤ 0.05) considering the two rootstocks independently. C: Control plants; I: Inducer-treated plants; M: AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks.

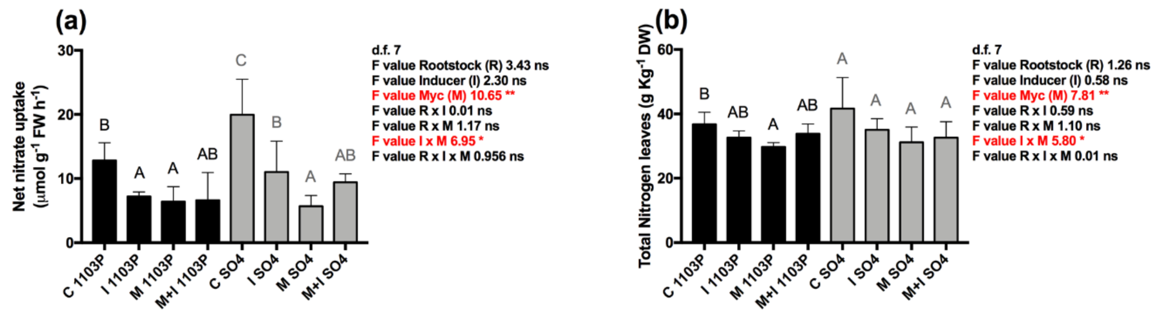

Fig. 2 Net nitrate uptake in roots and total N in leaves. a in vivo Net nitrate uptake. b Total N in leaves (g kg-1 DW). All data are expressed as mean SD (n = 3). ns, *, **, and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R x I x M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C: Control plants; I: Inducer-treated plants; M: AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks.

ABA Content and the Expression of ABA-related Genes

To complete the physiological characterization of the two genotypes in response to treatments, the concentration of ABA was quantified in roots and leaves (Fig. 3, Table S2). ABA levels showed a complex scenario in roots where all treatments led to higher ABA levels with respect to the control with the greater significant increase recorded in M SO4. Statistical analyses showed that factors influencing its level were R and M, alone or in the interactions with I (R x I, M x I, R x M x I) (Fig. 3a). ABA content in leaves was under the detection limit among the treatments (data not shown).

To better understand the role of ABA in our system, the expression of ABA-related genes was analyzed in both leaves and roots. Relative expression of: i) a gene encoding for a 9-cis-epoxycarotenoid dioxygenase potentially involved in ABA biosynthesis (*VvNCED3,* VIT_19s0093g00550 previously reported as *VvNCED1*); ii) a gene coding for an enzyme involved in conversion of ABA to 8'-hydroxy ABA (*VvABA8OH1*); iii) a β-glucosidase (BG) involved in free ABA biosynthesis *via* hydrolysis of ABA glucose ester to release the ABA active form (*VvBG1*; Jia et al. 2016); iv) a gene encoding an ABA glucosyltransferase (*VvGT*; Sun et al. 2010) were evaluated in leaves and roots. In leaves, *VvNCED3* expression was not affected by rootstock genotype whereas M samples showed significantly higher expression levels with respect to the other samples (Fig. 3b). No significant difference was detected for *VvABA8OH1* expression in leaves although 1103P generally showed higher values with respect to SO4 (Fig. 3c). By contrast, *VvNCED3* expression in roots was influenced by R, M

and I factors as well as by R x I interaction, and values for each rootstock genotype were lower in all treatments when compared to C plants (Fig. 3d). Similar to that observed in leaves, M+I treatment led to the significant lowest *VvNCED3* transcripts level in root samples (Fig. 3d). Two pathways promote free ABA accumulation: (1) NCED-mediated *de novo* synthesis (Qin and Zeevaart 1999) and (2) BG-mediated hydroxylation (Lee et al. 2006). Looking at *VvBG1* gene, its expression was significantly influenced by R and I in leaves, while the presence of the AMF was not significantly relevant. In roots all the factors and interactions, significantly affected *VvBG1* expression level, with the highest level in C SO4 samples (Fig. 3e,g). Finally, *VvGT* showed a trend similar to *VvBG1* in leaves where its expression was significantly influenced by R, I and I x M with the exception of SO4 samples where its expression was significantly higher only in M SO4 with respect to C SO4 (Fig. 3f). Conversely, in roots *VvGT* transcript levels were significantly lower in all the conditions with respect to the C 1103P plants (Fig. 3h).

Although *VvABA8OH1*, coding for an enzyme involved in ABA conversion, was not significantly regulated among genotypes and treatments in leaves, it results to be affected by all the considered factors and interactions in roots (Fig. 3i) where it appeared significantly upregulated in M 1103P, M SO4 and M+I SO4 plants with respect to their C (Fig. 3i). It is worth noting the low expression in I root samples, suggesting that the inducer may affect ABA catabolism independently from the genotype and the presence of the AM inoculum.

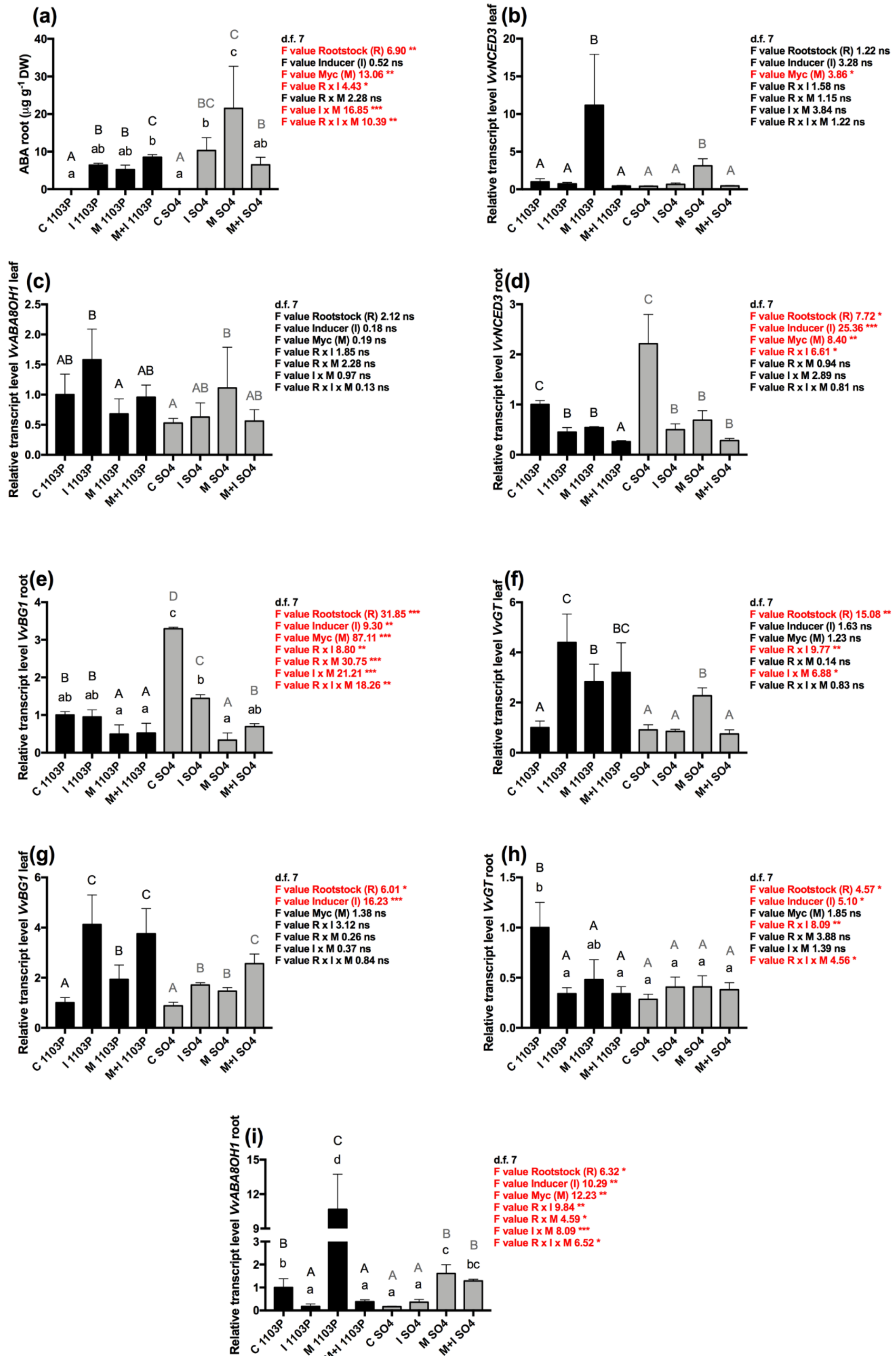
(a)
ABA root (µg g-1 DW)
d.f. 7
F value Rootstock (R) 6.90 **
F value Inducer (I) 0.52 ns
F value Myc (M) 13.06 **
F value R x I 4.43 *
F value R x M 2.28 ns
F value I x M 16.85 ***
F value R x I x M 10.39 **
(b)
Relative transcript level VvNCED3 leaf
d.f. 7
F value Rootstock (R) 1.22 ns
F value Inducer (I) 3.28 ns
F value Myc (M) 3.86 *
F value R x I 1.58 ns
F value R x M 1.15 ns
F value I x M 3.84 ns
F value R x I x M 1.22 ns
(c)
Relative transcript level VvABA8OH1 leaf
d.f. 7
F value Rootstock (R) 2.12 ns
F value Inducer (I) 0.18 ns
F value Myc (M) 0.19 ns
F value R x I 1.85 ns
F value R x M 2.28 ns
F value I x M 0.97 ns
F value R x I x M 0.13 ns
(d)
Relative transcript level VvNCED3 root
d.f. 7
F value Rootstock (R) 7.72 *
F value Inducer (I) 25.36 ***
F value Myc (M) 8.40 **
F value R x I 6.61 *
F value R x M 0.94 ns
F value I x M 2.89 ns
F value R x I x M 0.81 ns
(e)
Relative transcript level VvBG1 root
d.f. 7
F value Rootstock (R) 31.85 ***
F value Inducer (I) 9.30 **
F value Myc (M) 87.11 ***
F value R x I 8.80 **
F value R x M 30.75 ***
F value I x M 21.21 ***
F value R x I x M 18.26 **
(f)
Relative transcript level VvGT leaf
d.f. 7
F value Rootstock (R) 15.08 **
F value Inducer (I) 1.63 ns
F value Myc (M) 1.23 ns
F value R x I 9.77 **
F value R x M 0.14 ns
F value I x M 6.88 *
F value R x I x M 0.83 ns
(g)
Relative transcript level VvBG1 leaf
d.f. 7
F value Rootstock (R) 6.01 *
F value Inducer (I) 16.23 ***
F value Myc (M) 1.38 ns
F value R x I 3.12 ns
F value R x M 0.26 ns
F value I x M 0.37 ns
F value R x I x M 0.84 ns
(h)
Relative transcript level VvGT root
d.f. 7
F value Rootstock (R) 4.57 *
F value Inducer (I) 5.10 *
F value Myc (M) 1.85 ns
F value R x I 8.09 **
F value R x M 3.88 ns
F value I x M 1.39 ns
F value R x I x M 4.56 *
(i)
Relative transcript level VvABA8OH1 root
d.f. 7
F value Rootstock (R) 6.32 *
F value Inducer (I) 10.29 **
F value Myc (M) 12.23 **
F value R x I 9.84 **
F value R x M 4.59 *
F value I x M 8.09 ***
F value R x I x M 6.52 *
C 1103P
I 1103P
M 1103P
M+I 1103P
C SO4
I SO4
M SO4
M+I SO4

Fig. 3 Expression changes of ABA-related genes and metabolite quantification in both root and leaf tissues. a ABA content in roots. b VvNCED3 in leaf. c VvABA8OH1 in leaf. d VvNCED3 in root. e VvBG1 in root. f VvGT in leaf. g VvBG1 in leaf. h VvGT in root. i VvABA8OH1 in root. All data are expressed as mean SD (n = 3). ns, *, **, and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering R x I x M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$) considering the two rootstocks independently. C: Control plants; I: Inducer-treated plants; M: AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks.

Defense

Stilbenes are the main defense-related metabolites synthesized in grapevine. In this study *trans*-resveratrol and viniferin levels were measured in leaves among the several conditions tested (Fig. 4, Table S2). Particularly, resveratrol was only affected by the MxI interaction, showing in parallel significantly higher levels in I and M plants, independently from genotype, with respect to M+I and C plants (Fig. 4a). Viniferin, which was not detectable in C plants, was affected by the M x I interaction and by the I factor alone. I, M and M+I treated plants presented in fact significantly higher values of viniferin than C plants in both rootstocks (Fig. 4b). To correlate biochemical data with molecular responses, expression levels of genes coding for two stilbene synthases (*VvSTS1* and *VvSTS48*) were assessed. Results showed that in both rootstocks *VvSTS1* was upregulated mainly in M 1103P whereas in SO4 plants was observed an upregulation in both I and M with respect to the other treatments (Fig. 4c). *VvSTS48* expression was influenced by all the factors and their interactions, with the highest expression value in leaves of I-treated SO4 plants (Fig. 4d). Looking independently at each rootstock, in 1103P only I and M induced significant overexpression of *VvSTS48* while in SO4 plants all the treatments showed enhanced gene expression compared to their controls (Fig. 4d).
RT-qPCR was also applied to detect the expression levels of several target genes as markers of diverse defense response pathways (Fig. S1, Table S2). Two genes were studied both in leaves and roots (a sugar transporter, *VvSPT13* and a class III chitinases, *VvChitIII*), three genes only in leaves (a callose synthase, *VvCAS2*; a lipoxygenase *VvLOX,* and the Enhanced Disease Susceptibility 1, *VvEDS1*) (Fig. S1a-g). Expression of all the considered genes were influenced by I factor, while influence by M was more variable, suggesting a different impact of the treatments on plant metabolism. Among these genes, *VvSTP13*, encoding a sugar transporter, in leaves of both rootstocks was significantly upregulated in all treatments with respect to their C plants (Fig. S1a) while in root only M-treated plants showed significantly higher expression

values (Fig. S1). *VvChitIII* showed a different pattern in leaves and roots. In leaves, *VvChitIII* transcript was significantly induced in M- and M+I-treated plants (Fig. S1c) while in roots an upregulation was observed only in M-treated ones (Fig. S1d). *VvCAS2*, coding for a callose synthase (Santi et al. 2013), showed a downregulation in all the treatments, while *VvLOX* gene, encoding a lipoxygenase involved in the jasmonic acid biosynthesis, was upregulated in all the treatments: among them, the lowest value was observed in M SO4 plants (similar to the C 1103P leaves), suggesting a different response to symbioses in the two genotypes (Fig. S1e-f). *VvEDS1*, selected as marker of Systemic Acquired Responses (SAR) mediated by Salicylic Acid (SA), was influenced by I and M, showing an upregulation trend in I-treated leaves. Conversely, this gene was downregulated in M-treated plants (Fig. S1g).

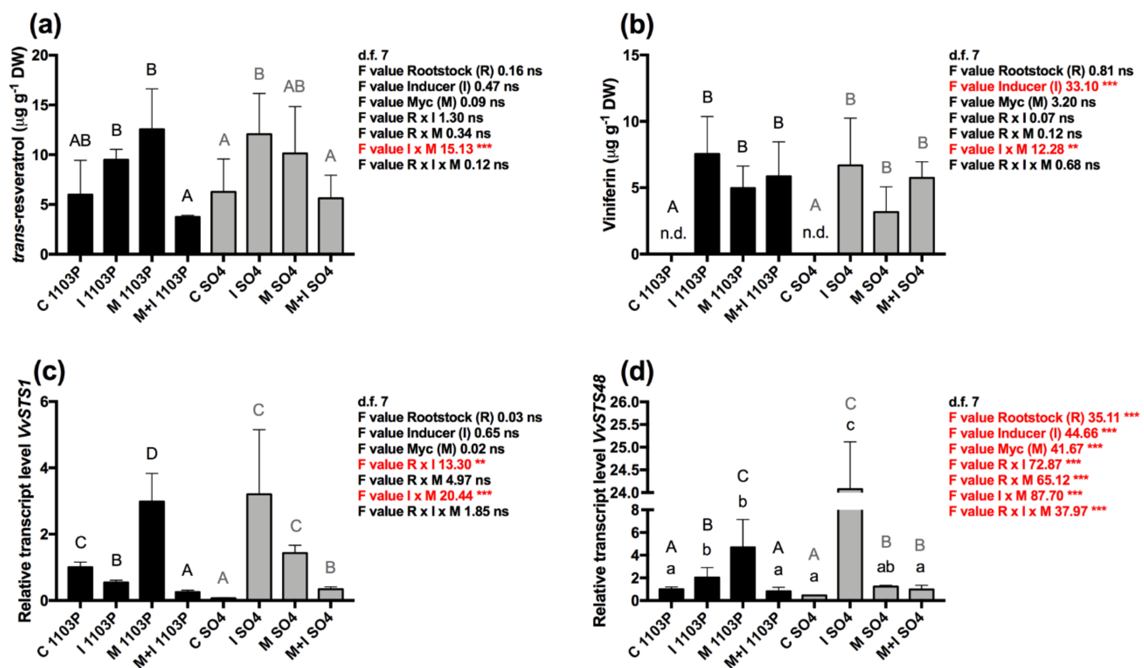

Fig. 4 Expression changes of stilbenes-related genes and metabolites quantification in leaf tissues. a trans-resveratrol quantification. b Viniferin quantification. c VvSTS1 gene expression changes. d VvSTS48 gene expression changes. All data are expressed as mean □ SD (n = 3). ns, *, **, and ***: non-significant or significant at P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test (P ≤ 0.05), considering R x I x M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test (P ≤ 0.05) considering the two rootstocks independently. C: Control plants; I: Inducer-treated plants; M: AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks.

Rhizoplane metaphylogenomic analyses

Bacterial community was analyzed at both order and genus level: the number of retained sequences after chimera removal and taxonomical assignment was always above 35,000 (detailed results of sequencing are reported in Table S3). Shannon index diversity indicated that the only significant difference was observed for the I SO4 samples which show higher index values (Table S4). No significant differences were observed among samples comparing the Shannon index on the fungal community (Table S5). Similar to Shannon index, non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity matrixes showed that the bacterial community (Fig. 5a) is more affected by treatments than the fungal one (Fig. S2).

The bacteria community composition for each sample type at order and genus levels are reported in Table S6. Statistical results of pairwise comparisons among genera are reported in Table S7. To simplify, results are described for the orders and genera that represent at least the 1% of the bacterial community (Fig. 5b). Comparison of the bacterial community between the two rootstocks (1103P *vs* SO4) revealed that 1103P has a significant higher relative abundance of *Pseudomonas* species whereas SO4 has a significant higher relative abundance of *Bacillus* species. Among the bacterial genera, which display significant differences among the treatments, M 1103P vines stimulated the presence of *Bacillus* species but repressed the interaction with *Pseudomonas* ones. In parallel, when comparing treatments on SO4 rootstock, a positive interaction between the mycorrhizal inoculation and the *Pseudomonas* abundance was observed, whereas the inducer treatment showed a negative impact on *Flavobacterium* abundance. The fungal community composition for each sample type at order and genus levels are reported in Table S6. Statistics of the pairwise comparisons among genera are reported in Table S8. Results for the fungal orders and genera that represent at least the 1% of the fungal community are reported in Fig. S3. Focusing on AMF, results confirm the presence of *Rhizophagus* and *Funneliformis* in inoculated plants. However, AMF were detected also in the I-treated plants (Fig. 6a). Despite the presence of AMF associated to these roots, gene expression analysis on fungal PT genes showed the presence of *RiPT* and *FmPT* transcripts only in M-inoculated plants. Surprisingly, absent or low expression levels were detected in I-treated plants (Fig. 6b,c; Table S2). Indeed, fungal *PT* genes were expressed in a different way in the two genotypes, suggesting a different symbiosis efficiency of the two rootstocks. This finding was further confirmed by a plant PT gene (*VvPT1-3*), which expression level was mainly affected by R and M factors, and by 'R x I' interaction. It was up-regulated in 1103P roots, independently by treatment, with respect to C 1103P and strongly up-regulated in M SO4 ones (Fig. 6d, Table S2).

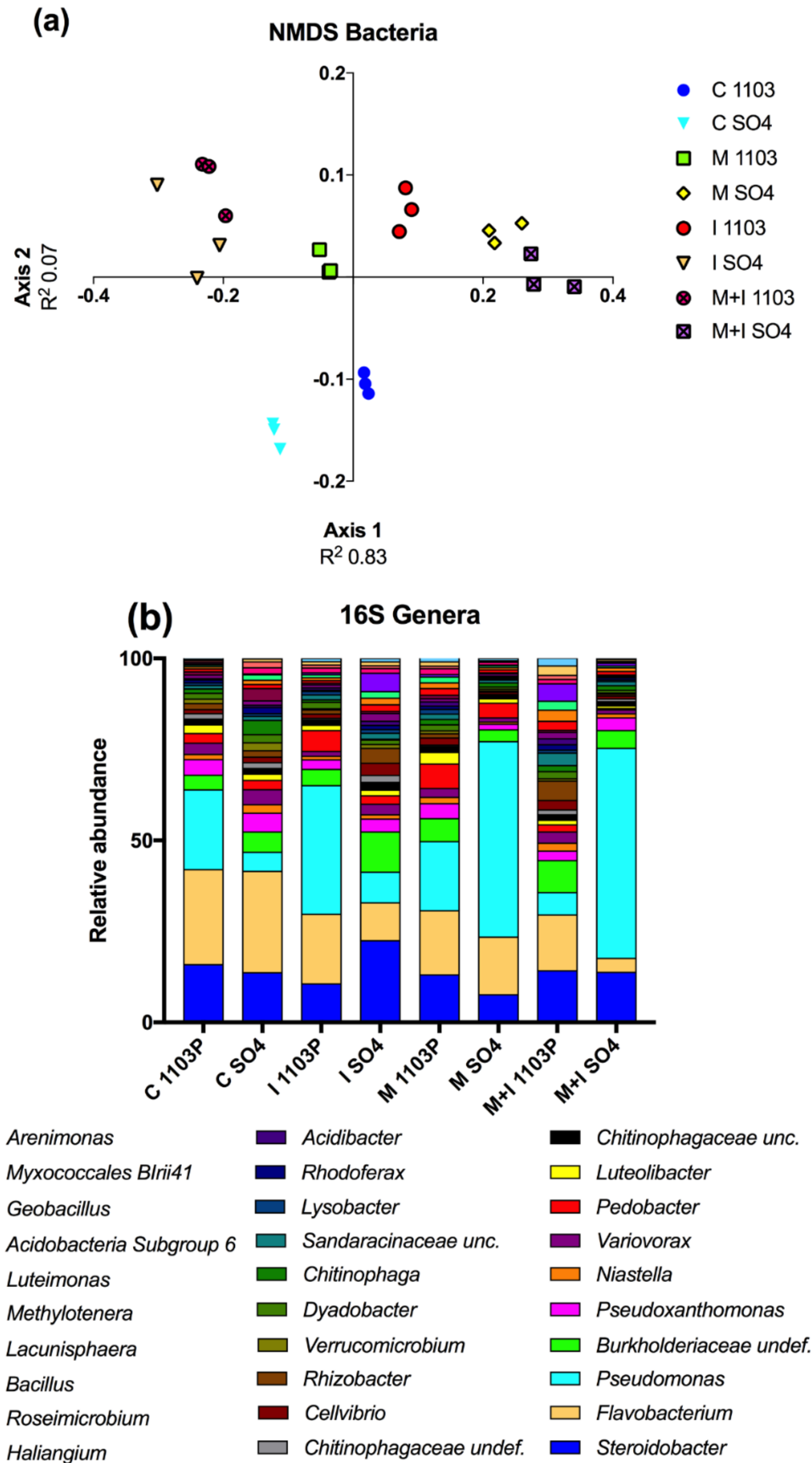

Fig. 5 Distinct root associated-bacteria community composition among treatments. NMDS algorithm based on Bray-Curtis distances matrixes was used to reduce into a bi-dimensional scaling data obtained for bacteria community (a). Relative abundance of bacterial genera (b) among treatments. Only genera representing at least the 1% over the total number of classified amplicons were retained (n = 3). C: Control plants; I: Inducer-treated plants; M: AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks.

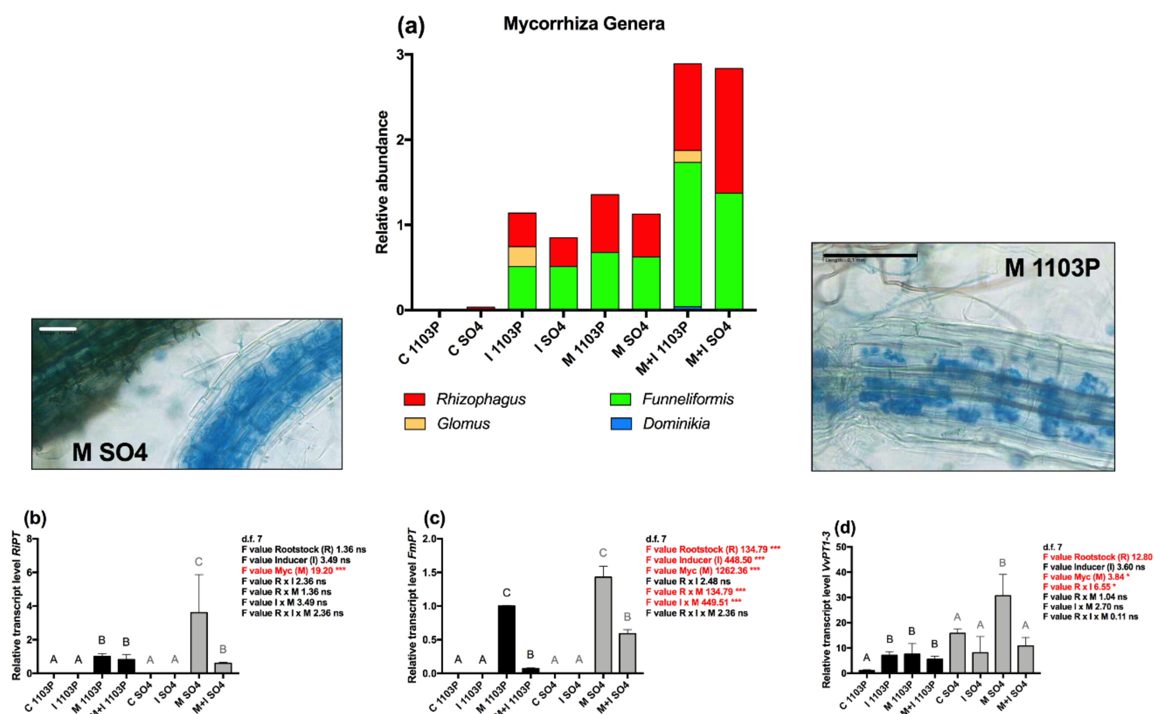

Fig. 6 Mycorrhiza genera and expression changes of plant and fungus Phosphate Transporter (PT) genes as markers of functional symbioses. a Relative abundances of mycorrhiza genera (n = 3). b RiPT. c FmPT. d VvPT1-3. Gene expression data are expressed as mean SD (n = 3). ns, *, **, and ***: non-significant or significant at P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test (P ≤ 0.05), considering R x I x M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test (P ≤ 0.05) considering the two rootstocks independently. C: Control plants; I: Inducer-treated plants; M: AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks. Insets: Microscope images of typical AM symbioses structures in 1103P and SO4 M-colonized roots.

Comparing the fungal composition in C, 24 genera with significant differences of relative abundance were observed. Among the analyzed genera, *Clonostachys* displayed a significant negative correlation with all the treatment in both rootstock genotypes. Focusing on significant genera, usually involved in pathogenic interaction, such as *Fusarium*, *Rhizoctonia* and *Ilyonectria* (Fig. S4), the concomitant use of mycorrhizal inocula with the inducer brought to a significant reduction of *Ilyonectria* in both rootstocks. Conversely, *Fusarium* abundance was stimulated in all treatments except for the inoculation with AMF in the 1103P rootstock. Finally, *Rhizoctonia* genus was positively influenced by the inducer, but only in the SO4 rootstock.

4.5 Discussion

Treatments and genotypes differently shape the root-associated bacterial and fungal communities

The importance of root-associated microbes was extensively demonstrated in several crops including grapevine, with the potential to exploit biocontrol strategies that rely on the beneficial traits of plant growth-promoting microorganisms (PGPBs) naturally associated with plants (Verbon and Liberman 2016; Marasco et al. 2018; Yu et al. 2019). Among them, AMF and their impacts on diverse plant species, including economically important crops, have been largely studied highlighting the importance of this relationship that can positively affect both growth and defense traits (Jacott et al. 2017). However, despite these advantages, grapevine breeders normally focus their attention more on phenotypic or metabolic peculiarities rather than on the improvement of the interactions with root-associated microbes (Marín et al. 2021).

Grapevine roots are commonly colonized by different AMF taxa depending on the considered environment, season and soil management making them relevant in defining the 'microbial terroir' of a specific grape cultivar (Massa et al. 2020). Svenningsen et al. (2018) reported that AMF ecosystem services might be suppressed by some bacterial groups belonging to Acidobacteria, Actinobacteria, Firmicutes, Chitinophagaceae, and Proteobacteria. Our results showed an inverse correlation between the presence of some of these bacteria (*i.e.*, Acidobacteria, genus *Vicinamibacter* and Actinobacteria genus *Gaiella*) and AMF "functionality", although ITS sequencing showed a similar level in term AMF abundance. It is also necessary to consider that, ITS was used in the present work as universal fungal marker (Schoch et al. 2012; Lindahl et al. 2013) to better define the overall fungal population despite ribosomal large subunit (LSU) region consistently shows greater utility for taxonomic resolution for AMF (Xue et al. 2019a). Despite the latter approach can give better results, it has rarely been used in environmental studies of AMF because of sequencing and bioinformatics challenges (Delavaux et al. 2021). Similarly, for a better description of the AMF population, it was recently reported that, the use of AMF specific primers, coupled to nested PCR, can greatly help in better define the AMF population (Suzuki et al. 2020).

Additionally, results obtained from the microbiome analysis confirm that the response of microbial communities to the different treatments are genotype dependent (Marasco et al. 2018). This is particularly clear for the bacterial community, where the addition of the mycorrhizal inoculum promoted the *Pseudomonas* genus in 1103P and the *Bacillus* genus in SO4. It is important to remind that both these genera were largely investigated in grapevine because

of their ability to protect vine plants against several fungal pathogens. *Pseudomonas* genus was studied for its ability to impair *Botrytis*, *Neofusiccocum*, *Ilyonectria*, *Aspergillus*, *Phaeomoniella* and *Phaeoacremonium* genera, which are all well-known grape fungal pathogens (Andreolli et al. 2019; Niem et al. 2020). On the other hand, *Bacillus* species were studied for their ability to reduce the impact of black foot disease (mainly due to infection by *Cylindrocarpon* and *Ilyonectria* species) and downy mildew on grapes (Zhang et al. 2017; Russi et al. 2020). These studies well fit with our data where we observed the lower *Ilyonectria* abundance in M+I 1103P and concomitantly the higher abundance of *Bacillus* species. Looking at the fungi, all the treatments promoted the presence of different AMF species, suggesting the recruitment of native AM fungal communities by the I-treated roots, independently from the rootstock genotypes. In detail, it is worth noting a higher diversity in AMF colonization in I 1103P with respect to I SO4 plants, independently from the presence of the AMF inoculum, confirming a diverse recruitment pattern for the two genotypes. Interestingly, *Clonostachys* genus negatively correlated with all the treatments. This genus was extensively studied for its promising exploitation as biological control agents against soil and root pathogens (Nygren et al. 2018; Sun et al. 2020). Considering that in all treatments the *Rhizophagus* genus was more abundant than in C, we can confirm that a mutual exclusion between *Clonostachys* and *Rhizophagus* genera is present. Although a full explanation for this reciprocally inhibitory interaction is still missing, the complex microbial community modulation mediated by the AM fungi could impair the ability of *Clonostachys* to endophytically colonize the host plant (Ravnskov et al. 2006; Akyol et al. 2018; Xue et al. 2019b). These findings, in accordance with the increase in defense-related metabolites and the expression data on defense-related genes, well fit with the concept of mycorrhizal-induce resistance (MIR) (Cameron et al. 2013) as a cumulative effect of direct and indirect (i.e. mediated by mycorrhizosphere associated microorganisms) defense responses. Recently, Emmett et al. (2021) also demonstrated that a conserved community is associated to AMF extraradical hyphae, suggesting an influence on the plant-fungal symbiosis.

<u>AM fungi and root-associated microbes balance rootstocks growth traits showing a different pattern of functional symbioses</u>

The impact of the different treatments on two different rootstock genotypes was evaluated. The selected rootstocks (i.e. 1103P and SO4) were well characterized at both agronomic and molecular level (Chitarra et al. 2017), showing opposite growth and defense attitudes. Among rootstock features, fine root development and density, imparting vigor to the scion, varied considerably with an impact on water and nutrient uptake as well as on the interaction with

soil microorganisms. AM colonization showed that SO4 consistently presented higher levels of root colonization, together with Kober 5BB and Ruggieri 140, with respect to the others (Chitarra et al. 2017). This is in agreement with previous works (Bavaresco and Fogher 1996; Bavaresco et al. 2000), who showed a variation in the range of AM-colonized grape rootstocks among genotypes, which could be considered the main factor driving AM recruitment. However, functional symbiosis was strongly influenced also by scion requirements, soil fertility and soil pH (Bavaresco and Fogher 1996; Bavaresco et al. 2000). Here, both rooting and growth parameters, and partially the CCI, clearly showed a compensation effect in the less vigorous SO4 with respect to 1103P, reaching similar values in all the treatments. A role could be attributed to AMF particularly in SO4. To attest this hypothesis, considering that high-affinity PTs in AM have been characterized and it has clearly been demonstrated that plants possess a symbiotic Pi uptake pathway (Berruti et al. 2016), AM fungal PT genes (*RiPT* and *FmPT*) have been tested showing a highly expression in M SO4 for both, and also in M+I SO4 for *FmPT*. Similarly, the plant gene *VvPT1-3*, homolog of mycorrhiza-inducible inorganic phosphate transporters such as *LePT4* and *OsPT11* (Balestrini et al. 2017), was significantly up-regulated in M SO4. The positive effects exerted by AM symbiosis in growth and physiological features were largely documented in several plants (*e.g.*, Chitarra et al. 2016; Balestrini et al. 2020). Surprisingly, although the ITS sequencing showed a certain abundance of AM genera in both I and M+I, the inducer seemed to lower the expression of plant and fungal genes generally involved in symbiosis functioning. This should be related to presence of bacteria reported to diminish AMF functionality (Svenningsen et al. 2018). As well, an impact of the inducer on the number of fine roots, which are those colonized by AMF, cannot be excluded also considering that IAA was not detectable in I samples. Looking at the whole microbial community, in addition to a selection based on the rootstock genotype, it is worth noting that I treatment (particularly I SO4) was able to significantly increase diversity of the microbiota (Table S4). Samples treated with the inducer showed higher bacterial diversity hosting many groups of PGPBs such as *Burkholderiaceae* that might be linked to potassium (K) and phosphorous (P) solubilization and availability (Gu et al. 2020); *Pseudomonas* and *Bacillus* spp. able to produce siderophores, auxin, cytokinins and characterized as phosphate-solubilizing bacteria (Saad et al. 2020; Subrahmanyam et al. 2020) (Table S7). These findings could explain the bacteria-mediated growth effects in I treatments particularly for the SO4 genotype. By contrast, the whole fungal diversity was not significantly affected among the treatments.

Nitrogen (N) is an essential element for all grapevine processes and N transporters were found among the genes upregulated by both a single AMF and a mixed bacterial-fungal inoculum through transcriptomics in grapevine roots (Balestrini et al. 2017). However, although AMF may positively influence plant N compound uptake and transport (Balestrini et al. 2020), negative, neutral or positive AMF effects on N nutrition has been reported (Bücking and Kafle 2015). Due to the fact that several nitrate transporters were found to be regulated by an AMF inoculum (Balestrini et al. 2017), the attention was mainly focused on nitrate uptake. Lower values of nitrate uptake with respect to controls were observed among all treatments, independently from the considered genotypes. Furthermore, any relevant effect on N accumulation in leaves was observed, suggesting that a positive correlation between N content and growth is not relevant in our system or likely due to a biomass dilution effect since the higher growth index recorded particularly in SO4-treated plants. AMF have been reported to show NH_4^+ preference to be assimilated in extraradical mycelium and translocated to plant roots after completion of the GS-GOGAT cycle (Balestrini et al. 2020). In this respect, to the plants side the lower NNU observed in M inoculated plants suggest a role of AMF in regulating root N uptake strategies helping plants in acquire N.

The plant hormone ABA is a chemical signal involved in the plant response to various abiotic environmental factors, but it can also play a role in interactions with phytopathogens by modulating tissue colonization depending on microorganism type, site and time of infection (Ton et al. 2009). An impact of ABA on AMF colonization has been also reported at diverse colonization stages (Bedini et al. 2018). A role for ABA in the mechanisms by which AM symbiosis influences stomata conductance under drought stress was also suggested (Chitarra et al. 2016). Here, ABA levels were affected by both the genotype and the AMF inoculum. A significant effect of the M treatment was found on the expression of a key gene involved in the ABA synthesis in leaves *(VvNCED3*), showing a positive correlation with the ABA levels in roots. Our result is in accordance with the fact that ABA produced in leaves is then translocated in roots where it might act as a signal to promote root growth (McAdam et al. 2016). AMF presence led to higher ABA content in M SO4 roots, despite the fact that generally SO4 rootstock was reported to have a low endogenous content (Chitarra et al. 2017), suggesting a potential enhanced tolerance to abiotic stresses in M SO4. As already reported by (Ferrero et al. 2018), the relationship between biosynthetic and catabolic processes may be complex and diverse in the different plant organs. Our results showed a different expression pattern of most of the considered genes involved in ABA synthesis and catabolism in leaves and roots. A gene coding for an ABA 8'-hydroxylase (*VvABA8OH1*), belonging to the

CYP707A gene family and with a primary role in ABA catabolism, showed an opposite trend in M and I root apparatus, in agreement with the ABA root accumulation. Overall, obtained data are in accordance with that reported by Martín-Rodríguez et al. (2016) showing that both ABA biosynthesis and catabolism are finely tuned in AM-colonized roots. Although with the activation of different mechanisms depending on the treatment, an impact on ABA homeostasis can be suggested particularly in SO4 genotype.

AM symbiosis triggers defence-related transcripts and metabolites more in 1103P than in SO4 rootstock

Plants finely tune the immune system to control both pathogen infection and beneficial microorganism accommodation. Soil bacteria and fungi play a double role in promoting growth and defense response, helping in maintaining the homeostasis in the whole microbial communities associated to the roots through the Induced Systemic Resistance (ISR) pathways (Liu et al. 2020). In grapevine, stilbenes are phytoalexins with proved antifungal activities (Chalal et al. 2014). Here, resveratrol content was higher in I and M leaves with respect to untreated controls, while viniferin, that is highly toxic for grape foliar pathogens such as downy and powdery mildew (Chitarra et al. 2017), has a similar trend in all the treatments while it was not detected in C plants. These patterns clearly highlight a stimulating effect mediated by root-associated microbes (native or inoculated), with differences that might be related to the diverse microbiome composition. Among the genes involved in stilbene synthesis, *VvSTS48*, coding for a stilbene synthase reported as induced by downy mildew infection, showed the highest expression value in I SO4 plants, suggesting a different modulation among treatments and genotypes. Carbohydrate metabolism is also involved in plant defense responses against foliar pathogens (Sanmartín et al. 2020). In tomato, AM symbiosis was reported to be involved in *Botrytis cinerea* resistance through the mycorrhiza-induced resistance (MIR) mediated by callose accumulation. A tomato callose synthase gene (*PMR4*) was in fact upregulated by mycorrhization mainly upon biotic infection (Sanmartín et al. 2020). In the present study, attention has been focused on the homolog grape gene *VvCAS2*. Conversely to that previously observed, *VvCAS2* showed a downregulation trend in all the treatments with respect to control plants. These findings suggest a primary role in microbe-mediate stimulating of defense responses against biotic factors in grape. Since a correlation between MIR and sugar signaling pathway was reported (Sanmartín et al. 2020), the expression of a grapevine sugar transporter gene (*VvSTP13*), homolog to the *Arabidopsis STP13*, involved in intracellular glucose uptake and in *B. cinerea* resistance, was followed in

leaves and roots. Although total soluble carbohydrates were not affected by treatments in leaves, *VvSTP13* expression showed an upregulation trend in all the treatments, particularly in both I sample and M 1103P leaves, suggesting an effect of AMF inoculum in the susceptible genotype.

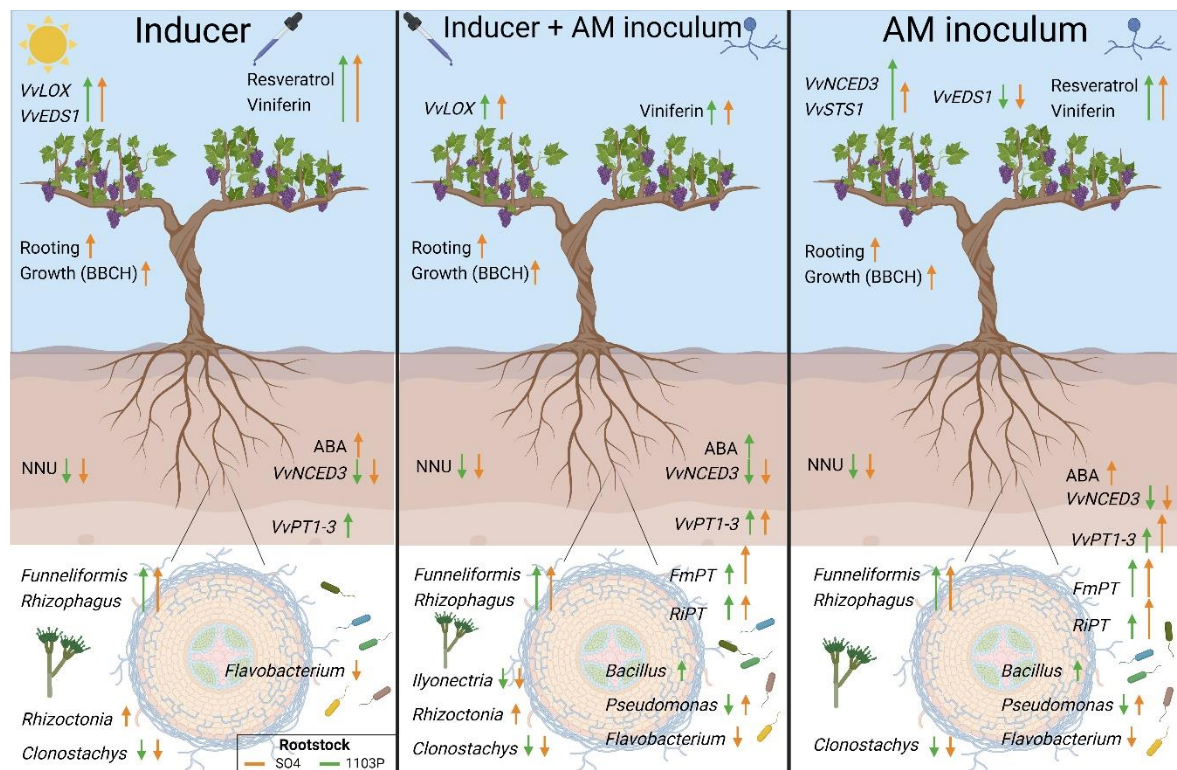

Fig. 7 Overview of phenotypic, biochemical, and molecular changes induced by the treatments. Green arrows indicate responses in 1103 Paulsen (1103P) rootstock whereas orange ones are referred to SO4 genotype. Upward arrows indicate an increase whereas downward arrows represent a decrease in content of metabolites or gene relative expression or relative abundance of microbial taxa with respect to Control (C) plants. NNU: Net Nitrate Uptake; ABA: Abscisic Acid.

Looking at the roots, *VvSTP13* upregulation trend was observed mainly in mycorrhizal roots, in agreement with the fact that expression of genes from the STP family was revealed in arbuscule-containing cells of *Medicago truncatula* (Hennion et al. 2019). The same trend observed for *VvSTP13* was also found for a gene coding for a class III chitinase (*VvChitIII*). Class III chitinases have been already reported to be markers of functional symbioses (Balestrini et al. 2017), being localized in arbuscule-containing cells (Hogekamp et al. 2011). Finally, the expression of two target genes (*VvLOX* and *VvEDS1*), respectively involved in ISR mediated by jasmonate and SAR mediated by salicylic acid, although differently

modulated by the inducer and AM fungi, confirmed the role of the whole microbiome on the plant immunity system in the scion of both rootstock genotypes (Cameron et al. 2013).

4.6 Conclusion

Overall, our results allowed to provide new insights into growth-defense tradeoffs responses in a model fruit crop (Fig. 7). Although molecular mechanisms at the basis of plant priming are still matter of debate, several hypotheses have been proposed. In this study, a finely tune regulation of growth and defence traits have been highlighted considering three main influencing factors, *i.e.,* the plant genotype, an AM inoculum and an oligosaccharide described as involved in AMF colonization induction. The attention has been focused on two rootstocks characterised by opposite trade-offs. Growth traits have been improved mainly in the low vigour genotype (SO4) by all the treatments probably through the activation of diverse pathways by the root associated microbes. It is worth noting that all the treatments shaped the microbial communities associated to the roots in both the genotypes. Looking at the defence response, a positive impact on immunity system has been revealed both by the AMF inoculum and the oligosaccharide, although with the activation of different pathways. Results suggest that AM symbiosis triggers a mycorrhiza-induced resistance (MIR) also in a model woody plant such as grapevine.

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4.8 Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and material

Sequences were deposited in NCBI database under the BioProject PRJNA718015, BioSamples SAMN18520793 to SAMN18520808 and SRR14089924 to SRR14089939.

Author contributions

WC, RMB and LN designed the experimental system. LN, GQ, GG, LM, NB, LL, RP, MGV, MS, FG, RMB and WC conducted the wet lab experiments and performed data elaboration. LN, GQ, RMB and WC performed RT-qPCR analyses. LN, GG and WC performed the microbiome data analysis of root endophytes. LN, RMB and WC wrote the first draft of the manuscript. All the authors carefully revised the final version.

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4.10 Supporting information

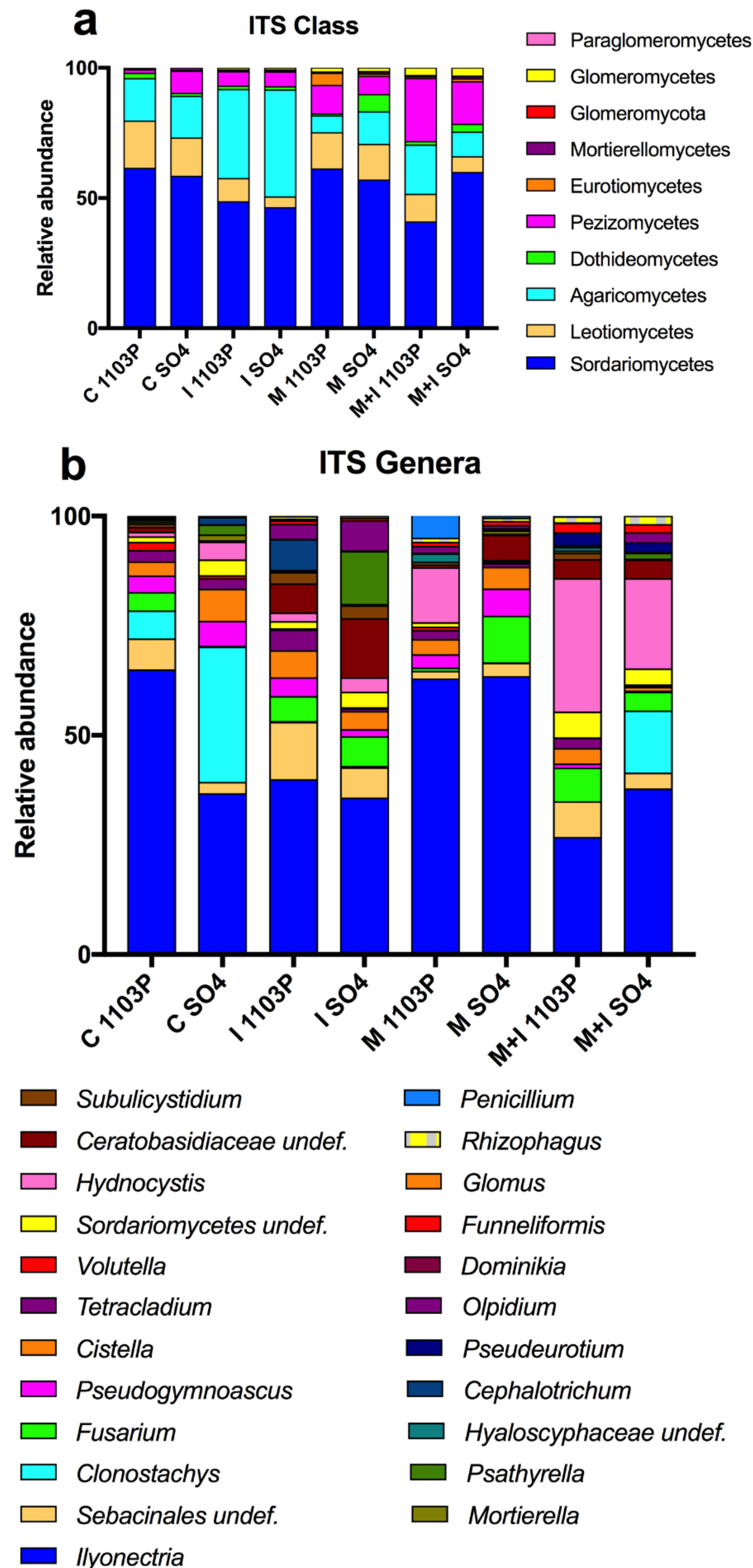

Fig. S1 Gene expression changes of defense-related target genes in both leaf and root. a VvSTP13 in leaf. b VvSTP13 in root. c VvChitIII in leaf. d VvChitIII in root. e VvCAS2 in leaf. f VvLOX in leaf. g VvEDS1 in leaf. h VvHNT1 in leaf. All data are expressed as mean SD (n = 3). ns, *, **, and ***: non-significant or significant

at P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively. Different lowercase letters above the bars indicate significant differences according to Tukey HSD test (P ≤ 0.05), considering R x I x M interaction. Analysis of variance on the single variables is reported in Table S2. Different uppercase letters above the bars indicate significant differences according to Tukey HSD test (P ≤ 0.05) considering the two rootstocks independently. C: Control plants; I: Inducer-treated plants; M: AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks.

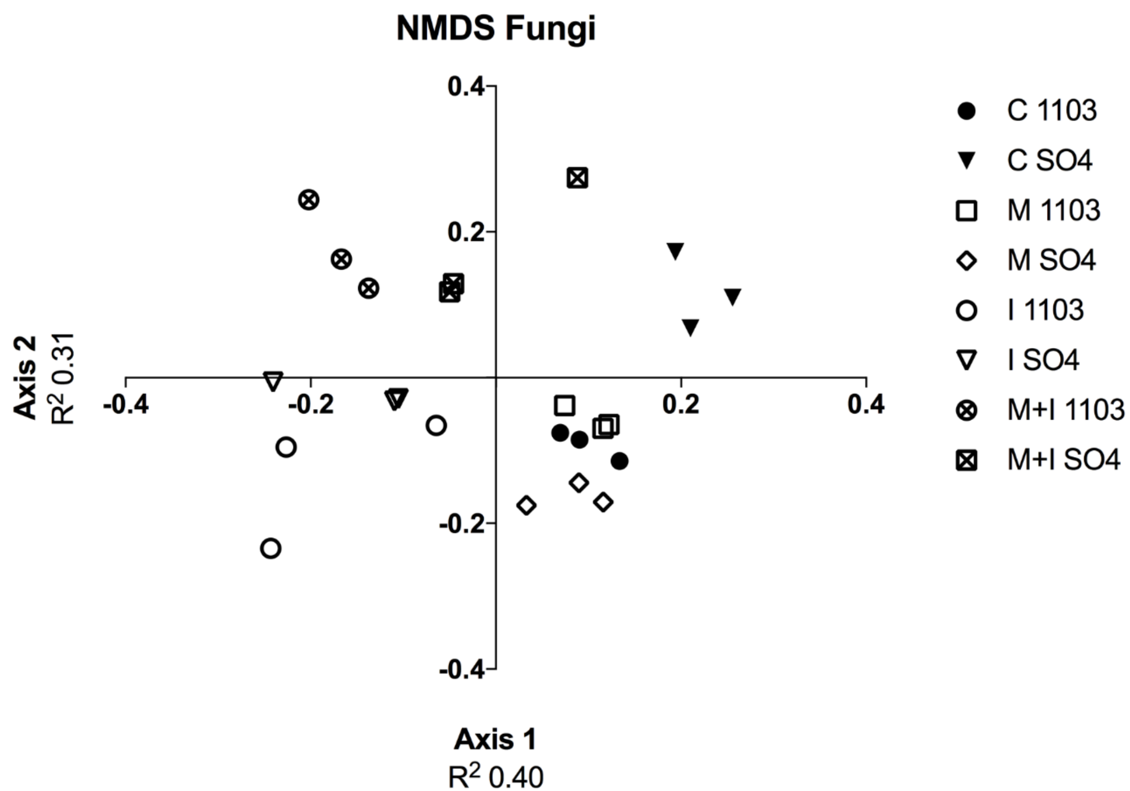

Fig. S2 NMDS of root-associated fungal communities. NMDS algorithm based on Bray-Curtis distances matrixes was used to reduce into a bi-dimensional scaling data obtained for and fungi community (n=3).

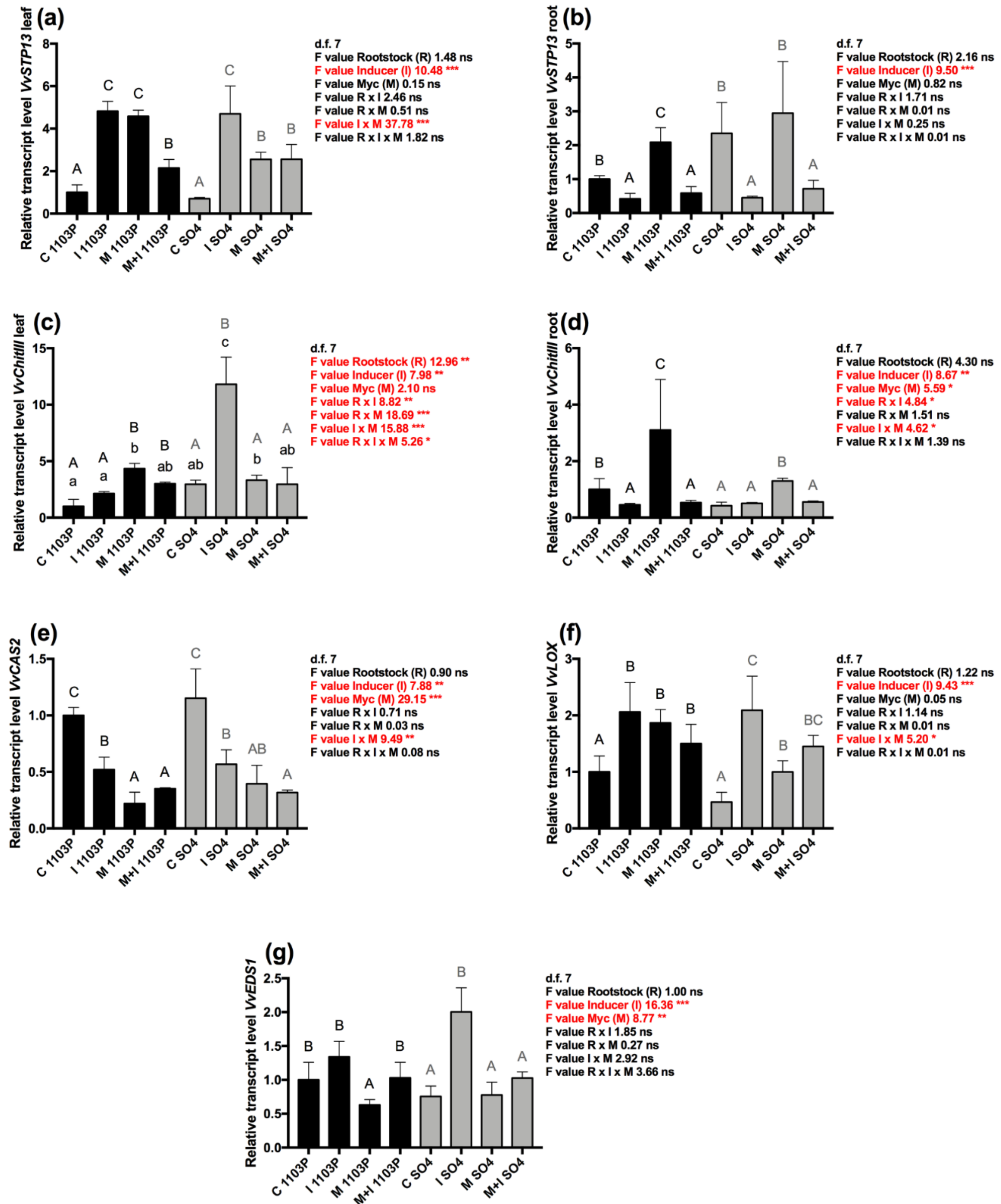

Fig. S3 Distinct root associated-fungal community structure among treatments. Relative abundances of bacterial class (a) and genera (b) among treatments. Only genera representing at least the 1% over the total number of classified amplicons were retained (n = 3). C: Control plants; I: Inducer-treated plants; M: AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks.

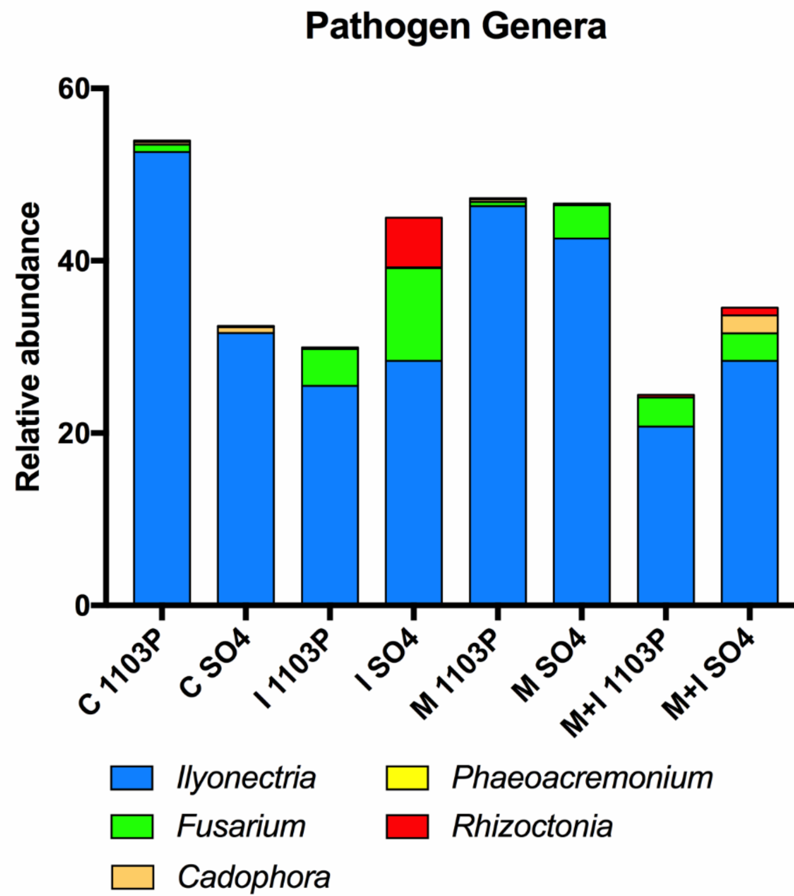

Fig. S4 Relative abundances of fungal pathogens genera. C: Control plants; I: Inducer-treated plants; M: AMF mixed inoculum-treated plants; M+I AMF mixed inoculum + Inducer-treated plants for 1103P and SO4 selected rootstocks (n = 3).

Table S1 Oligonucleotides used in this study.

Name	Primer sequence (5'-3')	Gene annotation or Target	Gene ID	Reference
VvACT1_F *VvACT1_R*	GCCCCTCGTCTGTGACAATG CCTTGGCCGACCCACAATA	Actin	VIT_04s0044g00580	Chitarra et al., 2017
VvEF1-a_F *VvEF1-a_R*	GAACTGGGTGCTTGATAGGC AACCAAAATATCCGGAGTAAAAGA	Elongation Factor 1-alpha	VIT_06s0004g03240	Balestrini et al., 2017
VvUBI_F *VvUBI_R*	TCTGAGGTTCGTGGTGGTA AGGCGTGCATAACATTTGCG	Ubiquitin	VIT_16s0098g00580	Chitarra et al., 2017
VvChitIII_F *VvChitIII_R*	TGCCAAAATCGAGGCACTAAGG TGGCCGAGACGATGATTTTC	Chitinase III	VIT_16s0050g02210	Balestrini et al., 2017
VvPT1-3_F *VvPT1-3_R*	GCACAAATCGAGAAATGGT GCGAGCACAGAATTAATACGAC	Phosphate transporter 1-3	VIT_16s0050g02370	Balestrini et al., 2017
VvNCED3_F *VvNCED3_R*	GCCCCAACCCCCAGTTC GCATGCCATCACCATCAAAG	9-cis-epoxycarotenoid dioxygenase	VIT_19s0093g00550	Chitarra et al., 2017
VvSTP13_F *VvSTP13_R*	GGGTACGGCAATGGATTCG CCCTCCCCATACACCACTAATCT	Sugar transporter 13	VIT_07s0151g00110	Chitarra et al., 2017
VvLOX_F *VvLOX_R*	CCATCTCTTGCACACTGGAA GCGAGCACAGAATTAATACGAC	Lipoxygenase	VIT_09s0002g01080	Balestrini et al., 2017
VvCAS2_F *VvCAS2_R*	TTCACCCCAGTTGCATTTCT CCGATCCTTCCTATGACCAC	Callose synthase	VIT_06s0004g01270	Chitarra et al., 2018*
VvSTS48_F *VvSTS48_R*	CTTGAAGGGGGAAAATGCT TTACTGCATTGAAGGGTAAACC	Stilbene synthase 48	VIT_16s0100g01200	Chitarra et al., 2017
VvEDS1_F *VvEDS1_R*	GGCTACTCGTCGGGTGCTCC GGGTGGGCTCTGATTGGGCT	Enhanced Disease Susceptibility 1	VIT_17s0000g07400	This study
VvSTS1_F *VvSTS1_R*	TGGCCCTGCAATTCTTGATG TTAGCACATGCCTCGTTGCTTC	Stilbene synthase 1	VIT_16s0100g01030	Balestrini et al., 2017
VvABA8OH1_F *VvABA8OH1_R*	ATGGACTTCCAGCCAGATTG GGACATCTCTCCAACCCAGA	ABA 8' Hydroxylase-1	VIT_18s0001g10500	This study
VvBG1_F *VvBG1_R*	TGATGGAACCGGGAAAATAA CCTGTCACCAAACTGCTGAA	Beta Glucosidase 1	VIT_01s0011g00760	This study
VvGT_F *VvGT_R*	CAAATGGGGAAGAAGGCGTG CAGGCCTGCTCATCAATGGA	Abscisate Beta-Glucosyltransferase-like	VIT_17s0000g07200	This study
RiTEF_F *RiTEF_R*	GCTATTTTGATCATTGCCGCC TCATTAAAACGTTCTTCCGACC	*R. irregularis* Elongation Factor		Volpe et al., 2018*
RiPT_F *RiPT_R*	AACACGATGTCAACAAAGCAAC AAGACCGATTCCATAAAAAGCA	*R. irregularis* Phosphate transporter		Volpe et al., 2018*
FmosEF_F *FmosEF_R*	GCAGAACGTGAGCGTGGTAT ACCAGTACCGGCAGCAATAA	*F. mosseae* Elongation Factor		Volpe et al., 2018*
FmPT_F *FmPT_R*	ACTGTTGGCGCTTAGTGCTTGG CAGCCCAACTTGATTTTGGTACG	*F. mosseae* Phosphate transporter		Volpe et al., 2018*

*Chitarra W, Cuozzo D, Ferrandino A, Secchi F, Palmano S, Perrone I, Boccacci P, Pagliarani C, Gribaudo I, Mannini F, Gambino G (2018). Dissecting interplays between *Vitis vinifera* L. and grapevine virus B (GVB) under field conditions. *Mol. Plant Pathol.*, doi: 10.1111/mpp.12735.

*Volpe V, Chitarra W, Cascone P, Volpe MG, Bartolini P, Moneti G, Pieraccini G, Di Serio C, Maserti B, Guerrieri E, Balestrini R (2018). The association with two different arbuscular mycorrhyzal fungi affects water stress tolerance in tomato. *Front. Plant Sci.*, vol. 9, Article 1480, doi: 10.3389/fpls.2018.01480.

Table S2 Analysis of variance (ANOVA) outcomes of target genes, metabolites and nitrogen content in leaf and root tissues. Different letters within each column indicate significant differences according to Tukey HSD test ($P \leq 0.05$). Rootstock (R), Inducer (I) and Myc (M) main effects were compared using the Student's t-test ($P \leq 0.05$).

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Table S3 General feature from sequencing results of MiSeq Illumina using specific 16S or ITS primers together with PNA.

Label	Sample	Total Bases	Read Count	GC (%)	AT (%)	Q20 (%)	Q30 (%)
1	C-1103_1	92.526.196	307.396	54,21	45,79	91,14	82,57
2	C-1103_2	82.972.456	275.656	55,94	44,06	90,24	81,25
3	C-1103_3	88.844.364	295.164	54,29	45,71	91,10	82,58
4	C-1103_4	89.865.958	298.558	54,68	45,32	91,49	82,98
5	C-SO4_1	88.074.406	292.606	54,77	45,23	91,37	82,75
6	C-SO4_2	80.221.316	266.516	54,35	45,65	91,34	82,77
7	C-SO4_3	86.717.498	288.098	54,69	45,31	91,49	83,02
8	C-SO4_4	89.400.010	297.010	54,58	45,42	91,64	83,25
9	M_1103_1	91.378.182	303.582	54,75	45,25	91,17	82,53
10	M_1103_2	96.838.924	321.724	54,97	45,03	90,73	81,91
11	M_1103_3	100.940.952	335.352	54,52	45,48	91,59	83,23
12	M_1103_4	93.794.610	311.610	54,79	45,21	91,35	82,84
13	M_SO4_1	91.224.672	303.072	52,94	47,06	91,83	83,57
14	M_SO4_2	100.704.366	334.566	53,75	46,25	91,40	82,96
15	M_SO4_3	85.050.560	282.560	54,38	45,62	91,29	82,72
16	M_SO4_4	106.910.384	355.184	54,57	45,43	91,29	82,82
17	M+I_1103_1	88.717.944	294.744	54,61	45,39	91,0	82,34
18	M+I_1103_2	74.859.904	248.704	54,53	45,47	90,48	81,53
19	M+I_1103_3	90.733.440	301.440	54,97	45,03	91,18	82,55
20	M+I_1103_4	92.878.366	308.566	54,60	45,40	91,26	82,73
21	M+I_SO4_1	94.303.902	313.302	55,12	44,88	90,79	82,09
22	M+I_SO4_2	87.233.412	289.812	53,97	46,03	91,47	83,03
23	M+I_SO4_3	88.372.396	293.596	54,53	45,47	91,73	83,43
24	M+I_SO4_4	97.129.088	322.688	54,60	45,40	91,73	83,53
25	I_1103_1	97.839.448	325.048	54,68	45,32	91,02	82,32
26	I_1103_2	89.237.470	296.470	54,79	45,21	90,75	81,96
27	I_1103_3	98.228.340	326.340	55,22	44,78	91,15	82,64
28	I_1103_4	97.925.534	325.334	54,73	45,27	91,56	83,18
29	I_SO4_1	90.788.222	301.622	54,23	45,77	92,07	83,93
30	I_SO4_2	88.157.482	292.882	53,88	46,12	91,32	82,85
31	I_SO4_3	84.787.486	281.686	53,91	46,09	91,23	82,79
32	I_SO4_4	93.466.520	310.520	54,49	45,51	91,23	82,80
1ITS	C-1103_1	108.871.700	361.700	46,87	53,13	88,95	80,89
2ITS	C-1103_2	96.846.750	321.750	47,79	52,21	87,88	79,48
3ITS	C-1103_3	109.028.220	362.220	46,67	53,33	88,73	80,91
4ITS	C-1103_4	95.314.660	316.660	46,80	53,20	89,55	81,81
5ITS	C-SO4_1	92.267.938	306.538	47,34	52,66	88,01	79,19
6ITS	C-SO4_2	96.267.024	319.824	46,97	53,03	87,99	79,73
8ITS	C-SO4_4	96.763.674	321.474	46,71	53,29	87,64	79,21
9ITS	M_1103_1	99.763.440	331.440	47,26	52,74	86,99	78,40
10ITS	M_1103_2	112.218.218	372.818	45,89	54,11	89,47	82,0
11ITS	M_1103_3	98.793.618	328.218	46,25	53,75	88,75	80,67
12ITS	M_1103_4	100.945.166	335.366	46,01	53,99	89,36	81,68
13ITS	M_SO4_1	101.018.610	335.610	47,19	52,81	89,21	81,30
14ITS	M_SO4_2	94.037.216	312.416	46,48	53,52	88,33	80,27
15ITS	M_SO4_3	110.278.574	366.374	45,33	54,67	89,51	81,82
16ITS	M_SO4_4	115.818.780	384.780	46,95	53,05	87,78	79,25
17ITS	M+I_1103_1	89.400.010	297.010	47,03	52,97	88,53	80,20

Table S4. Shannon index for bacterial (16S) communities sampled among the different treatments. Statistical ANOVA was conducted to detect significant differences, different letter in each row means significant differences according to Tukey's HSD test.

	Mean		SD	
C-1103	9,312	±	0,027	a
C-SO4	9,268	±	0,033	a
M-1103	9,284	±	0,243	a
M-SO4	9,446	±	0,087	a
M+I-1103	9,376	±	0,197	a
M+I-SO4	9,257	±	0,194	a
I-1103	9,263	±	0,188	a
I-SO4	9,573	±	0,167	b

Table S5. Shannon index for fungal (ITS) communities sampled among the different treatments. Statistical ANOVA was conducted to detect significant differences, different letter in each row means significant differences according to Tukey's HSD test.

	Mean		SD	
C-1103	4,151	±	0,807	a
C-SO4	3,854	±	0,879	a
M-1103	3,858	±	0,993	a
M-SO4	4,501	±	0,552	a
M+I-1103	4,532	±	0,456	a
M+I-SO4	4,492	±	1,147	a
I-1103	4,472	±	1,058	a
I-SO4	4,495	±	0,369	a

The following tables:

Table S6 Summary of bacterial and fungal communities composition among treatments.

Table S7 Statistical analysis of the bacterial community among the different treatments.

Table S8 Statistical analysis of the fungal community among the different treatments.

are available at:

https://link.springer.com/article/10.1007/s00374-021-01607-8#Sec21

CHAPTER 5

Novel sustainable strategies to control Plasmopara viticola in grapevine, unveil new insights on priming responses and arthropods ecology

Research Article

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CHAPTER 5

5. Novel sustainable strategies to control *Plasmopara viticola* in grapevine, unveil new insights on priming responses and arthropods ecology

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5.1 Abstract

BACKGROUND: Reduction of fungicides consumption in agriculture is globally recognized as a priority. Government authorities are fostering research to achieve a reduction of risks associated with conventional pesticides and promoting the development of sustainable alternatives. To address these issues, in the present study, alternative protocols for the control of downy mildew infection in grapevine were compared to the standard protocol. In the first protocol, only resistance inducers were used, composing a single formulation with Acibenzolar S-methyl, laminarin and disodium-phosphonate. The second and third protocols followed the standard protocol but substituting phosphonates with phosphorus pentoxide and *Ecklonia maxima* extract.

RESULTS: The results showed that at véraison downy mildew incidence and severity in all tested protocols were significantly reduced compared to non-treated controls on both canopy and bunches. Expression analysis of key genes involved in plant stress response, indicated that the two protocols for phosphites substitution induced a remodulation of salicylic acid and jasmonic acid, with positive impact on yields. Analysis of the first protocol revealed that the primed state induced a short delay in bunch ripening, with a shift of carbohydrate metabolism to boost the plant defences, involving an up-regulation of defence related-gene, SAR response and a decreased ROS detoxification. Additionally, analysis on the arthropods populations, in parallel to the positive results achieved using alternatives to conventional fungicides, were enriched by those showing the potential of naturally occurring predators of spider mites.

CONCLUSION: This study provides practical solutions to reduce environmental impact of treatments for the control downy mildew in viticulture.

Keywords: biological control, spider mites, resistance induction, phosphites, grapevine, environmental impact

5.2 Introduction

Development of agricultural practices that are more respectful for the environment, with reduced agrochemical needs is globally acknowledged as a priority in the next future (Siebrecht, 2020). In this line, the European Union (EU) is fostering the research and the transition toward a more sustainable agriculture through its common agricultural policy (CAP, https://ec.europa.eu/info/food-farming-fisheries/key-policies/common-agricultural-policy/cap-glance_en), especially considering the ongoing climate change (IPCC, 2019). The concept of agricultural sustainability still lacks a clear definition (Siebrecht, 2020; Velten et al., 2015): rising of major concerns in the agricultural context come from the necessity to develop new practices and technologies that in addition to meeting the social and economic goals (Velten et al., 2015), are not detrimental for the environment. They also have to reduce the risk for farmers and consumers, sustaining food production to meet the world demand and the safeguard of natural resources (Béné et al., 2019; Pretty, 2008). To achieve these goals, the European Union adopted a strictly regulating framework for the use of agrochemicals, marketability of plant protection products (PPPs) as well fixing residue levels in food and feed products (European Commission report 204/2020). Specifically, the adoption of the Directive 2009/128/EC also foster the implementation of Integrated Pest Management (IPM), encouraging the evaluation of all plant protection strategies and the adoption of the most appropriated agricultural practices to control infection of plant pathogens, minimizing pesticides consumptions together with the associated risks, even through the adoption of non-chemically synthesized and/or natural substances. Limits for agrochemicals and copper compounds use (Council Regulation (EC) No 834/2007 and Commission implementing regulation (EU) 2018/1981) are even more restrictive in case of organic farming and a revision of 2009/128/EC directive is also under evaluation given the limited effectiveness of the adopted measures (European Commission report 204/2020).

Grapevine (*Vitis vinifera*) is one of the most important crops in the EU with 3.4 millions of cultivated ha in 2019, accounting for the 50% of the grapevine cultivated area in the world (Faostat, http://www.fao.org/faostat/en/#data/QC). To meet a high-quality standard of wine and fruit products as well to prevent severe yield losses caused by pathogens spread, multiple pesticides applications are required across the growing season, making grapevine cultivation one of the crop systems with the higher agrochemicals demand and with a great environmental impact (Provost & Pedneault, 2016). Downy mildew, caused by the oomycete *Plasmopara viticola* (Berk. e Curt.) Berl. e De Toni, is native from North America where it is endemic in wild *Vitis* species (Gessler et al., 2011).

Downy mildew is one of the biggest threats for grapevine cultivation worldwide, including Europe and Italy, causing critical yield losses in the absence of appropriate controlling measures (Gessler et al., 2011; Rantsiou et al., 2020). Such treatments are mainly constituted by the use of synthetic fungicides and copper compounds, usually scheduled with the aid of meteorological predictions and decision support systems (DSS) (Gessler et al., 2011). Pesticide sells in the EU has been stable in the last 10 years, but fungicides applications are expected to increase in specific pathosystem, including downy mildew – grapevine, due to the ongoing climate change (Bois et al., 2017; M. Pugliese et al., 2011a). Since temperatures and carbon dioxide concentration greatly influence disease evolution, a study aimed at clarifying this relationship, showed that the rising of these variables determines an higher incidence of downy and powdery mildew (*Erysiphe necator*) in grapevine (M. Pugliese et al., 2011b). Even if agricultural practices and cultivation of resistant genotypes have been adopted to limit the spread of fungal pathogens, this context enlightens the pressing need for the development of alternative treatments, including biostimulants, elicitors and resistance inducers. This heterogenous group of products is mainly composed by natural and naturally derived compounds that modulates plant growth and defense responses (Jamiołkowska, 2020). Other ones are chemically synthetized and constitutes functional analogs of natural molecules. Acibenzolar-S methyl is a functional analogue of silicic acid (SA), discovered in 1990s, along with other inducers of the plant's Systemic Acquired Response (SAR). Compared to other related SAR inducers it showed a lower phytotoxicity and its impact on the environment is sensibly lower than many pesticides commonly adopted by farmers (Tripathi et al., 2010) (and references therein) . These characteristics have stimulated numerous studies on its applications, action mechanisms and diffusion on the agrochemicals market. Even if its mechanism of action is not yet fully understood, a study in tobacco plants hypothesized that Acibenzolar-S methyl is converted into Acibenzolar by SABP2 enzyme in cells cytoplasm, leading to a shift in redox potential and the consequent activation of transcription factors involved in defence signalling, like the well-studied SAR marker PR-1 (Tripathi et al., 2010).

Phytoalexins are a broad group of secondary metabolites with antimicrobial activity, accumulated soon after pathogen attacks. In grapevine they belong to the stilbene family, a group of phenylpropanoid molecules that includes resveratrol, viniferin and piceids (Ahuja et al., 2012; Schnee et al., 2008).

A growing attention in the last years has been posed on commercial formulations containing phosphites and organo-phosphonates, determining presence of phosphonic acid residuals in wines and horticultural products. Phosphonates $[PHO_3]^{2-}$ are the anions deriving from

phosphonic acid dissociation, however carbon-containing phosphonic acid compounds also falls under this nomenclature. The current residue definition for phosphonates in the EU is reported in the Regulation (EC) No 396/2005 as 'Fosetyl-Al (sum of fosetyl, phosphonic acid and their salts, expressed as fosetyl)'. Usually, they are commercialized in formulations as salt complexes of sodium and potassium representing a common choice in viticulture to control downy and powdery mildew spread, thanks to the great efficacy and their cheap prices.

Laminarin, a glucan derived from the brown algae *Laminaria digitata* has been shown to stimulate activation of defence mechanisms in numerous plants, including grapevine where its application showed to sensibly reduce botrytis (*Botrytis cinerea*) and downy mildew infection, inducing accumulation of SA, activation of stilbenoid patways, mitogen-activated protein kinase (MAPK) and PR proteins and of the oxidative burst (Aziz et al., 2003). Similarly to *Laminaria digitata*, another widely adopted brown algae extract is obtained from *Ecklonia maxima*, a species growing in west South African costs, that is rich in phytoregulators (Rengasamy et al., 2014; Stirk et al., 2014), secondary metabolites, sugar and polyphenols, improving plant nutrition and resilience to both biotic and abiotic stresses (Kocira et al., 2020).

The identification of alternatives to insecticides and acaricides to be used in vineyards is still a priority in the EU. Two main research lines have been followed, the first focused on the biological control of pests and the second devoted to biotechnics. Biological control strategies are inspired to mechanisms of natural control and they aim at exploiting the potential of natural resources such as predators, parasitoids and pathogens of arthropod pests. Several interesting case-studies in viticulture are reported (Carlo Duso et al., 2012; Furlan et al., 2021; Pertot et al., 2017) and those related to the control of spider mites are probably the most successful (e.g., Duso et al., 2012). Considering this, observations on a number of arthropod species occurring on the selected treatments were carried out with special emphasis on relationships between phytophagous mites and their natural enemies.

The present study aimed at investigating the efficacy of newly alternative and environmentally sustainable protocols for the control of downy mildew infection in grapevine, including treatments without phosphonates (A and B) or mainly based on resistance inducers (M), comparing their effectiveness with the standard protocol adopted by a commercial winery (ST) and a non-treated control group (CTRL).

5.3 Materials and Methods

Experimental design, sampling and vineyard location

The experiment was repeated for three years, between April and July of 2019, 2020 and 2021 in open field condition, in a vineyard located in Loncon, Venice area, Veneto, Italy. The vineyard was cultivated with Glera varieties grafted on Kober 5BB rootstock.
In the experiment three alternative protocols for the control of downy mildew diffusion were compared to the standard treatment protocol adopted by the winery "ST" and to a non-treated control group "CTRL". The "M" protocol consisted of resistance inducers only mixed in a single formulation following the standard winery schedule of treatments. The "A" and "B" protocols followed the standard winery protocol but substituting phosphonates, using a combination of two different dosages and application schedule.
Given the similar results obtained in 2019 and 2020 seasons by A and B protocol, only protocol B has been evaluated in the season 2021. Moreover since 2021 was characterized by a more severe pathogen pressure, to avoid the complete loss of the CTRL plants and the risk of compromising the production also for the following years, chemical treatment has been applied as reported in Supplementary Table 1.
For each protocol three replicates with 100 plants have been used (300 plants in total), adopting a randomised block design. Position of the blocks changed every year from 2019 to 2021 to avoid blocks overlapping across years. An outline of the adopted protocols is reported in Supplementary Table 1, the experimental products being tested were applied according to the guidelines EPPO/OEPP PP 1/31. Along the vegetative season, concomitant to downy mildew symptoms evaluation, three samples were collected for each protocol, one sample constituting a biological replicate. For each sample, pools of 5 fully developed leaves were collected, freeze-dried and stored at -20°C degrees for further analysis.

Yields, musts and SPAD evaluations

At ripening (BBCH stage 89)(Lorenz et al., 1995) bunches from single plants were collected, the total weight and number of bunches were recorded. Ten bunches for each protocol were then randomly selected and squeezed manually for musts evaluation. Total soluble solids concentration (°Brix) was determined with the aid of a refractometer while pH was measured using a pHmeter. Titratable acidity was measured by titration as described in OIV-MA-AS313-01 method and expressed as g L^{-1} of tartaric acid.

The chlorophyll content measure was conducted with a Minolta SPAD 502 (Chiyoda, Tokyo, Japan) portable spectrophotometer. Measures were taken at véraison (BBCH 81)(Lorenz et al., 1995), selecting randomly 33 leaves at a similar development stage for each treatment.

Evaluation of downy mildew symptoms

Visual scouting of downy mildew symptoms on leaves and bunches were conducted across the vegetative season (BBCH stages from 12-13 to 81) to evaluate the treatments' effectiveness. The disease indexes (incidence and severity) scoring were conducted using the Grape Assess Mobile App (University of Adelaide) using the downy mildew custom assessment for leaf and berries, the raw data are reported in the Supplementary Table 2. Incidence represents the ratio between symptomatic observations over the total number of observed leaves or bunches, while severity represents the infected surface area as percentage. For each condition three biological independent group of observations on leaves and three independent group of observations on bunches were made. Each biological replicate (both for leaves and bunches) is made by at least 60 observations (in total at least 180 observations for each treatments on leaves and 180 observations on bunches).

Sample processing for targeted biochemical and molecular analyses

For the quantification of stilbenoid compounds in leaves (piceid, trans-resveratrol and viniferin), about 100 mg of freeze-dried sample (leaf) were used. The quantification was made by external calibration method as previously reported using an Agilent 1220 Infinity LC system HPLC-DAD apparatus (Agilent R, Waldbronn, Germany) (Mannino et al., 2020; Pagliarani et al., 2020). The analysis was conducted adopting three biological replicates for each protocol; data are expressed as μg g^{-1} of dry mass.
A selected set of target genes involved in stress-related defence and signalling pathways were analysed to deepen our understanding of the main molecular responses induced by the applied protocols. Total RNA was isolated from the same leaf samples used for HPLC-DAD analysis using the Spectrum Plant Total RNA Kit (Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions. The RNA quantity was checked using a NanoDrop one spectrophotometer (Thermo Fisher Scientific). Before the retrotranscription, RNA samples were treated with DNase I (Invitrogen, Thermo Fisher Scientific Waltham, MA, USA) and absence of genomic DNA contamination was checked before cDNA synthesis by qPCR using *VvUBI* primers. Retrotranscription and quantitative Real-Time PCR (qPCR) reactions were carried out as previously reported using iTaq universal SYBR Green super-mix (Bio-

Rad)(Pagliarani et al., 2020). Thermal cycling conditions were as follows: an initial denaturation phase at 95 ◦C for 3 mins, followed by 40 cycles at 95 ◦C for 10 s and 60 ◦C for 30s. Melting curves were run at the end of amplification cycles. Melting curves for each gene have been added as Supplementary Figure 1. The geometric mean of the expression ratios of two housekeeping genes (actin and cytochrome oxidase, *VvACT* and *VvCOX*) was used for expression level normalization ($2^{-\Delta\Delta Ct}$)[25]. The list of primers used in the present work is available in Supplementary Table 3.

Arthropods occurring on different treatments

Arthropods occurring on the four treatments were sampled from mid-July to early September 2020. In early July the winery applied an insecticide (tau-fluvalinate) against *Scaphoideus titanus* Ball, the most important vector of the phytoplasma agent of Flavescence dorée. Protocol M was not treated with this insecticide, and this allowed to compare arthropod diversity and abundance on insecticide treated and untreated plots. Arthropod diversity and abundance were estimated by removing 30 leaves per replicate from 20 vines (4 replicates per treatment, 120 leaves in total form 80 plants). Leaf samples were transported to the laboratory where arthropods were identified under a dissecting microscope.

Meteorological data

Meteorological data including precipitations, relative humidity and temperature were collected by the meteorological station located in the vineyard. Leaf wetness data for the year 2020 were obtained by the meteorological station of the regional agency for environmental protection (ARPA) located in Loncon, Venice area, Veneto, Italy (longitude: 12.280561808984 latitude: 45.952328733587).

Statistical analysis

Statistical analysis has been performed with a one-way analysis of variance (ANOVA) in R ver. 4.0.5 to analyse the treatments effects on disease indexes, must characteristics (TSS, titratable acidity, pH), foliar stilbenes content, expression levels of target genes and on the area under disease pressure curve (AUDPC). For significative F-test ($p\text{-value}<0.05$), multiple comparisons between means were conducted using Tukey's honestly significant difference (HSD) post-hoc test, with an alpha=0.05. Differences in the averages of productivity data (yield, number of bunches per plant and average bunch weight) and SPAD measurements

were evaluated using Welch's ANOVA followed by Games-Howell post hoc test with an alpha=0.05.

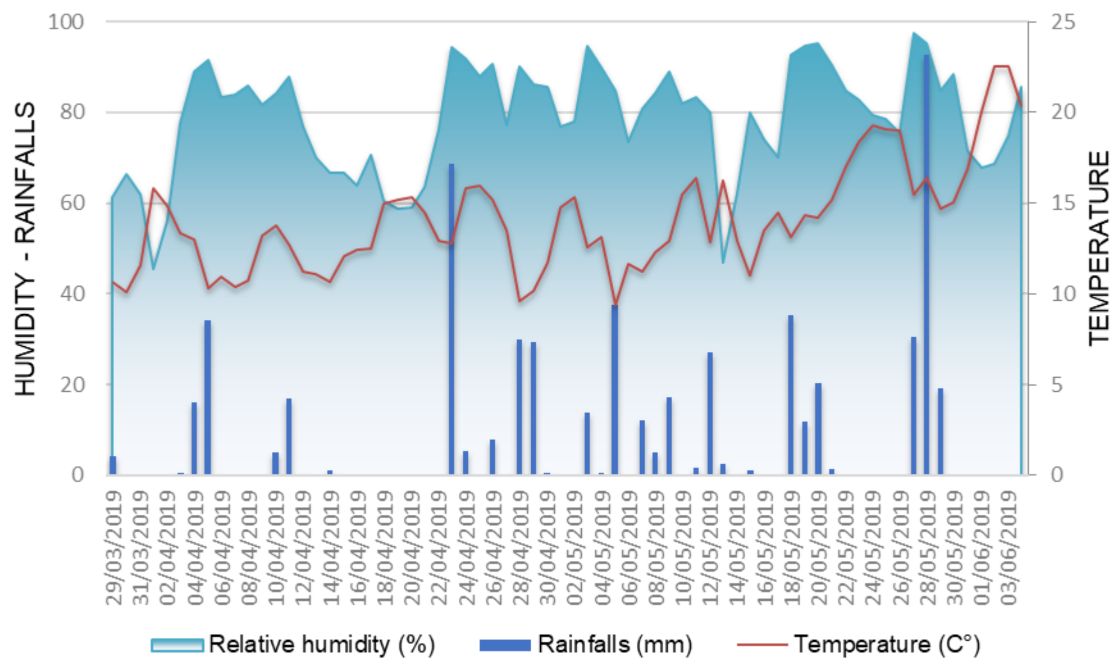
The effects of treatment (i.e., insecticide application) on the abundance of the most common arthropods (spider mites, predatory mites, predatory insects) detected on the grapevine canopy have been analyzed with a repeated measures linear mixed model with the MIXED procedure of SAS® (ver. 9.3; SAS Institute Inc., Cary, NC). In the analysis, the effect of treatment, time of sampling, and their interaction were considered as sources of variation and tested with an F test ($\alpha = 0.05$). Comparisons between vineyard management on each date were performed using Tukey's test ($\alpha = 0.05$) on the least-square means. The degrees of freedom were estimated with Kenward-Roger method. Before the analysis, data were checked for model assumptions, and arthropod numbers were transformed to log (n+1). Untransformed data are shown in the figures.

5.4 Results

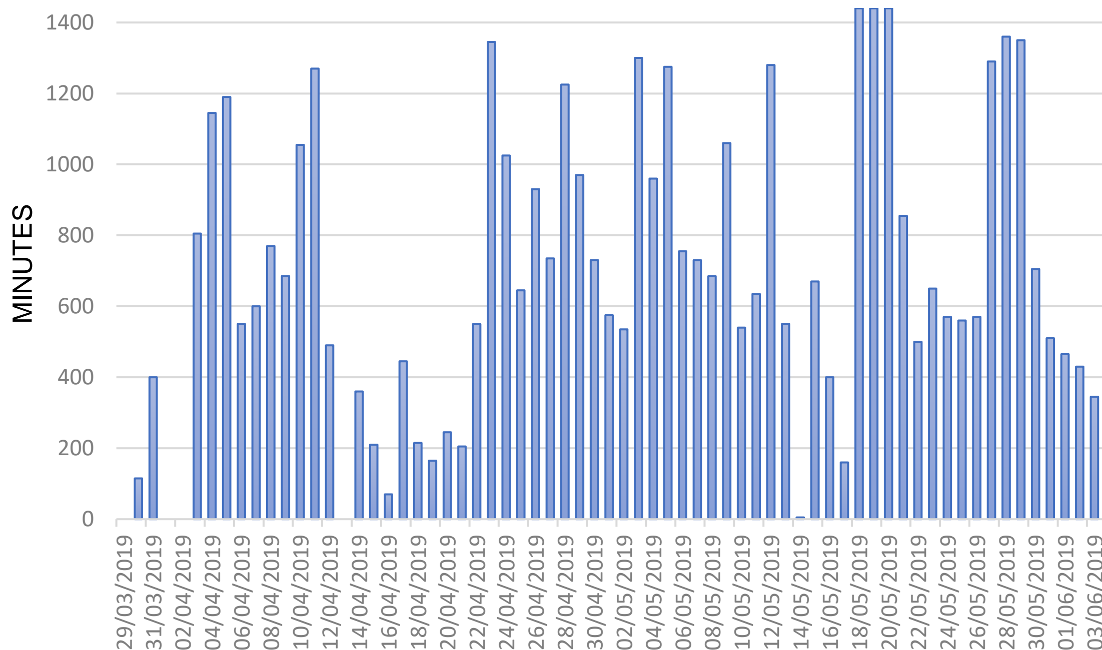
Disease indexes

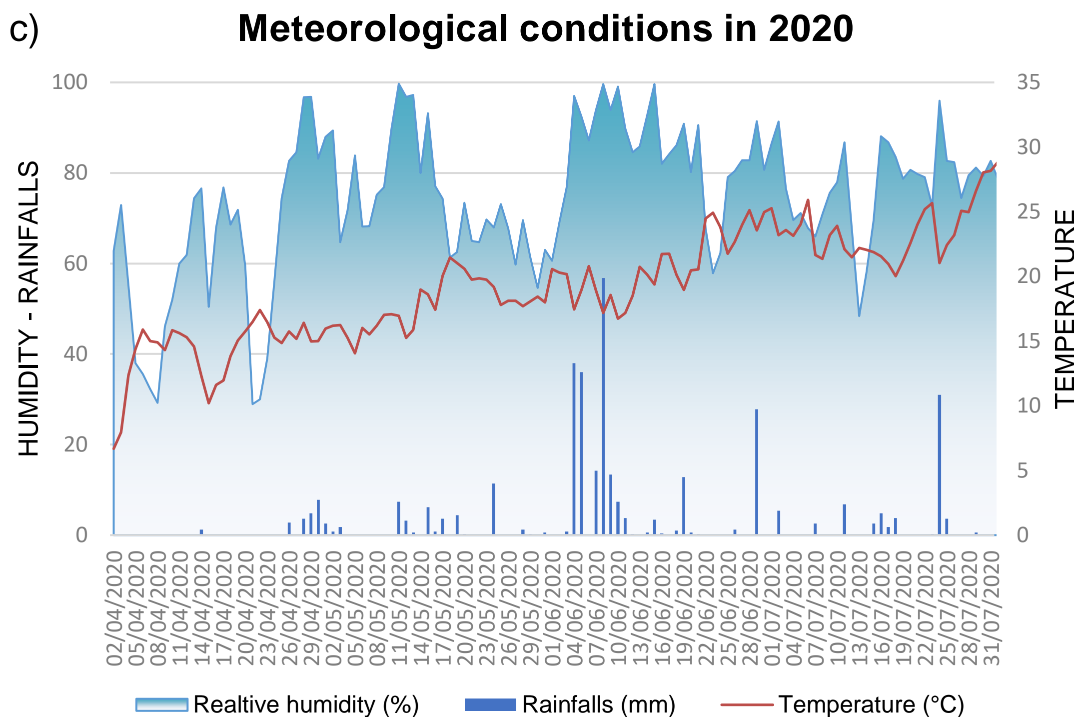
In 2019 it was possible to observe 2nd and 3rd leaves unfolded (BBCH stages 12-13) on 20th of April(Lorenz et al., 1995). However, given the abundant rainfalls of May summing up to 329.8 mm, and cold temperatures (fig.1), early flowering (BBCH stages 63, on more than 50% of the plants) was observed only on the 6th of June. In the days between 18th-31st May, an average temperature of 16.2°C with concomitant high humidity and long leaf wetness time were recorded (fig.1).

a)



b)





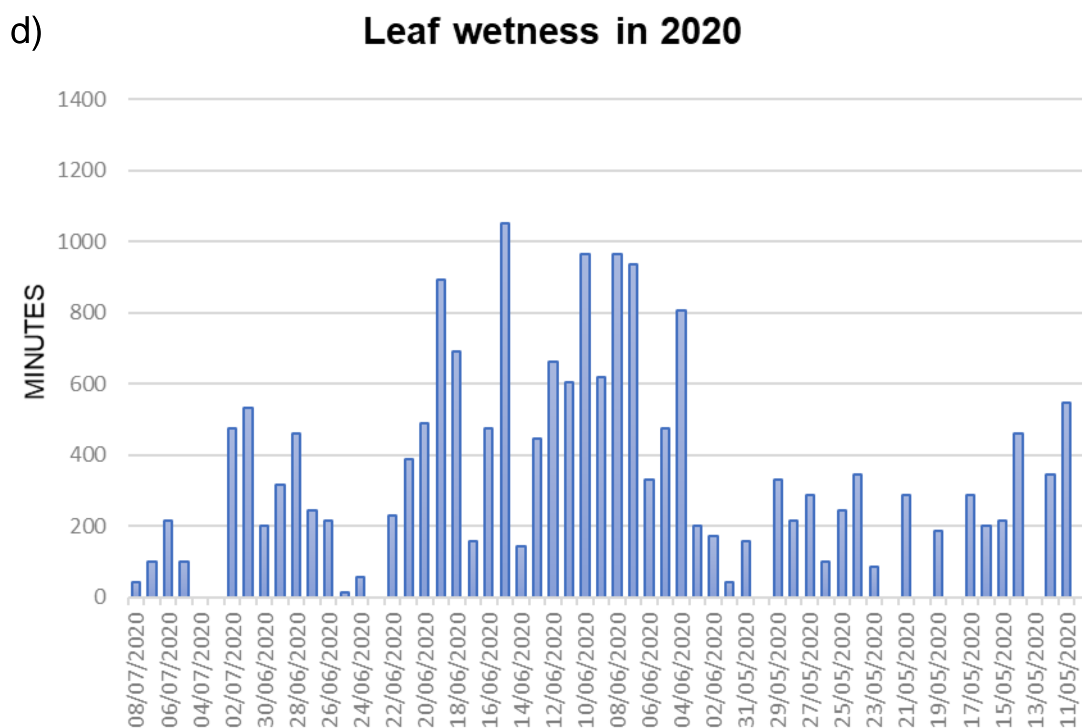


Figure 1. Meteorological conditions during 2019 and 2020 vegetative seasons. **a** Relative humidity (%, left scale), rainfalls (mm, left scale) and temperature (C°, right scale) in 2019. **b** Leaf wetness (min) in 2019. **c** Relative humidity (%, left scale), rainfalls (mm, left scale) and temperature (C°, right scale) in 2020. **b** Leaf wetness (min) in 2020.

Primary infection manifested on the 30th of May on the leaves of non-treated control group (CTRL) with an incidence of 25% and 1% severity, while no symptoms were observed on the plants of the remaining treatments (fig.2 a-c). Later in the season (June 17th) symptoms also appeared on bunches of M treatment, with an incidence 22.7% and 2.7% severity. On the 8th of July disease indexes showed a slight increase of symptoms for CTRL group and M protocol, in the latter case however the treatment was able to limit the disease to an incidence of 1% on the leaves and 29.1% on bunches.

In 2020, May was much dryer compared to 2019, with only 45 mm of rain. Across the period spanning from the 10th of May to the beginning of July the most abundant precipitations were concentrated between the 4th and 11th of June for a total of 169 mm of rain. At the same time, the averages of temperature and relative humidity were of 18°C and 94% respectively (fig.1), with a leaf wetness that went from 56% on the 4th of June to the 67% on 8th and 10th, with an average of 50% (fig.1). During the growing season of 2020, elongated shoots, and clearly visible inflorescences (BBCH stages 16 and 53), were already observable on the 23rd of April while end of flowering and fruit setting (BBCH 68-69-71) were reached on the 29th of May. The first symptoms of downy mildew infection were observed on the 12th of June (BBCH stage 75), on CTRL, with only trace presence (less than 1% of observations with symptoms) of primary infections. All the protocols under evaluation were able to greatly contain downy mildew diffusion, in contrast, the CTRL plants displayed a growing trend in both incidence and severity of *P. viticola* infections (fig.2 b-d). Downy mildew symptoms on bunches were observed only on the CTRL group with only trace presence (less than 1% of observations with symptoms) of the pathogen until the 1st of July, while the incidence increased rapidly up to the 60% on the 10th of July with a severity of 8%, significantly higher than treated plants. At véraison, on the 16th of July, the CTRL group showed an incidence slightly above 80% and a severity of 37% on canopies, significantly different from all the other protocols. Similar results were obtained for protocols A and M that showed an incidence of 17% and 13% and disease severity of 1% and 1.5% on leaves. Infection of berries compromised almost the totality of the harvest for CTRL group with 99% of bunches affected and roughly 47% of severity while no symptomatic evidence was reported for the other protocols.

Since protocols A and B performed similarly in 2019 and 2020, we decided to repeat for the third year the field trial only with protocol B because it requires less input with respect to protocol A (Supplementary Table 1) according also to the winery management strategy. In 2021, the third year in which the study has been repeated, precipitations were of 179 mm in May with an average temperature of 15,4 °C and an average humidity of 80% (Supplementary

Figure 2), setting the ideal condition for the pathogen spread. The primary infections manifested the 27th of May on CTRL plants with single spots affecting leaves in less the 1% of the plants. Even if June 2021 was characterised by the almost complete absence of precipitations, humidity remained high, with an average of 72% and a warm temperature of 23°C, promoting a rapid increase of downy mildew symptoms on the canopy: 4 days after the incidence on the canopy of CTRL plants was of 18,3% with a severity of 0,8% (Supplementary Figure 3). On the 10th of June these values raised to 76,6% and 7,5% respectively, while the first symptoms appeared also on bunches, confirming for the 3rd year a high disease pressure in the area. Incidence and severity for the canopy of CTRL plants reached 85% and 7,7% on the 16th of June, at this point considering that the ongoing season was the last relative to the present study to avoid the risk of complete loss of the plants and the compromission of the yield for the next seasons, the CTRL plants were treated with fungicides as reported in Supplementary Table 1. Even if symptoms were present on plants treated with the M protocol, a maximum incidence of 25% on the canopy and 34,4% on bunches was recorded. In addition, the disease severity was significantly lower than that of CTRL for the whole season (Supplementary Figure 3).

For a better representation of the treatments effect on disease development the area under disease progress curve (AUDPC) has been evaluated and the results are presented in Supplementary Table 4.

Evaluation of stilbenes accumulation in leaf

In order to evaluate the biochemical response to *P.viticola* infection and application of resistance inducers, three major stilbenoids were quantified in leaves by high pressure liquid chromatography (HPLC-DAD). The results for the years 2019 and 2020 are represented in (fig.3) and reported as the average ± standard error. In 2019 on the 16th of May, in the first phases of the growing season, piceide levels varied from 4.33±0.11 μg g^{-1} for CTRL, to the 52.39±0.05 μg g^{-1} for ST treatment (fig.3 a). The M treatment was significantly different from any other protocol showing a significantly higher accumulation. The great differences in piceid levels that were present at the beginning of the season reduced with time toward véraison, where treatments split between two statically different groups, the first formed by CTRL and M with a higher content and the second with lower content including ST, B and A treatments. While an increase of resveratrol was initially observed for all the treatments, in the period going from 31st of May till véraison on 17th of June, its content remained stable for A, B and M protocols (fig.3 c). Resveratrol content increased over time for the ST protocol reaching

138.42±10 μg g^{-1} at véraison, the highest level among treatments. In 2019 an initial increase in viniferin content was observed for CTRL, B and M. For the CTRL group the levels of viniferin was significantly higher than the other protocols for all the season (fig.3 e). A decrease in viniferin content was observed at véraison for A, B and M treatments, while its levels remained stable for ST treatment across the whole season.

In 2020, Piceide levels, despite diffused fluctuations, had an overall increase advancing with the growing season (fig.3 b). At véraison the CTRL group reported sensibly higher levels of this secondary metabolite compared to the other protocols. The largest significative differences in resveratrol levels were observed on the 22nd of June where the ST protocol showed the lowest content with 5.1±0.81 μg g^{-1} followed by protocols M and A, while the highest values, significantly different, were observed for B and CTRL, slightly above 26 μg g^{-1} (fig.3 d). At véraison, resveratrol content of CTRL group was the highest, statistically different from plants treated with protocol A, but not from the remaining protocols. Variability of viniferin content, among treatments, was lower compared to piceide and resveratrol (fig.3 f). The ST protocol kept the average viniferin content under 2 μg g^{-1} for the whole season, while at véraison protocols A, B and M had a slightly higher viniferin content compared to the 0.9±0.14 μg g^{-1} of ST treatment. The CTRL group, severely affected by downy mildew, presented the highest viniferin content of 9.4±0.3 μg g^{-1}.

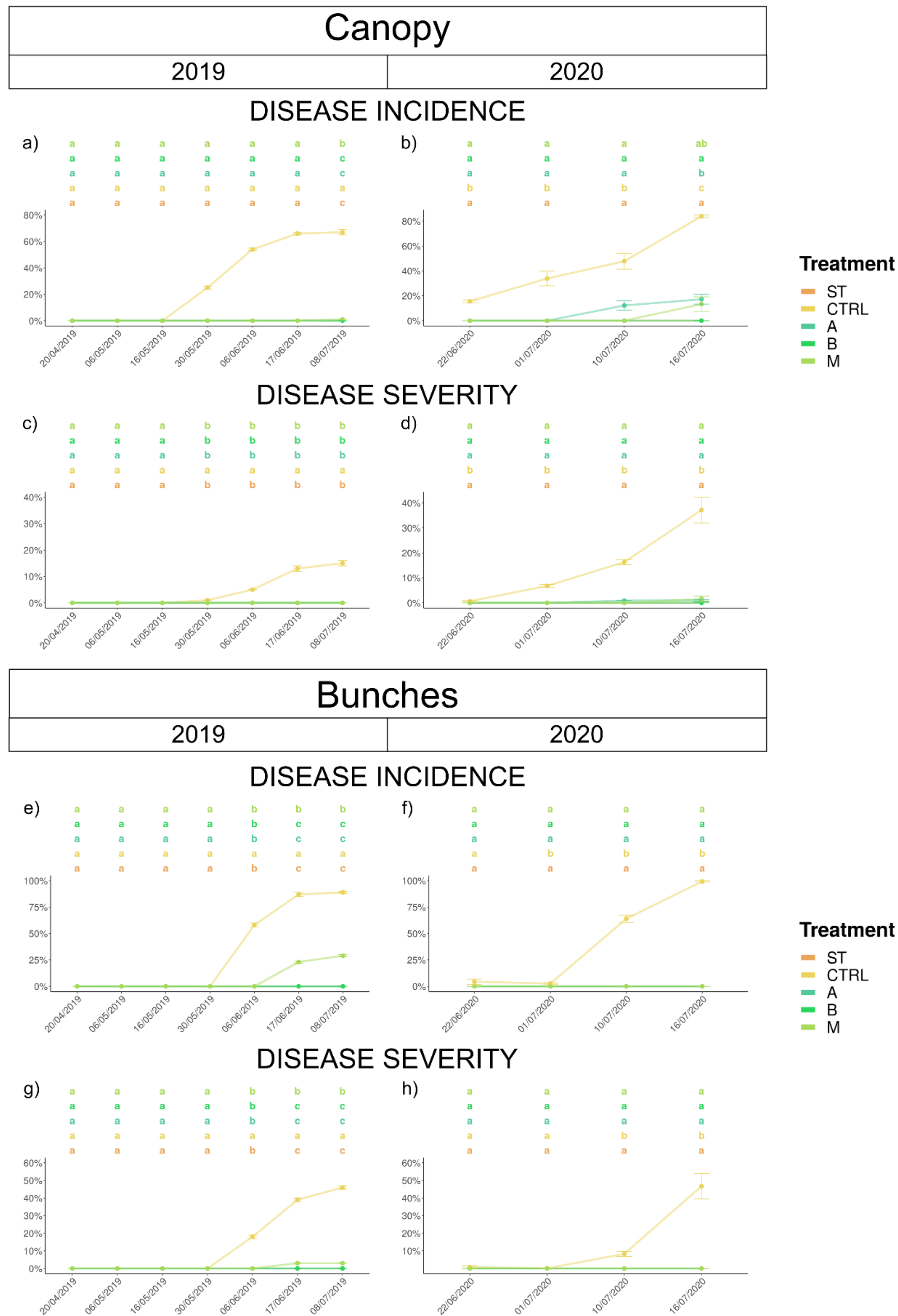


Figure 2. Disease indexes trends in 2019 and 2020, incidence represents the ratio between symptomatic observations over the total number of observed leaves or bunches, while severity represents the infected surface area as percentage. For each treatment, the average of three biological replicates ± standard error is reported. Different letters indicate that treatments are significantly different for Tukey's HSD test $p<0.05$. **a-b** Disease incidence on canopy in 2019 and 2020. **c-d** Disease severity on canopy in 2019 and 2020. **e-f** Disease incidence on bunches in 2019 and 2020. **c-d** Disease severity on bunches in 2019 and 2020.

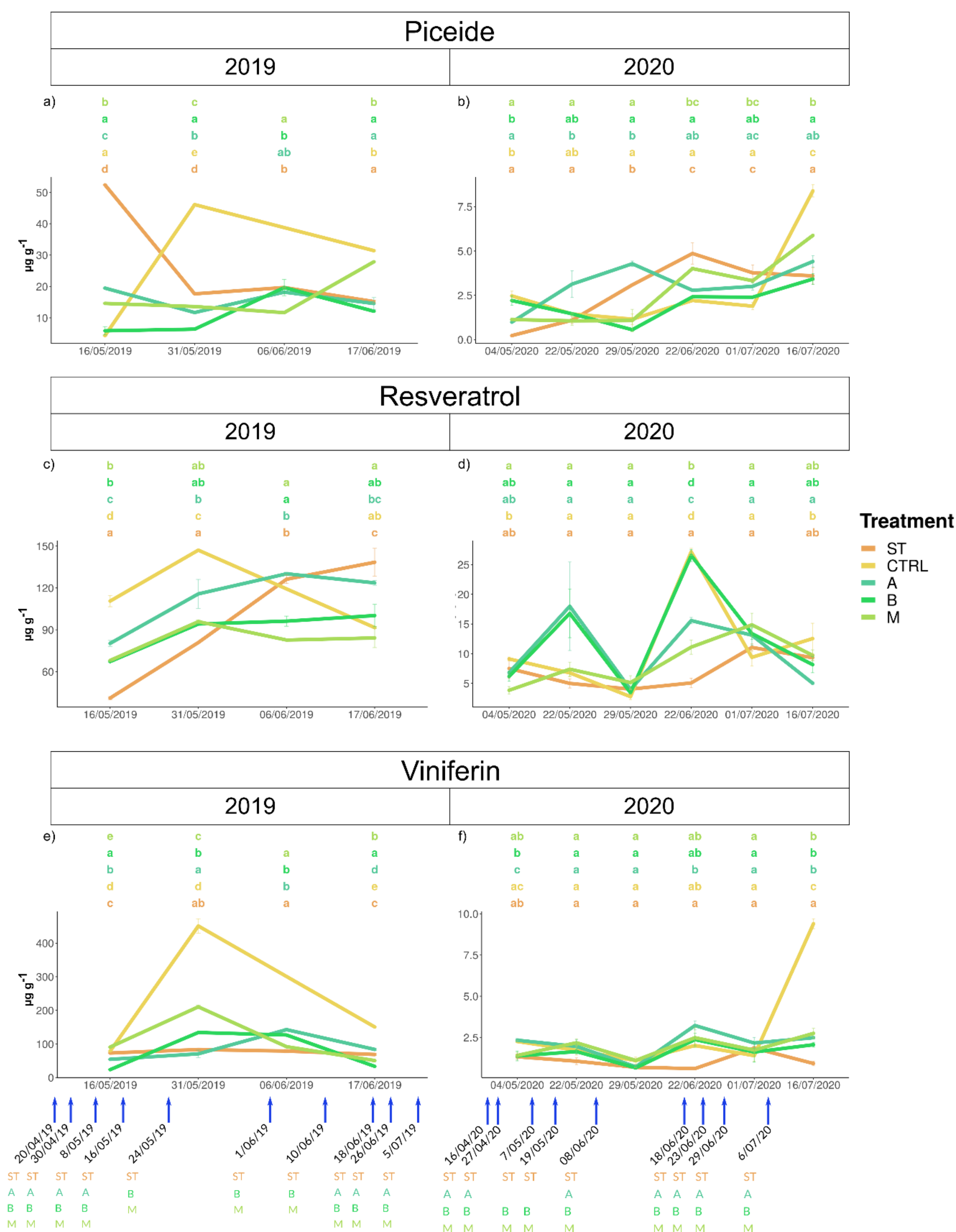


Figure 3. Trends of stilbenes content in leaves during the vegetative seasons of 2019 and 2020. The adopted treatments schedules are reported with blue arrows at the bottom of the figure indicating the application date for each protocol. For each treatment, the average of three biological replicates ± standard error is reported. Different letters indicate that treatments are significantly different for Tukey's HSD test $p<0.05$. **a-b** Foliar content of piceide in 2019 and 2020. **c-d** Foliar content of resveratrol in 2019 and 2020. **e-f** Foliar content of viniferin in 2019 and 2020.

Expression levels of target genes involved in stress and defence responses

To shed light on the grapevine response mechanisms taking place under the different assayed conditions, we studied the expression profiles of key genes involved in SAR, Induced Systemic Response (ISR), Pattern triggered immunity (PTI), synthesis of stilbenoid, Reactive Oxigen Species (ROS) detoxification, phenylpropanoid pathway and biosynthesis of Abscisic acid (ABA). Expression of such genes was evaluated for samples of 2020 before the onset of downy mildew primary infections (May 29th - T1), after primary infections manifestation (June 22nd - T2) and at véraison when the pathogen pressure was at the highest level (T3) (fig.4). Expression levels of the ST treatment at T1 were used as reference. To better understand the treatment's effects on the activation of SAR, the *VvNPR1* gene was considered (fig.4 a). Expression of *VvNPR1* one of the main regulators of SAR and ISR, is sensibly upregulated by ST protocol at T2, with a 3-fold increase compared to the remaining treatments and CTRL. At T3 an increase of expression levels for CTRL, A, B and M was observed. No differences between the treatments were found for *VvEFR1*, a gene involved in PTI, its levels although increase at T3 compared to reference (fig.4 c). Activation of ISR signal cascade was assayed following expression levels of lipoxygenase *VvLOX*, a gene catalysing the synthesis of jasmonate precursor. Before the spread of primary infections, *VvLOX* was initially downregulated in all the treatments compared to the reference and it remained low during T2 for all protocols, its levels increased in T3 for ST, B and A (fig.4 d). Stilbene synthase *VvSTS* expression was consistently higher during T3 with respect to T1 and T2. CTRL and M showed close expression levels significantly upregulated compared to the other protocols, at a level of more than 25 times the reference (fig.4 b). *VvNCED* gene expression (fig.4 e), catalysing the first step of ABA biosynthetic pathway, was studied to evaluate if major effects on ABA stress related responses were present. The reference level represented the highest expression level for this gene, while the remaining treatments at T1 as well as for all the treatments at T2 and T3 were sensibly downregulated. ROS detoxification activity was studied looking at the expression levels of ascorbate peroxidase *VvAPX2* and monodehydroascorbate reductase *VvMDHAR*. At T1 *VvAPX2* was upregulated in the M group, its expression decreased at T2 for the M treatment, resulting not different from ST and B whereas it was upregulated in CTRL and for a lesser extent in the A protocol. At T3 the M group showed the lowest expression level although not significantly different from A, that in turn presented a minor expression of this gene compared to ST and B (fig.5 a). While *VvMDHAR* expression did not presented a high variability between T1 and T2, its level had a slight increase at T3 for ST, CTRL and B while the expression decreased for the M treatment resulting significantly

different from the CTRL group (fig.5 b). Phenylalanine amino lyase, *VvPAL*, the enzyme catalysing the first reaction of the phenyl propanoind biosynthetic pathway, interestingly showed its highest expression level for the M treatment at T3 with a relative expression above 2.5, significantly higher compared to the other protocols (fig.5 c).

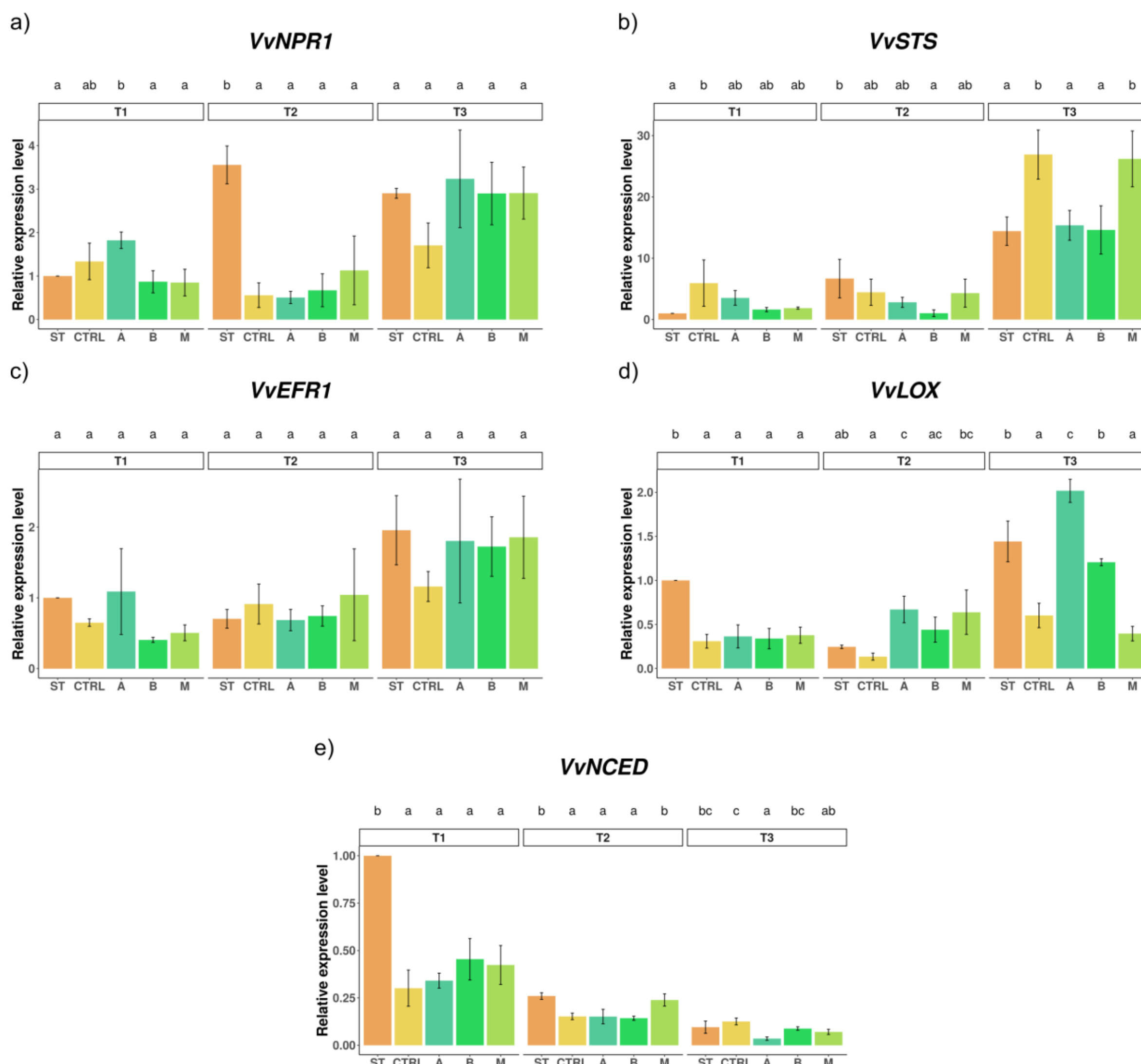


Figure 4. Relative expression of candidate genes involved in grapevine stress and defence responses before downy mildew primary infections (**T1**), after primary infections manifestation (**T2**) and during véraison at the highest pathogen pressure (**T3**). For each treatment, the average of three biological replicates ± standard deviation is reported. Different letters indicate that treatments are significantly different for Tukey's HSD test $p<0.05$. Expression levels at T1 for the ST treatment were set as reference. **a** Non-Expressor of PR genes 1, *VvNPR1*; **b** Stilbene synthase, *VvSTS*; **c** LRR receptor-like serine/threonine-protein kinase EFR, *VvEFR1*; **d** Lipoxygenase *VvLOX*; **e** 9-cis-epoxycarotenoid dioxygenase, *VvNCED*.

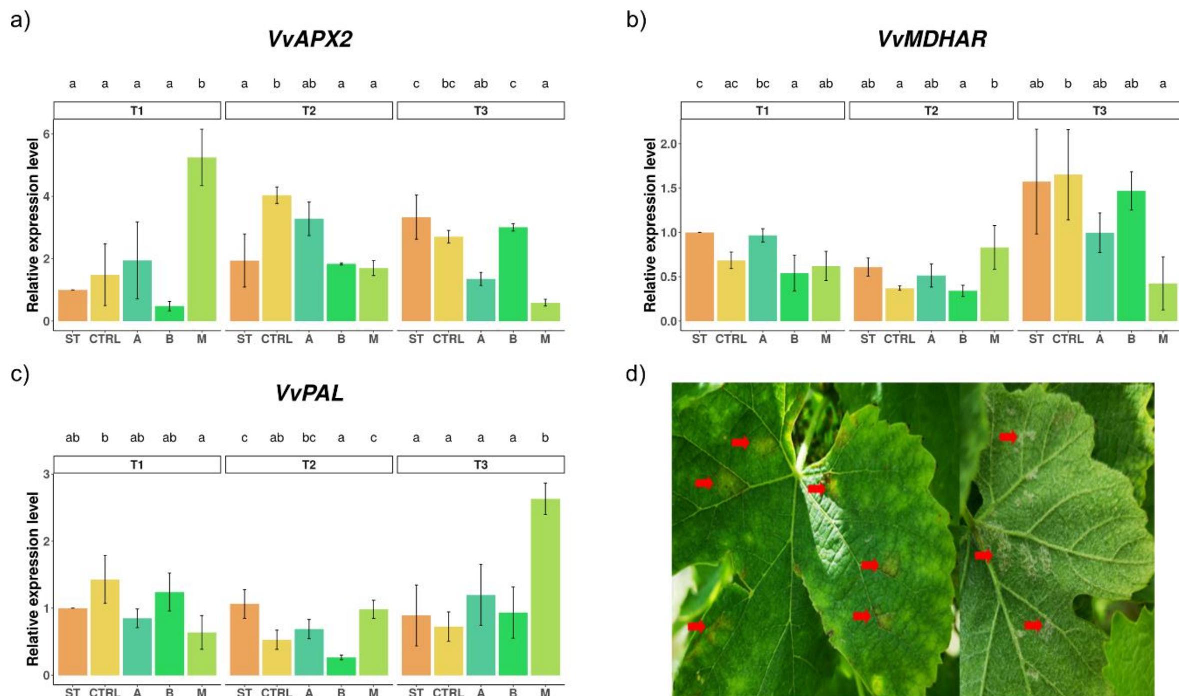


Figure 5. Relative expression of candidate genes involved in grapevine stress and defence responses before downy mildew primary infections (**T1**), after primary infections manifestation (**T2**) and during véraison at the highest pathogen pressure (**T3**). For each treatment, the average of three biological replicates ± standard deviation is reported. Different letters indicate that treatments are significantly different for Tukey's HSD test p<0.05. Expression levels at T1 for the ST treatment were set as reference. **a** Ascorbate peroxidase, *VvAPX2*; **b** Monodehydroascorbate reductase, *VvMDHAR*; **c** Phenylalanine amino lyase, *VvPAL*; **d** lignin deposition observable on leaves of M treatment.

Yield and SPAD analyses

While in 2019 was not possible to evaluate the productivity given the severe yield losses caused by adverse meteorological events occurred soon after véraison, in 2020 the analysis was conducted evaluating yield, number of bunches and the average bunch weight (Table 1). The yield of the CTRL group was greatly lower than the other protocols with an average of 3.5±0.46 Kg, p<0.05 for all the comparisons. The M protocol with 6.13±0.91 Kg was significantly different from all the other treatments while A and ST performed similarly with a yield exceeding 7 kg. The highest yield was obtained with treatment B 10.13±1.95 Kg, p<0.05 for all comparisons.

No differences among the treatments were observed for the bunches number (Kruskal-Wallis chi-squared = 8.8145, p-value = 0.06591) while an effect was present in case of the average weight of the bunch (Welch F test = 7.8E-05). The CTRL group with 338±197g was the

treatment with the lowest average bunch weight, significantly different from all the remaining treatments, while the highest value was observed for the B protocol with 578±107g. Between these values the A treatment with 544±124g was not statistically different from B, ST 475±120g and M 468±119g, whereas ST and M protocols had significantly lower values than B.

In 2021, ST and B protocols showed the highest yield with 5,13 ± 0,73 Kg and 4,45 ± 0,9 Kg, significantly different from the remaining protocols (Supplementary Table 5), while the lowest yield was observed for the CTRL plants. The M protocol, similarly to what observed in 2020, performed significantly better than CTRL with a yield of 3,12 ± 0,53 Kg being significantly different from both protocols ST and B. The lowest bunches number in 2021 (Supplementary Table 5), was observed for protocol M with an average of 11,25 ± 3,17 bunches per plant, not statistically different from CTRL, while the highest value of 17,67 ± 5,63 was obtained for the ST protocol. B plants had values comprised between M and ST, not statistically different from both these protocols. Concerning the average bunch weight, ST, B and M protocols performed similarly with significantly higher weight than CTRL bunches (Supplementary Table 5).

To estimate the leaf nitrogen content in 2020 the plants were evaluated using a SPAD chlorophyll meter, (Table 2). Statistical analysis indicated the presence of differences among protocols as indicated by the Welch ANOVA F statistics, equal to 31.1 with p-value of 1.63e-15. The highest value was observed for the B treatment with 38.96 ± 2.95, that according to Games Howell test is not statistically different from ST and A. The M treatment was significantly lower than B and ST while the CTRL group had the lowest value, significantly different from the other treatments.

In 2021 the leaf chlorophyll estimation (Supplementary Table 6) revealed the highest value for CTRL plants with 42,37 ± 3,34, followed by ST (38,80 ± 2,96) and B (36,96 ± 2,84) treatments, both resulting statistically different from CTRL. The M protocol showed the lowest value of 32,91 ± 2,90 statistically different from the other protocols.

Protocol	Yield (Kg)	Bunches number	Average bunch weight (Kg)
ST	7.19 ± 1.58 c	16.70 ± 7.23 a	0.475 ± 0.120 b
CTRL	3.51 ± 0.46 a	13.13 ± 6.21 a	0.339 ± 0.197 a
A	7.71 ± 1.59 c	15.21 ± 6.23 a	0.544 ± 0.124 bc
B	10.13 ± 1.95 d	18.27 ± 5.47 a	0.579 ± 0.108 c
M	6.13 ± 0.91 b	14.20 ± 5.05 a	0.468 ± 0.119 b

Table 1 Production indexes analysis for the year 2020: yield (Kg), number of bunches per plant and average bunch weight (Kg). Data are reported as average of three biological replicates ± standard deviation. Different letters correspond to treatments that differ for Tukey's HSD test for $p < 0.05$.

Protocol	**Mean** (n=33)
ST	38.09 ± 2.75 c
CTRL	27.82 ± 5.05 a
A	37.04 ± 2.79 bc
B	38.69 ± 2.95 c
M	36.08 ± 2.97 b

Table 2 SPAD analysis of leaf chlorophyll content in 2020. For each treatment, the average of three biological replicates ± standard deviation is reported, different letters indicate that treatments are significantly different for Games-Howell test for $p < 0.05$.

Musts evaluation

The treatments effects on musts biochemical characteristics were studied analysing the total soluble solids content (TSS), titratable acidity, pH, malic and tartaric acid content (fig.6 and Table 3). The highest TSS value 16.8 ± 0.62 % was observed for CTRL group, statistically different from the M treatment with 15 ± 0.46 % that showed the lowest value among all treatments while ST, B and A values resulted not different from both CTRL and M (fig.6 a). The M protocol showed the highest content of total acids with 6.4 ± 0.1 mg L^{-1} expressed as tartaric acid equivalents, not significantly different from ST, A and CTRL while the B treatment had the lowest content of total acids with 5.2 ± 0.4 mg L^{-1} , statistically different from ST and M but not from A and CTRL (fig.6 b). Only minor variability was observed in pH values that ranged from 3.22 ± 0.015 for ST to 3.36 ± 0.055 for B protocol, although a significant difference was found between the B protocol, and both CTRL and ST (fig.6 c).

Malic and tartaric acid concentrations were then quantified by HPLC. The CTRL group showed the lowest content of malic acid of 2.27 ± 0.23 mg L^{-1} not significantly different from A and B protocols but significantly lower than ST and M, that had the highest concentration of 3.34 ± 0.13 mg L^{-1} (Table 3). At the same time the CTRL group had the highest content of tartaric acid with 5.3 ± 0.14 mg L^{-1}, statistically different from all the remaining treatments that ranged from 4.52 ± 0.26 mg L^{-1} for ST to 4.77 ± 0.01 mg L^{-1} for the M protocol.

Results of musts characteristics showed, in 2021 (Supplementary Figure 4), a similar trend to 2020, even if no significant differences were observed for TSS, the M protocol showed, as previously described for 2020, the lowest sugar content and the highest TA, significantly different if compared to the remaining protocols. No differences were observed in pH values. As per musts evaluation of 2020, also for 2021 must of M protocol showed the highest value of malic acid as reported in Table 3.

Protocol	**Malic acid mg L-1**								**Tartaric acid mg L-1**							
	2020				2021				2020				2021			
ST	2.99	±	0 .14	bc	6,45	±	0,21	a	4 .52	±	0 .27	a	7,43	±	0,20	a
CTRL	2.28	±	0.24	a	6,68	±	1,05	a	5.30	±	0.15	b	7,81	±	0,29	ab
A	2.52	±	0.34	ab	\	±			4.61	±	0.14	a	\	±		
B	2.63	±	0.24	ab	6,17	±	0,56	a	4.63	±	0.15	a	7,46	±	0,29	a
M	3.35	±	0.14	c	9,57	±	0,76	b	4.77	±	0.02	a	8,28	±	0,10	b

Table 3 Determination of malic and tartaric acid concentrations in musts. For each treatment, the average of three biological replicates ± standard deviation (mg L^{-1}) is reported. Different letters indicate that treatments are significantly different for Tuckey's HSD test $p< 0.05$.

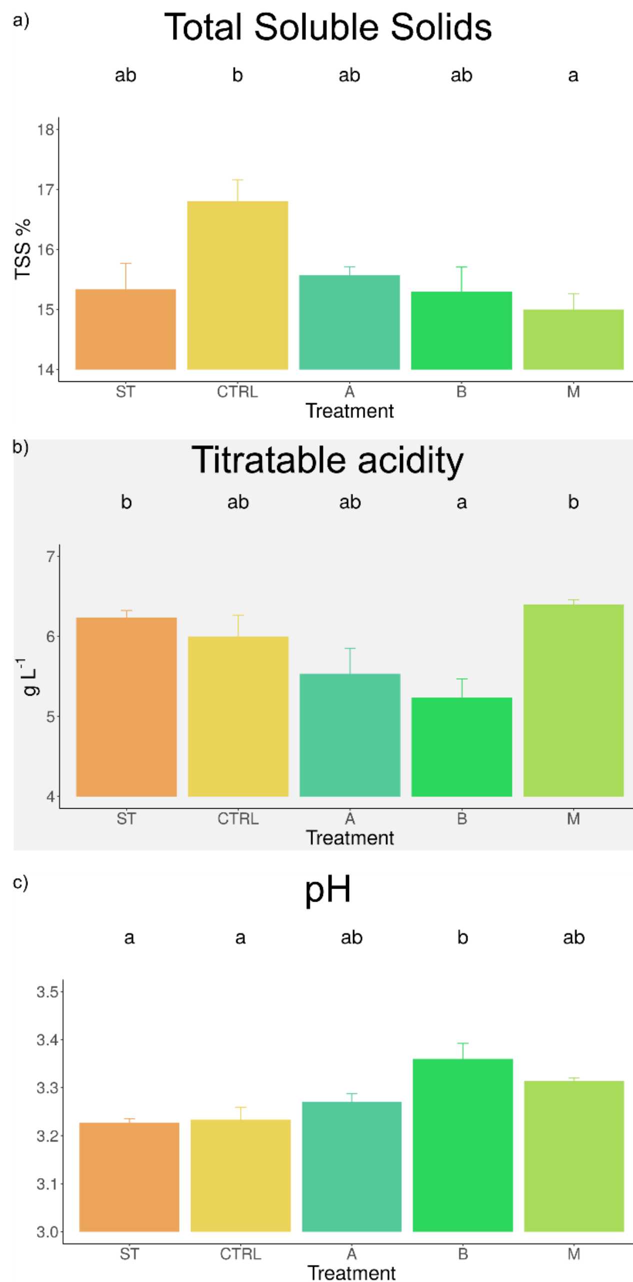


Figure 6. Musts analysis. **a** Total soluble solids (°Brix); **b** Titratable acidity expressed as mg L^{-1} of tartaric acid; **c** pH. For each treatment, the average of three biological replicates ± standard error is reported. Different letters indicate that treatments are significantly different for Tukey's HSD test $p<0.05$.

Arthropods occurring on experimental plots

In insecticide treated plots, predatory mite (mainly *Phytoseius finitimus* Ribaga) densities declined at negligible levels while they remained at moderate levels on treatment M ($F = 137.59$; $DF = 3, 19{,}6$; $p< 0.0001$), fig.7). At the same time, population densities of the red spider mite *Panonychus ulmi* (Koch) increased on insecticide treated plots while they occurred at negligible levels on treatment M ($F = 147.18$; $DF = 3, 60$; $p< 0.0001$; fig.7). These spider mite densities attracted a number of predators, in particular the phytoseiid mite *Amblyseius andersoni* (Chant) and the coccinellid *Stethorus punctillum* (Weise) from outside. They preyed actively upon spider mites reducing their levels in insecticide treated plots. Obviously, densities of these predators

reached higher levels on insecticide treated plots compared to treatment M (F = 8.09; DF = 3, 60; p = 0.0001; F = 5.86; g. d. l. = 3, 60; p = 0.0014 for *A. andersoni* and *S. punctillum*, respectively; fig.7).

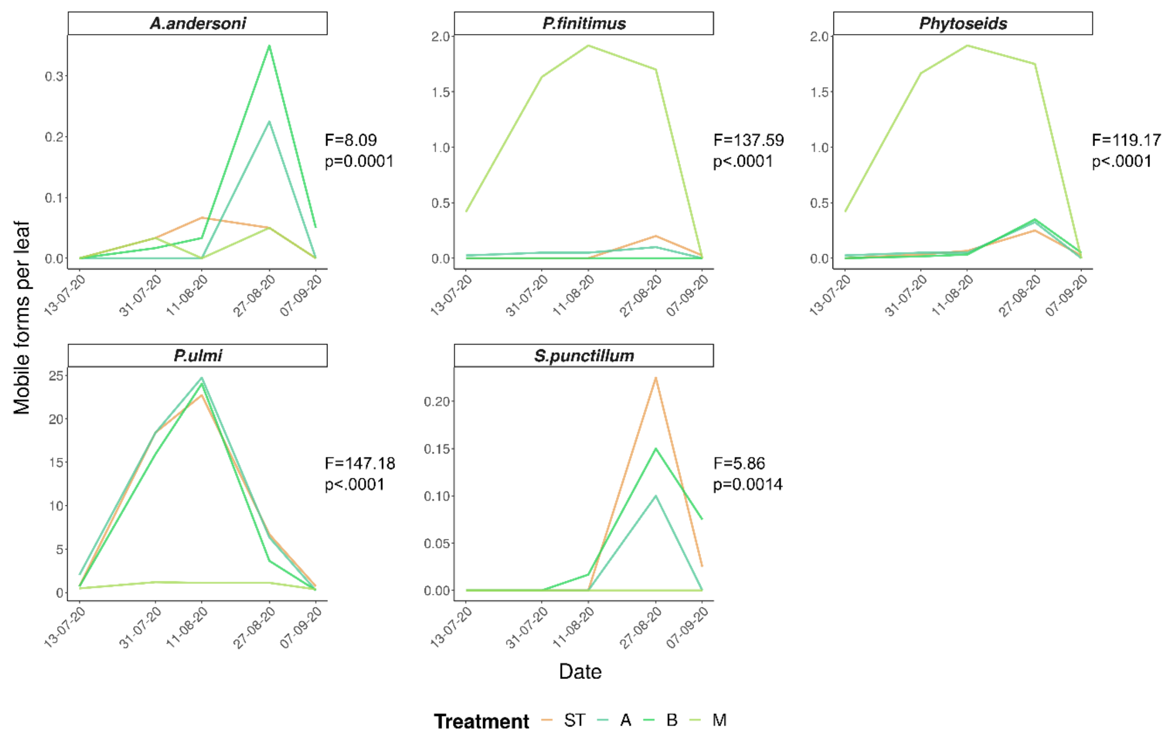


Figure 7. Arthropods occurring on the four treatments from mid-July to early September 2020. Mean number of *A.andersoni*, *P.finitimus*, *Phytoseids*, *P.ulmi* and *S.punctillum* observed on leaves collected in the vineyard.

5.5 Discussion and conclusions

Among the three experimental seasons, 2019, 2020 and 2021, extremely high disease pressure was observed on non-treated control. In parallel, no disease symptoms were observed on the plants of ST and B protocol. Although the treatment A did not present any symptoms on leaves and berries in 2019, in 2020 at véraison, an incidence of 17% with 1% severity were observed on canopy, while the bunches remained free of symptoms and no differences were observed on the productivity compared to the ST protocol. Notably in 2020, a higher yield was observed for protocol B, significantly different from the remaining treatments, together with a higher average bunch weight that was matched only by protocol A. These results confirm the positive effects of *E. maxima* extract on plant growth and productivity as already demonstrated in previous studies (Kocira et al., 2020; Rengasamy et al., 2014). Interestingly in

2020 the application of laminarin, acibenzolar S-methyl and disodium phosphonate in a single formulation (protocol M) showed no difference from the ST protocol for disease incidence and severity, with comparable productivity for the bunches number per plant and for the average bunch weight. In 2021 the M protocol confirmed the great capability of containing *P.viticola* growth and diffusion offering a sustainable alternative to the conventional protocol.
Notably, this is to our knowledge, the first report of the simultaneous application in open field condition of these plant protection products, which showed a remarkably increased efficacy compared to the results obtained by previous studies on downy and powdery mildew where the same resistance inducers have been tested singularly (Massimo Pugliese et al., 2018; Rantsiou et al., 2020; Romanazzi et al., 2016).
The analysis of real time qPCR data showed that the ST treatment led to an early upregulation of *VvNPR1* suggesting that the effect of the protocol on downy mildew could be also partially related to a quick activation of the SAR-related responses. Two of the considered genes, *VvPAL* and *VvSTS*, concern the biosynthesis of phytoalexins. Lignin, flavonoids and stilbenes are among the secondary metabolites involved in structural and defence functions that in plants are produced through the phenylpropanoid pathway, whose first reaction is catalysed by phenylalanine ammonia lyase *VvPAL* (Zhao et al., 2021). In grapevine a very large family of stilbene synthase has been reported (Parage et al., 2012), these enzymes are involved in the biosynthesis of stilbenes, a small class of phenylpropanoids including resveratrol, piceide and viniferin which are accumulated at the infection site to restrict pathogen diffusion (Ciaffi et al., 2019; Schnee et al., 2008) but also in response to a broad range of stimuli and stress conditions (Vannozzi et al., 2012). Moreover, synthesis of stilbenoids is one of the key measures, among the biochemical responses evolved by grapevine to counteract fungal infections (Alonso-Villaverde et al., 2011). In our experimental conditions, the higher stilbenes levels observed in 2019 could be explained by the higher rainfalls observed in this year, that registered 329.8 mm of rain in May, compared to only 45mm in 2020 in the same period. Indeed, several studies demonstrated that the concentration of these molecules is highly dependent from climatic conditions, plant genotype and disease pressure, showing that longer periods with high humidity and precipitations foster the accumulation of this secondary metabolites (Guerrero et al., 2020; Viret et al., 2018).
At véraison, concomitantly with an increased pressure of downy mildew, while *VvPAL* was overexpressed only in the protocol M, expression of stilbene synthase (*VvSTS*) increased for all the protocols, with the CTRL group and M treatment showing significantly higher accumulation than ST, B and A protocols. Transcription levels of *VvSTS* well explain the

higher piceide and viniferin concentration observed in the CTRL group, while for the M treatment only piceide accumulation resulted statistically different compared to ST and B. The M protocol however, despite presenting similar expression of *VvSTS* to CTRL, manifested only minor symptoms on the plants.

At the same timepoint, an opposite expression pattern was observed for *VvLOX* with respect to *VvSTS*: lipoxigenase catalyse the formation of fatty acid hydroperoxides, the first step of several fatty acid signalling pathways including jasmonate (Podolyan et al., 2010), and was significantly more expressed in the A treatment, followed by ST and B, while being less induced in M and CTRL. It has been previously shown in resistant genotypes a participation of jasmonic acid in the plant pathogen interaction (Figueiredo et al., 2015; Guerreiro et al., 2016) and our results show that ST, A and B protocols may stimulate this signalling pathway, especially at higher dosage of *E. maxima* extract. Since synergistic or antagonistic effects of the salicilic acid and jasmonic acid interaction seems dependent from their relative abundance (Guerreiro et al., 2016), the lack of an early activation of *VvNPR1* for A and B protocols, but observed for ST, could be explained by the activation of JA pathway, as demonstrated by an higher *VvLOX* expression at T2 compared to ST, even if not significant in case of B treated plants that had a lower dose of the biostimulant application.

The M protocol, in addition, showed a reduced expression of *VvAPX2* and *VvMDHAR* two enzymes involved ROS detoxification, indicating that accumulation of these molecules could have a major role in this treatment as demonstrated for laminarin and phosphonic acid in previous studies (Dann & McLeod, 2021; Gauthier et al., 2014; Tripathi et al., 2019). Laminarin application, indeed, has been shown to induce PAL and LOX activation, ROS burst, SA accumulation, phytoalexins synthesis and expression of specific PR genes (Aziz et al., 2003; Gauthier et al., 2014; Tripathi et al., 2019). Phosphonic acid stimulates plant immunity through a complex activation of several hormone pathways, including ethylene, salicylic and jasmonic acid, inducing ROS accumulation, deposition of callose, and production of secondary metabolites (Dann & McLeod, 2021) (and references therein).

Furthermore, the accumulation of H_2O_2 could be involved in the plant defence response against *P. viticola* as already reported in previous studies (Cruz-Silva et al., 2021; Xiang et al., 2016). More in detail, it was demonstrated that, specific downy mildew effectors (e.g. *PvRxLR28*) are involved in the regulation of ROS, impairing H_2O_2 accumulation and reducing the transcriptional levels of defence-related genes, and allowing an easier accommodation of the pathogens (Cruz-Silva et al., 2021). In our experimental set-up we observed a downregulation of genes involved in H_2O_2 detoxification (i.e. *VvAPX2* and *VvMDHAR*)

(Fimognari et al., 2020; Yildiztugay et al., 2020), suggesting a possible accumulation of this molecule which could be at the basis of the hypersensitive response observed (Hernández et al., 2016; Zechmann, 2020). Additionally, the overexpression of *VvPAL*, which is related to lignin biosynthesis, is another possible molecular response triggered by the elicitor application which could explain the impairment of *P. viticola* (Dai et al., 1995). All together these data could also explain the necrosis observed in the M protocols, which could be the result of both hypersensitive response and lignin deposition (fig.5 d).

It has been reported that acibenzolar S-methyl stimulate the plant defences inducing SAR downstream salicylic acid accumulation (Tripathi et al., 2019) (and references therein), moreover new evidences suggest that long term responses involve an extensive reprogramming of photosynthesis and carbohydrate metabolism, allocating more resources toward the defence metabolism with a modification of the growth-defence trade-off (Pagliarani et al., 2020). This hypothesis is supported by our observations, indeed even if no differences were observed for the bunch number and the average bunch weight, the yield obtained for the M treatment resulted significantly lower than ST, A and B.

The analysis of leaf chlorophyll content has been used in grapevine for the estimation of nitrogen content in leaves (Casanova-Gascón et al., 2018) (and references therein), however it also offer an estimate of the efficiency of the photosynthetic apparatus and pigmentation changes determined by biotic and abiotic stresses. SPAD measures of CTRL plants reported the lowest values in 2020, statistically different from all the remaining treatments, thus, given the high incidence of the pathogen on this treatment, is reasonable to hypothesize a severe impairment of the photosynthetic apparatus caused by the pathogen growth. In 2021 CTRL plants were treated with fungicides, this allowed to contain the symptoms progression on the canopy to a severity below 10%, preserving at least in part the photosynthetic apparatus as evidenced by the highest values. This result, in contrast to what observed in 2020, is due to the fact that, SPAD data were recorded a month after the application of the antifungal compounds that allowed plants to recovery and produce new leaves. Otherwise, the difference observed for the M protocol, that in 2020 had values significantly higher than CTRL but either in 2020 and 2021 showed a lower chlorophyll content than B and ST protocols offer new insights on the extent of the metabolic shift determined by resistance inducers, supporting our observations on protocols productivity.

The high values observed in the must of CTRL plants for sugars (soluble solids) and tartaric acid accumulated in berries during ripening are well explained by the severe yield loss determined by the pathogen spread on bunches with the effect of an increased flux of these

metabolites for the remaining bunches that act as sinks. The reprogramming of carbohydrate metabolism (trade-off) in the M protocol, is also supported by a significantly higher content of malic acid, both in 2020 and 2021, an intermediary product synthesized in the Krebs cycle(Sweetman et al., 2009),. Such increased level, could be indeed the symptom, of a slight delay in the ripening process, due to a reduced activity of the malic enzyme, responsible of malic acid degradation and reduction of acidity the in berry (Ruffner et al., 1984).

Overall, these results evidence that in a standard winery protocol for the control of downy mildew, the substitution of phosphites with a formulation of *E. maxima* extract and phosphoric pentoxide determines a new balance between SA and JA defence responses, with positive impact on yields and reduced risks for consumers. The application of a resistance inducers formulation containing acibenzolar S-methyl, laminarin and disodium phosphonate in a single formulation, suggests that pathogen infection is limited by priming the plant defence through the up-regulation of stilbenes related enzymes, activation of SAR response and decreased ROS detoxification allowing lignin deposition and hypersensitive like cell death responses on pathogen infection.

Observations carried out on arthropods occurring on the four treatments confirmed: i) the risks for natural antagonists associated to pesticide use, and ii) the potential of biocontrol agents in controlling spider mites. A single application of a pyrethroid promoted a spider mite outbreak due to its negative side-effects on predatory mites. This phenomenon, well-known for a number of pyrethroids synthetized in the 1970s, was also noticed for tau-fluvalinate but at higher doses than those used in viticulture (Carlo Duso et al., 2014). More studies are needed to confirm the importance of this finding. On the other hand, data suggest an effective role of *Ph. finitimus* in controlling spider mites as seen in treatment M. These observations are particularly interesting as the role of this species as biocontrol agent is poorly known despite its large distribution in the Mediterranean Basin (Ahmad et al., 2015; C Duso & Vettorazzo, 1999). The high populations densities of spider mites attracted naturally occurring predators. The response by *A. andersoni* and *S. punctillum* reduced spider mite numbers in a few weeks. This is another interesting example of natural regulation of arthropod populations associated to vineyards. More interestingly, these species are commercially reared and thus can used to manage spider mites representing an alternative to acaricide use.

In summary, the results obtained in the present work show that is possible to reduce phosphites and organo-phosphonate residuals in grapes and wines substituting these family of molecules in a standard protocol for the control of downy mildew with an algal derived biostimulant containing *E. maxima* extracts and phosphoric pentoxide. Moreover, in the light

of an increasing agricultural sustainability and of the restrictive regulation framework adopted by the European Union, the protocol M, composed mainly of resistance inducers, was remarkably efficient in the control downy mildew diffusion, representing a promising alternative to traditionally adopted strategies with higher environmental impact.

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Conflict of interest

The authors declare that they have no competing interests.

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5.7 Supplementary files

Supplementary Table 1

List of the protocols tested in the present study for 2019, 2020 and 2021 seasons

Protocol	Commercial product	Producer	Active ingredients		Dosage	Treatment schedule		
						2019	2020	2021
ST	ISONET® L PLUS	CBC	(E,E)-7,9-DODECADIEN-1-YL ACETATE	72%	500 diffusers ha^{-1}, 213 mg of active ingredients per diffuser	20/04/2019	06/04/2020	15/04/2021
			(Z)-9-DODECEN-1-YL ACETATE	8,40%				
	QUARTET	SYNGENTA	DISODIUM PHOSPHONATE	36,50%	1 L ha^{-1}	30/04/2019	27/04/2020	29/04/2021
	TIOVIT JET	SYNGENTA	SULPHUR	80%	2.8 Kg ha^{-1}			
	KARATHANE STAR	CORTEVA AGRISCIENCE	MEPTYLDINOCAP	35,71%	0.5L ha^{-1}	08/05/2019	07/05/2020	06/05/2021
	QUARTET	SYNGENTA	DISODIUM PHOSPHONATE	36,50%	1.5L ha^{-1}			
	POLYRAM DF	BASF	METIRAM	70%	2 Kg ha^{-1}			
	TIOGOLD DISPERSS	UPL	SULPHUR	80%	3 Kg ha^{-1}	16/05/2019	19/05/2020	11/05/2021
	QUADRIS	SYNGENTA	AZOXYSTROBIN	22,70%	1 L ha^{-1}			
	FLARE GOLD R WG	SYNGENTA	COPPER	14,19%	4.5 Kg ha^{-1}			
			METALAXYL-M	2%				
	QUARTET	SYNGENTA	DISODIUM PHOSPHONATE	36,50%	2 L ha^{-1}			
	ZORVEC VINABEL	DU PONT DE NEMOURS	ZOXAMIDE	30%	0.5 L ha^{-1}	24/05/2019	29/05/2020	16/05/2021
			OXATHIAPIPROLIN	4%				
	BOGARD	SYNGENTA	DIFENOCONAZOLE	23,60%	0.2 L ha^{-1}			
	POLYRAM DF	BASF	METIRAM	70%	2 Kg ha^{-1}	01/06/2019	08/06/2020	21/05/2021
	SILBOT 500 SC	ADAMA	DIMETHOMORPH	44%	0.5 L ha^{-1}			
	CUPROTEK DISPERSS	UPL	COPPER	20%	3 Kg ha^{-1}			
	TIOGOLD DISPERSS	UPL	SULPHUR	80%	2.3 Kg ha^{-1}	10/06/2019	18/06/2020	28/05/2021
	QUADRIS	SYNGENTA	AZOXYSTROBIN	22,70%	1 L ha^{-1}			
	SILBOT 500 SC	ADAMA	DIMETHOMORPH	44%	0.5 L ha^{-1}			
	QUARTET	SYNGENTA	DISODIUM PHOSPHONATE	36,50%	2.3 L ha^{-1}			
	FOLVIT 80 WDG	ADAMA	FOLPET	80%	1.4 Kg ha^{-1}			
	EPIK SL	SIPCAM ITALIA	ACETAMIPRID	4,67%	1.4 L ha^{-1}	18/06/2019	23/06/2020	04/06/2021
	MOXYL 45 WG	UPL	CYMOXANIL	45%	0.23 Kg ha^{-1}			
	TIOGOLD DISPERSS	UPL	SULPHUR	80%	2.3 Kg ha^{-1}			
	VIVANDO	BASF	METRAFENONE	42,37%	0.02 L ha^{-1}			
	FLEXITY	BASF	METRAFENONE	42,37%	0.23 L ha^{-1}			
	POLTIGLIA DISPERSS	UPL	COPPER	20%	0.5 Kg ha^{-1}		29/06/2020	14/06/2021
	TIOGOLD DISPERSS	UPL	SULPHUR	80%	2.3 Kg ha^{-1}			
	CIDELY	SYNGENTA	CYFLUFENAMID	5,10%	0.5 L ha-1	26/06/2019	06/07/2020	21/06/2021
	AMPEXIO	SYNGENTA	ZOXAMIDE	24%	0.5 Kg ha-1			
			MANDIPROPAMID	25%				
	COPPERLAND NEW	PHOENIX-DEL	COPPER	20%	1 Kg ha-1	05/07/2019	16/07/2020	05/07/2021
	SILBOT R WG	ADAMA	COPPER	40%	3.4 Kg ha-1			
			DIMETHOMORPH	6%				
	TIOGOLD DISPERSS	UPL	SULPHUR	80%	4.5 kg ha-1			
	EVURE PRO	ADAMA	TAU-FLUVALINATE	21,40%	0.27 L ha-1			
	CIDELY	SYNGENTA	CYFLUFENAMID	5,10%	0.5 L ha-1			
	ROMEO	SUMITOMO	CEREVISANE	94,10%	0.1 Kg ha-1		23/07/2020	13/07/2021
	QUADRIS	SYNGENTA	AZOXYSTROBIN	22,70%	1 L ha-1			
	COPPERLAND	PHOENIX-DEL	COPPER	20%	2.85 Kg ha-1			

	NEW							
	MOXYL 45 WG	UPL	CYMOXANIL	45%	0.6 Kg ha-1		29/07/2020	21/07/2021
	CUPROTEK DISPERSS	UPL	COPPER	20%	3 Kg ha-1			
	TIOGOLD DISPERSS	UPL	SULPHUR	80%	3 Kg ha-1			
	CUPROTEK DISPERSS	UPL	COPPER	20%	3 Kg ha-1		07/08/2020	
	TIOGOLD DISPERSS	UPL	SULPHUR	80%	4.5 Kg ha-1			
	TIOVIT JET	SYNGENTA	SULPHUR	80%	4.5 Kg ha-1		14/08/2020	
CTRL	CIDELY	SYNGENTA	CYFLUFENAMID	5,10%	0.5 L ha-1			19/06/2021
	AMPEXIO	SYNGENTA	ZOXAMIDE MANDIPROPAMID	24% 25%	0.5 Kg ha-1			
	COPPERLAND NEW	PHOENIX-DEL	COPPER	20%	1 Kg ha-1			05/07/2021
	SILBOT R WG	ADAMA	COPPER DIMETHOMORPH	40% 6%	3.4 Kg ha-1			
	TIOGOLD DISPERSS	UPL	SULPHUR	80%	4.5 kg ha-1			
	EVURE PRO	ADAMA	TAU-FLUVALINATE	21,40%	0.27 L ha-1			
	CIDELY	SYNGENTA	CYFLUFENAMID	5,10%	0.5 L ha-1			
	ROMEO	SUMITOMO	CEREVISANE	94,10%	0.1 Kg ha-1			13/07/2021
	QUADRIS	SYNGENTA	AZOXYSTROBIN	22,70%	1 L ha-1			
	COPPERLAND NEW	PHOENIX-DEL	COPPER	20%	2.85 Kg ha-1			
	MOXYL 45 WG	UPL	CYMOXANIL	45%	0.6 Kg ha-1			21/07/2021
	CUPROTEK DISPERSS	UPL	COPPER	20%	3 Kg ha-1			
	TIOGOLD DISPERSS	UPL	SULPHUR	80%	3 Kg ha-1			
M	BION 50WG	SYNGENTA	Acibenzolar S-metyl	50%	200 g ha^{-1}	20/04/2019 30/04/2019 08/05/2019 16/05/2019 24/05/2019 01/06/2019 10/06/2019 18/06/2019 26/06/2019 05/07/2019	06/04/2020 27/04/2020 07/05/2020 19/05/2020 29/05/2020 08/06/2020 18/06/2020 23/06/2020 29/06/2020 06/07/2020 16/07/2020 23/07/2020 29/07/2020 07/08/2020 14/08/2020	29/04/2021 06/05/2021 11/05/2021 16/05/2021 21/05/2021 28/05/2021 04/06/2021 14/06/2021 21/06/2021 05/07/2021 13/07/2021 21/07/2021
	Vacciplant	LABORATOIRES GOËMAR	Laminarin	5%	2 L ha^{-1}			
	QUARTET	SYNGENTA	Disodium phosphonate	36,50%	2 L ha^{-1}			
A	As ST without phosphonates					20/04/2019 30/04/2019 08/05/2019 16/05/2019 18/06/2019 26/06/2019 05/07/2019	27/04/2020 29/05/2020 08/06/2020 18/06/2020 23/06/2020 29/06/2020 06/07/2020 16/07/2020	Protocol not tested in 2021
	Basfoliar	COMPO	*Ecklonia maxima;* Phosphorus pentoxide		3L ha^{-1}			
B	As ST without phosphonates					20/04/2019 30/04/2019 08/05/2019 16/05/2019 24/05/2019 01/06/2019 10/06/2019 18/06/2019 26/06/2019 05/07/2019	27/04/2020 07/05/2020 19/05/2020 29/05/2020 08/06/2020 18/06/2020 23/06/2020 29/06/2020 06/07/2020 16/07/2020	29/04/2021 06/05/2021 11/05/2021 16/05/2021 21/05/2021 28/05/2021 04/06/2021 14/06/2021 21/06/2021 05/07/2021 13/07/2021 21/07/2021
	Basfoliar	COMPO	*Ecklonia maxima;* Phosphorus pentoxide		1.5 L ha^{-1}			

Supplementary Table 2

Raw data used for disease index analyses, available at:

https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1002%2Fps.6860&file=ps6860-sup-0006-TableS2.xlsx

Supplementary Table 3

Primer list used in the present work.

Name	Primer sequence (5'-3')	Gene annotation or Target	Reference	Gene ID	Melting / Annealing	Amplicon length	Amplification efficiency
VvACT1_F	GCCCCTCGTCTGTGACAATG	Actin	1	VIT_04s0044g00580	66.0-65.1 / 60	98	0.92
VvACT1_R	CCTTGGCCGACCCACAATA						
VvAPX2_F	TTGGATTTGCTGATGCTGAGG	Ascorbate peroxidase	2	VIT_08s0040g03150	64.0-65.6 / 60	115	0.88
VvAPX2_F	ACCACAAACCCAAATCACCGA						
VvCOX_F	CGTCGCATTCCAGATTATCCA	Cyclooxygenase	3	VIT_03s0110g00190	63.2-64.7 / 60	107	0.94
VvCOX_R	CAACTACGGATATATAAGAGCCAAAACTG						
VvEFR1-like	TGCCTTACTCAGCGGGGATG	Ethylene response factor EFR1	This work	VIT_09s0002g00470	67.1-66.9 / 60	83	0.92
VvEFR1-like	ACTCAAACTGCAACGCTGGC						
VvLOX_F	CCATCTCTTGCACACTGGAA	Lipoxygenase	4	VIT_09s0002g01080	63.1-62.5 / 60	105	0.99
VvLOX_R	GCGAGCACAGAATTAATACGAC						
VvMDHAR_F	CACTGGTGTTGCTGCAGATACATTCA	Monodehydroascorbate reductase	2	VIT_08s0007g03610	67.3 64.9 / 60	99	0.90
VvMDHAR_R	AAGTCTTGCAGCTCCCTCAG						
VvNCED3_F	GCCCCAACCCCCAGTTC	9-cis-epoxycarotenoid dioxygenase	1	VIT_19s0093g00550	65.7-62.9 / 60	87	0.89
VvNCED3_R	GCATGCCATCACCATCAAAG						
VvNPR1_F	GGCGGTTTTGGGGTATTTGT	Non-Expressor of PR genes 1	This work	VIT_11s0016g01990	64.3-64.5 / 60	112	0.93
VvNPR1_R	AGAGCACCTCCACCATGAAA						
VvPAL_F	TCCTCCCGGAAAACAGCTG	Phenylalanine ammonia-lyase	5	VIT_16s0039g01360	65.0-63.0 / 60	103	0.94
VvPAL_R	TCCTCCAAATGCCTCAAATCA						
VvSTS1_F	TGGCCCTGCAATTCTTGATG	Stilbene synthase 1	4	VIT_16s0100g01030	64.1-66.5 / 60	91	0.91
VvSTS1_R	TTAGCACATGCCTCGTTGCTTC						

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Supplementary Table 4

Area under disease pressure curve (AUDPC) values for leaf and bunches in 2019, 2020 and 2021 seasons. Different letters indicate that treatments are significantly different for Tuckey's HSD test p< 0.05.

LEAF				BUNCH			
2019				**2019**			
Protocol	**Mean**	**SD**		**Protocol**	**Mean**	**SD**	
A	0,00	0,00	a	A	0	0	a
B	0,00	0,00	a	B	0	0	a
CTRL	422,14	30,38	b	CTRL	1184,380651	67,08521255	b
M	0,97	0,51	a	M	79,18055556	5,76046087	a
ST	0,00	0,00	a	ST	0	0	a
p-value: 8.816e-12				p-value: 9.69e-13			
2020				**2020**			
Protocol	**Mean**	**SD**		**Protocol**	**Mean**	**SD**	
ST	0	0	a	ST	0	0	a
CTRL	271,1393223	22,19671928	b	CTRL	179,2169565	39,85216428	b
A	8,827777778	1,318572812	a	A	0	0	a
B	0	0	a	B	0	0	a
M	3,888888889	4,967661627	a	M	0	0	a
p-value: 4.578e-11				p-value: 5.633e-07			
2021				**2021**			
Protocol	**Mean**	**SD**		**Protocol**	**Mean**	**SD**	
ST	0	0	a	ST	0	0	a
CTRL	385,3666667	6,457639748	c	CTRL	519,7722222	1,760550209	c
B	0	0	a	B	0	0	a
M	33,49031008	2,962002697	b	M	64,06388889	8,017525653	b
p-value: 2.518e-14				p-value: 7.836e-15			

Supplementary Table 5

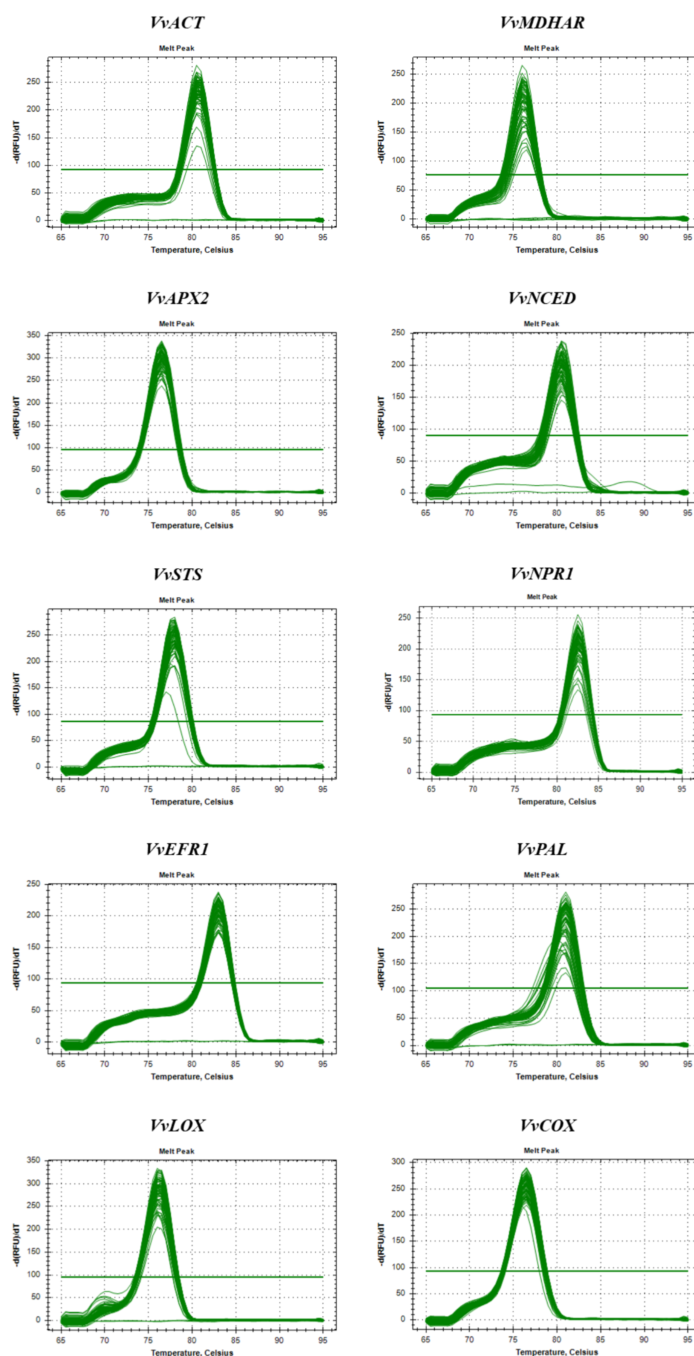
Production indexes analysis of the year 2021: yield (Kg), number of bunches per plant and average bunch weight (Kg). Data are reported as average of three biological replicates ± standard deviation. Different letters correspond to treatments that differ for Games-Howell post hoc test for $p < 0.05$.

Protocol	Yield (Kg)				Bunch number				Average bunch weight (Kg)			
ST	5,13	±	0,73	c	17,67	±	5,63	b	0,31	±	0,06	b
CTRL	2,25	±	0,26	a	12,33	±	4,75	a	0,21	±	0,08	a
B	4,45	±	0,90	c	15,83	±	3,35	ab	0,29	±	0,06	b
M	3,12	±	0,53	b	11,25	±	3,17	a	0,29	±	0,07	b

Supplementary Table 6

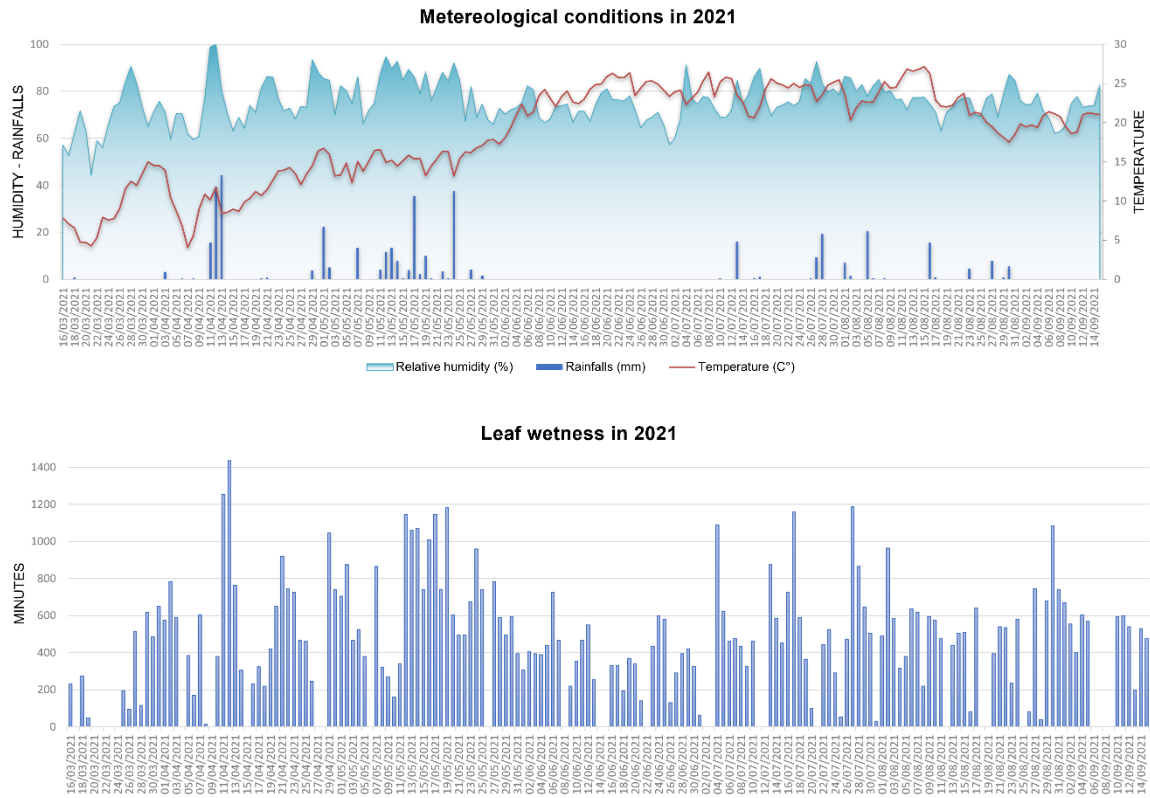
SPAD analysis of leaf chlorophyl content for the year 2021. For each treatment, the average of three biological replicates ± standard deviation is reported, different letters indicate that treatments are significantly different for Games-Howell post hoc test for $p < 0.05$.

Protocol	Mean			
ST	38,80	±	2,96	b
CTRL	42,37	±	3,34	c
B	36,96	±	2,84	b
M	32,91	±	2,90	a



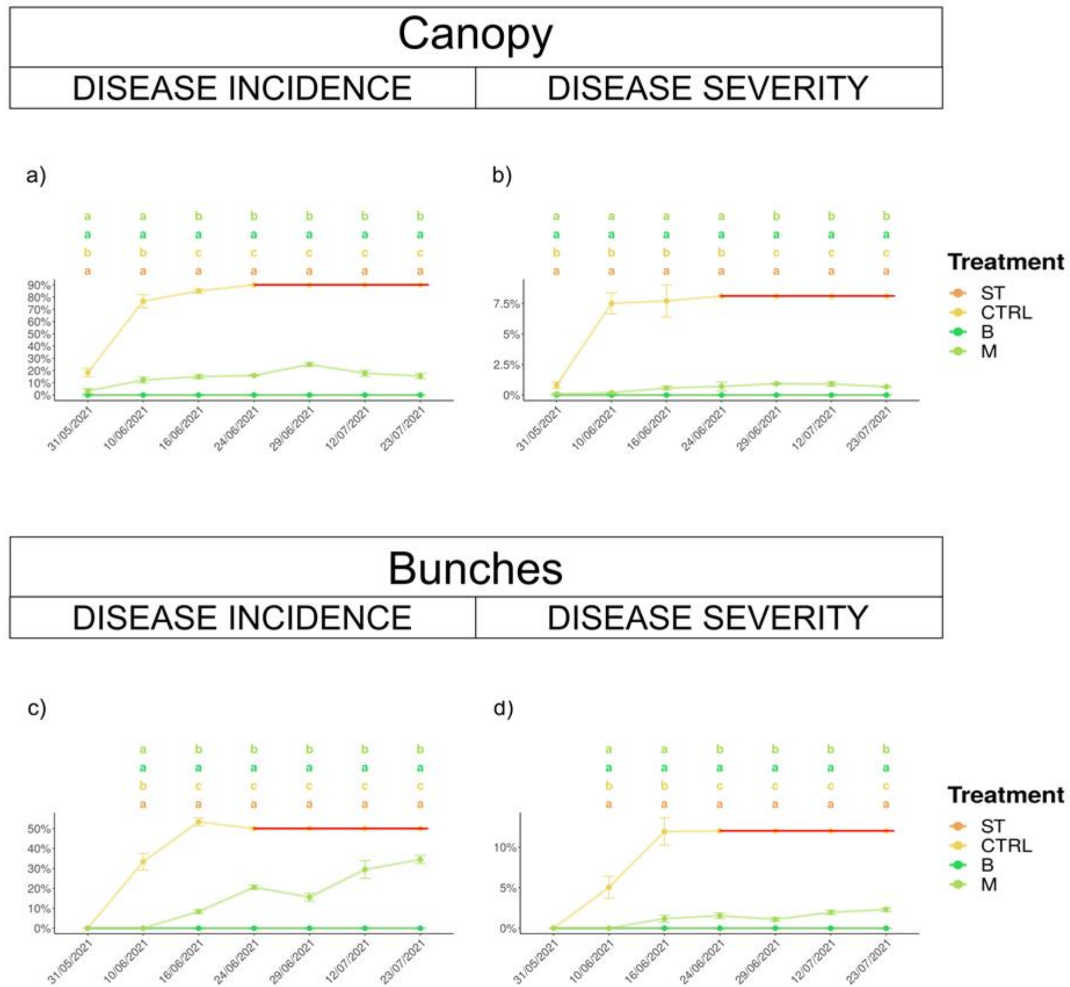
Supplementary Figure 1

Melting curves of housekeeping and candidate genes involved in grapevine stress and defence responses. VvACT, Actin; VvMDHAR, Monodehydroascorbate reductase; VvAPX2, Ascorbate peroxidase; VvNCED, 9-cis-epoxycarotenoid dioxygenase; VvSTS, Stilbene synthase; VvNPR1, Non-Expressor of PR genes 1; VvEFR1, LRR receptor-like serine/threonine-protein kinase EFR; VvPAL, Phenylalanine amino lyase; VvLOX, Lipoxygenase; VvCOX, Cytochrome oxidase.



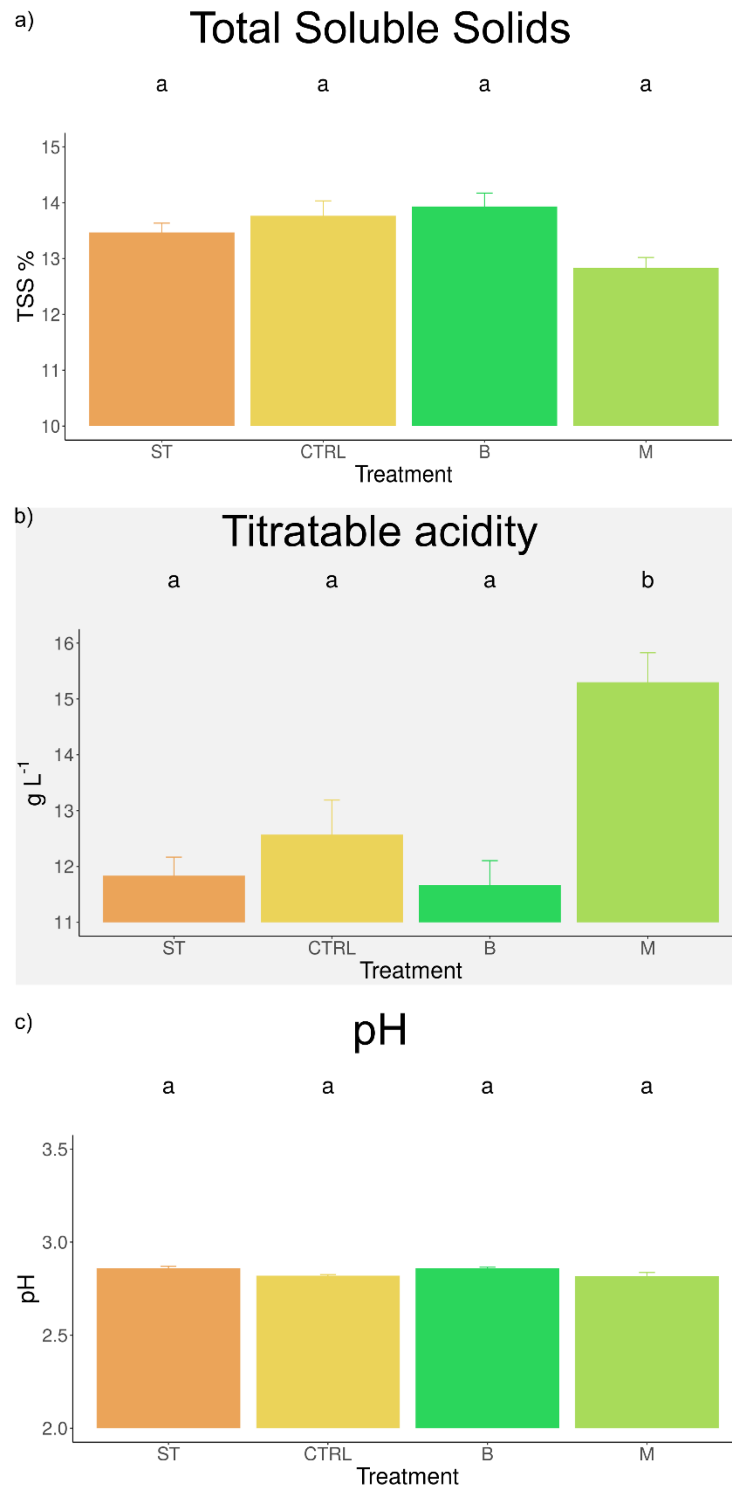
Supplementary Figure 2

Meteorological conditions during 2021 vegetative seasons. a Relative humidity (%, left scale), rainfalls (mm, left scale) and temperature (C°, right scale). b Leaf wetness (min).



Supplementary Figure 3

Disease indexes trends in 2021, incidence represents the ratio between symptomatic observations over the total number of observed leaves or bunches, while severity represents the infected surface area as percentage. For each treatment, the average of three biological replicates ± standard error is reported. Different letters indicate that treatments are significantly different for Tukey's HSD test $p<0.05$. **a)** Disease incidence on canopy in 2021. **b)** Disease severity on canopy in 2021. **c)** Disease incidence on bunches in 2021. **d)** Disease severity on bunches 2021.



<u>Supplementary Figure 4</u>

Musts analysis in 2021 a Total soluble solids (°Brix); b Titratable acidity expressed as mg L-1 of tartaric acid; c pH. For each treatment, the average of three biological replicates ± standard error is reported. Different letters indicate that treatments are significantly different for Tukey's HSD test p<0.05.

6. Conclusions

Considering the ongoing climate change scenario and the need of a transition toward a more sustainable agriculture, the reduction of fungicide environmental impact in viticulture represents a priority. Since viticulture is one of the most treated cropping systems, the development of novel, environmentally friendly strategies for the control of grapevine downy mildew is of particular relevance.

By using a cisgenic strategy, based on recombinase excision systems, is possible to transfer selected genes into woody plant elite varieties, maintaining its quality traits and avoiding the presence of selective markers - particularly adverse to the consumers opinion - in the genome of the target species. Such approach was adopted in the present thesis work to transfer *TNL2a* and *TNL2b* genes, belonging to the RPV3-1 haplotype, which confer resistance to *Plasmopara viticola*, in the "Chardonnay" susceptible genotype. Regenerated cisgenic plants could allow a drastic reduction of fungicide application compared to the wild type plants, however a few treatments per year will be necessary to prevent the resistance breakdown. Pyramidization of resistance genes will strengthen the resistance durability in the future, opening the way to a further reduction of downy mildew control treatments.

Besides cisgenic approaches, the use of RNAi-based techniques for plant disease management offers multiple advantages to ensuring a sustainable food production. Since its discovery, a lot of studies have focused on RNAi mechanism of action leading to an overall good characterisation of the system, with the identification of several RNA constructs able to induce RNAi effects, including dsRNAs, hpRNAs and miRNAs (Rosa et al. 2018). The use of dsRNAs has however showed in some cases a greater efficacy compared to hpRNAs resulting the preferable form of induction molecule (Rosa et al. 2018). At the same time different delivery systems for the exogenous application of dsRNAs have been optimised and one of the most interesting solution is the possibility of directly applying the dsRNA molecules through spraying, namely Spray Induced Gene Silencing (SIGS) (Dalakouras et al. 2020). In this way RNAi treatments on plants could be administered by using the same machinery traditionally adopted by farmers for fungicides applications. Compared to traditional fungicides, dsRNAs are less mobile in the soil, less persistent within the plant and, by targeting mRNA molecules, they allow to achieve a great substrate specificity, hence reducing off-target risks. Considering the limitations posed to the cultivation of genetically modified plants and their acceptance by the society, the application of exogenous dsRNAs seems to be particularly promising. As supplementary activity to the studies presented in this thesis, a preliminary study was conducted for the silencing of two essential genes of *Plasmopara viticola,* the

outcomes will support the use of SIGS as a sustainable and effective technique to control pathogen attack in grapevine, providing data for the design of more complex studies. For the experiment elongation factor 2 (*EF2*) and tubulin (*TUB*) genes, were selected based on the fungicide action sites. *EF2* encodes a soluble protein required for the mRNA translation and catalysing the translocation of the ribosomal unit and is targeted by the sordarins family of fungicides. TUB is an essential protein required for microtubule polymerization, indispensable for regulation of cell morphology and growth, that is targeted by the benomyl fungicide and has already been used in other studies as RNAi target. To produce a single fragment containing the sequences of both genes, smaller fragments of the single genes (about 250-300 bp) were amplified using specific primers designed with overlapping sequences that allowed to obtain a single PCR amplicon. The PCR amplicon was then cloned into plasmid L4440, which contains a double and convergent T7 promoter that allowed the production of dsRNAs after induction with IPTG. The vector was then transferred into competent HT115(DE3) *E. coli* cells, a strain specifically designed for dsRNA production, harbouring a mutation in the *RNAseIII* gene that is responsible for degradation of the small RNAs. Along with the production of dsRNAs to silence *Plasmopara viticola* genes, dsRNAs targeting the Green Fluorescent Protein were produced to be used as control. Production of dsRNAs was tested in 1.5 L of culture medium allowing the retrieval of about 800 μg of dsRNA per liter of bacterial culture. The expected results will allow to prove whether downy mildew infection could successfully be controlled by SIGS. The control of *Plasmopara viticola* through SIGS could therefore represent a remarkable breakthrough among the defence strategies developed against fungal pathogens. Indeed, such approach could be easily implemented by farmers, who commonly apply treatments through spraying, moreover SIGS bypasses the scepticisms related to the cultivation of conventional GMOs, minimizing the environmental impact and side effects on unspecific targets.

A promising scenario in the sustainable fight against grapevine downy mildew can be hypothesized from the joint use of resistant cisgenic varieties and SIGS applications. Given the complementarity between these two strategies, it would be possible to maximize the resistance durability retaining all advantages of the SIGS treatment and minimizing at the same time the number of spray applications and its associated costs.

Despite molecular mechanisms at the basis of plant priming are still largely unknown and we are just starting to solve the puzzle of this complex regulation network, the third strategy analysed in the present work and concerning the exploitation of defence priming was proved to be another valuable tool for the reduction of agrochemical impact in viticulture. In the first

priming related study, the different response of "1103P" and "SO4" rootstocks to the mycorrhizal colonization with respect to the tested factors showed a fine regulation of plant defence and growth behaviours by AMF and root associated microbes. Particularly, all treatments promoted more robustly the growth of the "SO4" genotype, known to be less vigorous. For both genotypes, the oligosaccharide inducer fostered the recruitment of native AMF present in the soil. However, a clear genotype effect emerged for the root colonization, probably due to a different recruitment pattern, as showed by the higher AMF diversity in treatments "I" and "I+M" of "1103P" compared to "SO4" plants. Moreover, the recruitment of microbes either when stimulated by the oligosaccharide treatment or by the AMF inoculum, triggered the plant immunity, leading to stilbene accumulation. These findings suggest that also in grapevine is possible to exploit the mycorrhiza-induced resistance (MIR) to prime the plant defences against downy mildew.

In the second study concerning the priming of plant defences, we demonstrated that by using resistance inducers is possible to achieve a robust stimulation of the plant immunity with an efficient control of *Plasmopara viticola* diffusion and symptoms, providing a direct image of its efficacy in a commercial vineyard setup. The treatment with acibenzolar S-methyl, laminarin and disodium phosphonate in a single formulation (M protocol), induced the plants in a primed state, characterised by the upregulation of key genes involved in the synthesis of stilbenes (*VvSTS*) and phenylpropanoids (*VvPAL*), activation of the SAR response and a decreased detoxification of reactive oxygen species. When challenged by the pathogen, the plants showed lignin deposition and hypersensitive like cell death responses on pathogen infection sites. The analysis of chlorophyll content and must characteristics, according with previous studies on the effects of resistance inducers published in literature, also evidenced that the M protocol determines an in-depth transcriptional reprogramming of carbohydrate metabolism, allocating more resources toward defence processes due to a modification of the growth-defence trade-off, and a slight delay in fruit ripening.

All the strategies presented in this thesis provide practical solutions for the control of the pathogen in a sustainable manner, however there are issues that need to be addressed, including those associated with regeneration efficiency through somatic embryogenesis and large-scale production of dsRNAs. Regeneration of plants through somatic embryogenesis in grapevine is still time consuming, and genotype dependent, with major limitation for recalcitrant varieties as 'Glera'. Our knowledge about the molecular mechanisms regulating somatic embryogenesis is still lacky however new studies are opening the way for the development of new molecular targets and strategies to improve somatic embryogenesis

efficiency (Sugimoto et al. 2019; Dal Santo et al. 2022). Social acceptance and large scale production of dsRNAs with high associated costs constitute the major limits to the wide adoption of this technology, although their adoption is favoured since plants treated with dsRNA are not considered GMO (Nji et al. 2021). Economic sustainability however is expected to improve in the future, given the large investments of the last years for production of RNA based vaccines, with the possibility of converting some manufacturing platforms to dsRNA production with agricultural purposes (Nji et al. 2021). Moreover, the use of cisgenic plants and the application of RNAi in the field is currently hindered by the limits and lacks of the European law. Also, more studies are required to better clarify the mechanisms regulating the defence priming that could allow the development of stronger elicitation systems and selection of plants with enhanced responses against pest and diseases. The presented approaches pave the way for a new green revolution, however studies addressing the integration of these strategies will be necessary in the close future.

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