UNIVERSITÀ DEGLI STUDI DI BARI ALDO MORO

DEPARTMENT OF BIOSCIENCES, BIOTECHNOLOGY AND ENVIRONMENT

PhD Program in Biosciences and Biotechnology

Curriculum: Genetics, Microbiology and Molecular Evolution

Multi-target antimicrobial activity and molecular characterization of autochthonous bacteria strains from *Vitis vinifera* leaves to cope with biotic stresses

XXXVII CYCLE – Green and Innovation

Supervisors:

Dr. Maria Francesca Cardone

Prof. Carlo Pazzani

Dr. Antonio Domenico Marsico

PhD Student:

Camilla Mandorino

PhD Program Coordinator:

Prof. Giovanna Valenti

Summary

English Abstract

This thesis aimed to explore and study the autochthonous microbiome of *Vitis vinifera* phylloplane to identify microorganisms with antimicrobial properties and the potential to be used as Biological Control Agents (BCAs) to cope with biotic stresses. Downy Mildew, caused by *Plasmopara viticola* was selected as a case study for the identification of BCAs. *P. viticola* is a strictly biotrophic oomycete that can attack all the plant's green tissues in the right environmental conditions. Nowadays, to avoid yield losses, disease control is commonly carried out by using chemical products that are harmful to the environment and to human and animal health. The identification of BCAs against *P. viticola* could promote more sustainable practices in viticulture as an eco-friendly alternative to currently used chemical pesticides. The first step of the research activities was the selection of the *V. vinifera* varieties from which microorganisms would be isolated. The selection was carried out by evaluating the degree of tolerance/susceptibility of the different varieties to Downy Mildew on the bunches and leaves and finally, three varieties were selected: 'Dawn seedless', 'Blush seedless' and 'Argentina'. Focus was brought on the bacterial component of the leaves microbiome of the chosen varieties and epiphytic and endophytic bacteria were isolated through a microbiological analysis. Different assays were performed to identify candidate BCAs. Sixteen among the total isolated bacteria were tested on leaf discs to evaluate their antagonistic activity against *P. viticola* and different classes of efficacy were defined according to the percentage ratio of the leaf disc area covered by the oomycete sporulation. Five bacterial strains ('BLG_B1.1.1', 'BLG_B1.3', 'BLG_B2', 'BLG_B4', 'BLG_B5') were selected on the basis of their different efficacy in controlling Downy Mildew infection and were characterized for their morphological features and their species was identified. The five bacterial strains were tested through an *in vivo* assay on leaves to study a possible mechanism of action against *P. viticola*. In particular, their effect on the expression of *P. viticola* effector genes *PvRxLR28* and *PvRxLR67* was assessed by performing a Real-Time PCR. Among the five strains, three determined a moderate to high reduction of *PvRxLR28* expression. Moreover, their effectiveness against a wide range of fungal pathogens and their safety to human health was also evaluated. A bioinformatic comparative analysis between the strains 'BLG_B2' and 'BLG_B4' was performed, followed by an evaluation of their ability to counteract the growth of human pathogenic bacteria.

Future perspectives of the present work involve testing the identified potential BCAs on plants to evaluate their effect on *V. vinifera* resistance against plant diseases affecting the phylloplane and carposphere. A pilot field trial on plants was carried out testing a synthetic microbial community already characterized for its biocontrol properties. Results will allow the definition of the best protocols to replicate the trial using the selected candidate bacterial BCAs.

Italian Abstract

Il progetto di tesi si proponeva di esplorare e studiare il microbioma autoctono del filloplano di *Vitis vinifera* al fine di identificare microrganismi con proprietà antimicrobiche ed il potenziale per essere utilizzati come agenti di controllo biologico (BCA) nei confronti di stress biotici. La Peronospora, causata da *Plasmopara viticola*, è stata scelta come caso studio per l'identificazione di BCA. *P. viticola* è un oomicete strettamente biotrofico che, nelle giuste condizioni ambientali, può attaccare tutti i tessuti verdi della pianta. Ad oggi, per evitare perdite di produttività, il controllo della malattia viene comunemente effettuato utilizzando prodotti chimici che, tuttavia, risultano dannosi per l'ambiente e per la salute umana e animale. L'identificazione di BCA contro *P. viticola* potrebbe promuovere pratiche più sostenibili in viticoltura come alternativa ecologica ai pesticidi chimici attualmente utilizzati. La prima fase delle attività di ricerca ha riguardato la selezione delle varietà di *V. vinifera* da cui isolare i microrganismi. La selezione è stata effettuata valutando il grado di tolleranza/suscettibilità delle diverse varietà alla Peronospora sui grappoli e sulle foglie, con la selezione finale di tre varietà: “Dawn seedless”, “Blush seedless” e “Argentina”. L'attenzione si è concentrata sulla componente batterica del microbioma delle foglie delle varietà scelte con conseguente isolamento, tramite un’analisi microbiologica, dei batteri epifiti ed endofiti. Sono stati eseguiti diversi saggi per identificare i candidati BCA. 16 dei batteri isolati sono stati testati su dischi fogliari per valutare la loro attività antagonista contro *P. viticola* e sono state definite diverse classi di efficacia in base al rapporto percentuale dell'area del disco fogliare coperta da sporulazione dell'oomicete. Cinque ceppi batterici ('BLG_B1.1.1', 'BLG_B1.3', 'BLG_B2', 'BLG_B4', 'BLG_B5') sono stati selezionati sulla base della loro diversa efficacia nel controllo dell'infezione di Peronospora e sono stati caratterizzati per le loro peculiarità morfologiche con successiva identificazione della specie di appartenenza. I cinque ceppi batterici sono stati poi testati con un saggio *in vivo* su

foglie per studiare un possibile meccanismo d'azione contro *P. viticola*. In particolare, è stato valutato il loro effetto sull'espressione dei geni effettori di *P. viticola PvRxLR28* e *PvRxLR67* tramite esecuzione di una Real-Time PCR. Nello specifico, tra i cinque ceppi, si è osservato come tre determinassero una riduzione da moderata a elevata dell'espressione di *PvRxLR28.* È stata inoltre valutata la loro efficacia contro un'ampia gamma di patogeni fungini e la loro sicurezza per la salute umana. È stata infine eseguita un'analisi bioinformatica comparativa tra i ceppi 'BLG_B2' e 'BLG_B4', seguita da un saggio volto a valutare la loro capacità di contrastare la crescita di batteri patogeni per l'uomo. Le prospettive future del presente lavoro prevedono che i potenziali BCA identificati siano testati su piante per valutare il loro effetto sulla resistenza di *V. vinifera* a condizioni patologiche che colpiscono il filloplano e la carposfera. A tal fine, è stata condotta una prova pilota in campo su piante testando una comunità microbica sintetica già caratterizzata per le sue proprietà di biocontrollo. I risultati della prova consentiranno di definire i migliori protocolli per replicare in futuro la prova utilizzando i candidati BCA batterici selezionati.

1. INTRODUCTION

1.1 Microbiome

1.1.1 Definition

The term "microbiome" was coined in 1988 by Whipps and colleagues [1] and defined as "a characteristic microbial community occupying a reasonably well-defined habitat, which has distinct physico-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity" [2] [3]. However nowadays, the most popular and cited definition is the one given by Lederberg in 2001 which describes the microbiome as the community of commensal, symbiotic, and pathogenic microorganisms in a body space or other environment [3]. A group of international experts reunited in 2020, starting from the definition of Whipps *et al.* [1] proposed a new definition of microbiome that includes the most recent technological and research novelties. Currently, the microbiome is defined as a specific microbial community with its theatre of activity, occupying a well-defined habitat having distinct physio-chemical properties, which results in the creation of specific ecological niches. Based on this definition, the microbiome is a dynamic and interactive micro-ecosystem that changes in time and scale and that constitutes a macro-ecosystem with its eukaryotic hosts. Unlike the term *microbiota*, which refers

to the actual microorganisms belonging to different kingdoms, the term *microbiome* includes not only the microorganisms, but also their microbial structures, metabolites, mobile genetic elements and genetic material [2].

1.1.2 Plants as holobionts

Plants harbor a wide variety of microorganisms constituting a complex microbial ecosystem, known as *plant microbiome*, that closely interact with plants themselves [4] [5]. Thus, it is not correct to consider plants as independent entities, but as a supra-organism with the microorganisms colonizing them, also called "holobiont" [7]. Considering that the term "hologenome" refers to the host genome and associated microbiome [6] [7]. In 2008 the theory of the evolution of the hologenome emerged and four axioms were established: 1. all animals and plants form symbiotic relationships with microorganisms 2. symbiotic microorganisms are passed down through generations. 3. the fitness of the holobiont in its environment is influenced by the host-microbe interaction. 4. variations in the hologenome result from changes in either the host or microbiota genomes. Plants' microbiota includes all eucaryotic and procaryotic microorganisms, as well as viruses, colonizing plant surfaces and inner tissues, respectively defined as epiphytes and endophytes. [8] [7] [9]. The members of the plant microbiota comprise beneficial, neutral and pathogenic microorganisms, according to the effect that they produce on the host. [5] [10]. Beneficial effects can be direct, for example, through the increase of nutrient availability for plants (synthesis of siderophores for iron sequestration, phosphorous solubilization, and fixation of the atmospheric nitrogen), protection against both abiotic (salinity, drought, increase of CO_2 levels, high temperature) and biotic stress, through competition for nutrient and space, antibiosis and the production of hydrolytic enzymes. Indirect benefits include the enhancement of plants' defense mechanisms. On the other hand, some microorganisms may be deleterious for the plant and cause infectious episodes in the presence of three elements: a susceptible host-plant, a virulent pathogen and a favorable environment [5] [10]. Finally, plant-microbial associations are extremely dynamic and different factors such as plant species, age, developmental stage, physiology, environment, soil type, crop management, climatic variables, microbe-microbe interactions, and presence of plant pathogens may modify microbial composition and interactions with its host. [5] [11].

1.2 Vitis vinifera microbiome

Vitis vinifera is a perennial woody plant of high global economic and social importance whose cultivation in 2022 occupied 75.866 km^2 of hectares worldwide according to the FAO [12]. The grapevine hosts numerous microorganisms on its compartments that influence the plant's health status, plant growth and wine's organoleptic characteristics [13]. In microbiology, the different compartments of the plant are grouped into 'phyllosphere' and 'rhizosphere'. The phyllosphere is the total above-ground surface of a plant and can be further subdivided into 'phylloplane' (leaves), 'anthosphere' (flowers) and 'carposphere' (fruits). The rhizosphere is defined as the area of the soil surrounding the plant roots.

1.2.1 Origin of grapevine microbial community

Microorganisms fix themselves in and on plant tissues, finding different entryways through which they penetrate inside the plant [7]. The root system offers different entry points represented by epidermal intercellular junctions where root hairs or lateral roots emerge or wounds on the rhizoplane, also various gateways exist in the aerial parts of the plant such as leaf stomata, trichomes, surface micro-wounds or hydathodes [14] [15]. Once inside the plant, microorganisms must overcome the plant's innate immunity and finally colonize the root cortex or the vascular system and spread within the host [16] [17]. Another mechanism through which microbes enter the plant is direct injection into the vascular system performed by sucking insects [18] [19]. The environment surrounding the grapevine is a rich source of microbes that are dispersed by wind, water and animals from the nearby fauna and flora, including other grapevines. Soil is considered a reservoir for bacteria that spread up to the aerial part of the plant. Also, vineyards are subject to strong anthropization, making humans an additional source of microorganisms [7] [19].

1.2.2 Differences in the microbial community within plant departments

Marked differences exist among the microbiota colonizing grapevine belowground and aboveground departments, and this is true especially for epiphytes. These differences are coherent with the fact that the compartments are exposed to strong different environmental conditions such as temperature, moisture, light incidence and human contact. For example, the soil can keep the temperature and humidity more stable than the aerial parts of the grapevine and block UV radiation [20] [21]. As for the aerial parts of the grapevine, leaves and bunches have to tolerate temperature and

humidity fluctuation. Moreover, the leaves are irradiated by UV during the daytime, whilst some grape berries may be shielded by the leaves themselves [22] [21]. In addition, nutrient availability differs among plant's compartments. Indeed, grape berries contain sugars and other nutrients from exudation and the soil contains compounds that are rich carbon sources. In contrast, leaves are nutrient-limited [23] [21].

1.2.3 Roots and rhizosphere

The soil represents the principal microbial reservoir for the plant's belowground compartments. The rhizosphere of young plants is initially colonized by microorganisms, thanks to the release by roots of carbon-containing compounds, called rhizodeposits. These active compounds represent the substrate for the development of microbes and, as well as the root morphology, can influence rhizosphere microbial community composition [24]. Root exudates consist of ions, free oxygen and water, enzymes, mucilage and a diverse array of carbon-containing primary and secondary metabolites [25]. Examples of the primary substance are organic acids, sugars, amino acids, lipids, coumarins, flavonoids, proteins, enzymes, aliphatic and aromatic substances. In particular, flavonoids often serve as a signal in plant-microorganism interaction [26]. For example, the secretion of isoflavones in soybean roots attracts the beneficial bacterium *Bradyrhizobium japonicum* as well as the pathogens *Phytophtora sojae* [27]. On the other hand, plants produce and secrete a variety of secondary metabolites, which can be toxic to microorganisms. For example, walnut (*Juglans regia*), that is known to produce juglone, is colonized by specific microbial population that can degrade or detoxify metabolites via specific hydrolases [28].

Also, root morphology was shown to have a role in shaping rhizosphere microbial communities [29] [13] [30]. For instance, grooves near cell borders offer nutrient accumulation and physical protection from predation favoring bacterial colonization [31]. Also, fine roots with small diameters have higher contents of essential minerals such as phosphorus and potassium and recruit a more diverse bacterial community than large roots [32]. Moreover, plant roots with high specific root lengths and low root tissue densities stimulate the growth of fast-growing bacteria over that of fungi due to high levels of root exudation and abundant release of resources into the soil [33]. Another critical element seems to be the presence or absence of root hairs because of

their role in nutrient acquisition. It was hypothesized that an impairment of their development may trigger the recruitment of a microbiota able to compensate for these limitations. Indeed, in barley (*Hordeum vulgare*) colonization by arbuscular mycorrhizal fungi is enhanced in the hairless genotype [34]. Some rhizospheric microorganisms can then enter the root endosphere, that is to say its internal tissues, and some even reach the vascular system [30]. Multiple studies have analyzed the taxonomic composition of the grapevine rhizosphere and root microbiome, and they highlighted that the dominant bacterial phyla are: Actinobacteria, Acidobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes, Proteobacteria, Verrucomicrobia. While the most abundant genera are: *Bacillus, Blastococcus, Clostridium, Flavobacterium, Gaiella, Methylobacterium, Micrococcus, Nitrososphaera, Pseudomonas, Rhizobium, Steroidobacter and Sphingomonas*. As for the fungi, the most abundant phyla are Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Morticellomycota, Mucoromycota, Zygomicota. The most present genera are: *Alternaria, Archeospora, Aspergillus, Dactylonectria, Fusarium, Glomus, Martierella, Mucor, Paraglomus, Penicillium, Peziza, Phaeoacremonium, Sclerocystis, Trichoderma*. [7] [35] [36] [37] [38] [39] [40].

1.2.4 Woody parts: trunk, spurs and canes

The woody compartments of the plant have unique characteristics; in particular, they are not completely homogeneous, because of the coexistence of living and dead tissues. Consequently, the microbiota associated with the perennial woody parts is subjected to less variation than the microbiota of other arial parts [7]. Among other vine parts, such as roots, leaves and flowers, that might share their microbiota with grape skin and then influence both the sanitary state of grapes and the winemaking process, woody parts of grapevine received poor attention [37]. Interesting are the results obtained by Morrison-Whittle and co-workers [41] that, by using next-generation sequencing (NGS) to examine the roles of soil, bark and fruits as source-habitats of the fungal diversity of fermenters, showed that eukaryotic microbial populations increasingly resemble those present on vine bark as the fermentation proceeds.

The most abundant bacterial phyla colonizing perennial tissues are: Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Proteobacteria, Verrucomicrobia; and the most abundant genera: *Achromobacter, Bacillus, Bradyrhizobium, Cellulomonas,*

Cutobacterium, Pseudomonas, Sphingomonas, Xanthomonas. [7] [42] [43] [44] [45]. The fungal community is much less extensive and only two phyla predominate in microbiota: Ascomycota and Basidiomycota. The only colonizing genera result: *Cladosporium, Alternaria, Chaetomium, Aureobasidium* and *Penicillium* [7] [16] [42].

1.2.5 Reproductive organs: flowers, berries and seeds

The study of the microbial community associated with grapevine reproductive organs, such as flowers, grape berries, and seeds, revealed a close correlation with the microbiome composition present in the soil or in the woody parts. [7] [30] [47]. Some studies reported that several microorganisms associated with grape berries are present in the soil, suggesting that the latter may represent a source of primary inoculum, able to take part in the structure of the microbial community on the aerial parts of the vine. Two hypotheses have been formulated to demonstrate the passage of microbial community from the soil to the aerial part of the plant; while the endophytes that spread from the soil through xylem vessels, for the epiphytes, physical proximity, environmental agents such as rain, winds and mechanical soil management practices, might contribute to the migration of telluric microbes. Also, trunk bark may be a source of inoculum for flowers and grapes because of the physical closeness and the possibility of even coming in direct contact [48]. Bacterial phyla colonizing reproductive organs are: Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and the most abundant bacterial genera: *Bacillus, Blastococcus, Enterobacter, Erwinia, Gaiella, Massilia, Mathylobacterium, Micrococcus, Pseudomonas, Sphingomonas*. [7] [42] [44] [47] [49]. As for fungi, two phyla are the most abundant: Ascomycota and Basidiomycota. The most present fungal genera are: *Alternaria, Aureobasidium, Botrytis, Cladosporium, Cryptococcus, Davidiella, Guehomyces, Penicillium, Sporobolomyces, Rhodotorula* [7] [42] [50] [51].

1.2.6 Leaves

Microbial biodiversity inhabiting phylloplane is the most studied among the grapevine phyllosphere, since it represents the largest microbial habitat. The microbial community colonizing leaves notably differs from belowground compartments, such as the rhizosphere, because of the lower nutrient availability and exposition to external stress factors [5]. On the other hand, similarities were found especially with flowers and berries [52]. Grapevine leaves microbiome follows a cyclic course: it comes together in spring, changes during the vegetative season and disassembles during leaf

senescence [30]. Two factors influencing the diversity, structure, composition, and function of bacterial and fungal communities are leaf age and cultivar, and in particular young leaves show higher microbial diversity and richness [53]. Just like other aboveground compartments, leaves interact with soil microbes through internal transport between tissues but also receive many microbial colonizers by deposition via rainfall and wind dispersal [54]. Bacteria are the major leaves colonizers, followed by fungi [5] [22]. The most abundant bacteria phyla inhabiting leaves are: Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Gemmatimonadetes, Proteobacteria, while the most abundant genera are: *Bacillus, Arthrobacter, Blastococcus, Curtobacterium, Enterococcus, Flavobacterium, Methylobacterium, Pantoea, Pseudomonas, Sphingomonas, Streptococcus* [7] [5] [49] [55] [56] [57] [58]. As for the fungi, three major present phyla are: Ascomycota, Basidiomycota and Zygomicota and the most present genera: *Alternaria, Aureobasidium, Cladosporium, Guehomyces, Epicoccum, Mucor, Pandora, Rhiopus, Sporormiella* [5] [7] [44] [49] [56] [59].

1.2.7 Factors influencing grapevine microbial community

Grapevine microbiome is a very dynamic system and multiple factors can contribute to its modification. These factors can be endogenous, meaning those strictly related to the plant status, and exogenous regarding external elements that have an impact on the plant [7].

One of the endogenous factors influencing the composition of the microbiome is the genetics of the plant. Since the nineteenth century, *V. vinifera* varieties have been cultivated as scions and grafted onto the rootstock of the other *Vitis* species and hybrids to prevent vineyards from succumbing to Phylloxera pest. Although it is widely known that the rootstock genotype and the grape-associated microbiota affect the grapevine physiology, in grafted plants, the rootstock and, to a lower measure, the scion genotype influence the microbial community of grapevine compartments. [54] [60]. Few studies have been published in recent years on the effects of grapevine rootstock genotype on root and/or rhizosphere microbiota composition. It was observed that rootstock influences the size and complexity of the network among the microbial community of grape root system compartments which resulted in more extended and complex grafted than ungrafted plants. The rootstock genotype drives the diversity and richness of the microbial community, modeling phyla distribution in the root tissues and the genera in the rhizosphere [59] indeed, Dries *et al.* [62] observed that rootstocks 1103P, 140 Ru,

161-49C and Kober 5BB were able to assemble distinct bacterial microbiota at the rhizosphere level. Comparing *V. vinifera* 'Falanghina' cultivar grafted on '1103 Poulsen' and 'Kobber 5BB', Zuzolo *et al.* [63] showed that Actinomycetota was the marker phyla differentiating the rhizosphere microbial communities associated with the different rootstock types, while at the genus level, '1103 Poulsen' genotype rhizosphere was enriched in taxa belonging to Actinomycetota and Alphaproteobacteria classes. The selection of different plant microbiota in root endosphere and rhizosphere compartments by different rootstock genotypes has repercussions on plant physiology and health. Indeed, Zuzolo *et al.* [63] also investigated the functional profile of the microbial communities associated with the two different rootstock genotypes and could see that most key enzyme-encoding genes involved in N cycling were significantly more abundant in 'Kobber 5BB' rootstock rhizosphere soil and that '1103 Paulsen' rhizosphere was enriched in genes involved in C cycle and Plant Growth Promotion (PGP). Also, D'amico *et al.* [64] observed in 'Lambrusco' cultivar grafted on the '1103 Paulsen' rootstock a lack of potassium solubilizing microorganisms in root endosphere compared to the same cultivar grafted on 'Kobber 5BB' rootstock, affecting potassium absorption by the plant and root development. Marasco *et al.* [65] also showed that not only rootstock genotypes but also rootstock–scion combination contributes to shape diversity and composition of microbial communities associated with the grapevine root system, detecting 35.0% and 27.3% of bacterial and fungal OTUs respectively, as specifically associated with the rhizosphere of one or more rootstock–scion combinations.

Other elements influencing grapevine microbiome are the age and partially the phenological stage that influences bacterial endophytes, stem fungi, and rhizosphere soil bacteria. [7] [66] [67] [68]. Andreolli *et al*. [69] showed that three-year-old and 15-year-old plants of the 'Corvina' cultivar were differently colonized: Actinobacteria and Bacilli prevailed in the 3-year-old plants, while the older plants featured more Proteobacteria. Also, younger plants have a higher diversity of taxa and an abundance of the *Rhizobium* genus, while the old plants contain a higher presence of *Pantoea.* As for the phenological stage, the fungal community of winter soil samples harbors rapid-growing taxa specialized to weather; in contrast to those samples from summer, which contained slow-growing taxa [70]. Pinto *et al.* [71] considered the ten most abundant eukaryotic and prokaryotic communities of *V. vinifera* microbiome in May, June and

July. In May *Guignardia* was the most abundant phytopathogen. In June, *Sporormiella* and *Alternaria* showed an increase and a decrease of *Guignardia* from May to July was detected. Then, in July an increase of *Alternaria*, *Aureobasidium* and *Sporormiella* was observed. As for the prokaryotes, in May Pseudomonadaceae, Streptococcaceae, Sphingomonadaceae and Enterobacteriaceae dominated the microbial community. In June, Streptococcaceae was the most abundant family followed by Enterobacteriaceae, Moraxellaceae and Pseudomonadaceae. Finally, in July, the most abundant families were Enterobacteriaceae and Streptococcaceae.

Among exogenous factors that structure microbial diversity in grapevine, it is possible to recognize environmental elements such as weather, climate, and geographic features [72]. Perazzolli *et al.* [73] observed significant differences among the phyllosphere microbiota of grapevines in Italy in Udine and San Michele all'Adige. Differences were noticeable in terms of the abundance of Archaea, Deltaproteobacteria, Ascomycota, and Basidiomycota. The abundance of the bacterial and fungal taxa correlated significantly with the environmental parameters of grapevine locations, such as temperature and the amount of rainfall. For instance, precipitation and temperature have been found to correlate with the abundance of filamentous fungi such as *Cladosporium* and *Penicillium* spp. and of ubiquitous bacteria such as the members of the *Enterobacteriaceae* family, as well as of yeast populations particularly *Hanseniaspora* and *Metschnikowia* [72]. Also, drought was observed to influence the microbial community of root endosphere, rhizosphere, and soil, with a great impact on the fungal composition of root endosphere as described by Carbone *et al*. [74]. The diversity of OTUs significantly decreased in presence of severe water deficit, while an increase in arbuscular mycorrhizal fungi was observed. As for the geographic factors, microbial communities colonizing different grapevine compartments show different sensitivity to them; in particular, the terroir has a greater impact on perennial organs' microbiota than on leaves and berries [7]. Coller *et al.* [29] analyzed the microbiome of different vineyard soils located in Trentino and observed that the fungal component was more variable across the different sampling sites, resulting more influenced by geographic factors than the bacteria and archaea components. Indeed, out of 12,101 total OTUs, only 5 were present in all samples, dominated by *Ascomycota*, *Zygomycota*, and a small fraction of *Basidiomycota*, constituting the

core mycobiome. Soil chemical and physical characteristics shape the composition of its microbial community, this includes the soil pH, carbon-nitrogen (C/N) ratios, phosphorous content, soil texture (i.e., sand and clay), bulk density and soil moisture. [72] [75]. However, it seems that chemical factors play a more important role than physical ones. Indeed, the influence of the latter on the modulation of microbial communities seems to be more related to their effect on the soil microclimate to which soil microbes are exposed, including temperature fluctuations, gas concentrations and exchange, soil water potential and holding capacity, that may influence, to some extent, soil microbial community composition in the vineyard [75] [7]. Liang *et al*. [76] studied the correlation between bacterial and fungal community compositions and soil edaphic properties and observed that a predominance of *Proteobacteria* was positively correlated with pH, while soil pH and total phosphorus were negatively related to the relative abundance of *Actinobacteria*. Soil organic carbon contents, total phosphorous, C/N ratio and available phosphorus possessed strong and positive correlations with the abundances of *Verrucomicrobia*, *Planctomycetes* and *Bacteroidetes*, while negatively correlated with the relative abundance of *Firmicutes*. As for fungi, they were mostly influenced by soil organic carbon contents which contributed to shaping the fungal community.

Other factors influencing the composition of the grapevine microbial community are the management practices of the vineyard [5] [7]. Bacterial and fungal community composition and diversity differ with conventional, organic, and biodynamic vineyard management because of the direct or indirect influence of the types of pesticides and fertilizers that the different management practices require. In particular, vineyards cultivated using organic or biodynamic agriculture show a greater bacterial and fungal richness [7] [41] [77]. Campisano *et al.* [78] and Pancher *et al*. [79] studied, respectively, the differences among bacterial and fungal communities associated with *V. vinifera* cultivars ‘Merlot’ and ‘Chardonnay’ comparing organic and Integrated Pest Management (IPM) practices in Italy. Differences across the viticulture practices were found, though no significant differences were obtained between grapevine cultivars. Concerning bacterial microorganisms, a dominance of *Mesorhizobium, Caulobacter* and *Staphylococcus* genera was detected in the organic practices, whereas the *Ralstonia, Burkholderia* and *Stenotrophomonas* genera were the most abundant on IPM vineyards. As for the fungal endophytes, isolates of *Alternaria* sp, *Epicoccum*

nigrum and *Aureobasidium pullulans* were observed on both viticulture practices, while species such as *Botryosphaeria obtusa*, *Botryosphaeria dothidea*, *Truncatella angustata*, *Neofusicoccum parvum* or *Phoma herbarum* were isolated from IPM vineyards. This distribution can be explained by the use of fungicides in IPM practices and its role in shaping microbial communities [5]. Morrison *et al.* [41] observed that bark communities in biodynamically managed vineyards had 35.8% more species than those in conventionally managed ones and fruit communities 63.1% more species, with a greater abundance of Ascomycota for the bark and Dothideomycetes and Tremellomycetes for the fruit. The use of pesticides and chemical treatment was shown to affect microbial biodiversity, both of pathogens and beneficial microorganisms. Pinto *et al.* [71] observed that chemical treatments had a negative impact on the balance between phytopathogens and phytoprotectors in the *V. vinifera* microbiome, affecting mostly the eukaryotic community with a lower abundance of *Aureobasidium* genus and an increase in the phytopathogen genera *Rhizopus, Lewia, Alternaria, Diaporthe, Phomopsis, Cryptovalsa, Stemphylium, Ustilago and Botryotinia*. As for the prokaryotic population, the applied chemicals caused a decrease of the relative abundance of Enterobacteriaceae, Pseudomonadaceae, Comamonadaceae and Xanthomonadaceae families. Chemical treatments also affect the rhizosphere microbiota, especially the fungal community, causing the disappearance of certain species and reducing grapevine mycorrhization [7]. For instance, the use of herbicides was observed to reduce mycorrhization compared to mechanical weed control, also, more CFUs were identified in soil samples subjected to weed control treatments based on natural compounds rather than mechanical weeding and use of chemical herbicides [81]. However, the modification in microbial communities in response to chemical treatments seems to be transient over time [7] [70] [81]. Also, practices like tillage create shifts in soil nutrient availability and aggregate size, composition, and stability, changing the physical environment and resource availability of soil microorganisms [77].

1.3 Vitis vinifera-microbial interactions

V. vinifera interacts with beneficial, neutral and pathogenic microorganisms. These plant-microbial interactions are of prime importance because they are intimately linked to plant health status. These interactions can be harmful, beneficial or neutral. The first type of interaction leads to a disease scenario that compromises plant normal

physiology and vitality, while interaction with beneficial microorganisms positively affects the plant by promoting its growth, increasing nutrient availability and soil quality, and enhancing plant resistance against biotic stresses inducing its defense mechanisms or acting as Biological Control Agents (BCAs) [5] [7].

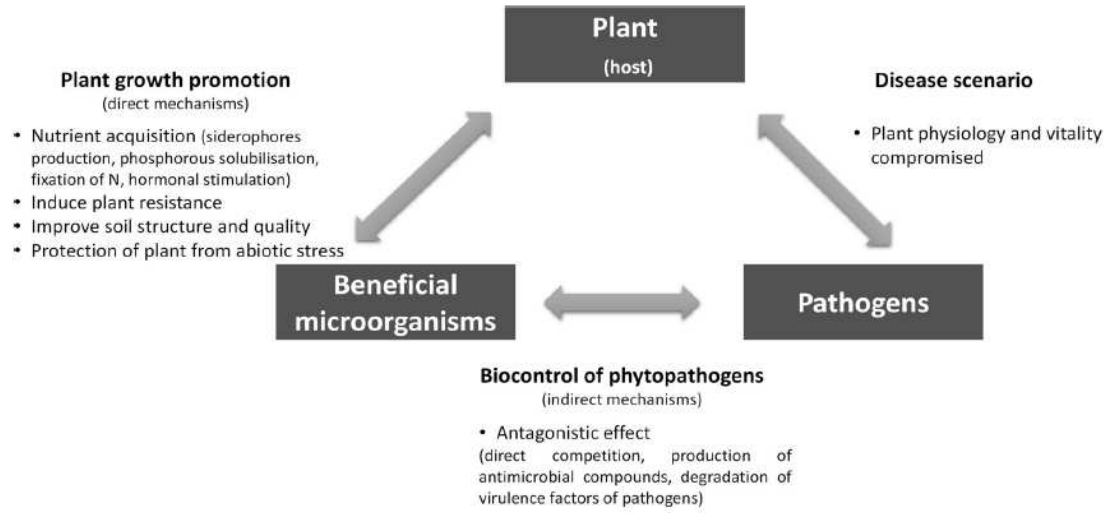

***Figure 1**: Schematic representation of plant-microbial interactions and effect of beneficial and pathogenic microorganisms [5].*

1.3.1 Deleterious microorganisms: plant diseases

Grapevine is exposed to a wide variety of pathogens, such as bacteria, viruses, fungi, oomycetes and nematodes, that can cause important crop losses during both pre- and post-harvest. Grapevine diseases also have a strong social and economic impact and their management requires a massive use of pesticides during the production cycle of the plant [7] [81].

According to their life cycle and infection strategies, pathogenic microorganisms can be classified as necrotrophics, biotrophics and hemibiotrophics. Necrotrophic pathogens feed on dead tissue, secreting lytic enzymes and phytotoxins to promote cell death into the host plant. Biotrophic pathogens, on the other hand, feed on living tissue developing structures in order to invade the cell and obtain metabolism products. Finally, hemibiotrophic pathogens start with a biotrophic infection phase and then turn to a final necrotrophic phase, killing its host at the end of the life cycle [82].

1.3.1.1 Necrotrophic pathogens

Botrytis cinerea is one of the highest broadly studied necrotrophic fungal pathogens. This fungus has the ability to live as a parasite in green tissue and as a saprophyte in

dead or decaying ones. This is the reason for its wide distribution in nature and host unspecificity. Particularly in *V. vinifera*, *B. cinerea* infection causes a disease known as "grey mold", both in pre- and post-harvest period. The fungus can affect complete berry clusters with the appearance of conidia on leaves and fruits [83] [84]. *B. cinerea* infects grapevine by two main mechanisms: (1) direct mycelium penetration through skin pores or injury, and (2) early invasion, where conidia infect mainly the flower receptacle remaining latent within the berry until maturity. When conidium receives nutrients from the host surface, it develops the appressorium that secretes lytic enzymes to cross cuticle and outer epithelial wall and penetrate the cell [81]. Fungicide application remains the common method to control *B. cinerea*. The average costs for chemical *Botrytis* control, for all crops and all countries, is about € 40 ha^{-1} [85]. Fungicides specially targeted against *Botrytis* ("botryticides") cost € 540 million (2021), representing 10% of the world fungicide market [86]. The wine and table grapes segment represent 50% of the value of the total market for botryticides [85].

Other necrotrophic fungal pathogens are *Penicillium* spp., *Alternaria* spp., *Rhizopus* spp., *Aspergillus* spp., *Cladosporium* spp. and *Aureobasidium* spp., that are responsible for secondary rot and grape damages and losses. These fungi are regarded as weak opportunistic pathogens of *V. vinifera*. As for *Alternaria* spp., *Rhizopus* spp. and *Aspergillus* spp., they infect grape berries after a prior wounding event; also, *Penicillium* spp. and *Cladosporium* spp., which produce respectively blue−white molds and dark green velvety molds, occur when grapes have already been damaged [87].

1.3.1.2 Biotrophic pathogens

The most important grapevine biotrophic microorganisms are *Erysiphe necator*, *P. viticola* and the three major recognized worldwide grapevine bacterial pathogens: *Xylophilus ampelinus*, *Xylella fastidiosa* and *Agrobacterium vitis*.

E. necator is the etiologic agent of the grapevine powdery mildew, so called because it appears as a white-grayish powder on the surface of the infected tissue, mostly leaves and stems. *E. necator* conidium attaches to the plant's cells tissue and forms a germ tube that differentiates into a specialized infectious structure (appressorium) that produces a mechanical pressure to penetrate and invade host cells. Finally, thanks to the formation of the haustorium, the fungus absorbs nutrients necessary to complete

its life cycle. Then, secondary hyphae spread along the infected tissue and finally asexual reproductive bodies emerge from them [81].

P. viticola is an oomycete obligate pathogen that causes the grapevine Downy Mildew (DM). The development of the disease happens in the presence of mild temperatures and high humidity. In this condition, all the plant's green organs, like leaves, shoots and unripened berries, can be attacked. When *P. viticola* oospores germinate, they release zoospores which move through the water film and encyst at the stomata on the lower surface of leaves or other green tissues. Then, zoospores germinate and develop the primary hyphae that penetrate the stomata, branch into the intercellular space of the mesophyll and form haustoria. Haustoria spread throughout the leaf parenchyma, making it possible for the pathogen to obtain nutrients, suppress the plant's defense responses, and use the host's metabolism to its favor. The first symptoms of grapevine DM include yellowish spots on the leaves, the so-called oil spots, and the appearance of sporangiophores from the stomata carrying sporangia that led to secondary infections. [81] [88] [89]. On a molecular level, *P. viticola* secrets effectors that play a crucial role in the insurgence of the infection. Among them several studies have been focused on RxLR effectors. The pathogen invasion can induce in grapevine multiple defense reactions, first PAMP-Triggered Immunity and secondly Effector-Triggered Immunity that may lead to hypersensitive response (HR) and programmed cell death (PCD) response in an attempt to control the spread of the infection. *P. viticola* can overcome these defense mechanisms through the secretion of effectors, such as RxLR. Effectors suppress plant immunity by impairing Hydrogen Peroxide (H_2O_2) accumulation, PCD and reducing the transcriptional levels of defense-related genes, allowing the infection to establish and progress. [90] [91] [92].

X. ampelinus enters the grapevine through wounds or natural openings like stomata causing the appearance of longitudinal red streaks on the stems that develop into cankers and necrotic symptoms; leaves can also be infected via the petioles and veins and consequently die [7] [93]. *X. ampelinus* is closely related to grapevine and currently no other host plant of intermediate vector has been described [93].

X. fastidiosa, responsible for Pierce's disease, is a xylem-limited bacterium that grows in warm environments. When the plant is infected, the bacterium creates a biofilm in the xylem vessels composed of pectin, cellulose, hemicellulose and proteins that cause

their occlusion and disrupt water and nutrients flow through them [81] [94]. Consequently, leaves start to dry out, become brown from the tip and margins progressing inward, and finally become necrotic and drop [7] [93]. The spreading of the disease and transmission of the pathogen to new host plants takes place thanks to insect vectors such as sharpshooters, leafhoppers and spittlebugs during xylem sap-feeding [81].

A. vitis causes crown gall, the most widely distributed grapevine bacterial disease [93]. The pathogen infects grapevine at injuries caused by twisting, freeze and other woundings of canes and induces the formation of tumorigenic structures at the site of infection for nutrient uptake [81] [95]. *A. vitis* contains Ti plasmids that carry *vir* genes and T-DNA sequences that allow it to transfer to the plant cell and stably integrate into the host plant's chromosomal genome. The expression of T-DNA genes into the plant cells results in an abnormal division of undifferentiated cells because of hormonal overproduction and the development of tumors of variable sizes following the wounds [7] [95] [93]. Nematodes promote grapevine infection by agrobacterium, however nowadays it is not known whether they can mediate the transmission of the bacterium from one plant to another [93].

1.3.1.3 Hemibiotrophic microorganisms

Among hemibiotrophic microorganisms, soil-borne fungi cause grapevine root diseases and can result in the loss of the whole plant. Grapevine root rots include black foot rot (caused by *Cylindrocarpon* spp.), verticillium wilt (caused by *Verticillium dahlia*), phytophthora root rot (caused by various *Phytophthora* spp.) and armillaria root rot (caused by *Armillaria* spp.) [96].

As for black foot rot, the pathogen invades roots of grapevines and quickly moves into the butt of the rootstock. The vascular tissue turns black and the xylem becomes occluded with fungal tissue, gums and tyloses. *V. dahlia* penetrates the roots in the region of elongation and the cortex is colonized. From the cortex, the hyphae invade the xylem vessels and conidia are formed. Vascular colonization occurs as conidia are transported into the plant along with water. The vascular system becomes plugged with fungal material and host reaction products, preventing water from reaching the upper parts of the plant. Leaves and stems deprived of water then exhibit symptoms of wilting and foliar chlorosis [97].

Phytophthora spp. survive in soil as oospores. The most common way of infection is by indirect germination and zoospore production, which then allows the movement of zoospores in water films, resulting in root or crown infection. Symptoms of the disease include grapevine stunting, decline and death associated with mild to severe root rot and/or crown rot [96] [98].

As for *Armillaria* spp. it infects grapevine roots, killing the cambium and decaying underlying xylem. Symptoms include stunted shoots, dwarfed leaves, wilting, premature defoliation and withering of berries [98].

Several fungal pathogens are also responsible for grapevine trunk diseases. These fungi live in and colonize the wood of the perennial organs causing wood necrosis, wood discoloration, vascular infections, and white decays [99]. Examples of grapevine trunk diseases are Eutypa dieback, Botryosphaeria dieback and Esca disease. Botryosphaeria is caused by fungi belonging to the family of Botryosphaeriaceae, Eutypa dieback by fungus *Eutypa lata*, while fungi *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* and *Fomitiporia mediterranea* are associated with Esca disease [99]. Spores are released from old, infected wood within 2 hours of wetting by rain or irrigation and continue to be released for up to 36 hours after rain has stopped. Occasionally, spores have been detected up to 2 weeks after a rainfall event. Spores are not always detected on days with rainfall, possibly due to the delay of 12 days before a new generation of spores is produced in fruiting bodies, ready for release. Spores of *E. lata* can be carried by wind, while spores of Botryosphaeriaceae are carried predominantly by rain. Spores then land on open wounds and germinate within the woody tissue. The fungi then grow, killing tissue and reducing the transport of water and nutrients to the leaves. Esca disease pathogens spores can germinate on the surface of the wounds or be carried by pruning instruments and penetrate inside the trunk through moist or pruning wounds. Once inside the plant, Esca disease pathogens and *E. lata*, can produce enzymes that degrade lignin, cellulose, and hemicellulose, making the wood soft and friable [100].

1.3.2 Beneficial microorganisms

Grapevine is also colonized by a wide variety of beneficial rhizospheric and endophytic microbes, which can promote plant growth and health. Among the beneficial microorganisms, arbuscular mycorrhizal fungi (AMF) and plant-growth-

promoting bacteria (PGPB) can be recognized [7]. PGPB are good biofertilizers because of their ability to make mineral nutrients bioavailable to the grapevine. Indeed, the mineral elements that the plant needs, such as phosphorus, nitrogen and phosphate, are naturally present in the soil but cannot be efficiently or directly assimilated by plants because they are complexed with other molecules. [30] [13]. Some PGPB produce enzymes or organic acids that transform organic phosphorus into soluble ionic phosphate. Other PGPB produce ammonium or convert nitrites to nitrates, that are easily absorbed by the plant, and can produce microbial siderophores, which bind with iron, and contribute to plant iron nutrition. [101] [102] [103]. These properties have been found in different bacteria genera associated with different grapevine compartments including *Pseudomonas, Enterobacter, Arthrobacter, Bacillus, Pantoea, Micrococcus* [7] [30] [105]. PGPB can also produce hormones that directly stimulate plant growth [30]. Also, some fungi are involved in phosphate solubilization, for example, those belonging to *Mortierella* genus [74] [72]. AMF associated with roots increase the exploitation of the soil and play an important role in water and nutrient absorption by activating phosphorus and nitrogen transporters in root cortical cells. Also, hyphae increase the exchange surface between grapevine roots and soil. The result is a better fitness of the plant and the increase of its aerial and root biomass [7] [30] [106]. Some endophytic bacteria limit the damage caused by biotic and abiotic stresses through the synthesis of ACC deaminase [30] [59]. In stressful environmental conditions, such as salt, drought, waterlogging, heavy metals, and pathogenicity, there is an increase in plants in the production of the stress hormone ethylene that may encourage defoliation and other cellular processes that lower crop production [107]. The enzyme ACC deaminase cleaves the ethylene precursor (1-aminocyclopropane-1-carboxylic acid), decreases ethylene concentration in plants enhancing their salt tolerance and mitigating the effects of drought stress [107] [108]. Bacteria genera that have been found to possess ACC deaminase activity are: *Achromobacter, Bacillus, Enterobacter, Pseudomonas and Rhizobium* [107] [109]. Some endophytes can produce compounds that intervene in reactive oxygen species (ROS) scavenging other protective molecules, such as melatonin, proline, carotenoids and volatile organic compounds (VOCs) which play an active defense role against abiotic stress [108]. Other PGPB, and some endophytic fungi, have a biostimulant effect through the synthesis of phytohormones that favor plant growth. PGPB have been found to produce indole-3-acetic acid (IAA), cytokines and abscisic acid (ABA) that promote

lateral root development, cell division, cell enlargement, and tissue growth, stimulate the activity of apical and axillary, facilitate cell division in the shoot. [107] [108] [110]. Bacteria genera showing biostimulant properties include *Bacillus, Pseudomonas, Enterobacter, Micrococcus* [7] [107] [108].

Some of these microbes can act as BCAs, thanks to their ability to prime plant immunity, known as Induced Systemic Resistance (ISR) and to their antagonism against pathogens.

1.3.2.1 Induced Systemic Resistance (ISR)

Grapevines' first line of defense against pathogens is represented by pattern-triggered immunity (PTI) or PAMPs-triggered immunity that becomes active when pathogen-associated molecular patterns (PAMPs), molecular structures of pathogens such as fungal chitin or bacterial flagellin, or damage-associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRRs). PTI activates numerous signaling pathways in the host cells. One of the rapid responses is an influx of extracellular Ca(2+) into the cytosol, followed by the activation of mitogen-activated protein kinases, ROS signaling, and other signaling molecules, such as ROS, lipids, callose, salicylic acid, n-hydroxypipecolic acid, jasmonic acid, ethylene, and cytokinin. To overcome PTI many pathogens secrete effector proteins to which the plant responds through a second layer of immune responses called effector-triggered immunity (ETI) resulting from the direct recognition of the effectors by nucleotide-binding (NB) and leucine-rich-repeat (LRR)-containing receptors (NLRs), or the indirect one, triggering downstream defense signaling pathways and responses including hypersensitive response (HR), a rapid and local cell death. [81] [111] [112].

Biological or chemical factors, including beneficial microorganisms, can determine the onset of induced resistance, a physiological "state of enhanced defensive capacity", against a broad spectrum of attackers. Indeed, like in pathogens, microbe-associated molecular patterns (MAMPs) or host-derived DAMPs are associated with beneficial microorganisms and the perception of these triggers the activation of the plant's immune response. Induced resistance is expressed locally at the induction site and systemically in plant parts spatially separated from the inducer [113] [114]. Induced resistance relies on signaling pathways regulated by plant hormones and the two most clearly defined forms of induced resistance are systemic acquired resistance (SAR), and induced systemic resistance (ISR), which can be differentiated based on the nature

of the elicitor and the regulatory pathways involved [115]. SAR relies on the activation of pathogenesis-related (PR) genes and accumulation of pathogenesis-related proteins, some of which have antimicrobial activity, and salicylic acid pathway. SAR initiation in distal organs takes place thanks to proteins acting as chaperones for the mobile SAR signals and metabolites like the methyl ester of salicylic acid (MeSA), diterpenoid dehydroabietinal (DA), a glycerol-3-phosphate (G3P)-dependent factor, azelaic acid (AzA), and pipecolic acid (Pip) involved in SAR long-distance signaling. [114]. ISR is activated by beneficial microbes, does not require pathogenesis-related proteins and is regulated by a network of interconnected signaling pathways in which the phytohormones jasmonic acid and ethylene play a major role. ISR leads to an increase in the level of basal resistance to several pathogens simultaneously through the potentiation of defense-related gene expression and augmentation of structural barriers [114] [115] [116]. As a result, the plant is in a primed state, meaning that it is prepared to better combat pathogen attack and is characterized by a faster and/or stronger activation of cellular defenses upon invasion, resulting in an enhanced level of resistance [114].

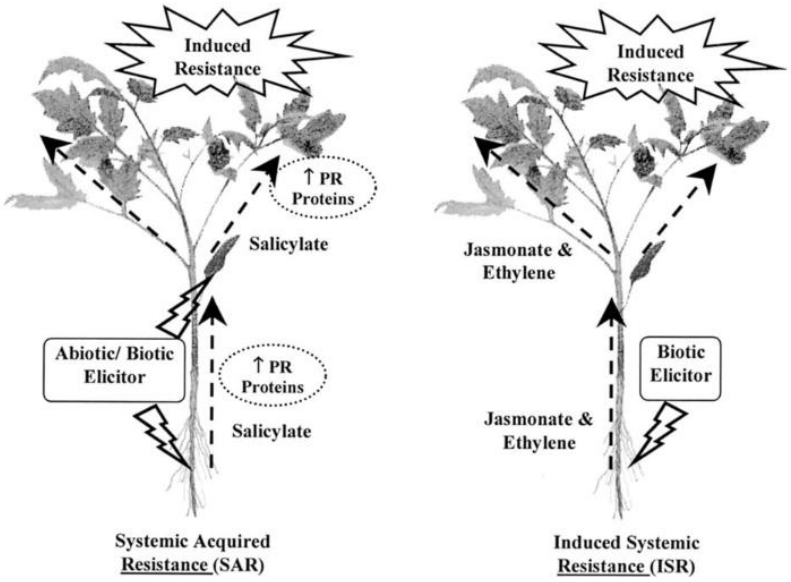

***Figure 2**: Representation of the two best characterized forms of induced resistance in plants. SAR resistance is dependent on the salicylic acid, and associated with the accumulation of pathogenesis-related (PR) proteins. ISR is dependent on the phytohormones ethylene and jasmonic acid and is not associated with the accumulation of PR proteins [113].*

There is a lot of current knowledge about MAMPs/DAMPs associated with beneficial microorganisms that trigger immune response in grapevine. Varnier and co-authors [117] demonstrated that rhamnolipids, produced by the bacteria *Psudomonas*

aeruginosa, act as MAMPs in grapevine, because from their perception several signaling events, such as the Ca(2+) influx, mitogen-activated protein kinase activation and ROS production, were activated. Furthermore, effective plant defenses against *B. cinerea* were induced, including the expression of a wide range of defense genes and a HR-like response. Lakkis and co-authors [118] studied the ability of endophytic bacterium *Psudomonas fluoresces* PTA-CT2 to induce ISR in grapevine against *P. viticola* and *B. cinerea* by using two cultivars differing in their susceptibility to DM (Pinot noir, as susceptible and Solaris as partially resistant). Their results demonstrated thar PTA-CT2 induced ISR against *P. viticola* and *B. cinera* by priming common and distinct defensive pathways. After *P. viticola* challenge, PTA-CT2 primers salicylic acid (SA)-related genes in both cultivars and HR-related gene in Solaris and abscisic acid (ABA) accumulation in Pinot noir. Against *B. cinerea* ISR was associated with downregulation of HR-related gene and accumulation of ABA and phytoalexins. *Streptomyces anaulatus* S37 isolated from wild *V. vinfera* is an endophytic PGP rhizobacterium (PGPR) that confers resistance against different pathogens, including *B. cinerea* [119]. It was demonstrated that *S. anulatus* S37 perception by grapevine cells tiggers early and late defence responses, such as ion fluxes, oxidative burst, extracellular alkalinization, activation of protein kinases, conduction of defence gene expression and phytoalexin accumulation. Moreover, *S. anulatus* S37-primed grapevine cells became refractory to infection by *B. cinerea*, showing a reduction in pathogen-induced cell death [120]. The biocontrol activity of *Bacillus subtilis* PTA-271 was demonstrated by Trotel-Aziz and co-authors [121] against Botryosphaeria dieback, caused by the fungus *Neofusicoccum parvum*. This fungus produces two main toxins (-)-terremutin and (R)-mellein that may suppress grapevine immunity and promote Botryosphaeria dieback symptoms. Interestingly, the beneficial bacterium *B. subtilis* PTA-271 not only antagonizes the pathogen but also primes host immune response and detoxifies both fungal phytotoxins.

1.3.2.2 BCAs and antagonistic activity against pathogens

The direct antagonistic activity of BCAs can manifest itself through three main mechanisms of action: 1. competition for space and nutrients.; 2. hyperparasitism; 3. antibiosis (production of enzymes and/or antibiotic metabolites, including volatile organic compounds) [122].

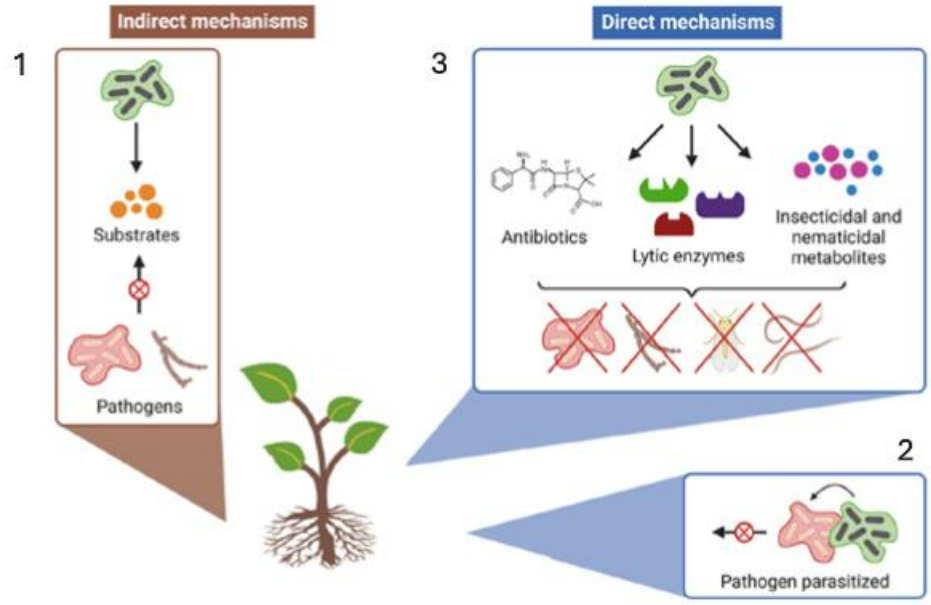

***Figure 3**: Main mechanisms of action of BCAs. 1. Competition; 2. Hyperparasitism; 3. Antibiosis. Modified from [123].*

1.3.2.3 Competition

Potential BCAs must be able to occupy plant tissues and rapidly consume nutrient sources essential for pathogen infection such as sugars, pollen and plant exudates on plant surfaces and in plant residues so that outcompeted pathogens will not be able to infect the host. In this way, the pathogen population will decline but will not be killed by the antagonist [124]. For example, yeasts can put into effect this mechanism of action because can rapidly invade specific niches, fastly multiply and consume a broad range of carbohydrates and nitrogen sources [125]. Some BCAs also act by producing a variety of low-molecular-weight siderophores with a high affinity for ferric iron that bind to it and subtract it to pathogens [126]. This mechanism has been investigated for isolates of *Pseudomonas* spp. and some fungi. [124] [127]. Iron depletion results effective in inhibiting development of pathogens like *B. cinerea*, *A. alternata.* Competitive antagonists may modulate growth conditions for the pathogen in the targeted niche not only through nutrient depletion but also by other mechanisms, for example, modulating leaf wetness periods and pH through the secretion of specific compounds. [124] [128].

1.3.2.4 Hyperparasitism

Parasitism is defined as the competitive interaction between two organisms in which one of them gains nutrients from the other. The interaction is defined as hyperparasitism when the host is a parasite, for example, a plant pathogen. This type

of interaction usually exists between fungi [124]. Hyperparasites can be biotrophic or necrotrophic. In the first case, the hyperparasite depends on the living host fungus and gains nutrients from the host cells via haustoria without killing the host; in the second case, the hyperparasite gains nutrients from dead host cells through the invasion of host spores or hyphal cells. Indeed, the hyperparasite secretes secondary metabolites, such as proteases and chitinases, that encounter the host cells leading to openings in the cell wall, degradation and subsequent disorganization of the cytoplasm [124] [129].

1.3.2.5 Antibiosis

Microorganisms can produce secondary metabolites belonging to heterogeneous groups of organic, low-molecular weight compounds that are deleterious to other microorganisms' growth or metabolic activities [124]. These molecules are released in small quantities in the environment and, currently, different microbes produce a great number of known antibiotics: actinomycetes (8700 different antibiotics), bacteria (2900) and fungi (4900) [124] [130]. The production of antimicrobial metabolites represents the most potent mechanisms of action of microbes against competitors since it gives them an advantage in resource-limited environments. Different bacteria genera have been identified as producers of antimicrobial metabolites with broad-spectrum activity such as *Agrobacterium*, *Bacillus*, *Pantoea*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Streptomyces*, and many other genera [124] [127]. The *Bacillus* genus has been studied in detail for the role of its secondary metabolites and especially the antimicrobial activity of lipopeptides (LPs), such as, iturin, surfactin, and fengycin has been investigated, together with the polyketide antibiotics bacillaene, difficidin, and macrolactin [124] [127]. Indeed, *Bacillus*-derived LPs have a great biological control potential against a broad range of agronomically important fungal pathogens. LPs through their fatty acid moiety can interact with the plasma membrane lipid bilayer of the pathogenic microorganisms, by inducing the formation of pores and ion channels, causing osmotic imbalance and leading to cell death. In particular, the fungitoxic activity of lipopeptides is related to the permeabilization of spore/conidia that results in membrane damage and inhibition of germination and hyphal cell perturbation [131] [132].

1.3.3 BCAs against V. vinifera pathogens

The use of BCAs in agriculture began in the second half of the 20th century and different candidates have been identified. In particular, *B. subtilis* is one of the most widely used biological fungicides that exerts its antagonistic activity against multiple pathogens such as *B. cinerea* and *E. necator* through antibiosis and resistance induction [133] [134]. While *Bacillus thuringensis* is used to control insects and mites [133]. Some *Streptomyces* strains show antagonist activity against *Fusarium* spp. through inhibition of fungal growth, normal branches and conidia of conidiophores, malformation and hyphae lysis [47]. *Pseudomonas* strains can act as BCAs against a wide range of plant pathogens, including *Agrobacterium tumefaciens* [135]. Also, the genus *Lactobacillus* has been investigated for its antimicrobial properties and one recent work pointed out the ability of one strain of *Fructobacillus fructosus* and one of *Lactobacillus plantarum* to counteract the growth on grapes of *B. cinerea* and *A. niger* [104]. Moreover, fungi belonging to *Trichoderma* genus, are reported as BCAs for pathogens responsible for Esca disease, acting via substrate competition, antibiosis, and mycoparasitism, and Eutypa Dieback, inhibiting mycelial growth through volatile and nonvolatile metabolites [133] [136]. AMF promote a better tolerance of grapevine to biotic stress and *Rhizophagus intaradices* could reduce the detrimental effects of the pathogenic fungus *Armillaria mellea.* [7] [137]. Furthermore, non-*Saccharomyces* yeasts, such as, *Rhodotorula glutinis*, *Rhodotorula mucilaginosa*, *Candida oleophila*, *Pichia* spp. *Metschnikowia* spp. have been discovered and patented as BCAs against post-harvest pathogens like *Penicillium* spp. and *Aspergillus* spp. [138]. Currently, there is a lack of commercial microbial biopesticides active against *P. viticola*. Some microorganisms have been investigated for their potential antagonistic activity against *P. viticola,* for example *Trichoderma harzianum* acts against the oomycete by increasing lignin, callose, and hydrogen peroxide, in addition to upregulating the defense enzymes phenylalanine ammonia-lyase, peroxidase, and 1,3-glucanase [139]. Also, *Ochrobactrum* sp. strain SY286, isolated from asymptomatic DM infected grapevine leaves, showed strong inhibitory activity against *P. viticola* [140]. In another study, among 239 tested bacterial endophytes isolated from grapevine leaves, two endophytic strains, identified as *B. subtilis* GLB191 and *Bacillus pumilus* strain GLB197 have proved their preventive effects for *P. viticola* both on leaf disks and during 2-year field trials [141]. Endophytic bacterium *Bacillus altitudinis* GLB197 isolated from grapevine leaves showed significant inhibition of DM both in leaf disk

test and in field experiments [142]. Also, endophytic fungi *Acremonium persicinum* and *Acremonium sclerotigenum*, isolated from asymptomatic grapevine, showed a hyperparasitism on *P. viticola* [143] [144] [145] and *E. nigrum*, isolated from *P. viticola*-infected leaves, inhibited sporangial germination [146] [147]. The management of *P. viticola* particularly requires the development and use of BCAs because of the occurrence of resistance in its populations resulting from the great use of chemical pesticides and synthetic fungicides [140].

1.3.4 BCAs application

BCAs can be applied to plants through different methodologies according to different factors such as: the mode of action of the BCA, the plant growth stage and the type of formulation. For instance, the method of application of wettable powders, water-dispersible granules, and liquid formulations differs from dry powders and granules. BCAs active against seed-borne and soil-borne pathogens are usually applied to the seeds in the form of powders and granules that, together with adhesive materials, constitute a coating that envelops the seeds. Differently, liquid inoculants are sprayed onto the seeds. Aerial application of BCAs is particularly indicated to protect plants from above-ground pathogens using spray equipment such as aerosol which represents a very effective application method. BCAs can also be directly applied to the soil usually in the formulation of granular inoculants, wettable powders, water-dispersible granules or liquid inoculants. Moreover, through drip irrigation and hydroponic systems, BCAs, especially when in liquid formulations, can be directly applied to the plant root zone [225]. Another application strategy involves microbial encapsulation using polymeric membranes which guarantees several advantages including an improved microenvironment for microbial survival, physical protection for a prolonged period to prevent a rapid decline of introduced inoculants, and increased shelf life [226] [61]. Soil application is commonly used when large populations of BCAs need to be introduced to the soil. Indeed, BCAs can be used in single-strain formulations or in the form of microbial consortia composed of multiple compatible beneficial microbes, having a better chance to survive and provide benefits to the host [226] [227].

1.4 Plant-associated bacteria as a rich source of bioactive molecules: biotechnological applications

In the last decade, plant sources have been explored extensively for novel chemical metabolic entities. Indeed, associated microbes produce a great variety of secondary metabolites belonging to different functional classes comprising terpenoids, alkaloids, steroids, phenolics, chinones, tannins, saponins, xanthones, tetralones, and others that are evolutionary relevant and strictly related to survival and adaptation. Approximately 80% of natural products are candidate drug molecules for the pharmaceutical industries, which is why plant-microbe associations represent a new field in generating bioactive compounds, with significance in drug discovery programs and healthcare. Microbes-produced secondary metabolites display a wide range of activities such as antitumoral, antidiabetic, antimicrobial and efficiency in bioremediation [148] [149].

Focusing on bacteria, different *Streptomyces* species and strains were reported to be able to produce molecules having antitumoral activity. For example, *Streptomyces* strains associated with *Trewia nudiflora* produced rifamycin B and geldanamycin having cytotoxic activity [150]. *Streptomyces* sp. strain Is9131 and *Streptomyces laceyi* strain MS53, respectively isolated from *Maytenus hookeri* and *Ricinus communis* inhibited human SGC7901 gastric, HL60 leukemia, BEL7402 liver, and A-549 lung tumor cell lines growth and human breast cancer cell line SKBR3 through the secretion of cytotoxic molecules maytansine and salaceyins. [151] [152]. Recently, biphenyls-producer *Streptomyces* sp. strain BO-07 and *Streptomyces cavourensis* strain YBQ59, isolated from *Boesenbergia rotunda* and *Cinnamomum cassia* showed antitumoral activity against human HepG2 and Huh7 liver cell lines, and HeLa cervical tumor cell lines and human lung adenocarcinoma EGFR-TKI-resistant cells A549 [153] [154]. Also, other genera such as *Actinomyces, Actinoplanes, Amycolatopsis, Micromonospora, Saccharopolyspora* are recognized as producers of molecules with antitumoral potential activity. For example, exopolysaccharides produced by *Bacillus amyloliquefaciens* isolated from *Ophiopogon japonicus* were reported to possess antitumor activity against the human gastric carcinoma cell lines MC-4 and SGC-7901 [155] [156].

The plant-associated microbial community also comprehends microorganisms that are effective in bioremediation and able to remove organic pollutants, hydrocarbons,

and heavy metals [148] [157]. Several species of bacterial endophytes have been found to degrade environmental contaminants. Different *Bacillus* species and strains are reported as good bioremediation agents. For example, *B. safensis* ZY16, *Bacillus* sp. SBER3, *B. pumilus* E2S2, *Bacillus* sp. E3 *B. thuringiensis* GDB-1, *Bacillus* sp. L14, *B. cereus,* isolated from *Chloris virgata, Populus deltoides, Sedum plumbizincicola, Pinus sylvestris, Solanum nigrum, Phragmites communis*, were reported to be involved in phytoremediation of oil and arsenic contaminated soils, biodegradation of polyaromatic hydrocarbons (PAH), bioremediation of heavy metals, degradation of chlorpyrifos and bifenthrin in polluted water, bioremediation of petroleum hydrocarbons. [158] [159] [160] [161] [162] [148]. Also, endophytic *Pseudomonas* species showed bioremediation properties: *P. fluorescens* G10, *P. aeruginosa* L10, *P. putida* W619-TCE were able to perform phytoextraction of lead (Pb), trichloroethylene degradation and biodegradation of petroleum hydrocarbons [163] [164] [165].

Plant-symbiontic microorganisms and their secondary metabolites have been taken into consideration in the perspective of finding new compounds active against antibiotic-resistant bacteria which represent a public health threat that has rapidly spread over decades due to the continuous and uncontrolled administration of antimicrobial medicines, becoming an increasing worldwide concern [149]. In particular, multidrug-resistant 'ESKAPE' organisms (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella* spp., *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) are strictly associated with high rates of morbidity and mortality, as well as an economic impact [149] [166]. The *Streptomyces* genus stands out from other microorganisms considering that more than 80% of the industrially produced antibiotics are processed by this bacterial genus [149] [167]. Numerous metabolites from endophytic *Streptomyces* species have been characterized and associated with antibiotic activity, such as kakadumycins, munumbicins, *p*-aminoacetophenonic acids, and xiamycins [168] [169] [170]. Another genus strongly associated with the production of secondary metabolites having antibacterial activity is that of *Bacillus* and different endophytic *Bacillus* species, for example, *B. atrophaeus, B. mojavensis, B. subtilis, B. thuringiensis, B. licheniformis, B. cereus* were reported to produce metabolites active against human pathogens. These bioactive molecules belong to different chemical classes such as: butanoic acid

derivates, diketopiperazines, proteins, phthalates, alkaloids, lactones and act as antibacterial against pathogenic bacteria *Klebsiella pneumoniae, Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* [171] [172] [166] [173] [174]. In light of the rapid rise of antibiotic resistance, endophytic bacteria represent a field that is worth exploring in this quest for alternative antibiotic agents and the increasing understanding of plant-microbes associations will also led to an increasing knowledge of the primary and secondary metabolites that these bacteria produce, their bioactive properties and how to benefit from them.

1.5 Aim of the study

The aim of this work, as part of the REVINE project, is to explore the microbiome of *V. vinifera* leaves, with a particular focus on bacteria, epiphytic and endophytic, in order to identify microorganisms with antimicrobial properties and the potential to be used as biocontrol agents and provide biological alternatives to chemical pesticides currently used in viticulture. In particular, DM caused by *P. viticola* was selected as a case study to identify potentially effective BCAs. Nowadays, to avoid yield losses, disease control is commonly carried out by using chemical products that are harmful to the environment and human and animal health. Because of that, the identification of new BCAs results an urgent necessity. Different *V. vinifera* cultivars, having different degrees of tolerance to *P. viticola*, were chosen as niches for the isolation of the epiphytic and endophytic bacteria colonizing their leaves. Selected bacterial strains were assessed through "*in semi-vivo"* tests on leaf discs to evaluate their potential antagonistic activity against *P. viticola* and further characterized for their mechanisms of action through *in vivo* tests on leaves. Bacterial strains were further investigated for some of the features that good biocontrol candidates should have, in particular, effectiveness against a wide range of pathogens and safety for human health. Moreover, a molecular characterization of the selected bacterial strains was performed with a further assessment of two of them for their antibacterial properties. The final goal is to promote sustainable practices in viticulture and valorize the autochthonous microbiome of *V. vinifera* identifying useful microorganisms to cope with biotic stresses.

2. MATERIALS AND METHODS

2.1 Selection of niches suitable for the isolation of microorganisms

Based on the results of previous works, in which a fair amount of success in terms of findings BCAs starting from genotypes tolerant to the target disease was reported [138] [174] we decided to select the niche for the microorganism isolation based on the level of tolerance against DM shown by different cultivars of *V. vinifera*. As a first step, we analyzed data relating to the degree of infection in bunches of 50 table grape varieties, collected at CREA-VE of Turi (BA) during the 2014 and 2018 productive years. Specifically, the bunches present on 5 plants per cultivar were divided and counted on the basis of the severity of symptoms, according to an empirical scale from 0 to 5 (where: 0=totally healthy bunch, 1=infection between 5 and 20% of the total surface of the bunch, 2= infection between 25 and 40% of the total surface of the bunch, 3= infection between 45 and 60% of the total surface of the bunch, 4= infection between 65 and 80% of the total surface of the bunch, 5= infection between 65 and 100% of the total surface of the bunch). The average incidence of the disease, as the percentage ratio between the number of infected bunches and the total number of bunches, and the McKinney index [175] expression of the average disease severity, were calculated for each cultivar. A cluster analysis of collected data was then carried out to observe any differences between the tested varieties. Based on the results of the cluster analysis, 19 of the 50 previously studied varieties were selected; 4 highly susceptible (‘Dawn seedless’, ‘Regal seedless’, ‘Supernova’ and ‘Black Pearl’), 8 moderately susceptible (‘Paula’, ‘Delight’, ‘Centenial’, ‘Sugraone’, ‘Blush’, ‘Pizzutello’, ‘Autumn seedless’ and ‘Melissa’) and 7 poorly susceptible (‘Argentina’, ‘Ruby seedless’, ‘Duca di Magenta’, ‘Queen Rs’, ‘Crimson seedless’, ‘Inzolia Imperiale’ and ‘Perlon’). For each selected cultivar, grafted vines (rootstock 1103 Paulsen) were realized, grown in pots in the absence of specific anti-DM treatments and then analyzed for their degree of tolerance/susceptibility to infection caused by *P. viticola* at the leaves. An artificial inoculation bioassay in environmentally controlled condition, using leaf discs was performed [224]. 114 fully developed leaves (six for each cultivar) were detached and kept at 21°C for two hours in the presence of light to allow the stomata to open. Seven leaf discs (21 mm diameter) were excised from each leaf using a cork borer, for a total of 399 leaf discs. Leaf discs were repeatedly washed under tap water to remove superficial materials, dried under a laminar flow hood and finally placed, abaxial side up, in Petri dishes on filter paper wetted with 3 ml of sterile

demineralized water. Each leaf disc was inoculated with 50 µl of a 2,5 x 10^4 sporangia ml^{-1} suspension of *P. viticola*. The DM pathogen *P. viticola* was isolated from naturally infected leaves of three-year- old new table grape genotypes, obtained in the breeding programs of CREA-VE, grown in an untreated vineyard in Turi (BA), Apulian region, Italy. The sporulation of the pathogen was collected using a vacuum pump and stored at -20°C [146]. The *P. viticola* inoculum was prepared by resuspending about 1 g of powder with distilled water. The sporangial suspensions were then adjusted to the desired concentration using a hemacytometer under a light microscope. Then, plates containing inoculated leaf discs were kept overnight in the dark at 21°C for 4 hours and then subjected to a 16-h photoperiod. After 24 hours from the inoculation, the inoculum was removed and 5 days after the inoculation the phenotypic evaluation of the leaf discs was carried out, using an empirical scale from 0 to 4 (Figure 4).

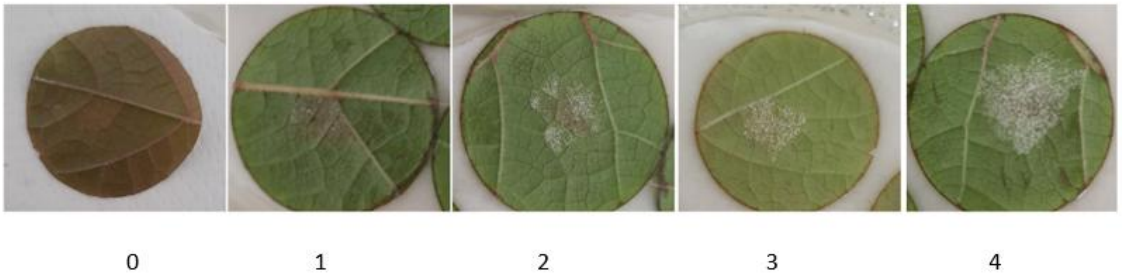

Figure 4*: Empirical scale used to phenotypically evaluate the foliar discs 5 days after the inoculation with 50 µl of a suspension of zoosporangia of P. viticola concentrated 2,5 x 104 CFU ml^{-1}, where 0= no infection, 1= slight necrosis, 2= limited sporulation with necrosis spots, 3= compact sporulation covering the inoculation site, 4= diffuse sporulation beyond the inoculation site.*

For each variety, we calculated:

- the average Disease Incidence (DI), as percentage ratio between the number of symptomatic leaf discs and the total of inoculated leaf discs;

- the average Disease Severity (DS), calculated using the McKinney's formula [175].

Collected data, together with those related to the susceptibility/tolerance to the infection on the bunches, were subjected to cluster analysis.

2.2 Isolation of bacterial strains

Three table grape varieties, 'Dawn seedless', 'Argentina' and 'Blush' were selected based on their tolerance or susceptibility to DM, both at the carposphere and phylloplane level. Ten grams of leaves, distinguished into old and young leaves, were collected from each variety. The leaves were cut (Figure 5b) and shaken for 30 minutes

in flasks containing 100 mL of Ringer's Solution (Figure 5c) (1L: NaCl 2,25 g, KCl 0,01 g, $CaCl_2$ 0,12 g, $NaHCO_3$ 0,05 g, Tween20 4 drops; pH 7) to dissolve the microbial component on the surface and inside the leaves. Three dilutions (1:10, 1:100, 1:1000) of the leaf solution were prepared (Figure 5d) and 100 µl from each one were collected and aseptically plated on Nutrient Agar (NA) (Peptone 5 g, Yeast extract 2 g, NaCl 5 g, Agar 16 g; pH 6,8) a general-purpose substrate for the cultivation of a wide variety of bacteria. After that, dilutions were incubated in a water bath at 80°C for 10 minutes to select bacteria belonging to *Bacillus* genus, able to survive at high temperatures, and again 100 µl from each one were collected and aseptically plated on Nutrient Agar (NA). Plates were incubated for three days at 25°C. Well-developed bacteria colonies were grouped based on their color and morphology. Representative colonies for each group were selected, grown on liquid Nutrient Broth (NB) (Peptone 5 g, Yeast extract 2 g, NaCl 5 g; pH 6,8) and then stored at -80°C in liquid NB with 15% (v/v) of glycerol.

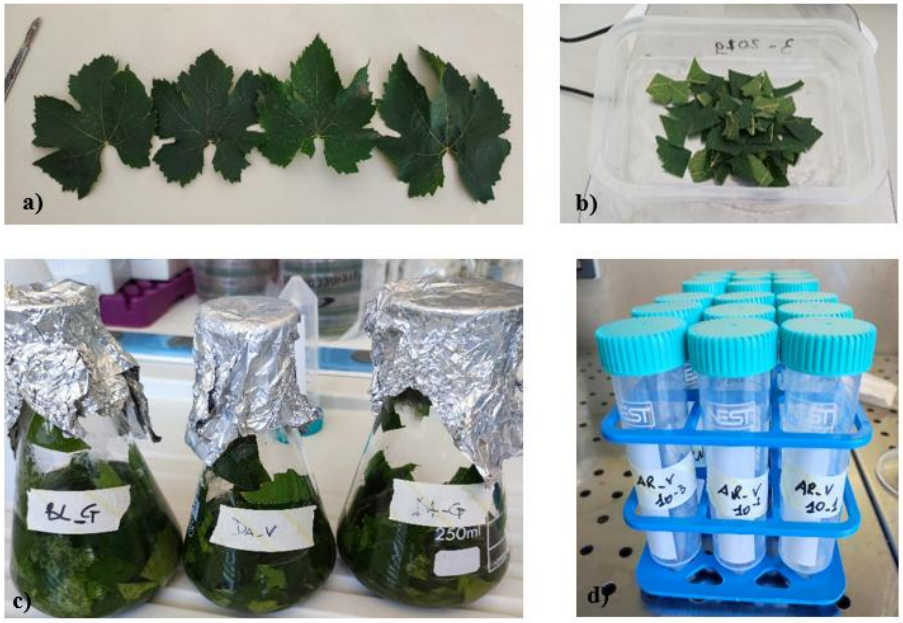

Figure 5*: Procedure for bacterial strains isolation: a) Leaves collected from the selected varieties; b) Box containing 10 grams of cut leaves; c) Flasks containing cut leaves in Ringer's solution; d) Dilutions of the leaves solution.*

2.3 Screening on leaf discs for antagonistic activity against P. viticola

Healthy leaves of susceptible cultivar 'Flame' were used to perform a leaf disc assay. Leaves were incubated at 21°C for two hours in the presence of light to allow the stomata to open. A total of 357 leaf disks, each having a diameter of 21 mm were cut out from the leaves using a cork borer. Their surface was sterilized by dipping in

sodium hypochlorite (3.5% active chloride) solution for 30 sec., washed in sterile water two times and then air dried. A total of 21 leaf disks (seven for each replicate) were soaked in 100 mL of a bacterial suspension for 30 min (Figure 6b). The fermentation broths of 16 bacterial isolates were prepared in Luria Bertani (LB) (1L: Tryptone 10g, Yeast extract 5g, NaCl 10 g) liquid medium in a shaker at 180 rpm for 48 hours at 25°C and diluted to 10^7 CFU mL^{-1} with sterile LB (Figure 6a). The same number of leaf disks soaked in sterile LB were used as controls. All the leaf disks were then transferred to Petri dishes with two-layer wet paper, closed with Parafilm to retain moisture and then incubated at 21°C for 48 h. 50 µL of *P. viticola* inoculum at the concentration of 2.5 x 10^4 sporangia mL^{-1} was applied onto the abaxial surface of each leaf disc as previously described (Figure 6d). Inoculated leaf disks were subsequently kept overnight in the dark at 21°C for 4 hours and then subjected to a 16-h photoperiod.

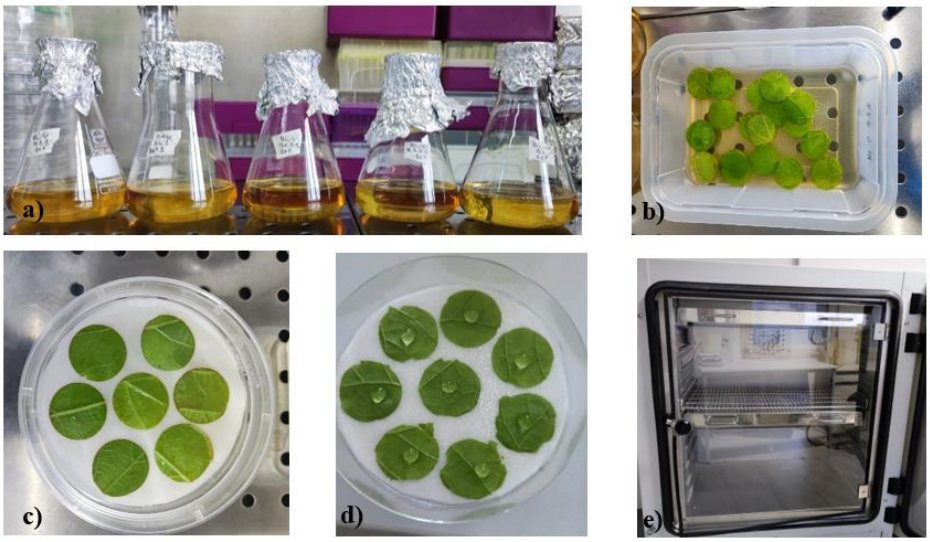

Figure 6*: Assay on leaf discs for the evaluation of the antagonistic activity of bacterial isolates against P. viticola: a) Flasks containing 10^7 CFU mL^{-1} bacterial suspensions; b) Box containing leaf discs soaked in bacterial suspension; c) Leaf discs placed in Petri dishes containing discs of moistened absorbent paper; d) Leaf discs inoculated with 50 µL of P. viticola inoculum at the concentration of 10^5 sporangia mL^{-1}; e) Incubator set at 21°C for 4 hours and 16-h photoperiod.*

The average percentage ratio of the leaf disc area covered by *P. viticola* sporulation was assessed 6 dpi, by using the graphical software ImageJ and the effectiveness (%) of each bacterial isolate to control DM was calculated using the following formula [176]:

$$E(\%) = (1-T1/C1) \times 100$$

where:

T1 = the average percentage ratio of the leaf area covered by *P. viticola* sporulation in treated leaf discs

C1 = the average percentage ratio of the leaf area covered by *P. viticola* sporulation in untreated leaf discs

2.4 Morphological and molecular identification of selected bacteria

Bacterial isolates 'BLG_B1.1.1', 'BLG_B1.3', 'BLG_B2', 'BLG_B4', 'BLG_B5' were selected to be further characterized. The morphological features of bacterial colony, such as size, shape, morphology of the edges, surface, appearance and color, were annotated and their molecular identification was performed through whole-genome-based taxonomic analysis following genome sequencing.

2.4.1 Genome sequencing

Genomic DNA of bacterial isolates 'BLG_B1.1.1', 'BLG_B1.3', 'BLG_B2', 'BLG_B4', 'BLG_B5' was extracted through the phenol-chloroform procedure obtaining samples having an amount ≥ 100 ng, a volume ≥ 20 µL, concentration ≥ 5 ng/µL and an OD260/280 = 1.8-2.0. Extracted DNA was sequenced at the Novogene Bioinformatics Technology Co., Ltd using Microbial Whole Genome Sequencing Service. The first step of the workflow involved samples quality control (Sample QC) where samples were evaluated for their quantity, integrity and purity through Agilent 5400 (Agilent). The second step was the library preparation. Genomic DNA samples were fragmented into short fragments. These DNA fragments were then end-polished, A-tailed, and ligated with full-length adapters for Illumina sequencing before further size selection. PCR amplification was then conducted. Purification was performed using the AMPure XP system (Beverly). The resulting library was assessed on the Agilent Fragment Analyzer System (Agilent) and quantified to 1.5 nM through Qubit (Thermo Fisher Scientific) and qPCR. The qualified libraries were pooled and sequenced on the Illumina platform carrying out a short read, paired-end 150 bp sequencing. The original fluorescence image files obtained from the Illumina platform were transformed into short reads (Raw data) by base calling and these short reads were recorded in FASTQ [179] format, which contains sequence information and corresponding sequencing quality information.

2.4.2 Sequencing data assembly and whole-genome-based species identification

Raw sequencing data in FASTQ format (R1 and R2) were uploaded to the Galaxy platform (https://usegalaxy.org/) for assembly. Assembly was performed using the Shovill tool, with the trimming option enabled to enhance data quality and remove low-quality sequences. The resulting assembled genome, in FASTA format containing multiple contigs, was used for downstream analyses. To identify the species based on whole-genome data, the assembled FASTA file was uploaded to the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de), a bioinformatics platform for whole-genome-based taxonomic analysis. TYGS employs a comprehensive methodology, including genome-wide comparisons and phylogenetic inferences, to determine species-level classifications. Briefly, closest type strain genomes were identified through two complementary approaches: MASH analysis, which approximates intergenomic relatedness to find the ten closest type strains and 16S rRNA gene-based BLAST searches using sequences extracted via RNAmmer, identifying type strains with the best matches for further precise distance calculations using the Genome BLAST Distance Phylogeny (GBDP) approach under the 'coverage' algorithm and distance formula d5. Accurate intergenomic distances among genome pairs were calculated using GBDP under the 'trimming' algorithm with distance formula d5. Digital DNA-DNA Hybridization (dDDH) values and confidence intervals were calculated using GGDC 4.0. Then, phylogenomic trees were constructed using a balanced minimum evolution approach with branch support derived from 100 pseudo-bootstrap replicates in FASTME 2.1.6.1, with midpoint rooting and visualization through PhyD3. Results, including species and subspecies assignments, were provided by TYGS, along with visualized phylogenetic trees and clustering analyses, which supported the identification of the query genome and its placement within defined species groups, for more details on the methods refer to the TYGS website.

2.5 Gene expression analysis

Bacterial strains 'BLG_B2', 'BLG_B4', 'BLG_B1.1.1', 'BLG_B1.3' and 'BLG_B5'were selected on the basis of their different effectiveness in controlling DM infection. An *in vivo* bioassay using grape leaves was performed to evaluate the ability of selected bacterial isolates to reduce the expression of two *P. viticola* effectors, *PvRxLR28* and *PvRxLR67*, respectively an early and late effector [90].

2.5.1 Leaves inoculation and samples collection

A single colony for each of the five selected bacteria was picked and left to grow overnight in 35 ml of NB liquid medium in a rotary shaker at 150 rpm and room temperature (Figure 7a). Bacteria liquid cultures were centrifuged at 5000 g for 10 minutes, the supernatant discarded, and the pellets washed twice with sterile saline physiological solution (NaCl 0,85%). Finally, pellets were re-suspended in sterile saline physiological solution and adjusted to a final concentration of 10^7 CFU ml^{-1} (Figure 7b). 30 fully developed leaves (six for each selected bacteria) were collected from the susceptible table grape cultivar 'Flame'. Six additional leaves were collected to be used as control. Leaves were then incubated at 21°C for two hours in the presence of light to allow the stomata to open. Leaf surface was sterilized by dipping in sodium hypochlorite (3.5% active chlorine) solution for 30 sec., washed in sterile water two times and then air dried. Each leaf was put in a Petri dish containing Water Agar at 1% (m/v), with the abaxial surface facing up and then sprayed with the bacteria suspension, until the whole abaxial surface was covered in drops (Figure 7d). Leaves were incubated at 21°C for 48h and then infected with a *P. viticola* 10^5 sporangia mL^{-1} inoculum, that was applied in the same way as bacterial inoculum (Figure 7c). Inoculated leaves were subsequently kept overnight in the dark at 20-21°C for 4 h and then subjected to a 16-h photoperiod. Three leaves sprayed only with *P. viticola* and three sprayed only with bacterial suspensions were used as positive and negative mock, respectively. Leaves were collected at two different time-points, 24 hours and 6 days after infection with *P. viticola*. Three leaves were collected at each time point, frozen with liquid nitrogen and pulverized using mortars and pestles. Approximately 50 mg of the pulverized material were used to perform total RNA extraction.

***Figure 7**: Assay on leaves for gene expression analysis: a) Bacteria overnight liquid cultures; b) Bacteria inocula at 10^7 CFU/ml concentration; c) P. viticola inoculum at 10^5 sporangia mL^{-1} concentration; d) Bacteria inocula ready to be sprayed on leaves; e) Sprayed leaves positioned in Petri dishes containing Water Agar at 1% (m/v) f) Leaves incubated at 21°C and then subjected to a 16-h photoperiod.*

2.5.2 Symptomatic manifestation evaluation

To confirm the antagonistic activity of the selected bacterial strains, the DS in both treated and untreated leaves was evaluated by using an empirical 0-to-4 rating scale (Figure 8) at the second time-point. The average DS and effectiveness of each bacterial strain to control DM were calculated as previously described.

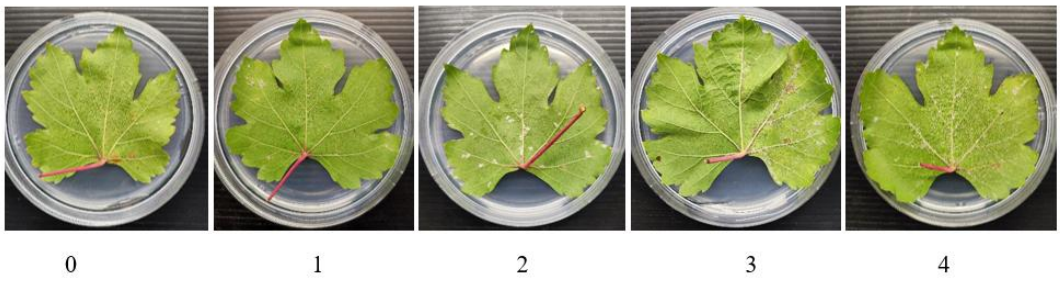

***Figure 8**: Empirical scale from 0 to 4 representing 5 grades of symptomatic manifestation of DM used to measure DS.*

2.5.3 RNA extraction, cDNA synthesis, Real-Time PCR

For each time point and treatment three biological replicates were used for total RNA extraction using Plant/Fungi total RNA purification Kit (Norgen Biotek Corp.) following the manufacturer's instructions. Total RNA was treated with DNase using DNase I (Sigma-Aldrich). RNA concentration and purity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The three biological replicates, for each time point and treatment, were then pooled together using the same amount of RNA for each replicate and cDNA synthesized using qScript™ cDNA Synthesis Kit (Quantabio) following the manufacturer's instructions. Gene expression analysis of two *P. viticola* effectors, *PvRxLR28* and *PvRxLR67* [90] respectively an

early and late effector, was performed through Real-Time PCR using specific primers [177] for their amplification and for the amplification of *P. viticola* housekeeping gene β-tubulin.

GENE	PRIMER FORWARD	PRIMER REVERSE
β-tubulin	GAGCACGAAGGAGGTTGA	CGTGGTGCTCATTTTCAGG
PvRxLR28	AACGTGGACGAAGATAAGGGA	AATTTTCAAAGGGTGGGATAC
PvRxxLR67	TGCACCAAGAATCCAAGAAGT	ATGCGGCGCTCAAACAATG

***Table 1**: Sequences of the forward and reverse primers used for the amplification through Real-Time PCR of housekeeping gene β-tubulin and effector genes PvRxLR28 and PvRxLR67.*

For each pool three technical replicates were performed. Each Real-Time PCR reaction had a total volume of 20 µl and was composed of 4 µl of a Reaction Mix containing 5X HOT FIREPol® MultiPlex Mix (Solis BioDyne) and 5mM Syto9, 0,4 µl of Primer Forward and 0,4 µl of Primer Reverse and a variable volume of cDNA, corresponding to 20-30 ng, and H_2O. Real-Time PCR started with an initial denaturation at 95 °C for 10 min, followed by 45 cycles of a 15 s denaturation at 95 °C and a 30 s annealing reaction at 60 °C. Gene expression values were relatively calculated as fold changes using $2^{-\Delta\Delta Ct}$ method [178].

2.6 Screening of the antagonistic activity of selected bacterial isolates against V. vinifera rot fungi

The spectrum of antagonistic activity of selected bacterial strains ('BLG_B1.1.1', 'BLG_B2', 'BLG_B4', 'BLG_B1.3', 'BLG_B5') against four phytopathogens (*Alternaria arborescens, Penicillum digitatum*, *Penicillum glabrum* and *Aspergillus niger*) was assessed on PDA plates by dual culture method. Plates measuring 9 cm in diameter, each containing 15-20 mL of Potato Dextrose Agar (PDA) (1L: Dextrose 20 g, Agar 15 g, Potato starch 4 g) medium, were used. 20 µL of a bacterial suspension [10^7 CFU mL^{-1}] were spotted in the center of each plate, which were incubated at 25°C for 48 hours. Two pathogen disks (5 mm diameter) were placed at 2.2 cm from the center of the PDA plate. Plates inoculated only with the pathogens were used as control. Each treatment was replicated five times. Following the inoculation, plates were incubated at 25°C in the dark. The antagonistic activity was expressed as the inhibition rates against mycelia growth compared to the control. It was calculated by the following formula:

$$PI(\%) = (1 - R1/R2) \times 100$$

where R1 and R2 were the mycelial radial growth of the pathogen in the control and in the presence of the antagonist, respectively.

2.7 Safety of bacterial isolates for human health: evaluation of hemolytic activity

Selected bacterial isolates were tested for their ability to produce hemolysin and a possible deleterious action on human red blood cells (erythrocytes) [185]. A plate assay was performed where bacteria were incubated on sheep blood agar 5% (v/v) (VWR BDH CHEMICALS-Blood Agar Base). A cell suspension of each bacterium was planted by a zig-zag streak with a sterile loop [188]. Three replicates for each isolate were set up and then incubated at 37°C (human body temperature). Three plates for each selected bacteria containing LB medium were used as control. After three days of incubation, β-hemolysis was observed by a clear zone around the bacterial colony, indicating erythrocyte breakage; α-hemolysis or partial hemolysis was represented by a color change to dark-green, indicating a reduction of red blood cells' hemoglobin to methemoglobin. Non-alteration over the medium (γ-hemolysis) indicated no damage to erythrocytes [186].

2.8 Sequence comparative analysis BLG_B2 vs BLG_B4

A comparative bioinformatic analysis was performed on the two most interesting isolates 'BLG_B2' and 'BLG_B4', both belonging to *Bacillus velezensis* species by employing a specific bioinformatic pipeline to compare the sequencing data obtained as described in paragraph *2.4.1* and identify genomic variants. Raw reads were aligned to the *B. velezensis* reference genome (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000960265.2/) using the BWA-MEM algorithm (version 0.7.17), with the default parameters. PCR duplicates were removed by means of Picard MarkDuplicates tool (version 3.1.1, http://broadinstitute.github.io/picard/). Variant calling analysis was performed using GATK (version 4.3.0.0) HaplotypeCaller command (-ERC GVCF, -ploidy 1 options). The individual gVCFs files obtained were merged and genotyped using the CombineGVCFs and GenotypeGVCFs commands, respectively. Finally, SnpEff (v5.2) (https://pcingola.github.io/SnpEff/) was used to annotate the list of variants. Particular attention was given to calls with differing genotypes between the two samples and marked as "HIGH" concerning their functional impact.

2.9 Screening of the antagonistic activity of selected bacterial isolates against bacteria

Bacterial strains 'BLG_B2' and 'BLG_B4' were assessed for the antagonistic activity against challenge multi-resistant bacteria such as *S. typhimurium*, methicillin-resistant *S. aureus* (MRSA), a multi-resistant strain of *E. coli, Vibrio parahaemolyticus* and in addition two non-pathogenic bacteria, such as *B. pumilus* and *E. coli* LE392. For each one of the five bacterial isolates and a *B. subtilis* ATCC6633, chosen as a control, an overnight liquid culture in NB medium was prepared together with overnight liquid cultures in LB medium of the challenge bacteria. Liquid cultures were incubated in a rotary shaker at 37 °C. The following day liquid cultures of the bacterial isolates and *B. subtilis* were centrifuged at 15500 rpm for 5 minutes (Figure 9a), the supernatants were collected and filtrated using Nalgene® filtering units having a 0,22 µm filter (Thermo Fisher Scientific) (Figure 9b) connected to a peristaltic pump, in order to eliminate any cellular residues (Figure 9c) Filtered supernatants were used as culture media for the growth of challenge bacteria. Briefly, 25 ml of the filtered supernatants were distributed in flasks, one for each challenge bacteria, and enriched with sterile glucose at 0,4% (m/v). Finally, 25 µl were collected from the overnight liquid cultures of challenge bacteria and released in the flasks containing the supernatants (Figure 9d). The liquid cultures were then incubated overnight in a rotary shaker at 37 °C and the following day Optical Density (OD) at 600 nm was measured using a spectrophotometer to evaluate bacterial growth.

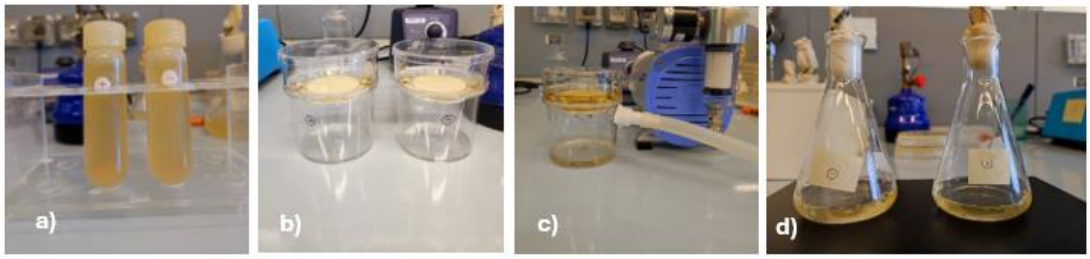

Figure 9*: Assay for the assessment of the antagonistic activity of selected bacterial isolates against multi-resistant bacteria: a) Overnight liquid cultures of the tested isolate and control ready to be centrifuged; b) Supernatants collected in Nalgene® filtering units; c) Filtered supernatant using peristaltic pump; d) Liquid cultures of the challenge bacteria in glucose enriched filtered supernatants.*

2.10 Pilot field trial

A field trial, part of the REVINE project, was performed at the company Ermes S.a.s di Giacomo Suglia & f.lli located in Noicattaro (BA) where part of the PhD was spent. A synthetic microbial consortium (SynCom) composed of 24 different species of

bacteria, fungi and actinomycetes isolated from the rhizosphere or internal tissues of grapevines and selected at CREA-VE for their biocontrol potential against various grapevine diseases, was tested *in vivo* by application on the roots to investigate its effect on the resilience capacities to biotic stresses of two table grape cv.: 'Autumn Crisp' and 'Anxa'.

2.10.1 SynCom inoculum preparation

Each one of the isolated microorganisms was plated on LB solid medium, for bacteria, and Czapek Yeast Autolysate Agar (CYA), for fungi, in order to isolate a single colony which was then inoculated in 100 ml of liquid medium, either LB or CYA. Liquid cultures were incubated in a rotary shaker at 28°C for a different time course, specific for each microbe. Microorganisms' growth was assessed by measuring the OD at 600 nm using Nanodrop (Thermo Fisher Scientific). Once the OD reached values between 1 and 4, liquid culture media were stored at 4°C for a few days before root application. Finally, the ready-to-be-applied inoculum was composed of a blend of the liquid culture medium of each microorganism proportionately mixed to obtain a final concentration of 10^6 CFU ml^{-1}.

2.10.2 Root inoculation

The SynCom was tested on two grapevine varieties: 'Autumn Crisp' grafted onto the 1103 P rootstock and 'Anxa' (a new seedless variety selected by CREA-VE) grafted onto the 140 Ruggeri rootstock. For each variety, 10 plants were treated with the SynCom and 10 plants were used as a control. The root system of the inoculated plants was immersed for 10 minutes in the SynCom suspension at 10^6 CFU ml^{-1}, while the root system of the control plants was immersed for 10 minutes in water. Subsequently, the plants were transplanted along the rows in the open field, for a total of three repetitions per thesis. 4 months after transplanting, soil sampling was carried out for the various thesis and repetitions, in correspondence with the rhizosphere of the plants and at approximately 10-15 cm depth, to carry out the metagenomic analyses. During the summer season of 2025, surveys of some agronomic parameters and main grapevine diseases such as DM, powdery mildew and gray mold will be performed.

Country	Region	Locality	GSM coordinates	
			N	E
Italy	Apulia	Noicattaro	41° 2' 2.86"	16° 58' 21.04"

***Figure 10**: REVINE experimental field and geographic coordinates at the company Ermes S.a.s di Giacomo Suglia & f.lli located in Noicattaro (BA).*

2.11 Statistical analysis

The data were analysed using R software (v. 4.2.2). Data normality, homoscedasticity and homogeneity of variances were evaluated by Shapiro-Wilk's test, Bartlett's test and Levene's test, respectively. Data collected to evaluate the degree of tolerance of grape cultivars were analysed using cluster analysis, whose basic idea consists of defining clusters so that the total intra-cluster variation is minimized. In detail, data set was preliminary analysed to assess the clustering tendency, using the Hopkins statistic [187] that measures the probability that a given data set is generated by a uniform data distribution. In other words, it tests the spatial randomness of the data. Internal measures, included in the clValid Rpackage, such as 'Connectivity', 'Average Silhouette width' and 'Dunn index', were performed to choose the appropriate clustering algorithms for our da-ta set. The two-way ANOVA test followed by the post-hoc Tukey test ($p < 0.05$) were performed to identify differences in the antagonistic activity of isolated bacterial strains against the different considered pathogens of grapevine. The Student's t-test ($p < 0.05$) was performed in order to evaluate the effectiveness of selected bacterial strains challenge multi-resistant bacteria.

3. RESULTS

3.1 Selection of niches suitable for the isolation of microorganisms

The main objective of this activity is to select a suitable niche for the isolation of microorganisms. The first step was to identify table grape cultivar with differential

behaviour in terms of tolerance/susceptibility against DM at the carposphere level. For this purpose, data related to the average DI and DS detached on the bunches of 50 table grape cultivars, during two productive years, were analyzed by performing a cluster analysis. Preliminary, the Hopkins statistic (D), using 0.5 as the threshold to reject the alternative hypothesis (the data set D is not uniformly distributed), is used to assess the clustering tendency of our data. Since the D value is close to 1), we can reject the null hypothesis (the dataset D is uniformly distributed) and conclude that our data set contains a meaningful cluster. To choose the appropriate clustering algorithms for our dataset, 'Connettivity', 'Average Silhouette width' and 'Dunn index' were calculated and Kmeans clustering with three clusters performs the best in each case. Based on the results of Kmeans clustering (Figure 11) the 50 table grape analyzed cultivars can be grouped in three clusters, whose details are reported in Table 2:

Cluster number		Cultivar
1	Moderately susceptible	Rubinia, Pansè de Malaga, Sernia Rs, Maxia, Paula, DeLight, Centenial, Blond, Sugraone, Autumn Royal, Red globe, Nerona, Perla di Yavola, Blush, Pizzutello, Florentia, Rossa del Magreb, Almeria, Autumn seedless
2	Highly susceptible	Dawn seedless, Regal seedless, Supernova, Black Pearl, Beograska, Palieri, Patrizia
3	Poorly susceptible	Coarna Negra, Incrocio Ceresa, Giada, Dimiat, Victoria, Argentina, Cardinal, Ruby seedless, Duca di Magenta, Incrocio Croyodero, Chasselas Dorè, Queen Rs, Resin d'Afrique, Duraca, Crimson seedless, Millenium, Inzolia Imperiale, Red Italia, Doradilla, Perlon, Thompson seedless, Carati, Emperor Rs, Italia.

***Table 2**: Report of the 50 selected table grape cultivars clustered in: moderately susceptible, highly susceptible, and poorly susceptible.*

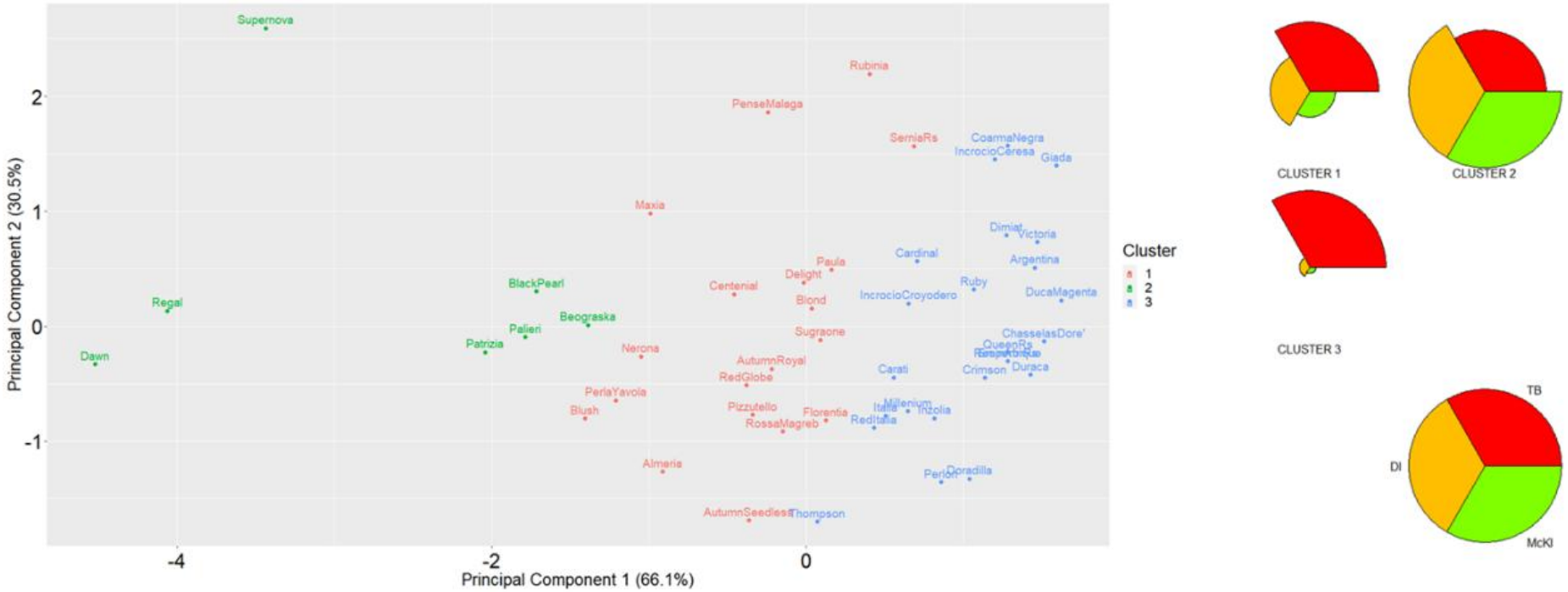

***Figure 11**: Cluster analysis of the data expressing the average DI and DS (McKinney Index) of DM on the bunches of the 50 selected table grape cultivars. McKI = average DS; DI = average DI; TB = total bunches.*

19 of the 50 studied varieties were selected; 4 highly susceptible (‘Dawn seedless’, ‘Regal seedless’, ‘Supernova’ and ‘Black Pearl’), 8 moderately susceptible (‘Paula’, ‘Delight’, ‘Centenial’, ‘Sugraone’, ‘Blush’, ‘Pizzutello’, ‘Autumn seedless’ and ‘Melissa’) and 7 poorly susceptible (‘Argentina’, ‘Ruby seedless’, ‘Duca di Magenta’, ‘Queen Rs’, ‘Crimson seedless’, ‘Inzolia Imperiale’ and ‘Perlon’), in order to evaluate their level of tolerance/susceptibility to DM infection at the phylloplane level. Susceptible varieties were included in the analysis to investigate whether the same degree of susceptibility to DM observed on the berries was also detectable on the leaves. Based on collected data using a leaf disc bioassay, the average DI and DS were calculated for each cultivar. This data, together with those related to the susceptibility/tolerance at the carposphere level, were used to perform a cluster analysis. Since also in this case the D value is close to 1, we can conclude that our data set contains a meaningful cluster. Based on the ‘Connettivity’, ‘Average Silhouette width’ and ‘Dunn’ indices Kmeans clustering with three clusters resulted the best clustering algorithm. The Kmeans clustering divided the 19 table grape cultivars in three clusters (Figure 12). In detail, the cultivars ‘Supernova seedless’, ‘Regal seedless’, ‘Melissa’, ‘Black Pearl’ and ‘Dawn seedless’ are characterized by a lower level of tolerance to *P. viticola* infection, at both the carphosphere and phylloplane levels (Cluster 2). Seven cultivars (‘Blush seedless’, ‘Delight’, ‘Pizzutella’, ‘Centenial’, ‘Sugraone’, ‘Paula’ and ‘Autumn seedless’) grouped in Cluster 1 and characterized by a high and medium level of tolerance to DM, at the phylloplane and carposphere levels, respectively. Finally, cultivars ‘Perlon’, ‘Ruby seedleess’, ‘Duca di Magenta’, ‘Crimson seedless’, ‘Queen’, ‘Inzolia Imperiale’ and ‘Argentina’ belonged to the Cluster 3 and showed a medium and high level of tolerance to DM at the phylloplane and carposphere levels, respectively. From the leaves of one cultivar for each cluster (‘Dawn seedless’, ‘Blush’ and ‘Argentina’), whose characteristics are reported in Table 3, the isolation activities of potentially candidate bacteria as good Biological Control Agents (BCAs) were carried out, as described in the Material and Methods. The choice of also including the susceptible cultivar ‘Dawn seedless’ was made in order to evaluate the hypothesis, reported in several works [138] [174], of a co-evolution of microorganisms colonizing certain niches, in this case leaves, and the genotype of the host plant in terms of tolerance to target pathogens through the analysis and comparison of the biocontrol activity of bacteria inhabiting susceptible and tolerant cultivars.

Cultivar	Cluster	Susceptibility on the leaves		Susceptibility on the bunches	
		Average DI	Average DS	Average DI	Average DS
Blush	1	High	High	Medium	Medium
Dawn seedless	2	Low	Low	High	High
Argentina	3	Medium	Medium	Low	Low

***Table 3**: Report of the characteristics of the 3 selected cultivars (average DI and average DS) expressing their degree of susceptibility to DM on leaves and bunches.*

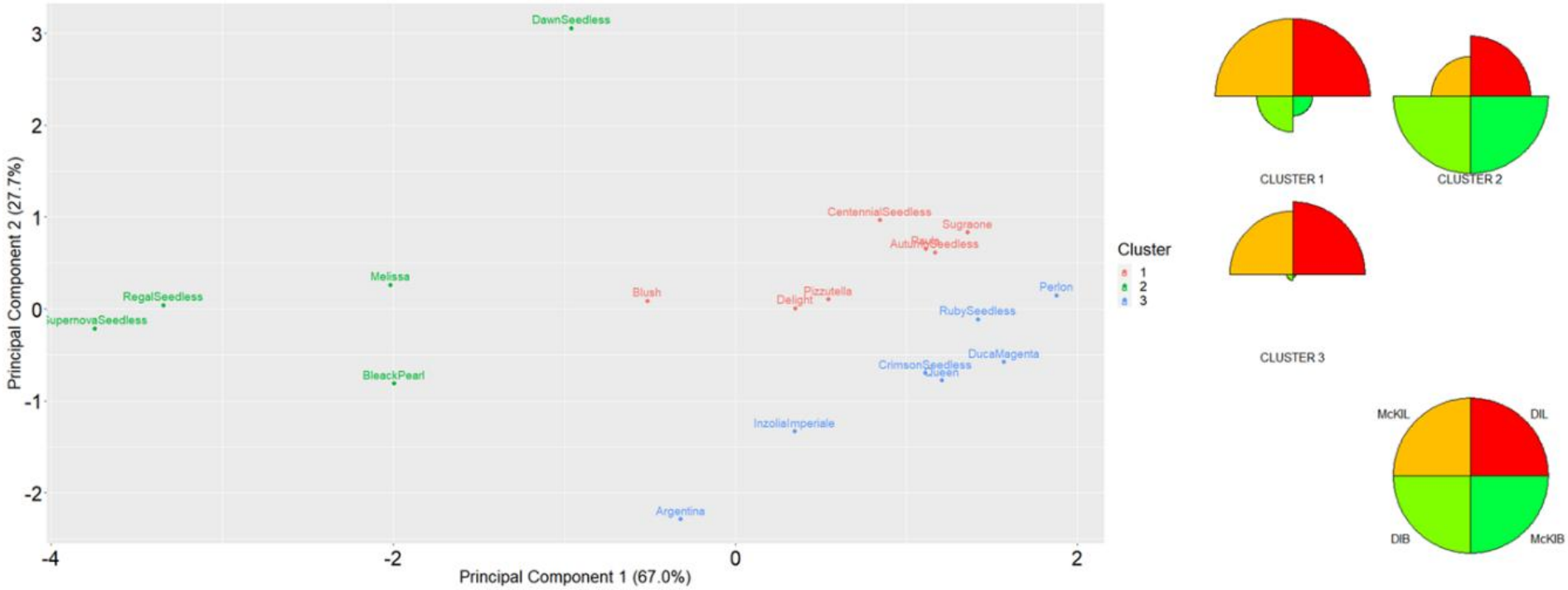

Figure 12: *Cluster analysis combining the data expressing the average DI and DS (McKinney Index) of DM on the bunches and leaves of the 19 selected cultivars. McKIL = average DS on leaves; DIL = average DI on leaves; McKIB = average DS on bunches; DIB = average DI on bunches.*

3.2 Isolation of bacterial strains

Bacterial isolates obtained from the leaves of 'Argentina', 'Blush' and 'Dawn seedless' varieties were divided into 26 different groups based on morphological features such as dimensions and shapes of the colonies, edges, rough or smooth surfaces, glossy or matt appearance and color. From this preliminary discrimination, a random selection of 1-2 bacterial colonies from each of 26 groups resulted in a total of 36 bacterial isolates, including *Bacillus* isolated using high temperature as a criterion of selection, which represented the starting point for the further studies conducted in this work (Table 4):

Size	**Shape**	**Morphology of the edges**	**Surface**	**Appearance**	**Colour**	**Bacteria isolate code***
Very small	Rounded	Defined	Smooth	Opaque	White with yellow shades	ARV_B3, ARV_B9
					Whitish	BLG_B1
				Glossy	Yellow	DAG_B4.1.2
			Wrinkled	Opaque	Yellow	DAG_B1.3, ARG_B2, ARG_B5
Small	Rounded	Defined	Smooth	Glossy	Yellow	BLG_B1.3
				Opaque	Yellow	BLG_B1.2 DAG_B4.1.1
					Whitish	ARV_B4, BLG_B2
					Cream white	BLG_B6
			Wrinkled	Opaque	Whitish	ARV_B4
	Oval	Poorly defined	Smooth	Opaque	Cream white	DAG_B5
Medium	Rounded	Defined	Smooth	Opaque	Whitish	ARV_B2
					Cream white	DAG_B6
		Poorly defined	Smooth	Glossy	Transparent	DAV_B1.1.1
	Slightly oval	Poorly defined	Smooth	Opaque	White with grey shades	ARV_B8
	Oval	Poorly defined	Smooth	Glossy/buttery	Yellow	BLG_B1.2, ARV_B7, BLG_B5
				Opaque	Cream white	BLG_B4
		Not defined	Smooth	Opaque	Cream white	DAG_B6.3.1, BLG_B2
Large	Rounded	Defined	Smooth	Opaque	Yellow	ARG_B3
			Wrinkled	Opaque	Yellow	DAV_B1.1.2, DAV_B3, DAV_B4
		Poorly defined	Smooth	Glossy	Transparent	DAV_B5
				Opaque	Cream white	DAV_B6
	Oval	Not defined	Smooth	Glossy/buttery	Cream white	ARG_B6
					White with yellow shades	DAG_B1

		Poorly defined	Smooth	Glossy	White with yellow shades	DAG_B2.2
				Glossy/buttery	White with yellow shades	DAG_B3
Very large	Oval	Poorly defined	Wrinkled	Opaque	Whitish	BLG_B1.1.1

Table 4: *Morphological characteristics of 36 bacterial isolates, obtained from the phylloplane of three table grape cultivar, showing a different degree of tolerance against DM.*
** ARV = indicates bacteria isolates obtained from old leaves collected from the cultivar 'Argentina';*
ARG = indicates bacteria isolates obtained from young leaves collected from the cultivar 'Argentina',
BLV = indicates bacteria isolates obtained from old leaves collected from the cultivar 'Blush seedless'
BLG = indicates bacteria isolates obtained from young leaves collected from the cultivar 'Blush seedless'
DAV = indicates bacteria isolates obtained from old leaves collected from the cultivar 'Dawn seedless'
DAG = indicates bacteria isolates obtained from young leaves collected from the cultivar 'Dawn seedless'

3.3 Screening on leaf discs for antagonistic activity against P. viticola

16 of the 36 bacterial isolates, chosen as a heterogeneous representation of the totality of isolates based on morphological characteristics, were tested for their ability to reduce the sporulation of *P. viticola "in semi-vivo*" condition on leaf discs obtained from leaves of the susceptible cultivar 'Flame'. Leaf discs were pre-treated with bacterial fermentation broth at 10^7 CFU mL^{-1} and then artificially inoculated with 50 µL of *P. viticola* inoculum at the concentration of 10^4 sporangia mL^{-1}. For each bacterial isolate, the percentage of the leaf disc area covered by *P. viticola* sporulation was measured and, according to that, three classes of effectiveness were defined.

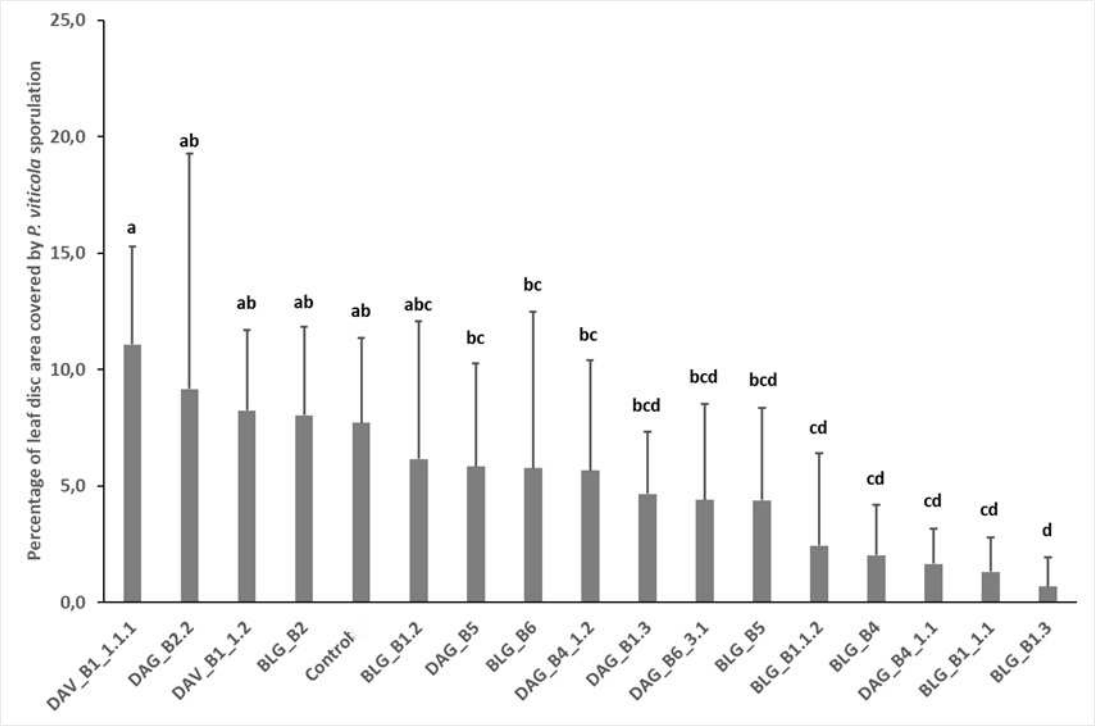

***Figure 13**: Graph bar representing the percentage of leaf discs area covered by P. viticola sporulation for each treatment with bacterial isolates. The normality and homoscedasticity of variances were determined through Shapiro-Wilk's test and Levene's test, respectively. Because normal distribution and homoscedasticity of variance were verified, the two-way Anova test was performed to analyse the variance ($p < 0.05$) followed by the post-hoc Tukey test ($p < 0.05$). Bars labelled with different letters are statistically significant.*

Inoculated leaf discs pre-treated with the bacterial isolates 'DAV_B1.1.1', 'DAG_B2.2', 'DAV_B1_1.2', 'BLG_B2' and 'BLG_B1.2' showed the same percentage of area covered by *P. viticola* sporulation as the one detected in the control. Leaf disc pre-treated with the bacterial isolates 'DAG_B5', 'BLG_B6', 'DAG_B41.2', 'DAG_B1.3', 'DAG_B6_3.1' and 'BLG_B5' showed a no-significant reduction in sporulation surface (between 24.3 and 43.1%) compared to the control. Finally, the leaf discs pre-treated with bacterial isolates 'BLG_B1.1.2', 'BLG_B4', 'DAG_B4_1.1', 'BLG_B1_1.1' and 'BLG_B1.3' showed a significant reduction in infection area (between 68.4 and 98.2%) compared to the control. The most effective bacterial strains 'BLG_B1.3', BLG_B1.1.1', and 'BLG_B4', together with the moderately effective bacterial strain 'BLG_B5' and the ineffective one 'BLG_B2', were selected for further characterization studies. Indeed, despite resulting ineffective, 'BLG_B2' belonged to the same genus (*Bacillus*) as 'BLG_B4' and showed similar morphological features, suspecting them to belong to the same species, its investigation was carried out to thoroughly study the differences in its behaviour compared to 'BLG_B4'.

3.4 Morphological and molecular identification of selected bacteria

The morphological characteristics of the five selected bacteria are reported in Figure 14 and include a visual analysis of the main characteristics of a bacterial colony, such as size, shape, morphology of the edges, surface, appearance and color.

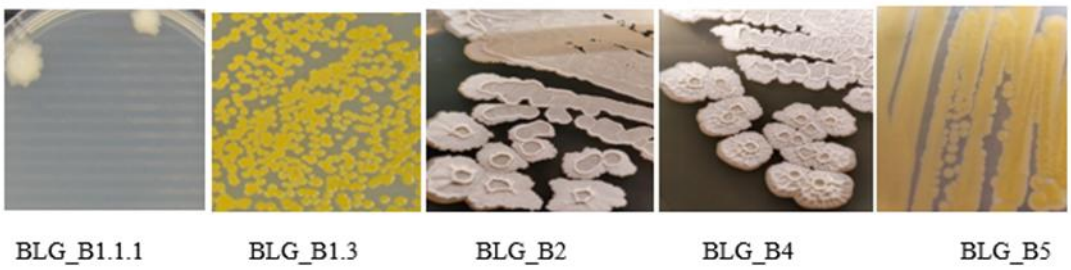

***Figure 14**: Bacterial isolates ('BLG_B1.1.1', 'BLG_B1.3', 'BLG_B2', 'BLG_B4', 'BLG_B5') selected as representatives of the three different classes of effectiveness against P. viticola.*

'BLG_B1.1.1' shows a very large oval colony, with poorly defined edges and a wrinkly/opaque surface, whitish in color. 'BLG_B1.3' is characterized by a small rounded colony, with defined edges and smooth/glossy surface, yellow in color. Bacterial isolate 'BLG_B2' shows a medium oval colony, with not defined edges and opaque surface, cream white in color. 'BLG_B4' shows a medium oval colony, with poorly defined edges and an opaque surface, cream white in color. Finally, bacterial isolate 'BLG_B5' is characterized by a medium oval colony, with poorly defined edges and a smooth glossy/buttery surface, yellow in color.

Moreover, to more precisely assess these five bacterial species, we performed whole-genome sequencing followed by assembly and a whole genome-base taxonomic classification using the TYGS platform. The sequencing data were processed and analyzed as described in the methods section, enabling accurate species identification. The analysis identified the following species: two *Bacillus velenzensis* isolates ('BLG_B2' and 'BLG_B4'), one *Pseudomonas psychrotolerans* isolate ('BLG_B1.3'), one *Bacillus subtilis* ('BLG_B1.1.1'), and one *Pantotea pleuroti* isolate ('BLG_B5'). The taxonomic identification was corroborated by phylogenomic analysis, which placed the isolates within their respective species groups, confirming their classification and highlighting their phylogenetic relationships. These results, supported by Digital DNA-DNA Hybridization (dDDH) values (Table 5) and phylogenetic trees, provided a comprehensive molecular and genomic characterization of the selected bacterial isolates.

BACTERIA ISOLATE	SPECIES	dDDH radius
BLG_B2	*Bacillus velezensis* KACC 13105	100%
BLG_B4	*Bacillus velezensis* KACC 13105	100%
BLG_B5	*Pantoea pleuroti* JZB 2120015	84,2%
BLG_B1.1.1	*Bacillus subtilis* ATCC 6051	91,5%
BLG_B1.3	*Pseudomonas psychrotolerans* DSM 15758	83,5%

***Table 5**: Whole-genome identification and dDDH radius values for bacterial isolates.*

Three out of the five selected bacteria belonged to different *Bacillus* species which is not surprising since a specific temperature-based selection for this genus of bacteria was carried out and several works have already reported its antimicrobial and biocontrol properties [124] [127] [132]. Just like *Bacillus*, *Pseudomonas* and *Pantoea* are renowned as effective biocontrol agents [203] [204] [205] [206].

3.5 Gene expression analysis

The selected bacterial strains (*B. velezensis* 'BLG_B2'and 'BLG_B4', *B. subtilis* 'BLG_B1.1.1', *P. psychrotolerans* 'BLG_B1.3' and *P. pleuroti* 'BLG_B5') were used to perform a bioassay, using whole leaves of the susceptible cultivar 'Flame seedlees', with the aim to both confirm their antagonistic activity against DM and to study a possible mechanism of action. We evaluated the effects of bacteria colonization on two *P. viticola* effector genes, *PvRxLR28* and *PvRxLR67*, respectively an early and a late effector.

Visual analysis of the treated leaves at 6 dpi revealed significant differences among bacterial isolates in terms of effectiveness against DM (Figure 15).

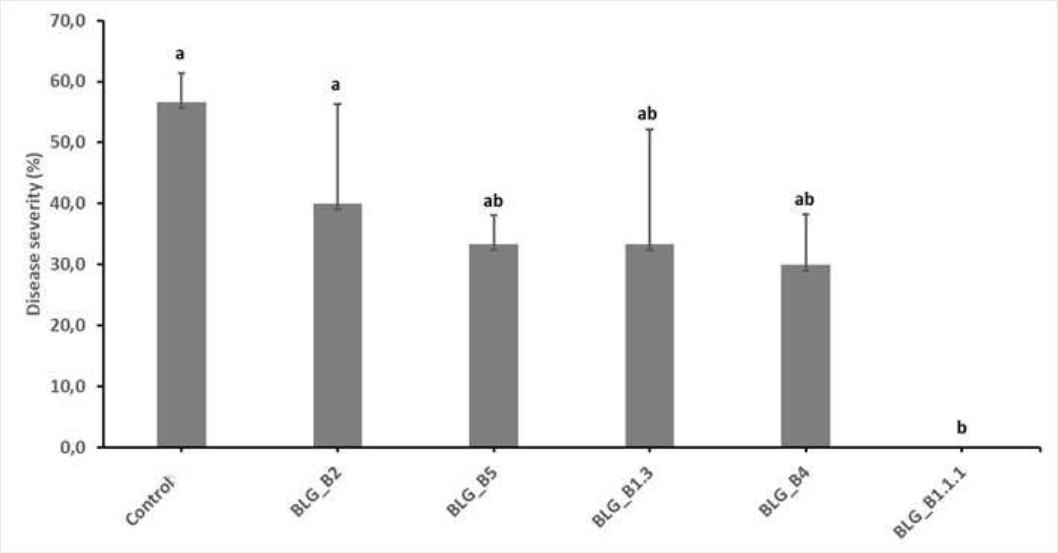

***Figure 15**: Graph bar representing the effect on DS, calculated using the McKinney's formula and resulting from phenotypical analysis and symptoms evaluation of treatment with each bacterial isolate. The normality and homoscedasticity of variances were determined through Shapiro-Wilk's test and Levene's test, respectively. Because normal distribution and homoscedasticity of variance were verified, the two-way Anova test was performed to analyse the variance ($p < 0.05$) followed by the post-hoc Tukey test ($p < 0.05$). Bars labelled with different letters are statistically significant.*

No significant differences were found in *B. velezensis* 'BLG_B2' in terms of DS compared to the control, confirming its ineffectiveness. Leaves treated with *P. pleuroti* 'BLG_B5', *P. psychrotolerans* 'BLG_B1.3' and *B. velezensis* 'BLG_B4' showed lower DS than control, but not statistically significant. Finally, leaves treated with *B. subtilis* 'BLG_B1.1.1' revealed a DS equal to zero, confirming their high antagonistic activity against *P. viticola*. In order to study the mechanism of action of the selected bacteria and in particular their ability to stimulate plant defense, the effect on the expression of two *P. viticola* effector genes, *PvRxLR28* and *PvRxLR67*, respectively an early and a late effector, was assessed by Real-Time PCR.

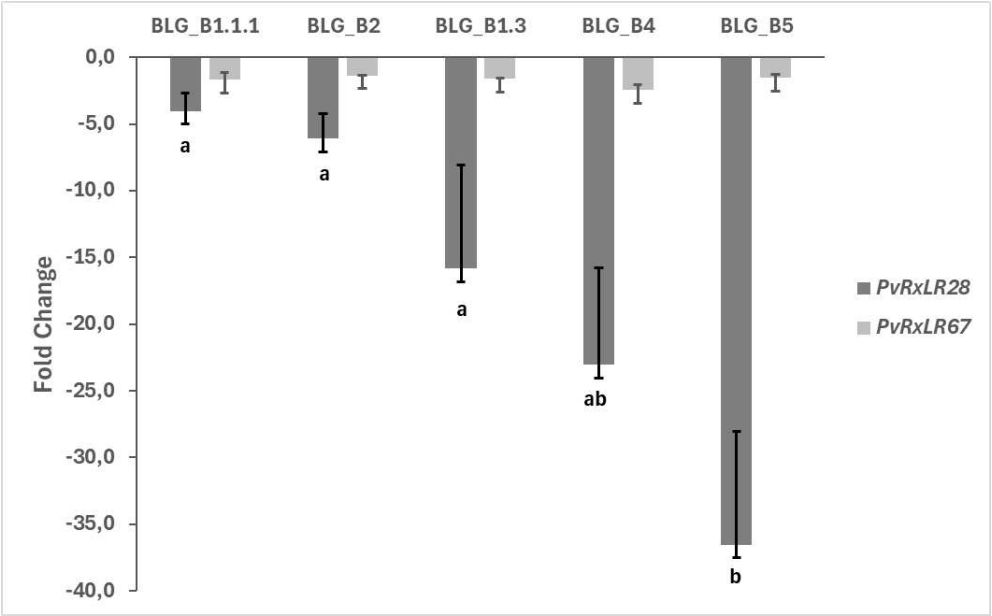

***Figure 16**: Graph bar representing the fold change in the expression levels of P. viticola effector genes PvRxLR28 and PvRxLR67 resulting from leaves treatment with each of the five bacterial isolates. The normality and homoscedasticity of variances were determined through Shapiro-Wilk's test and Levene's test, respectively. Because normal distribution and homoscedasticity of variance were verified, the two-way Anova test was performed to analyze the variance ($p < 0.05$) followed by the post-hoc Tukey test ($p < 0.05$). Bars labelled with different letters are statistically significant.*

Expression analysis revealed significant differences among bacterial strains in terms of expression of the early effector *PvRxLR28*; in contrast, all the tested bacteria similarly reduced the expression of the late effector *PvRxLR67* (Figure 16). Considering the effect of bacterial colonization on *P. viticola PvRxLR28* effector gene, *P. pleuroti* 'BLG_B5' resulted the most effective in reducing its expression, compared to the other. Otherwise, *B. subtilis* 'BLG_B1.1.1' and *B. velezensis* 'BLG_B2' resulted the less effective in terms of reducing *PvRxLR28* effector gene expression. Finally, *P. psychrotolerans* 'BLG_B1.3' and *B. velezensis* 'BLG_B4' resulted moderately effective in reducing the expression of the analyzed effector.

3.6 Screening of the antagonistic activity of selected bacterial isolates against V. vinifera rot fungi

The selected bacterial strains *B. velezensis* 'BLG_B2', *B. velezensis* 'BLG_B4', *B. subtilis* 'BLG_B1.1.1', *P. psychrotolerans* 'BLG_B1.3' and *P. pleuroti* 'BLG_B5') were further assessed for their ability to reduce the mycelia growth of four phytopathogens of grapevine (*A. arborescens*, *P. digitatum*, *P. glabrum* and *A. niger)*, performing a dual culture assay on PDA medium. The antagonistic activity was expressed as the inhibition rates against mycelia growth compared to the control.

Among the tested fungal pathogens, *P. digitatum* resulted sensitive to the presence of the strains *B. velezensis* 'BLG_B2', *B. velezensis* 'BLG_B4' and *P. pleuroti* 'BLG_5'. Specifically, the effectiveness of these bacterial strains in reducing the mycelia growth of the pathogen was found to be 84.3%, 51.5% and 47.7% respectively. The same bacterial strains showed low efficiency in reducing the mycelia growth of *A. arborescens*, that was found to be 21.4%, 19.2% and 24.4%, respectively. (Figure 17). No significant reduction in terms of mycelia growth was detected for the fungal pathogen *P. glabrum* and *A. niger* co-cultivated with all the selected bacteria.

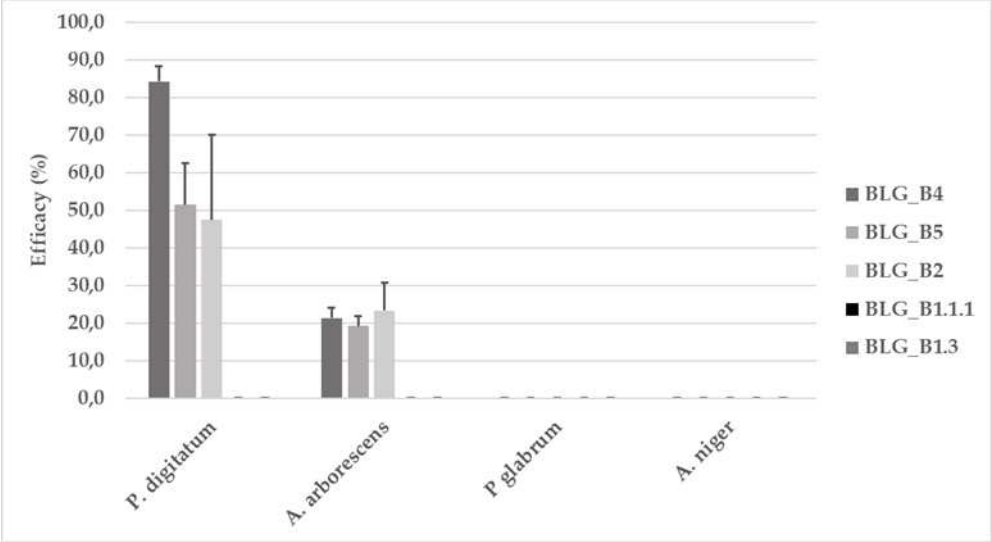

Figure 17: *Efficacy of selected bacteria in reducing in vitro mycelia growth of four fungal grapevine pathogens. Data are presented as the mean of five replicates with standard deviation (vertical bars).*

3.7 Safety for human health

In the perspective of potential use of the bacterial isolates as BCAs, *B. velezensis* 'BLG_B2', *B. velezensis* 'BLG_B4', *B. subtilis* 'BLG_B1.1.1', *P. psychrotolerans* 'BLG_B1.3' and *P. pleuroti* 'BLG_B5' were investigated for their ability to lyse the red blood cell membrane through hemolysin production. For this purpose, bacterial strains were grown on sheep blood agar at 37°C and on LB medium as control. As shown in Figure 18 all the tested bacterial strains were able to grow at the operating temperature when streaked on LB medium. Otherwise, only *B. subtilis* strain 'BLG_B1.1.1', *B. velenzensis* strain 'BLG_B4' and *P. psycotolerans* strain 'BLG_B1.3' resulted able to grow on sheep blood agar at the tested temperature, but no clear or brown areas were detected around their colony.

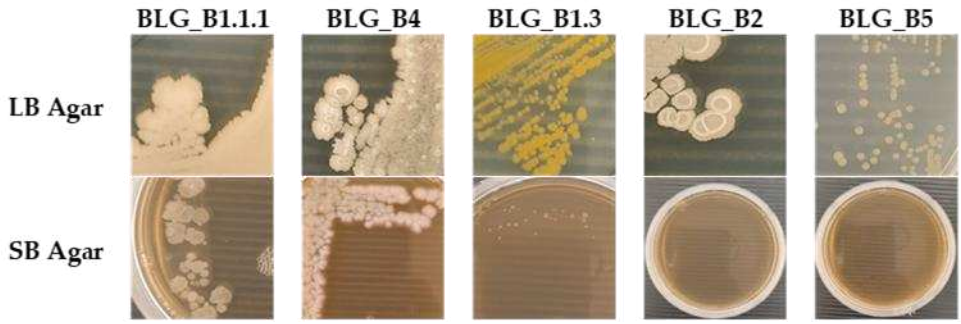

***Figure 18**: Selected bacteria strains grown on LB agar and Sheep Blood (SB) agar after three days of incubation at 37°C and detection of hemolytic activity.*

3.8 Bioinformatic and comparative analysis BLG_B2 vs BLG_B4

A comparative analysis between the genomes of the two bacterial strains belonging to the species *B. velenzensis* ('BLG_B2' and 'BLG_B4') was performed, to investigate the possible reasons accountable for their different behaviour showed during experiments conducted in this work. These two strains showed an opposite antagonistic activity against *P. viticola* "*in semi-vivo"* condition. Moreover, gene expression analysis of *P. viticola* effector genes *PvRxLR28* and *PvRxLR67* also revealed differences between the two bacterial isolates. Treatment with *B. velenzensis* strain 'BLG_B4' determined a higher and statistically significant reduction in the levels of expression of both effector genes compared to *B. velenzensis* strain 'BLG_B2' (Figure 19).

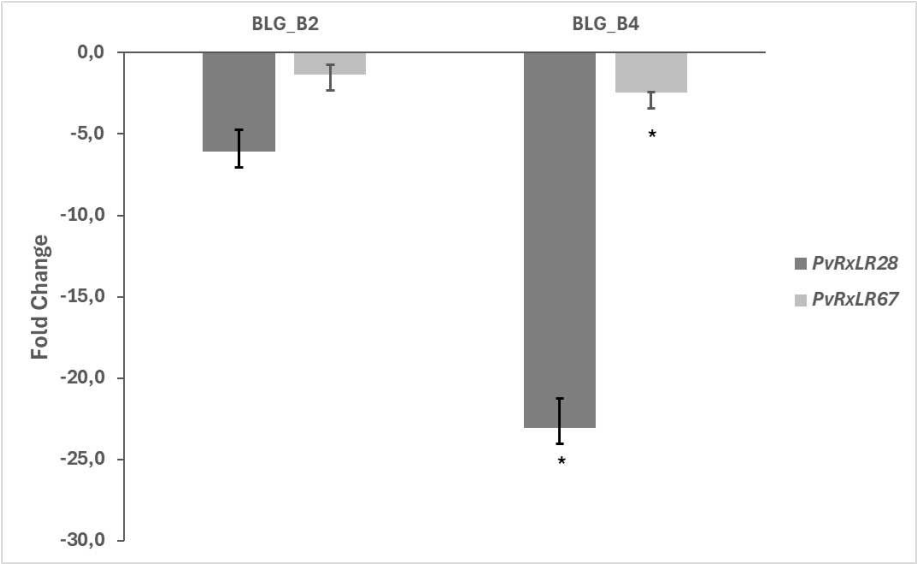

***Figure 19**: Graph bar representing the fold change in the expression levels of P. viticola effector genes PvRxLR28 and PvRxLR67 resulting from leaves treatment with B. velezensis strains 'BLG_B4' and 'BLG_B2'. Stars indicate the presence of a statistically significant difference between the two isolates in the effect on the levels of expression of the two effectors according to Student's t-test ($p < 0.05$).*

Comparative analysis of *B. velezensis* strain 'BLG_B2' and strain 'BLG_B4' genomes was performed searching for unique or common genomic variants between the two genomes. Therefore, each bacterial isolate's genome was compared to the reference genome of the identified species and a peculiar map of variants, such as single nucleotide polymorphism, insertion/deletion, and copy number variation was defined.

The resulting variants are reported in Table 6. Further, the variants between the two genomes were compared. Among the 15 identified variants, 9 were found both in *B. velezensis* strains 'BLG_B2' and 'BLG_B4', while 6 were not common between the two bacterial isolates and could be found only in the genome of one of them. We focused on the analysis of the 6 variants not in common. In detail, taking into account the differences in the behaviours and properties of *B. velezensis* 'BLG_B2' and 'BLG_B4', the variants that could functionally explain these dissimilarities were 2 affecting in *B. velezensis* 'BLG_B2' the genes PV82_RS12005 and PV82_RS13580. As for PV82_RS12005, it carries a frameshift mutation in position 546645 of the chromosome NZ_JTKJ02000014.1 that determines the change TTTGC > T. PV82_RS13580 carries a stop-gained mutation in position 853695 of the chromosome NZ_JTKJ02000014.1 that determines the change C > A causing the generation of a stop codon and production of a truncated protein. PV82_RS12005 and PV82_RS13580 are respectively the *yeeF* and *yxdJ* genes. *yeeF* encodes a polymorphic toxin, a metal-dependent DNase which may play an important role in antibacterial competition [189] [190]. *yxdJ* encodes a response regulator that binds to DNA and that together with a histidine kinase, constitutes a two-component system controlling the transcription of the neighbouring *yxdLMyxeA* operon encoding an ABC transporter involved in the secretion of antifungal metabolites [191] [192].

	CHROM	POS	REF	ALT	QUAL	FORMAT	BL_G_B2	BL_G_B4	
1	NZ_JTKJ02000002.1	1253530	G	A	21437.06	GT:AD:DP:GQ:PL	1:0,334:334:99:14174,0	1:0,170:170:99:7277,0	
2	NZ_JTKJ02000013.1	17	A	AG	2657.03	GT:AD:DP:GQ:PL	1:4,56:60:99:1840,0	1:7,31:38:99:831,0	
3	NZ_JTKJ02000014.1	723775	CT	C	9726.59	GT:AD:DP:GQ:PL	1:1,320:321:99:9737,0	0:21,0:21:99:0,924	PV82_RS09660
4	NZ_JTKJ02000014.1	2048001	T	G	9213.63	GT:AD:DP:GQ:PL	0:227,0:227:99:0,1800	1:0,217:217:99:9224,0	
5	NZ_JTKJ02000014.1	3810112	A	G	28541.06	GT:AD:DP:GQ:PL	1:0,402:402:99:17063,0	1:0,268:268:99:11492,0	
6	NZ_JTKJ02000014.1	546645	TTTGC	T	16092.59	GT:AD:DP:GQ:PL	1:0,359:359:99:16103,0	0:67,0:67:99:0,1800	PV82_RS12005

7	NZ_JTKJ02000014.1	8536 95	C	A	18880.63	GT:AD:DP:GQ:PL	1:0,439:439:99:18891,0	0:67,0:67:99:0,1800	PV82_RS13580
8	NZ_JTKJ02000017.1	92	G	A	14026.06	GT:AD:DP:GQ:PL	1:3,188:191:99:8325,0	1:2,129:131:99:5715,0	
9	NZ_JTKJ02000017.1	93	C	T	14026.06	GT:AD:DP:GQ:PL	1:3,188:191:99:8325,0	1:2,129:131:99:5715,0	
10	NZ_JTKJ02000017.1	97	A	G	14139.06	GT:AD:DP:GQ:PL	1:3,189:192:99:8358,0	1:3,132:135:99:5795,0	
11	NZ_JTKJ02000017.1	191	G	A	18973.06	GT:AD:DP:GQ:PL	1:7,276:283:99:11292,0	1:11,182:193:99:7695,0	
12	NZ_JTKJ02000017.1	192	C	T	19068.06	GT:AD:DP:GQ:PL	1:7,277:284:99:11387,0	1:11,182:193:99:7695,0	
13	NZ_JTKJ02000017.1	40702	A	ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG	7733.59	GT:AD:DP:GQ:PL	0:216,0:216:99:0,1800	1:51,68:129:99:7744,0	
14	NZ_JTKJ02000017.1	54133	G	A	16205.63	GT:AD:DP:GQ:PL	1:0,378:378:99:16216,0	0:51,0:51:99:0,1800	PV82_RS17180
15	NZ_JTKJ02000017.1	36627 2	A	G	16624.06	GT:AD:DP:GQ:PL	1:4,262:266:99:10438,0	1:3,163:166:99:6200,0	

Table 6: *Summary of the identified variants resulting from the comparative analysis of the genomes of B. velezensis strains 'BLG_B2' and 'BLG_B4' with the reference genome and between the two isolates. The table reports the chromosome where the variants were identified, the position, the sequence of the reference genome, the identified alteration, the quality and format, the indication of the isolate hosting the variants and the codes of the interested genes in the reference genome.*

	INFO
1	AC=2;AF=1.00;AN=2;DP=523;FS=0.000;MLEAC=2;MLEAF=1.00;MQ=60.00;QD=25.36;SOR=0.855;ANN=A|missense_variant|MODERATE|PV82_RS01840|PV82_RS01840|transcript|PV82_RS01840|protein_coding|1/1|c.1087G>A|p.Ala363Thr|1087/1821|1087/1821|363/606||WARNING_TRANSCRIPT_NO_START_CODON,A|upstream_gene_variant|MODIFIER|PV82_RS01830|PV82_RS01830|transcript|PV82_RS01830|protein_coding|c.-3734C>T||||3734|,A|upstream_gene_variant|MODIFIER|cheV|PV82_RS01850|transcript|PV82_RS01850|protein_coding|c.-2479G>A||||2479|,A|downstream_gene_variant|MODIFIER|PV82_RS01825|PV82_RS01825|transcript|PV82_RS01825|protein_coding|c.*4546G>A||||4546|WARNING_TRANSCRIPT_NO_START_CODON,A|downstream_gene_variant|MODIFIER|PV82_RS01835|PV82_RS01835|transcript|PV82_RS01835|protein_coding|c.*1264G>A||||1264|WARNING_TRANSCRIPT_NO_START_CODON,A|downstream_gene_variant|MODIFIER|PV82_RS01845|PV82_RS01845|transcript|PV82_RS01845|protein_coding|c.*759C>T||||759|,A|downstream_gene_variant|MODIFIER|PV82_RS19735|PV82_RS19735|transcript|PV82_RS19735|protein_coding|c.*2129C>T||||2129|,A|downstream_gene_variant|MODIFIER|PV82_RS01855|PV82_RS01855|transcript|PV82_RS01855|protein_coding|c.*3433C>T||||3433|,A|downstream_gene_variant|MODIFIER|PV82_RS01860|PV82_RS01860|transcript|PV82_RS01860|protein_coding|c.*4020C>T||||4020|
2	AC=2;AF=1.00;AN=2;BaseQRankSum=2.09;DP=422;FS=23.909;MLEAC=2;MLEAF=1.00;MQ=59.20;MQRankSum=1.74;QD=27.11;ReadPosRankSum=-8.320e-01;SOR=5.926;ANN=AG|upstream_gene_variant|MODIFIER|pdaB|PV82_RS08910|transcript|PV82_RS08910|protein_coding|c.-282_-281insG||||281|WARNING_TRANSCRIPT_NO_START_CODON,AG|upstream_gene_variant|MODIFIER|gerD|PV82_RS08920|transcript|PV82_RS08920|protein_coding|c.-1751_-1750insG||||1750|,AG|upstream_gene_variant|MODIFIER|PV82_RS08940|PV82_RS08940|transcript|PV82_RS08940|protein_coding|c.-4821_-4820insG||||4820|,AG|downstream_gene_variant|MODIFIER|kbaA|PV82_RS08915|transcript|PV82_RS08915|protein_coding|c.*1046_*1047insC||||1046|,AG|downstream_gene_variant|MODIFIER|PV82_RS08925|PV82_RS08925|transcript|PV82_RS08925|protein_coding|c.*2340_*2341insC||||2340|,AG|downstream_gene_variant|MODIFIER|cwlD|PV82_RS08930|transcript|PV82_RS08930|protein_coding|c.*3472_*3473insC||||3472|,AG|downstream_gene_variant|MODIFIER|PV82_RS08935|PV82_RS08935|transcript|PV82_RS08935|protein_coding|c.*4246_*4247insC||||4246|,AG|intergenic_region|MODIFIER|CHR_START-pdaB|CHR_START-PV82_RS08910|intergenic_region|CHR_START-PV82_RS08910||n.17_18insG|||||
3	AC=1;AF=0.500;AN=2;BaseQRankSum=-3.040e-01;DP=371;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=60.00;MQRankSum=0.00;QD=30.30;ReadPosRankSum=1.53;SOR=0.324;ANN=C|frameshift_variant|HIGH|PV82_RS09660|PV82_RS09660|transcript|PV82_RS09660|protein_coding|1/1|c.764delA|p.Lys255fs|764/1224|764/1224|255/407||,C|upstream_gene_variant|MODIFIER|PV82_RS09635|PV82_RS09635|transcript|PV82_RS09635|protein_coding|c.-2638delA||||2638|,C|upstream_gene_variant|MODIFIER|comX|PV82_RS19210|transcript|PV82_RS19210|protein_coding|c.-2451delA||||2451|,C|upstream_gene_variant|MODIFIER|PV82_RS09640|PV82_RS09640|transcript|PV82_RS09640|protein_coding|c.-1622delA||||1622|WARNING_TRANSCRIPT_NO_START_CODON,C|upstream_gene_variant|MODIFIER|degQ|PV82_RS09645|transcript|PV82_RS09645|protein_coding|c.-1274delA||||1274|WARNING_TRANSCRIPT_NO_START_CODON,C|downstream_gene_variant|MODIFIER|PV82_RS09655|PV82_RS09655|transcript|PV82_RS09655|protein_coding|c.*467delT||||467|,C|downstream_gene_variant|MODIFIER|PV82_RS09665|PV82_RS09665|transcript|PV82_RS09665|protein_coding|c.*893delA||||893|,C|downstream_gene_variant|MODIFIER|PV82_RS09670|PV82_RS09670|transcript|PV82_RS09670|protein_coding|c.*2377delA||||2377|,C|downstream_gene_variant|MODIFIER|PV82_RS09675|PV82_RS09675|transcript|PV82_RS09675|protein_coding|c.*3025delA||||3025|,C|downstream_gene_variant|MODIFIER|PV82_RS09680|PV82_RS09680|transcript|PV82_RS09680|protein_coding|c.*3489delA||||3489|,C|downstream_gene_variant|MODIFIER|PV82_RS09685|PV82_RS09685|transcrip

	t|PV82_RS09685|protein_coding|c.*3809delA||||3809|,C|downstream_gene_variant|MODIFIER|PV82_RS09690|PV82_RS09690|transcript|PV82_RS09690|protein_coding|c.*4092delA||||4092|;LOF=(PV82_RS09660|PV82_RS09660|1|1.00)
4	AC=1;AF=0.500;AN=2;DP=449;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=60.00;QD=28.73;SOR=0.702;ANN=G|missense_variant|MODERATE|PV82_RS10325|PV82_RS10325|transcript|PV82_RS10325|protein_coding|1/1|c.310T>G|p.Phe104Val|310/678|310/678|104/225|,G|upstream_gene_variant|MODIFIER|PV82_RS10305|PV82_RS10305|transcript|PV82_RS10305|protein_coding|c.-3523A>C||||3523|,G|upstream_gene_variant|MODIFIER|PV82_RS10320|PV82_RS10320|transcript|PV82_RS10320|protein_coding|c.-586A>C||||586|,G|upstream_gene_variant|MODIFIER|PV82_RS10330|PV82_RS10330|transcript|PV82_RS10330|protein_coding|c.-365T>G||||365|,G|upstream_gene_variant|MODIFIER|PV82_RS10335|PV82_RS10335|transcript|PV82_RS10335|protein_coding|c.-2092T>G||||2092|,G|upstream_gene_variant|MODIFIER|PV82_RS10350|PV82_RS10350|transcript|PV82_RS10350|protein_coding|c.-4762T>G||||4762|,G|downstream_gene_variant|MODIFIER|PV82_RS10310|PV82_RS10310|transcript|PV82_RS10310|protein_coding|c.*2548T>G||||2548|,G|downstream_gene_variant|MODIFIER|PV82_RS10315|PV82_RS10315|transcript|PV82_RS10315|protein_coding|c.*1994T>G||||1994|,G|downstream_gene_variant|MODIFIER|spxO|PV82_RS19590|transcript|PV82_RS19590|protein_coding|c.*1749A>C||||1749|,G|downstream_gene_variant|MODIFIER|fumC|PV82_RS10340|transcript|PV82_RS10340|protein_coding|c.*3004A>C||||3004|,G|downstream_gene_variant|MODIFIER|PV82_RS10345|PV82_RS10345|transcript|PV82_RS10345|protein_coding|c.*4460A>C||||4460|WARNING_TRANSCRIPT_NO_START_CODON
5	AC=2;AF=1.00;AN=2;DP=690;FS=0.000;MLEAC=2;MLEAF=1.00;MQ=60.00;QD=30.97;SOR=0.699;ANN=G|stop_lost|HIGH|PV82_RS11215|PV82_RS11215|transcript|PV82_RS11215|protein_coding|1/1|c.1519T>C|p.Ter507Glnext*?|1519/2274|1519/2274|507/757|,G|upstream_gene_variant|MODIFIER|pgmB|PV82_RS11205|transcript|PV82_RS11205|protein_coding|c.-2430T>C||||2430|,G|upstream_gene_variant|MODIFIER|PV82_RS11210|PV82_RS11210|transcript|PV82_RS11210|protein_coding|c.-748T>C||||748|,G|downstream_gene_variant|MODIFIER|PV82_RS11195|PV82_RS11195|transcript|PV82_RS11195|protein_coding|c.*4004A>G||||4004|,G|downstream_gene_variant|MODIFIER|clpP|PV82_RS11200|transcript|PV82_RS11200|protein_coding|c.*3164A>G||||3164|,G|downstream_gene_variant|MODIFIER|PV82_RS11220|PV82_RS11220|transcript|PV82_RS11220|protein_coding|c.*1496T>C||||1496|,G|downstream_gene_variant|MODIFIER|PV82_RS11225|PV82_RS11225|transcript|PV82_RS11225|protein_coding|c.*2385T>C||||2385|,G|downstream_gene_variant|MODIFIER|PV82_RS11230|PV82_RS11230|transcript|PV82_RS11230|protein_coding|c.*3222T>C||||3222|,G|downstream_gene_variant|MODIFIER|PV82_RS11235|PV82_RS11235|transcript|PV82_RS11235|protein_coding|c.*4572T>C||||4572|
6	AC=1;AF=0.500;AN=2;DP=438;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=60.00;QD=27.24;SOR=0.893;ANN=T|frameshift_variant|HIGH|PV82_RS12005|PV82_RS12005|transcript|PV82_RS12005|protein_coding|1/1|c.1649_1652delGCAA|p.Ser550fs|1652/1737|1649/1737|550/578|,T|upstream_gene_variant|MODIFIER|PV82_RS11965|PV82_RS11965|transcript|PV82_RS11965|protein_coding|c.-4849_-4846delGCAA||||4846|,T|upstream_gene_variant|MODIFIER|PV82_RS11975|PV82_RS11975|transcript|PV82_RS11975|protein_coding|c.-3041_-3038delGCAA||||3038|,T|upstream_gene_variant|MODIFIER|PV82_RS11980|PV82_RS11980|transcript|PV82_RS11980|protein_coding|c.-2295_-2292delGCAA||||2292|,T|upstream_gene_variant|MODIFIER|PV82_RS20015|PV82_RS20015|transcript|PV82_RS20015|protein_coding|c.-1681_-1678delGCAA||||1678|WARNING_TRANSCRIPT_MULTIPLE_STOP_CODONS,T|upstream_gene_variant|MODIFIER|PV82_RS11990|PV82_RS11990|transcript|PV82_RS11990|protein_coding|c.-1283_-1280delGCAA||||1280|,T|upstream_gene_variant|MODIFIER|PV82_RS12000|PV82_RS12000|transcript|PV82_RS12000|protein_coding|c.-109_-106delGCAA||||106|,T|downstream_gene_variant|MODIFIER|PV82_RS11970|PV82_RS11970|transcript|PV82_RS11970|protein_coding|c.*3842_*3845delTTGC||||3842|,T|downstream_gene_variant|MODIFIER|PV82_RS12010|PV82_RS12010|transcript|PV82_RS12010|protein_coding|c.*1668_*1671delGCAA||||1671|,T|downstream_gene_variant|MODIFIER|PV82_RS12015|PV82_RS12015|transcript|PV82_RS12015|protein_coding|c.*1928_*1931delGCAA||||1931|,T|downstream_gene_variant|MODIFIER|PV82_RS12020|PV82_RS12020|transcript|PV82_RS12020|protein_coding|c.*2642_*2645delGCAA||||2645|WARNING_TRANSCRIPT_NO_START_CODON,T|downstream_gene_variant|MODIFIER|PV82_RS12025|PV82_RS12025|transcript|PV82_RS12025|protein_coding|c.*3494_*3497delGCAA||||3497|;LOF=(PV82_RS12005|PV82_RS12005|1|1.00)
7	AC=1;AF=0.500;AN=2;DP=517;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=60.00;QD=28.20;SOR=0.865;ANN=A|stop_gained|HIGH|yxdJ|PV82_RS13580|transcript|PV82_RS13580|protein_coding|1/1|c.451G>T|p.Glu151*|451/690|451/690|151/229|,A|upstream_gene_variant|MODIFIER|PV82_RS13560|PV82_RS13560|transcript|PV82_RS13560|protein_coding|c.-3988G>T||||3988|,A|upstream_gene_variant|MODIFIER|yxdM|PV82_RS13565|transcript|PV82_RS13565|protein_coding|c.-2106G>T||||2106|,A|upstream_gene_variant|MODIFIER|yxdL|PV82_RS13570|transcript|PV82_RS13570|protein_coding|c.-1358G>T||||1358|,A|upstream_gene_variant|MODIFIER|yxdK|PV82_RS13575|transcript|PV82_RS13575|protein_coding|c.-236G>T||||236|,A|upstream_gene_variant|MODIFIER|PV82_RS13595|PV82_RS13595|transcript|PV82_RS13595|protein_coding|c.-3001C>A||||3001|,A|upstream_gene_variant|MODIFIER|PV82_RS13600|PV82_RS13600|transcript|PV82_RS13600|protein_coding|c.-3909C>A||||3909|,A|upstream_gene_variant|MODIFIER|PV82_RS13605|PV82_RS13605|transcript|PV82_RS13605|protein_coding|c.-4684C>A||||4684|,A|downstream_gene_variant|MODIFIER|PV82_RS13555|PV82_RS13555|transcript|PV82_RS13555|protein_coding|c.*4376C>A||||4376|,A|downstream_gene_variant|MODIFIER|PV82_RS13585|PV82_RS13585|transcript|PV82_RS13585|protein_coding|c.*600G>T||||600|,A|downstream_gene_variant|MODIFIER|PV82_RS13590|PV82_RS13590|transcript|PV82_RS13590|protein_coding|c.*2036G>T||||2036|
8	AC=2;AF=1.00;AN=2;BaseQRankSum=-3.560e-01;DP=1021;FS=24.257;MLEAC=2;MLEAF=1.00;MQ=60.00;MQRankSum=0.00;QD=25.00;ReadPosRankSum=2.39;SOR=5.113;ANN=A|upstream_gene_variant|MODIFIER|PV82_RS20080|PV82_RS20080|transcript|PV82_RS20080|protein_coding|c.-1952G>A||||1952|,A|upstream_gene_variant|MODIFIER|PV82_RS16915|PV82_RS16915|transcript|PV82_RS16915|protein_coding|c.-3248G>A||||3248|WARNING_TRANSCRIPT_NO_START_CODON,A|upstream_gene_variant|MODIFIER|thiC|PV82_RS16920|transcript|PV82_RS16920|protein_coding|c.-4228G>A||||4228|,A|intragenic_variant|MODIFIER|rrf|PV82_RS16825|gene_variant|PV82_RS16825||n.92G>A|||||
9	AC=2;AF=1.00;AN=2;BaseQRankSum=1.86;DP=1060;FS=24.257;MLEAC=2;MLEAF=1.00;MQ=60.00;MQRankSum=0.00;QD=29.56;ReadPosRankSum=2.87;SOR=5.113;ANN=T|upstream_gene_variant|MODIFIER|PV82_RS20080|PV82_RS20080|transcript|PV82_RS20080|protein_coding|c.-1951C>T||||1951|,T|upstream_gene_variant|MODIFIER|PV82_RS16915|PV82_RS16915|transcript|PV82_RS16915|protein_coding|c.-3247C>T||||3247|WARNING_TRANSCRIPT_NO_START_CODON,T|upstream_gene_variant|MODIFIER|thiC|PV82_RS16920|transcript|PV82_RS16920|protein_coding|c.-4227C>T||||4227|,T|intragenic_variant|MODIFIER|rrf|PV82_RS16825|gene_variant|PV82_RS16825||n.93C>T|||||
10	AC=2;AF=1.00;AN=2;BaseQRankSum=2.07;DP=1201;FS=32.359;MLEAC=2;MLEAF=1.00;MQ=60.00;MQRankSum=0.00;QD=30.62;ReadPosRankSum=2.77;SOR=4.959;ANN=G|upstream_gene_variant|MODIFIER|PV82_RS20080|PV82_RS20080|transcript|PV82_RS20080|protein_coding|c.-1947A>G||||1947|,G|upstream_gene_variant|MODIFIER|PV82_RS16915|PV82_RS16915|transcript|PV82_RS16915|protein_coding|c.-3243A>G||||3243|WARNING_TRANSCRIPT_NO_START_CODON,G|upstream_gene_variant|MODIFIER|thiC|PV82_RS16920|transcript|PV82_RS16920|protein_coding|c.-4223A>G||||4223|,G|intragenic_variant|MODIFIER|rrf|PV82_RS16825|gene_variant|PV82_RS16825||n.97A>G|||||
11	C=2;AF=1.00;AN=2;BaseQRankSum=2.10;DP=2029;FS=31.008;MLEAC=2;MLEAF=1.00;MQ=60.00;MQRankSum=0.00;QD=28.17;ReadPosRankSum=-2.937e+00;SOR=1.762;ANN=A|upstream_gene_variant|MODIFIER|PV82_RS20080|PV82_RS20080|transcript|PV82_RS20080|protein_coding|c.-1853G>A||||1853|,A|upstream_gene_variant|MODIFIER|PV82_RS16915|PV82_RS16915|transcript|PV82_RS16915|protein_coding|c.-3149G>A||||3149|WARNING_TRANSCRIPT_NO_START_CODON,A|upstream_gene_variant|MODIFIER|thiC|PV82_RS16920|transcript|PV82_RS16920|protein_coding|c.-4129G>A||||4129|,A|intragenic_variant|MODIFIER|rrf|PV82_RS16825|gene_variant|PV82_RS16825||n.191G>A|||||
12	AC=2;AF=1.00;AN=2;BaseQRankSum=2.19;DP=2024;FS=30.874;MLEAC=2;MLEAF=1.00;MQ=60.00;MQRankSum=0.00;QD=26.80;ReadPosRankSum=-2.836e+00;SOR=1.748;ANN=T|upstream_gene_variant|MODIFIER|PV82_RS20080|PV82_RS20080|transcript|PV82_RS20080|protein_coding|c.-1852C>T||||1852|,T|upstream_gene_variant|MODIFIER|PV82_RS16915|PV82_RS16915|transcript|PV82_RS16915|protein_coding|c.-3148C>T||||3148|WARNING_TRANSCRIPT_NO_START_CODON,T|upstream_gene_variant|MODIFIER|thiC|PV82_RS16920|transcript|PV82_RS16920|protein_coding|c.-4128C>T||||4128|,T|intragenic_variant|MODIFIER|rrf|PV82_RS16825|gene_variant|PV82_RS16825||n.192C>T|||||
13	AC=1;AF=0.500;AN=2;BaseQRankSum=1.06;DP=460;FS=7.391;MLEAC=1;MLEAF=0.500;MQ=59.78;MQRankSum=-1.152e+00;QD=26.00;ReadPosRankSum=5.27;SOR=1.356;ANN=ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|disruptive_inframe_insertion|MODERATE|PV82_RS17110|PV82_RS17110|transcript|PV82_RS17110|protein_coding|1/1|c.203_204insCACAGACAAAGCCCGGCATTCACAAACCGGCTCCATGCAGCCTGCTGACCTGATTCAACAGGCGTTCAGCGGGGAATCCGGCGGACAGCAGCACACGGACAAAGCCCGGCATTCACAAACCGGCTCTACGCAGCCTGCCGAGCTGATCAAACAGGCTTTCGGCGGGGAATCCGGCGGACAGCAGCA|p.His68_Glu69insThrAspLysAlaArgHisSerGlnThrGlySerMetGlnProAlaAspLeuIleGlnGlnAlaPheSerGlyGluSerGlyGlyGlnGlnHisThrAspLysAlaArgHisSerGlnThrGlySerThrGlnProAlaGluLeuIleLysGlnAlaPheGlyGlyGluSerGlyGlyGlnGlnHis|203/393|203/393|68/130|,ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|upstream_gene_variant|MODIFIER|cspB|PV82_RS17090|transcript|PV82_RS17090|protein_coding|c.-4147_-4146insCACAGACAAAGCCCGGCATTCACAAACCGGCTCCATGCAGCCTGCTGACCTGATTCAACAGGCGTTCAGCGGGGAATCCGGCGGACAGCAGCACACGGACAAAGCCCGGCATTCACAAACCGGCTCTACGCAGCCTGCCGAGCTGATCAAACAGGCTTTCGGCGGGGAATCCGGCGGACAGCAGCA||||4147|,ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|upstream_gene_variant|MODIFIER|PV82_RS17100|PV82_RS17100|transcript|PV82_RS17100|protein_coding|c.-1838_-1837insCACAGACAAAGCCCGGCATTCACAAACCGGCTCCATGCAGCCTGCTGACCTGATTCAACAGGCGTTCAGCGGGGAATCCGGCGGACAGCAGCACACGGACAAAGCCCGGCATTCACAAACCGGCTCTACGCAGCCTGCCGAGCTGATCAAACAGGCTTTCGGCGGGGAATCCGGCGGACAGCAGCA||||1838|,ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|upstream_gene_variant|MODIFIER|PV82_RS17115|PV82_RS17115|transcript|PV82_RS17115|protein_coding|c.-348_-347insTGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG||||347|,ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|upstream_gene_variant|MODIFIE

	R|PV82_RS17120|PV82_RS17120|transcript|PV82_RS17120|protein_coding||c.-1008_-1007insTGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG||||1007|,ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|upstream_gene_variant|MODIFIER|PV82_RS17130|PV82_RS17130|transcript|PV82_RS17130|protein_coding||c.-2770_-2769insTGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG||||2769|,ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|upstream_gene_variant|MODIFIER|PV82_RS17135|PV82_RS17135|transcript|PV82_RS17135|protein_coding||c.-4821_-4820insTGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG||||4820|,ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|downstream_gene_variant|MODIFIER|PV82_RS17085|PV82_RS17085|transcript|PV82_RS17085|protein_coding||c.*4438_*4439insTGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|downstream_gene_variant|MODIFIER|PV82_RS17095|PV82_RS17095|transcript|PV82_RS17095|protein_coding||c.*2933_*2934insTGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG||||2934|,ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|downstream_gene_variant|MODIFIER|PV82_RS17105|PV82_RS17105|transcript|PV82_RS17105|protein_coding||c.*294_*295insTGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG||||295|,ATGCTGCTGTCCGCCGGATTCCCCGCCGAAAGCCTGTTTGATCAGCTCGGCAGGCTGCGTAGAGCCGGTTTGTGAATGCCGGGCTTTGTCCGTGTGCTGCTGTCCGCCGGATTCCCCGCTGAACGCCTGTTGAATCAGGTCAGCAGGCTGCATGGAGCCGGTTTGTGAATGCCGGGCTTTGTCTGTG|downstream_gene_variant|MODIFIER|PV82_RS17125|PV82_RS17125|transcript|PV82_RS17125|protein_coding||c.*1910_*1911insCACAGACAAAGCCCGGCATTCACAAACCGGCTCTACGCAGCCTGCCGAGCTGATCAAACAGGCTTTCGGCGGGGAATCCGGCGGACAGCAGCACACGGACAAAGCCCGGCATTCACAAACCGGCTCTACGCAGCCTGCCGAGCTGATCAAACAGGCTTTCGGCGGGGAATCCGGCGGACAGCAGCA||||1910|WARNING_TRANSCRIPT_NO_START_CODON
14	AC=1;AF=0.500;AN=2;DP=438;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=60.00;QD=30.02;SOR=0.979;ANN=A|stop_gained|HIGH|glpK|PV82_RS17180|transcript|PV82_RS17180|protein_coding|1/1|c.500G>A|p.Trp167*|500/1491|500/1491|167/496||,A|upstream_gene_variant|MODIFIER|glpD|PV82_RS17185|transcript|PV82_RS17185|protein_coding||c.-1130G>A||||1130|,A|upstream_gene_variant|MODIFIER|PV82_RS17190|PV82_RS17190|transcript|PV82_RS17190|protein_coding||c.-2937G>A||||2937|,A|upstream_gene_variant|MODIFIER|PV82_RS17195|PV82_RS17195|transcript|PV82_RS17195|protein_coding||c.-4825G>A||||4825|,A|downstream_gene_variant|MODIFIER|PV82_RS17160|PV82_RS17160|transcript|PV82_RS17160|protein_coding||c.*3703G>A||||3703|,A|downstream_gene_variant|MODIFIER|PV82_RS17165|PV82_RS17165|transcript|PV82_RS17165|protein_coding||c.*2108G>A||||2108|,A|downstream_gene_variant|MODIFIER|PV82_RS17170|PV82_RS17170|transcript|PV82_RS17170|protein_coding||c.*1516G>A||||1516|,A|downstream_gene_variant|MODIFIER|PV82_RS17175|PV82_RS17175|transcript|PV82_RS17175|protein_coding||c.*516G>A||||516|
15	AC=2;AF=1.00;AN=2;BaseQRankSum=4.30;DP=461;FS=7.538;MLEAC=2;MLEAF=1.00;MQ=59.37;MQRankSum=0.00;QD=31.98;ReadPosRankSum=-2.230e-01;SOR=0.712;ANN=G|synonymous_variant|LOW|PV82_RS18795|PV82_RS18795|transcript|PV82_RS18795|protein_coding|1/1|c.954A>G|p.Glu318Glu|954/1458|954/1458|318/485||WARNING_TRANSCRIPT_NO_STOP_CODON,G|downstream_gene_variant|MODIFIER|PV82_RS18760|PV82_RS18760|transcript|PV82_RS18760|protein_coding||c.*4648A>G||||4648|,G|downstream_gene_variant|MODIFIER|PV82_RS18765|PV82_RS18765|transcript|PV82_RS18765|protein_coding||c.*4148A>G||||4148|,G|downstream_gene_variant|MODIFIER|PV82_RS18770|PV82_RS18770|transcript|PV82_RS18770|protein_coding||c.*3705A>G||||3705|,G|downstream_gene_variant|MODIFIER|PV82_RS18775|PV82_RS18775|transcript|PV82_RS18775|protein_coding||c.*3499A>G||||3499|WARNING_TRANSCRIPT_NO_START_CODON,G|downstream_gene_variant|MODIFIER|PV82_RS18780|PV82_RS18780|transcript|PV82_RS18780|protein_coding||c.*2102A>G||||2102|,G|downstream_gene_variant|MODIFIER|PV82_RS18785|PV82_RS18785|transcript|PV82_RS18785|protein_coding||c.*1657A>G||||1657|,G|downstream_gene_variant|MODIFIER|PV82_RS18790|PV82_RS18790|transcript|PV82_RS18790|protein_coding||c.*1135A>G||||1135|,G|downstream_gene_variant|MODIFIER|PV82_RS19675|PV82_RS19675|transcript|PV82_RS19675|protein_coding||c.*941A>G||||941|

***Table** 7: Report of the information related to each one of the variants resulting from the comparative analysis.*

3.9 Screening of the antagonistic activity of selected bacterial isolates against bacteria

Comparative analysis between *B. velezensis* strains 'BLG_B2' and 'BLG_B4' revealed the existence in *B. velezensis* 'BLG_B2' of two mutations in two genes involved in antimicrobial processes. In particular, a frameshift mutation in the antibacterial toxin encoding *yeeF* gene could interfere with antibacterial competition. Because of that *B. velezensis* strains 'BLG_B2' and 'BLG_B4' were assessed for the antagonistic activity against challenge bacteria, including multi-resistant bacteria and two non-pathogenic bacteria. For each isolate the filtered supernatant of overnight liquid cultures enriched with 0,4% glucose was used as the liquid culture medium for the growth of challenge bacteria. The filtered supernatant of a liquid culture of *B. subtilis* ATCC6633 was used as a control. The experiment was repeated three times for all the challenge bacteria. *B. velezensis* 'BLG_B4' was the first bacteria to be tested giving the following results:

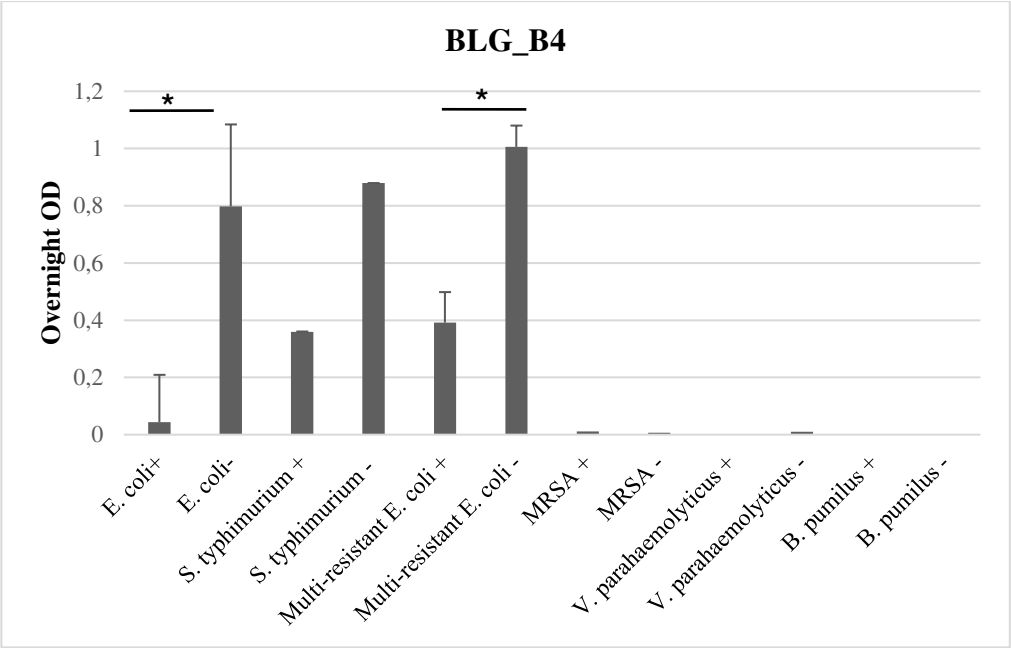

***Figure 20**: Graph bar representing the mean OD values of overnight liquid cultures of challenge bacteria in enriched B. velezensis 'BLG_B4' and B. subtilis ATCC6633 supernatants. The symbols + and – indicate whether the challenge bacteria were inoculated in the filtered supernatant of B. velezensis 'BLG_B4' or B. subtilis ATCC6633, respectively. The star indicates the existence of a statistically significant difference between the OD values of the two liquid cultures according to Student's t-test (p-value < 0.05).*

The filtered supernatant of *B. velezensis* 'BLG_B4' statistically significantly inhibited the growth of both multi-resistant *E. coli* and non-pathogenic *E. coli* compared to *B. subtilis* ATCC6633 (Student's t-test $p < 0.05$). As for *S. typhimurium, B. velezensis* 'BLG_B4' supernatant noticeably inhibited its growth compared to *B. subtilis* ATCC6633 even though to a non-statistically significant level (Student's t-test $p = 0,07$). Finally, for both the tested supernatants no growth was detectable for MRSA, *V. parahaemolyticus* and *B. pumilus*.

The same experiment was performed for *B. velezensis* 'BLG_B2' giving the following results:

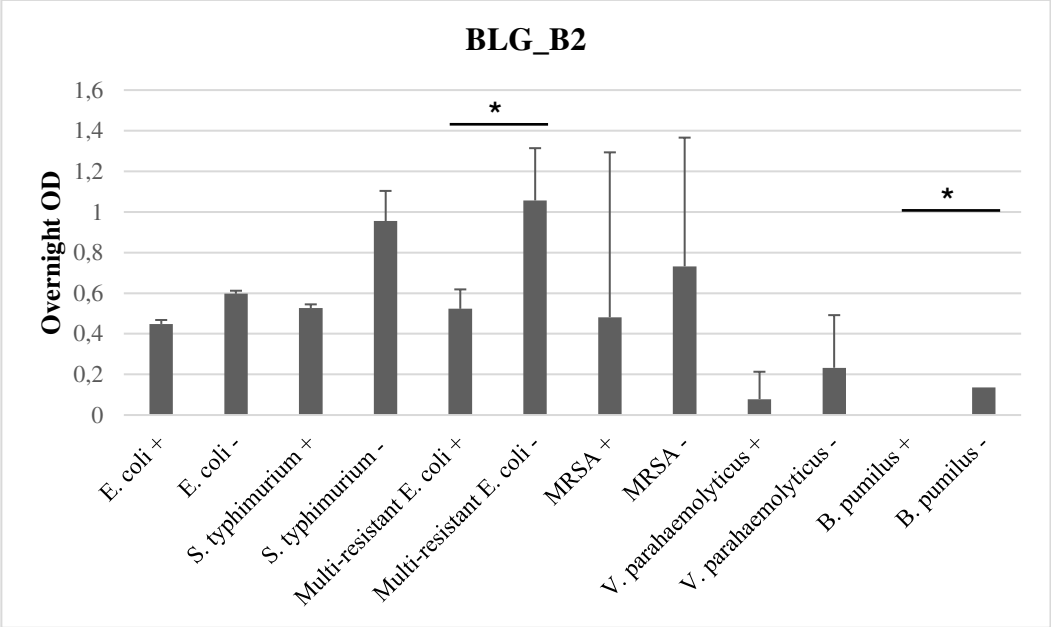

***Figure 21**: Graph bar representing the mean OD values of overnight liquid cultures of challenge bacteria in enriched B. velezensis 'BLG_B2' and B. subtilis ATCC6633 supernatants. The symbols + and – indicate whether the challenge bacteria were inoculated in the filtered supernatant of B. velezensis 'BLG_B2' or B. subtilis ATCC6633, respectively. The star indicates the existence of a statistically significant difference between the OD values of the two liquid cultures according to Student's t-test (p-value < 0.05).*

The experiment was repeated three times for all the challenge bacteria. *B. velezensis* 'BLG_B2' supernatant statistically significantly inhibited the growth of multi-resistant *E. coli* and non-pathogenic *B. pumilus* (Student's t-test $p < 0.05$). As for *S. typhimurium, B. velezensis* 'BLG_B2' supernatant noticeably inhibited its growth compared to *B. subtilis* ATCC6633 even though to a non-statistically significant level (Student's t-test $p = 0,08$). No remarkable or significant differences were found between the effect of the supernatants of *B. velezensis* 'BLG_B2' and *B. subtilis* ATCC6633 on the growth of non-pathogenic *E. coli,* MRSA, and *V. parahaemolyticus*.

Moreover, a preliminary investigation of the antibacterial properties of *B. subtilis* 'BLG_B1.1.1', *P. psychrotolerans* 'BLG_B1.3' and *P. pleuroti* 'BLG_B5' was prepared. Preliminary results, indicating the ineffectiveness of this bacterial strains against the challenge bacteria, were reported in Table 8:

	OPTICAL DENSITY (OD_{600nm}) of overnight liquid cultures					
	Experiment 1		Experiment 1		Experiment 1	
	BLG_B1_1.1	***B. subtilis* ATCC6633**	**BLG_B1.3**	***B. subtilis* ATCC6633**	**BLG_B5**	***B. subtilis* ATCC6633**
MRSA	1,012	0,005	1,194	1,288	0,844	0,966
V. parahaemolyticus	0,376	0,331	0,366	0,334	0,266	0,284
multi-resistant *E. coli*	1,120	1,536	1,300	0,685	1,092	0,860
S. typhimurium	0,928	0,921	0,964	0,751	0,948	0,788
E. coli	0,640	0,875	0,730	0,895	0,969	0,513
B. pumilus	0,122	0,109	0,303	1,010	0,165	0,131

***Table 8**: Summary of the OD values of overnight liquid cultures of challenge bacteria in enriched B. subtilis 'BLG_B1.1.1', P. psychrotolerans 'BLG_B1.3' and P. pleuroti 'BLG_B5' and B. subtilis ATCC6633 supernatants. The same experiment was performed only once for each tested bacterial isolate.*

3.10 Pilot field trial

Future perspectives of the present work include the *in vivo* inoculation on plants to evaluate the effect of the selected bacteria on *V. vinifera* resistance against plant diseases affecting the phylloplane and carposphere, as a part of the objective of the REVINE project. In this regard, part of the PhD was spent in the company Ermes S.a.s di Giacomo Suglia & f.lli located in Noicattaro (BA) where a pilot field trial was performed. The trial aimed to investigate the effect of the treatment with a microbial consortium, a synthetic community (SynCom), selected at CREA-VE, and applied to the roots, on the resilience capacities to biotic stresses of two table grape cv.: 'Autumn Crisp' and 'Anxa'. The SynCom was composed of 24 different species of bacteria, fungi and actinomycetes isolated from the rhizosphere or internal tissues of grapevines and selected for their biocontrol potential against various grapevine diseases. The analyses of this trial are ongoing and include the metagenomic analysis of rhizosphere samples collected from the treated plants four months after the application of the microbial consortium and surveys of agronomic parameters and major grapevine diseases such as DM, powdery mildew and grey mold that will be performed during summer season of 2025. Results will allow the definition of protocols to apply potential BCAs alone or in SynCom directly on vineyards.

4. DISCUSSION

Plants harbor a broad spectrum of microorganisms colonizing their internal and external tissues and organs constituting their microbiome and playing a central role in multiple aspects of nature and human practices including planet health, natural vegetation, and agricultural production systems up to human health [2]. Plant microbiomes have attracted researchers' attention because of their ability to synthesize various metabolites with relevant biotechnological interests. [148][149]. Indeed, plant microbiomes have been widely explored for their antagonistic activity against various phytopathogens with the aim of employing them as eco-friendly alternatives to chemical pesticides. [193] [148] [194] [195]. This work aimed to explore and valorize the autochthonous microbiome of *V. vinifera*, with a particular focus on the bacterial component, in order to identify microorganisms with antimicrobial activity to cope with biotic stresses, choosing the DM, caused by *P. viticola,* as a case study. The first step in developing BCAs is the isolation in their natural environments, where they compete with plant pathogens [125] [196]. *P. viticola* is a strictly biotrophic oomycete that can invade all the green host tissues, such as leaves, flowers and unripened berries [197]. For this reason, in the present work, bacteria were isolated from the phylloplane of three table grape varieties selected based on their degree of tolerance/susceptibility to DM, evaluated both at the carposphere and phylloplane level. This choice was made based on what previously reported in several works, in which a fair amount of success in terms of finding BCAs, starting from genotypes with high levels of tolerance towards the target disease was registered [138] [174].

4.1 Bacteria isolation and characterization and study of mechanism of action

The preliminary characterization study of different *V. vinifera* varieties, both at the carposphere and phylloplane levels, has shown a wide variability of behaviour in terms of tolerance/susceptibility to DM infections, in line with what previously reported by other authors [198] [199]. Based on the collected data three grape varieties were selected: the cultivar ‘Dawn seedless’, very susceptible both at the phylloplane and carposphere level, the cultivar ‘Blush seedless’, characterized by a high and medium level of tolerance to DM, at the phylloplane and carposphere levels and the cultivar ‘Argentina’, characterized by a medium and high level of tolerance to DM respectively at the phylloplane and carposphere levels. 36 bacterial isolates, selected based on their morphological features, were isolated from the phylloplane of the aforementioned

varieties and 16 were evaluated for their antagonistic activity against *P. viticola*, using leaf discs from the susceptible cultivar 'Flame', treated with a bacterial suspension and infected with the pathogen. Among the highly effective isolates, four (*B. subtilis* 'BLG_B1.1.1', *P. psycotolerans* 'BLG_B1.3', *B. velenzensis* 'BLG_B4' and *P. pleuroti* 'BLG_B5') out of five were obtained from the phylloplane of the most tolerant cultivar 'Blush seedless', suggesting the hypothesis of a co-evolution of microorganisms within a certain niche and the genotype of the host plant. In fact, a lower success rate in terms of finding BCAs was obtained in similar works, where the isolation activity was based on a random approach. For example, among 239 bacterial endophytes isolated from grapevine leaves, only two endophytic strains, identified as *B. subtilis* GLB191 and *B. pumilus* GLB197 have proved their preventive effect against *P. viticola* both on leaf disks and during 2-year field trials [141]. In the same way, Furuya and co-workers isolated one hundred bacteria from healthy berries of different *V. vinifera* cultivars. Among these, a *B. subtilis* strain KS1, selected for their ability to reduce the mycelial growth of *B. cinerea* in *in vitro* condition, decreased the incidence of DM on berries and leaves in vineyard conditions [200]. Finally, among 247 bacterial colonies, collected from the shoot xylem of the grapevine cultivar 'Koshu', an endophytic *B. velenzensis* strain KOF112 showed biocontrol activities against *B. cinerea*, *Colletotrichum gloeosporioides* and *P. viticola* [201]. Further, we investigate a possible mechanism of action of the selected bacterial isolates. Microorganisms act as biocontrol agents employing strategies such as: direct antibiosis, competition for niches and nutrients, interference with pathogen signaling or by inducing plant resistance [202] [203]. Information on the mechanisms of action of the antagonists is essential to develop appropriate formulation and methods of application, and to register and select new effective microorganisms [125]. *Bacillus* genus is a great producer of many antimicrobials and *Bacillus* species such as *B. subtilis*, *B. velezensis, B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, *B. sphaericus*, *B. mojavensis*, *B. pasteurii* and *B. pumilus* are very efficient inhibitors of plant pathogens [132]. Extensive studies on the *Bacillus* genus reported lipopeptides, together with polyketide antibiotics, as the principal produced antimicrobial compounds active against agronomically important fungal pathogens including *B. cinerea*, *Fusarium oxysporum* and *Aspergillus* spp [124] [127] and human pathogens like *K. pneumoniae*, *S. typhymurium*, *E. coli* [49] [171] [172] [166] [173]. Several *Pantoea* species are already

known as very effective biocontrol agents against *B. cinerea*, *Penicillium spp*., *Rhizopus spp*., *F. oxysporum*, *A. alternata* and more, being able to synthesize antimicrobial volatile organic compounds, antibiotics and biosurfactants and stimulating plant innate immunity conferring plant resistance to phytopathogens at the local and/or systemic level [203] [204]. Bacteria belonging to *Pseudomonas* genus are renowned for their role in plant protection biotechnology by serving as antibacterial and antifungal agents, also including already commercialized products based on *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, and *P. syringae* [205]. These bacteria produce a wide variety of antibiotic secondary metabolites, among them phenazine co-compounds, volatile organic compounds and lipopeptides play a crucial role in combating various fungal pathogens like *B. cinerea*, *P. expansum, P. italicum, R. solanacearum* [206].

As possible mechanism of action, in this work we decided to explore the ability of selected bacterial strains to interfere with pathogen signaling and to decrease the expression of two main *P. viticola* effectors, *PvRxLR28* and *PvRxLR67*, that can compromise the plant defense. Effectors are low molecular weight molecules, secreted by pathogenic fungi, bacteria and oomycets, including *P. viticola*, that play a crucial role in the insurgence of the infection. When plants are attacked by pathogens activate a first line of defense called by PTI and a second line called ETI. The induction of PTI is associated with MAP kinase signaling, transcriptional induction of pathogen-responsive genes, production of ROS and deposition of callose to reinforce the cell wall at the sites of infection, all of which contribute to the prevention of microbial growth [207]. PTI is effective against non-adapted pathogens but not against adapted pathogens, because these are able to secrete effectors [208]. Effectors can act in the apoplast or the cytoplasm, like the RxLR effectors. *P. viticola* RxLR effectors are differentially expressed during the stages of the infection and suppress plant immunity. [177] [90] [91] [92]. In the interactions between *P. viticola* and grapevine, the high and early expression of the effector gene *PvRxLR28* may be crucial for the onset and progress of the infection. Indeed, this effector is able to impair plant defenses, such as the H_2O_2 accumulation in infected tissues by down-regulating plant genes encoding ROS-producing enzymes (*RbohA* and *RbohB*) [90]. Furthermore, this effector resulted able to repress plant defense-related genes, such as the salicylic acid responsive pathogenesis-related genes PR1 and PR2, the jasmonic acid responsive gene LOX and

the ethylene responsive gene EFR1 [90]. Results showed that all the tested bacterial isolates were able to reduce the expression of the early effector gene *PvRxLR28* with detectable significant differences among the isolates. In contrast, they similarly reduced the expression of the late effector gene *PvRxLR67.* This suggests that the antagonistic activity of the selected bacteria occurs in the early stages of the infection process. Combining the data resulting from gene expression analysis together with those related to the phenotypical manifestation of the disease, we can observe that *B. velenzensis* 'BLG_B2' significantly reduced the expression of the *PvRxLR28* effector, but to a level insufficient to determine effective disease control. This suggests that effector suppression alone may not be sufficient for biocontrol, as other pathogenicity factors or effectors could compensate for *PvRxLR28* suppression. By contrast, *B. subtilis* strain 'BLG_B1.1.1', which in all experiments conducted in this work proved to be the most effective against *P. viticola*, did not differ from *B. velenzensis* strain 'BLG_B2' in terms of its effect on effector expression. These data suggest that the antagonistic activity of this strain may rely on additional mechanisms, such as the production of antimicrobial compounds, or competition for resources. Finally, *P. psychrotolerans* strain 'BLG_B1.3', *B. velenzensis* strain 'BLG_B4', and *P. pleuroti* strain 'BLG_B5' showed higher levels of reduction in the *PvRxLR28* effector than *B. velenzensis* strain 'BLG_B2', which in the case of *P. pleuroti* strain 'BLG_B5' were statistically significant. However, these bacterial strains did not show a marked antagonistic activity against the pathogen. This discrepancy suggests that the interference with effector expression observed with these bacterial strains may have resulted in a delay in the appearance of symptoms rather than complete disease control. This may still represent an advantage for the plant, especially in the context of integrated management of the disease. Indeed, combining or alternating the use of these microorganisms with anti-DM pesticides with a lower environmental impact, such as those based on copper, could lead to increasing the effectiveness of the latter, which in general have lower efficacy than those based on systemic active ingredients. For instance, the prophylactic application of *Lysobacter capsici* AZ78 alone to grapevine leaves reduced DM to the same degree as a copper-based fungicide. Moreover, combining *L. capsici* AZ78 with low-dose copper treatments significantly enhanced disease control compared to full-dose copper treatments alone [228].

The interference with the expression of phytopathogens' effector genes and the mechanism underlying it is still not a much-investigated action of BCAs, indeed there is a lack of literature reporting this type of study. Cruz-Silva *et al.* [177] reported that in susceptible 'Cabernet Sauvignon' grapevines inoculated with AMF and artificially infected with *P. viticola,* effector *PvRxLR28* was significantly down-regulated at 6 hours post-infection compared to the non-mycorrhizal plants and supposed that this might be the result of the priming of defense mechanisms in susceptible cultivars that could decrease pathogenicity in the first hours of infection The bacterial strains tested in this work (in particular *P. pleuroti* strain 'BLG_B5', *B. velenzensis* strain 'BLG_B4' and *P. psychrotolerans* strain 'BLG_B1.3') also appear to interfere with the expression of this effector during the first hours post-infection, suggesting a partial priming of host defense. Regarding the mechanism underlying the interference with the effector genes expressions, one hypothesis could be the activation of jasmonic acid defense pathway, that was previously reported for different strains of *B. velenzensis*, both in grapevine leaves inoculated with *P. viticola* [209] and tomato fruit infected with *B. cinerea* [210]. In the same way, *B. subtilis* strain PTA-271 can efficiently attenuate Botryosphaeria dieback by enhancing some host immune responses, such as the expression of salicylic acid- and jasmonic acid responsive gene [121].

We focused our attention on the two bacterial strains 'BLG_B4' and 'BLG_B2'. Indeed, we observed that, despite they belonged to the same species (*B. velenzensis*) they did not behave in the same way, both in terms of antagonistic activity against *P. viticola*, and effect on the expression of the two tested *P. viticola* effector genes. We supposed that the noticed differences could be due to genomic variants existing between the two bacterial isolates that might be functionally related to their different antimicrobial properties and to their impairment in *B. velenzesis* 'BLG_B2'. To test this hypothesis, after the performance of a variant calling analysis aligning raw reads resulting from genome sequencing with *B. velenzensis* reference genome, calls with differing genotypes between the two samples were underscored taking into account also their functional impact. In detail, two variants in *B. velenzensis* 'BLG_B2' genome caught our attention: a frameshift mutation affecting gene PV82_RS12005 and a stop-gained mutation affecting PV82_RS13580. PV82_RS12005 and PV82_RS13580 are respectively the *yeeF* and *yxdJ* genes. *yeeF* encodes a polymorphic toxin involved in the contact-dependent growth antagonism which may play an important role in

interbacterial competition. YeeF protein C-terminal domain is a metal-dependent DNase that requires Mg^{2+} or Mn^{2+} ion(s) for its activity and results active in diverse conditions like elevated temperature, alkaline pH and low salt conditions. [189] *yxdJ* encodes a response regulator which binds to DNA and that, together with a histidine kinase, constitutes a two-component system (TCS) that positively regulates the expression of the *yxdLMyxeA* operon by direct interaction with its promoter region. The *yxdLMyxeA* operon encodes an ABC transporter, specifically an extruder, involved in the secretion of antibiotic and antifungal metabolites [191] [192]. Considering the functions of the proteins encoded by the two genes, the frameshift mutation in the *yeeF* gene and the stop-gained mutation in *yxdJ* in our opinion, might determine the production of non-functional proteins in *B. velenzensis* 'BLG_B2' affecting its antimicrobial properties and potentially explain the differences observed between *B. velenzensis* 'BLG_B2' and 'BLG_B4' despite belonging to the same species. This hypothesis is partially confirmed by results obtained in the antagonistic tests against antibiotic-resistant bacteria. Bacterial strains were tested using their filtered supernatants of overnight liquid cultures as the culture medium for the growth of challenge multi-resistant bacteria (MRSA, *V. parahaemolyticus, S. typhimurium,* multi-resistant *E. coli)* and non-pathogenic bacteria (*B. pumilus* and *E. coli*) and the supernatant of a culture of *B. subtilis* ATCC6633 as a control. *B. velezensis* 'BLG_B4' supernatant statistically significantly inhibited the growth of multi-resistant *E. coli* and non-pathogenic *E. coli* compared to *B. subtilis* ATCC6633 suggesting the potential production and export in the supernatant by *B. velezensis* 'BLG_B4' of compounds affecting *E. coli* growth. Differently, *B. velezensis* 'BLG_B2' supernatant statistically significantly inhibited only the growth of multi-resistant *E. coli*. The efficacy of these two strains against the multidrug-resistant *E. coli* strain represents a noticeable result, as the management of antibiotic-resistant bacteria has become a public health threat due to the widespread use of antibiotics. [149] In particular, multidrug-resistant 'ESKAPE' organisms are associated with high rates of morbidity and mortality. [149]. *B. velezensis* has already demonstrated in recent works its potential in counteracting multi-resistant bacteria. Baharudin and co-workers [215] reported the cell-free supernatant of two *B. velezensis* strains, isolated from stingless bees, was able to inhibit the growth of *V. parahaemolyticus* and different strains of MRSA when added to solid culture medium. Priyanto and co-workers [216] observed that metabolite extract

obtained from a *B. velezensis* endophytic strain isolated from *Archidendron pauciflorum* exerted an anti-MRSA activity while performing a disc-diffusion assay.

4.2 Potential technological development of selected BCA candidates

Selected bacterial strains were evaluated for their application development to define their possibility of use and potential in field utilization. To be considered a good BCA, a microorganism must possess some features, such as effectiveness at reasonable doses and against a wide range of pathogens, being able to survive in formulations easy to distribute and with long shelf-life and safe to human health [125]. In this work we evaluated the effectiveness of the selected bacterial strains against several grapevine fungal pathogens and their ability to degrade blood erythrocytes at a temperature like that of a human internal temperature. Results of the *in vitro* antagonism tests performed in this work seem to show a specific activity of the tested bacterial strains. *B. subtilis* 'BLG_B1.1.1', highly effective against *P. viticola*, resulted unable to control other tested fungal pathogens. Otherwise, the ineffective *B. velenzenzis* 'BLG_B2', showed a good ability to contain the *in vitro* growth of *P. digitatum*. The antagonistic activity of both the other strains belonging to the species *B. velenzensis* ('BLG_B4') and *P. pleuroti* ('BLG_B5') seem to be interesting, as in addition to having a fair amount of efficacy in contrasting *P. viticola*, they showed a high aptitude in reducing the mycelia growth of *P. digitatum*. Human health risk assessment is a prerequisite for the application of a microorganism as BCA [211] [214]. In this work we used the ability to degrade erythrocytes to evaluate the potential toxicity of the bacterial strains studied, which has previously been proposed as a useful biological model to study potential human health risks [212]. In the experiment conducted in this work, the selected bacterial strains, despite their ability to grow at 37°C (human temperature) on LB medium, a commonly used bacterial culture medium in the laboratory [213], showed different behaviour when growing on a blood-containing medium. While *B. velenzenzesis* 'BLG_B2' and *P. pleuroti* 'BLG_B5' resulted unable to grow in this kind of medium, *B. subtilis* 'BLG_B1.1.1', *B. velenzensis* 'BLG_B4' and *P. psycotolerans* strain 'BLG_B1.3' did not show hemolytic activity. Our results, although in contrast with those obtained by other authors [217] [218] [222] [223] [219] [220] [221], provide a preliminary indication of the low risk concerning the risk of using these bacterial strains for human health.

The process of characterization of new BCAs requires the *in vivo* test of the candidate microorganisms [125]. Indeed, the *in vivo* inoculation on plants to evaluate the effect of the selected bacteria on *V. vinifera* resistance against plant diseases affecting the phylloplane and carposphere is one of the future perspectives of the present work, as a part of the objective of the REVINE project. In this regard, part of the PhD was spent in the company Ermes S.a.s di Giacomo Suglia & f.lli located in Noicattaro (BA) where a pilot field trial was performed consisting of the application of a synthetic microbial community (SynCom) of 24 different species of microorganisms with already proven biocontrol properties selected at CREA-VE, to the roots of plants of the table grape cv. 'Autumn Crisp' and 'Anxa', to evaluate their resilience capacities to biotic stresses. The analysis of the trial results is ongoing and will be useful in setting up a protocol for the preparation of the microbial inoculum and deciding the mode of testing our candidate bacterial BCAs, alone or in SynCom directly on vineyards.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, we screened 16 different bacterial strains, isolated from the phylloplane of three table grape cultivars, to develop biological control alternatives against the DM of grapevine. Based on the *in vivo* experiment we proposed four bacterial strains as suitable BCAs against *P. viticola*: *B. subtilis* 'BLG_B.1.1.1', *B. velenzensis* 'BLG_B4', *P. pleuroti* 'BLG_B5' and *P. psycotolerans* 'BLG_B1.3'. These strains, as demonstrated by gene expression analysis, were able to reduce the expression of the early effector gene *PvRxLR28* with significant differences among one another and to reduce, to a similar level, the expression of the late effector gene *PvRxLR67*. In particular, *B. subtilis* 'BLG_B.1.1.1', that in all experiments showed a high efficacy in containing *P. viticola*, could be considered as a valid alternative for the current chemical pesticides. Otherwise, the other strains could find the right application in integrated control strategies of the disease. Furthermore, we identified a strain belonging to the species *B. velenzensis* ('BLG_B2') that, despite its ineffectiveness in containing grapevine DM, could have interesting developments as a BCA in the control of other fungal pathogens, such as *P. digitatum*, the causal agent of 'green mold'. Furthermore, another noteworthy aspect that can be detected in this work is represented by the versatility demonstrated by some of the selected bacterial strains and, in particular, by the two strains belonging to the species *B. velenzensis* ('BLG_B2' and 'BLG_B4'). Indeed, both have not only shown that they can be potentially

developed as BCAs against plant phytopathogens, but also as agents against the multi-drug-resistant strain of *E. coli*.

The molecular characterization of the bacterial strains pointed out the existence in 'BLG_B2' of two mutations in genes directly and indirectly involved in antimicrobial processes that could explain its different behaviour observed in the performed assays against *P. viticola*, compared to the one of the strain 'BLG_B4', belonging to the same species. The functional effect of these mutations was also noticeable in the different antibacterial activity showed by the two strains. All the proposed bacterial strains showed a preliminary predisposition for technological development thanks to a partial efficiency against a wide range of fungal pathogens and their inability to cause hemolysis of red blood cells. Further perspectives of the present work involve research covering (i) evaluation in field conditions and at low concentration, (ii) ability to survive at inexpensive, easy to distribute and with a long *shelf-life* formulation and (iii) biotechnological aspects of large-scale production and formulation, which are needed to develop suitable bioproducts. First insights into the testing of the bacterial strains in field condition will be obtained from the results of the performed pilot field trial. Results demonstrated that the autochthonous microbiome of *V. vinifera* represents an important reservoir of microorganisms with the potential to be employed in different fields including agriculture, as eco-friendly alternatives to chemical pesticides, making *V. vinifera* microbiome a resource worth valorizing.

6. BIBLIOGRAPHY

[1] J. Whipps, K. Lewis, R. Cooke, Mycoparasitism and plant disease control. In: Burge M, editor. Fungi Biol Control Syst. Manchester University Press; 1988. p. 161-187.

[2] G. Berg *et al.*, «Microbiome definition re-visited: old concepts and new challenges», *Microbiome*, vol. 8, fasc. 1, p. 103, dic. 2020, doi: 10.1186/s40168-020-00875-0.

[3] M. T. Parker, «An Ecological Framework of the Human Virome Provides Classification of Current Knowledge and Identifies Areas of Forthcoming Discovery».

[4] P. Vandenkoornhuyse, A. Quaiser, M. Duhamel, A. Le Van, e A. Dufresne, «The importance of the microbiome of the plant holobiont», *New Phytologist*, vol. 206, fasc. 4, pp. 1196–1206, giu. 2015, doi: 10.1111/nph.13312.

[5] C. Pinto e A. C. Gomes, «Vitis vinifera microbiome: from basic research to technological development», *BioControl*, vol. 61, fasc. 3, pp. 243–256, giu. 2016, doi: 10.1007/s10526-016-9725-4.

[6] R. Guerrero, L. Margulis, e M. Berlanga, «Symbiogenesis: the holobiont as a unit of evolution», *International Microbiology*, fasc. 16, pp. 133–143, 2013, doi: 10.2436/20.1501.01.188.

[7] P. Bettenfeld *et al.*, «The microbiota of the grapevine holobiont: A key component of plant health», *Journal of Advanced Research*, vol. 40, pp. 1–15, set. 2022, doi: 10.1016/j.jare.2021.12.008.

[8] I. Zilber-Rosenberg e E. Rosenberg, «Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution», *FEMS Microbiol Rev*, vol. 32, fasc. 5, pp. 723–735, ago. 2008, doi: 10.1111/j.1574-6976.2008.00123.x.

[9] M. Youle, N. Knowlton, F. Rohwer, J. Gordon, e D. A. Relman, «Superorganisms and Holobionts: Looking for a term for the functional entity formed by a macrobe and its associated symbiotic microbes and viruses? The term is "holobiont"», *Microbe Magazine*, vol. 8, fasc. 4, pp. 152–153, apr. 2013, doi: 10.1128/microbe.8.152.1.

[10] P. Trivedi, J. E. Leach, S. G. Tringe, T. Sa, e B. K. Singh, «Plant–microbiome interactions: from community assembly to plant health», *Nat Rev Microbiol*, vol. 18, fasc. 11, pp. 607–621, nov. 2020, doi: 10.1038/s41579-020-0412-1.

[11] K. M. G. Dastogeer, F. H. Tumpa, A. Sultana, M. A. Akter, e A. Chakraborty, «Plant microbiome–an account of the factors that shape community composition and diversity», *Current Plant Biology*, vol. 23, p. 100161, set. 2020, doi: 10.1016/j.cpb.2020.100161.

[12] FAO. 2024. *Land statistics 2001–2022 – Global, regional and country trends*. FAOSTAT Analytical Briefs, No. 88. Rome.

[13] R. Darriaut *et al.*, «Grapevine rootstock and soil microbiome interactions: Keys for a resilient viticulture», *Horticulture Research*, vol. 9, p. uhac019, gen. 2022, doi: 10.1093/hr/uhac019.

[14] J. Mercado-Blanco, «Life of Microbes Inside the Plant», in *Principles of Plant-Microbe Interactions*, B. Lugtenberg, A c. di, Cham: Springer International Publishing, 2015, pp. 25–32. doi: 10.1007/978-3-319-08575-3_5.

[15] N. Kim *et al.*, «Cytotoxic 6-Alkylsalicylic Acids from the Endophytic».

[16] E. M. Bucci, «Xylella fastidiosa , a new plant pathogen that threatens global farming: Ecology, molecular biology, search for remedies», *Biochemical and Biophysical Research Communications*, vol. 502, fasc. 2, pp. 173–182, lug. 2018, doi: 10.1016/j.bbrc.2018.05.073.

[17] R. S. Chandi, «Integrated Management of Insect Vectors of Plant Pathogens», *AG*, fasc. Of, dic. 2020, doi: 10.18805/ag.R-1982.

[18] G. Del Frari, A. Gobbi, M. R. Aggerbeck, H. Oliveira, L. H. Hansen, e R. B. Ferreira, «Characterization of the Wood Mycobiome of Vitis vinifera in a Vineyard Affected

by Esca. Spatial Distribution of Fungal Communities and Their Putative Relation With Leaf Symptoms», *Front. Plant Sci.*, vol. 10, p. 910, lug. 2019, doi: 10.3389/fpls.2019.00910.

[19] D. Bozoudi e D. Tsaltas, «Grape Microbiome: Potential and Opportunities as a Source of Starter Cultures», in *Grape and Wine Biotechnology*, A. Morata e I. Loira, A c. di, InTech, 2016. doi: 10.5772/64806.

[20] J. H. J. Leveau e J. J. Tech, «GRAPEVINE MICROBIOMICS: BACTERIAL DIVERSITY ON GRAPE LEAVES AND BERRIES REVEALED BY HIGH-THROUGHPUT SEQUENCE ANALYSIS OF 16S RRNA AMPLICONS», *Acta Hortic.*, fasc. 905, pp. 31–42, ago. 2011, doi: 10.17660/ActaHortic.2011.905.2.

[21] S. Zhang, X. Chen, Q. Zhong, Z. Huang, e Z. Bai, «Relations among epiphytic microbial communities from soil, leaves and grapes of the grapevine», *Frontiers in Life Science*, vol. 10, fasc. 1, pp. 73–83, gen. 2017, doi: 10.1080/21553769.2017.1365776.

[22] S. E. Lindow e M. T. Brandl, «Microbiology of the Phyllosphere».

[23] D. V. Badri, T. L. Weir, D. Van Der Lelie, e J. M. Vivanco, «Rhizosphere chemical dialogues: plant–microbe interactions», *Current Opinion in Biotechnology*, vol. 20, fasc. 6, pp. 642–650, dic. 2009, doi: 10.1016/j.copbio.2009.09.014.

[24] G. Berg e K. Smalla, «Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere: Plant species, soil type and rhizosphere communities», *FEMS Microbiology Ecology*, vol. 68, fasc. 1, pp. 1–13, apr. 2009, doi: 10.1111/j.1574-6941.2009.00654.x.

[25] H. P. Bais, T. L. Weir, L. G. Perry, S. Gilroy, e J. M. Vivanco, «THE ROLE OF ROOT EXUDATES IN RHIZOSPHERE INTERACTIONS WITH PLANTS AND OTHER ORGANISMS», *Annu. Rev. Plant Biol.*, vol. 57, fasc. 1, pp. 233–266, giu. 2006, doi: 10.1146/annurev.arplant.57.032905.105159.

[26] L. J. Shaw, P. Morris, e J. E. Hooker, «Perception and modification of plant flavonoid signals by rhizosphere microorganisms», *Environmental Microbiology*, vol. 8, fasc. 11, pp. 1867–1880, nov. 2006, doi: 10.1111/j.1462-2920.2006.01141.x.

[27] P. F. Morris, E. Bone, e B. M. Tyler, «Chemotropic and Contact Responses of *Phytophthora sojae* Hyphae to Soybean Isoflavonoids and Artificial Substrates1», *Plant Physiology*, vol. 117, fasc. 4, pp. 1171–1178, ago. 1998, doi: 10.1104/pp.117.4.1171.

[28] H. Rettenmaier e F. Ljngens, «Purification and some Properties of two Isofunctional Juglone Hydroxylases from Pseudomonas putida Jl».

[29] E. Coller et al., «Microbiome of vineyard soils is shaped by geography and management», Microbiome, vol. 7, fasc. 1, p. 140, dic. 2019, doi: 10.1186/s40168-019-0758-7.

[30] P. Fournier *et al.*, «The functional microbiome of grapevine throughout plant evolutionary history and lifetime», in *Advances in Ecological Research*, vol. 67, Elsevier, 2022, pp. 27–99. doi: 10.1016/bs.aecr.2022.09.001.

[31] C. H. Herms, R. C. Hennessy, F. Bak, D. B. Dresbøll, e M. H. Nicolaisen, «Back to our roots: exploring the role of root morphology as a mediator of beneficial plant–microbe interactions», *Environmental Microbiology*, vol. 24, fasc. 8, pp. 3264–3272, ago. 2022, doi: 10.1111/1462-2920.15926.

[32] Z. H. Pervaiz *et al.*, «Root microbiome changes with root branching order and root chemistry in peach rhizosphere soil», *Rhizosphere*, vol. 16, p. 100249, dic. 2020, doi: 10.1016/j.rhisph.2020.100249.

[33] X. Wan, X. Chen, Z. Huang, e H. Y. H. Chen, «Contribution of root traits to variations in soil microbial biomass and community composition», *Plant Soil*, vol. 460, fasc. 1–2, pp. 483–495, mar. 2021, doi: 10.1007/s11104-020-04788-7.

[34] S. Robertson-Albertyn *et al.*, «Root Hair Mutations Displace the Barley Rhizosphere Microbiota», *Front. Plant Sci.*, vol. 8, p. 1094, giu. 2017, doi: 10.3389/fpls.2017.01094.

[35] S. Hacquard et al., «Microbiota and Host Nutrition across Plant and Animal Kingdoms», Cell Host & Microbe, vol. 17, fasc. 5, pp. 603–616, mag. 2015, doi: 10.1016/j.chom.2015.04.009.

[36] J. Sasse, E. Martinoia, e T. Northen, «Feed Your Friends: Do Plant Exudates Shape the Root Microbiome?», *Trends in Plant Science*, vol. 23, fasc. 1, pp. 25–41, gen. 2018, doi: 10.1016/j.tplants.2017.09.003.

[37] N. Vitulo *et al.*, «Bark and Grape Microbiome of Vitis vinifera: Influence of Geographic Patterns and Agronomic Management on Bacterial Diversity», *Front. Microbiol.*, vol. 9, p. 3203, gen. 2019, doi: 10.3389/fmicb.2018.03203.

[38] V. Gayevskiy e M. R. Goddard, «Geographic delineations of yeast communities and populations associated with vines and wines in New Zealand», *The ISME Journal*, vol. 6, fasc. 7, pp. 1281–1290, lug. 2012, doi: 10.1038/ismej.2011.195.

[39] J. Drumonde-Neves, R. Franco-Duarte, T. Lima, D. Schuller, e C. Pais, «Association between Grape Yeast Communities and the Vineyard Ecosystems», *PLoS ONE*, vol. 12, fasc. 1, p. e0169883, gen. 2017, doi: 10.1371/journal.pone.0169883.

[40] J. F. Swift, M. E. Hall, Z. N. Harris, M. T. Kwasniewski, e A. J. Miller, «Grapevine Microbiota Reflect Diversity among Compartments and Complex Interactions within and among Root and Shoot Systems», *Microorganisms*, vol. 9, fasc. 1, p. 92, gen. 2021, doi: 10.3390/microorganisms9010092.

[41] P. Morrison-Whittle, S. A. Lee, e M. R. Goddard, «Fungal communities are differentially affected by conventional and biodynamic agricultural management approaches in vineyard ecosystems», *Agriculture, Ecosystems & Environment*, vol. 246, pp. 306–313, ago. 2017, doi: 10.1016/j.agee.2017.05.022.

[42] M. Andreolli, S. Lampis, e G. Vallini, «Diversity, Distribution and Functional Role of Bacterial Endophytes in Vitis vinifera», in *Endophytes: Biology and Biotechnology*, vol. 15, D. K. Maheshwari, A c. di, in Sustainable Development and Biodiversity, vol. 15. , Cham: Springer International Publishing, 2017, pp. 233–266. doi: 10.1007/978-3-319-66541-2_10.

[43] F. Bekris *et al.*, «Grapevine wood microbiome analysis identifies key fungal pathogens and potential interactions with the bacterial community implicated in grapevine trunk disease appearance», *Environmental Microbiome*, vol. 16, fasc. 1, p. 23, dic. 2021, doi: 10.1186/s40793-021-00390-1.

[44] G. ELENA, E. BRUEZ, P. REY, e J. LUQUE, «Microbiota of grapevine woody tissues with or without esca-foliar symptoms in northeast Spain», *Phytopathologia Mediterranea*, vol. 57, fasc. 3, dic. 2018, doi: 10.14601/Phytopathol_Mediterr-23337.

[45] M. Paolinelli, G. Escoriaza, C. Cesari, S. Garcia-Lampasona, e R. Hernandez-Martinez, «Characterization of Grapevine Wood Microbiome Through a Metatranscriptomic Approach», *Microb Ecol*, vol. 83, fasc. 3, pp. 658–668, apr. 2022, doi: 10.1007/s00248-021-01801-z.

[46] RP. Schreiner, KL., The diversity of arbuscular mycorrhizal fungi amplified from grapevine roots (Vitis vinifera L.) in Oregon vineyards is seasonally stable and influenced by soil and vine age. Mycologia. 2009 Sep-Oct;101(5):599-611. doi: 10.3852/08-169. PMID: 19750939.

[47] S. Compant, B. Mitter, J. G. Colli-Mull, H. Gangl, e A. Sessitsch, «Endophytes of Grapevine Flowers, Berries, and Seeds: Identification of Cultivable Bacteria, Comparison with Other Plant Parts, and Visualization of Niches of Colonization», *Microb Ecol*, vol. 62, fasc. 1, pp. 188–197, lug. 2011, doi: 10.1007/s00248-011-9883-y.

[48] G. Martins *et al.*, «Characterization of Epiphytic Bacterial Communities from Grapes, Leaves, Bark and Soil of Grapevine Plants Grown, and Their Relations», *PLoS ONE*, vol. 8, fasc. 8, p. e73013, ago. 2013, doi: 10.1371/journal.pone.0073013.

[49] P. Singh, S. Santoni, P. This, e J.-P. Péros, «Genotype-Environment Interaction Shapes the Microbial Assemblage in Grapevine's Phyllosphere and Carposphere: An NGS Approach», *Microorganisms*, vol. 6, fasc. 4, p. 96, set. 2018, doi: 10.3390/microorganisms6040096.

[50] L. M. Manici, M. L. Saccà, F. Caputo, A. Zanzotto, M. Gardiman, e G. Fila, «Long-term grapevine cultivation and agro-environment affect rhizosphere microbiome rather than plant age», *Applied Soil Ecology*, vol. 119, pp. 214–225, ott. 2017, doi: 10.1016/j.apsoil.2017.06.027.

[51] C. M. O. Longa *et al.*, «Soil microbiota respond to green manure in organic vineyards», *J Appl Microbiol*, vol. 123, fasc. 6, pp. 1547–1560, dic. 2017, doi: 10.1111/jam.13606.

[52] I. Zarraonaindia *et al.*, «The Soil Microbiome Influences Grapevine-Associated Microbiota», *mBio*, vol. 6, fasc. 2, pp. e02527-14, mag. 2015, doi: 10.1128/mBio.02527-14.

[53] S. Cui, L. Zhou, Q. Fang, H. Xiao, D. Jin, e Y. Liu, «Growth period and variety together drive the succession of phyllosphere microbial communities of grapevine», *Science of The Total Environment*, vol. 950, p. 175334, nov. 2024, doi: 10.1016/j.scitotenv.2024.175334.

[54] J. F. Swift, Z. Migicovsky, G. E. Trello, e A. J. Miller, «Grapevine bacterial communities display compartment-specific dynamics over space and time within the Central Valley of California», *Environmental Microbiome*, vol. 18, fasc. 1, p. 84, nov. 2023, doi: 10.1186/s40793-023-00539-0.

[55] D. Bulgari *et al.*, «Endophytic bacterial diversity in grapevine (Vitis vinifera L.) leaves described by 16S rRNA gene sequence analysis and length heterogeneity-PCR», *J Microbiol.*, vol. 47, fasc. 4, pp. 393–401, ago. 2009, doi: 10.1007/s12275-009-0082-1.

[56] M. D. P. Martínez-Diz, M. Andrés-Sodupe, R. Bujanda, E. Díaz-Losada, A. Eichmeier, e D. Gramaje, «Soil-plant compartments affect fungal microbiome diversity and composition in grapevine», *Fungal Ecology*, vol. 41, pp. 234–244, ott. 2019, doi: 10.1016/j.funeco.2019.07.003.

[57] D. Thoms, Y. Liang, e C. H. Haney, «Maintaining Symbiotic Homeostasis: How Do Plants Engage With Beneficial Microorganisms While at the Same Time Restricting Pathogens?», *MPMI*, vol. 34, fasc. 5, pp. 462–469, mag. 2021, doi: 10.1094/MPMI-11-20-0318-FI.

[58] C.-J. Dong, L.-L. Wang, Q. Li, e Q.-M. Shang, «Bacterial communities in the rhizosphere, phyllosphere and endosphere of tomato plants», *PLoS ONE*, vol. 14, fasc. 11, p. e0223847, nov. 2019, doi: 10.1371/journal.pone.0223847.

[59] R. Marasco, E. Rolli, M. Fusi, G. Michoud, e D. Daffonchio, «Grapevine rootstocks shape underground bacterial microbiome and networking but not potential functionality», *Microbiome*, vol. 6, fasc. 1, p. 3, dic. 2018, doi: 10.1186/s40168-017-0391-2.

[60] S. N. Vink, F. Dini-Andreote, R. Höfle, A. Kicherer, e J. F. Salles, «Interactive Effects of Scion and Rootstock Genotypes on the Root Microbiome of Grapevines (Vitis spp. L.)», Applied Sciences, vol. 11, fasc. 4, p. 1615, feb. 2021, doi: 10.3390/app11041615.

[61] Jones, D. Gareth. (1993). Exploitation of Microorganisms || Formulation of biological control agents. , 10.1007/978-94-011-1532-2(Chapter 16), 411–439. doi:10.1007/978-94-011-1532-2_16

[62] L. Dries *et al.*, «Rootstocks Shape Their Microbiome—Bacterial Communities in the Rhizosphere of Different Grapevine Rootstocks», *Microorganisms*, vol. 9, fasc. 4, p. 822, apr. 2021, doi: 10.3390/microorganisms9040822.

[63] D. Zuzolo *et al.*, «The rootstock shape microbial diversity and functionality in the rhizosphere of Vitis vinifera L. cultivar Falanghina», *Front. Plant Sci.*, vol. 14, p. 1205451, ago. 2023, doi: 10.3389/fpls.2023.1205451.

[64] F. D'Amico *et al.*, «The Rootstock Regulates Microbiome Diversity in Root and Rhizosphere Compartments of Vitis vinifera Cultivar Lambrusco», *Front. Microbiol.*, vol. 9, p. 2240, set. 2018, doi: 10.3389/fmicb.2018.02240.

[65] R. Marasco *et al.*, «Rootstock–scion combination contributes to shape diversity and composition of microbial communities associated with grapevine root system»,

Environmental Microbiology, vol. 24, fasc. 8, pp. 3791–3808, ago. 2022, doi: 10.1111/1462-2920.16042.

[66] C. Berlanas *et al.*, «The Fungal and Bacterial Rhizosphere Microbiome Associated With Grapevine Rootstock Genotypes in Mature and Young Vineyards», *Front. Microbiol.*, vol. 10, p. 1142, mag. 2019, doi: 10.3389/fmicb.2019.01142.

[67] E. Bruez, K. Baumgartner, S. Bastien, R. Travadon, L. Guérin-Dubrana, e P. Rey, «Various fungal communities colonise the functional wood tissues of old grapevines externally free from grapevine trunk disease symptoms: Fungal microflora of GTD-free old vines», *Australian Journal of Grape and Wine Research*, vol. 22, fasc. 2, pp. 288–295, giu. 2016, doi: 10.1111/ajgw.12209.

[68] W. Ji *et al.*, «Characterization of rhizosphere bacterial community and berry quality of Hutai No.8 (*Vitis vinifera* L.) with different ages, and their relations», *J Sci Food Agric*, vol. 99, fasc. 10, pp. 4532–4539, ago. 2019, doi: 10.1002/jsfa.9690.

[69] M. Andreolli, S. Lampis, G. Zapparoli, E. Angelini, e G. Vallini, «Diversity of bacterial endophytes in 3 and 15 year-old grapevines of Vitis vinifera cv. Corvina and their potential for plant growth promotion and phytopathogen control», *Microbiological Research*, vol. 183, pp. 42–52, feb. 2016, doi: 10.1016/j.micres.2015.11.009.

[70] M. M. Hernandez e C. M. Menéndez, «Influence of seasonality and management practices on diversity and composition of fungal communities in vineyard soils», *Applied Soil Ecology*, vol. 135, pp. 113–119, mar. 2019, doi: 10.1016/j.apsoil.2018.11.008.

[71] C. Pinto, D. Pinho, S. Sousa, M. Pinheiro, C. Egas, e A. C. Gomes, «Unravelling the Diversity of Grapevine Microbiome», *PLoS ONE*, vol. 9, fasc. 1, p. e85622, gen. 2014, doi: 10.1371/journal.pone.0085622.

[72] D. Liu, Q. Chen, P. Zhang, D. Chen, e K. S. Howell, «The Fungal Microbiome Is an Important Component of Vineyard Ecosystems and Correlates with Regional Distinctiveness of Wine», *mSphere*, vol. 5, fasc. 4, p. 10.1128/msphere.00534-20, ago. 2020, doi: 10.1128/msphere.00534-20.

[73] M. Perazzolli *et al.*, «Resilience of the Natural Phyllosphere Microbiota of the Grapevine to Chemical and Biological Pesticides», *Appl Environ Microbiol*, vol. 80, fasc. 12, pp. 3585–3596, giu. 2014, doi: 10.1128/AEM.00415-14.

[74] M. J. Carbone *et al.*, «Drought Influences Fungal Community Dynamics in the Grapevine Rhizosphere and Root Microbiome», *JoF*, vol. 7, fasc. 9, p. 686, ago. 2021, doi: 10.3390/jof7090686.

[75] K. L. Steenwerth, R. E. Drenovsky, J.-J. Lambert, D. A. Kluepfel, K. M. Scow, e D. R. Smart, «Soil morphology, depth and grapevine root frequency influence microbial communities in a Pinot noir vineyard», *Soil Biology and Biochemistry*, vol. 40, fasc. 6, pp. 1330–1340, giu. 2008, doi: 10.1016/j.soilbio.2007.04.031.

[76] H. Liang, X. Wang, J. Yan, e L. Luo, «Characterizing the Intra-Vineyard Variation of Soil Bacterial and Fungal Communities», *Front. Microbiol.*, vol. 10, p. 1239, mag. 2019, doi: 10.3389/fmicb.2019.01239.

[77] K. N. Burns *et al.*, «Vineyard soil bacterial diversity and composition revealed by 16S rRNA genes: Differentiation by vineyard management», *Soil Biology and Biochemistry*, vol. 103, pp. 337–348, dic. 2016, doi: 10.1016/j.soilbio.2016.09.007.

[78] A. Campisano, L. Antonielli, M. Pancher, S. Yousaf, M. Pindo, e I. Pertot, «Bacterial Endophytic Communities in the Grapevine Depend on Pest Management», *PLoS ONE*, vol. 9, fasc. 11, p. e112763, nov. 2014, doi: 10.1371/journal.pone.0112763.

[79] M. Pancher *et al.*, «Fungal Endophytic Communities in Grapevines (Vitis vinifera L.) Respond to Crop Management», *Appl Environ Microbiol*, vol. 78, fasc. 12, pp. 4308–4317, giu. 2012, doi: 10.1128/AEM.07655-11.

[80] J. G. Zaller *et al.*, «Herbicides in vineyards reduce grapevine root mycorrhization and alter soil microorganisms and the nutrient composition in grapevine roots, leaves, xylem sap and grape juice», *Environ Sci Pollut Res*, vol. 25, fasc. 23, pp. 23215–23226, ago. 2018, doi: 10.1007/s11356-018-2422-3.

[81] G. Armijo, R. Schlechter, M. Agurto, D. Muñoz, C. Nuñez, e P. Arce-Johnson, «Grapevine Pathogenic Microorganisms: Understanding Infection Strategies and Host Response Scenarios», *Front. Plant Sci.*, vol. 7, mar. 2016, doi: 10.3389/fpls.2016.00382.

[82] J. Glazebrook, «Contrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens», *Annu. Rev. Phytopathol.*, vol. 43, fasc. 1, pp. 205–227, set. 2005, doi: 10.1146/annurev.phyto.43.040204.135923.

[83] S. Rosa *et al.*, «Game-changing alternatives to conventional fungicides: small RNAs and short peptides», *Trends in Biotechnology*, vol. 40, fasc. 3, pp. 320–337, mar. 2022, doi: 10.1016/j.tibtech.2021.07.003.

[84] M. A. Hevia, P. Canessa, e L. F. Larrondo, «Circadian clocks and the regulation of virulence in fungi: Getting up to speed», *Seminars in Cell & Developmental Biology*, vol. 57, pp. 147–155, set. 2016, doi: 10.1016/j.semcdb.2016.03.021

[85] D. Steiger, (2007) Global economic importance of Botrytis protection. In: Book of Abstracts, 14th International Botrytis Symposium, Cape Town, South Africa, 21–26 October 2007 [P. Fourie and M. Vivier, eds.), Cape Town, South Africa: AFRICAN SUN MeDIA Pty (Ltd.)].

[86] UIPP (2002) *Annual Report. Paris*, France: Union des Industries de la Protection des Plantes

[87] C. C. Steel, J. W. Blackman, e L. M. Schmidtke, «Grapevine Bunch Rots: Impacts on Wine Composition, Quality, and Potential Procedures for the Removal of Wine Faults», *J. Agric. Food Chem.*, vol. 61, fasc. 22, pp. 5189–5206, giu. 2013, doi: 10.1021/jf400641r.

[88] C. Gouveia, R. B. Santos, C. Paiva-Silva, G. Buchholz, R. Malhó, e A. Figueiredo, «The pathogenicity of Plasmopara viticola: a review of evolutionary dynamics,

infection strategies and effector molecules», *BMC Plant Biol*, vol. 24, fasc. 1, p. 327, apr. 2024, doi: 10.1186/s12870-024-05037-0.

[89] M. F. Kumaşoğlu, N. Özer, B. Aktürk, e H. İ. Uzun, «Plasmopara viticola infection process and accumulation of important stilbenes in some grapevine varieties grown in Turkey and in individuals for brined leaves», *J Plant Dis Prot*, vol. 129, fasc. 4, pp. 967–979, ago. 2022, doi: 10.1007/s41348-021-00561-9.

[90] J. Xiang, X. Li, J. Wu, L. Yin, Y. Zhang, e J. Lu, «Studying the Mechanism of Plasmopara viticola RxLR Effectors on Suppressing Plant Immunity», *Front. Microbiol.*, vol. 7, mag. 2016, doi: 10.3389/fmicb.2016.00709.

[91] X. Lan et al., «Plasmopara viticola effector PvRXLR131 suppresses plant immunity by targeting plant receptor-like kinase inhibitor BKI1», Molecular Plant Pathology, vol. 20, fasc. 6, pp. 765–783, giu. 2019, doi: 10.1111/mpp.12790.

[92] P. Mestre, M.-C. Piron, e D. Merdinoglu, «Identification of effector genes from the phytopathogenic Oomycete Plasmopara viticola through the analysis of gene expression in germinated zoospores», Fungal Biology, vol. 116, fasc. 7, pp. 825–835, lug. 2012, doi: 10.1016/j.funbio.2012.04.016.

[93] E. Szegedi e E. L. Civerolo, «Bacterial diseases of grapevine», *Int. j. hortic. sci.*, vol. 17, fasc. 3, giu. 2011, doi: 10.31421/IJHS/17/3/956.

[94] D. L. Hopkins e A. H. Purcell, «*Xylella fastidiosa* : Cause of Pierce's Disease of Grapevine and Other Emergent Diseases», *Plant Disease*, vol. 86, fasc. 10, pp. 1056–1066, ott. 2002, doi: 10.1094/PDIS.2002.86.10.1056.

[95] T. J. Burr, C. Bazzi, S. Süle, e L. Otten, «Crown Gall of Grape: Biology of *Agrobacterium vitis* and the Development of Disease Control Strategies», *Plant Disease*, vol. 82, fasc. 12, pp. 1288–1297, dic. 1998, doi: 10.1094/PDIS.1998.82.12.1288.

[96] D. C. Mundy, «Ecology and control of grapevine root diseases in New Zealand a review», *NZPP*, vol. 68, pp. 396–404, gen. 2015, doi: 10.30843/nzpp.2015.68.5818.

[97] Berlander, I.; Powelson, M.L. Verticillium wilt. Plant Health Instructor. 2000. https://doi.org/10.1094/PHI-I-2000-0801-01.

[98] W. D. Gubler et al., «Root diseases of grapevines in California and their control», Austral. Plant Pathol., vol. 33, fasc. 2, p. 157, 2004, doi: 10.1071/AP04019.

[99] V. Mondello et al., «Grapevine Trunk Diseases: A Review of Fifteen Years of Trials for Their Control with Chemicals and Biocontrol Agents», Plant Disease, vol. 102, fasc. 7, pp. 1189–1217, lug. 2018, doi: 10.1094/PDIS-08-17-1181-FE.

[100] J. Kenfaoui *et al.*, «A Panoramic View on Grapevine Trunk Diseases Threats: Case of Eutypa Dieback, Botryosphaeria Dieback, and Esca Disease», *JoF*, vol. 8, fasc. 6, p. 595, giu. 2022, doi: 10.3390/jof8060595.

[101] E. Baldan *et al.*, «Beneficial Bacteria Isolated from Grapevine Inner Tissues Shape Arabidopsis thaliana Roots», *PLoS ONE*, vol. 10, fasc. 10, p. e0140252, ott. 2015, doi: 10.1371/journal.pone.0140252.

[102] S. Asghari *et al.*, «Induction of systemic resistance to *Agrobacterium tumefaciens* by endophytic bacteria in grapevine», *Plant Pathology*, vol. 69, fasc. 5, pp. 827–837, giu. 2020, doi: 10.1111/ppa.13175.

[103] A. V. Mardanov *et al.*, «Complete Genome Sequences of Endophytic Bacilli Isolated from Grapevine Plants», *Microbiol Resour Announc*, vol. 8, fasc. 48, pp. e01265-19, nov. 2019, doi: 10.1128/MRA.01265-19.

[104] N. De Simone *et al.*, «Antimicrobial Properties, Functional Characterisation and Application of Fructobacillus fructosus and Lactiplantibacillus plantarum Isolated from Artisanal Honey», *Probiotics & Antimicro. Prot.*, vol. 15, fasc. 5, pp. 1406–1423, ott. 2023, doi: 10.1007/s12602-022-09988-4.

[105] A. Samad, F. Trognitz, S. Compant, L. Antonielli, e A. Sessitsch, «Shared and host-specific microbiome diversity and functioning of grapevine and accompanying weed plants», *Environmental Microbiology*, vol. 19, fasc. 4, pp. 1407–1424, apr. 2017, doi: 10.1111/1462-2920.13618.

[106] S. Trouvelot, L. Bonneau, D. Redecker, D. Van Tuinen, M. Adrian, e D. Wipf, «Arbuscular mycorrhiza symbiosis in viticulture: a review», *Agron. Sustain. Dev.*, vol. 35, fasc. 4, pp. 1449–1467, ott. 2015, doi: 10.1007/s13593-015-0329-7.

[107] M. A. Khoso *et al.*, «Impact of plant growth-promoting rhizobacteria (PGPR) on plant nutrition and root characteristics: Current perspective», *Plant Stress*, vol. 11, p. 100341, mar. 2024, doi: 10.1016/j.stress.2023.100341.

[108] D. Pacifico *et al.*, «The Role of the Endophytic Microbiome in the Grapevine Response to Environmental Triggers», *Front. Plant Sci.*, vol. 10, p. 1256, ott. 2019, doi: 10.3389/fpls.2019.01256.

[109] H. M. Rafique *et al.*, «Converging alfalfa (*Medicago sativa* L.) and petroleum hydrocarbon acclimated ACC-deaminase containing bacteria for phytoremediation of petroleum hydrocarbon contaminated soil», *International Journal of Phytoremediation*, vol. 25, fasc. 6, pp. 717–727, mag. 2023, doi: 10.1080/15226514.2022.2104214.

[110] N. Torres, N. Goicoechea, A. M. Zamarreño, e M. Carmen Antolín, «Mycorrhizal symbiosis affects ABA metabolism during berry ripening in Vitis vinifera L. cv. Tempranillo grown under climate change scenarios», *Plant Science*, vol. 274, pp. 383–393, set. 2018, doi: 10.1016/j.plantsci.2018.06.009.

[111] Q.-M. Nguyen, A. B. B. Iswanto, G. H. Son, e S. H. Kim, «Recent Advances in Effector-Triggered Immunity in Plants: New Pieces in the Puzzle Create a Different Paradigm», *IJMS*, vol. 22, fasc. 9, p. 4709, apr. 2021, doi: 10.3390/ijms22094709.

[112] P. Balint-Kurti, «The plant hypersensitive response: concepts, control and consequences», *Molecular Plant Pathology*, vol. 20, fasc. 8, pp. 1163–1178, ago. 2019, doi: 10.1111/mpp.12821.

[113] G. E. Vallad e R. M. Goodman, «Systemic Acquired Resistance and Induced Systemic Resistance in Conventional Agriculture», *Crop Sci.*, vol. 44, fasc. 6, pp. 1920–1934, nov. 2004, doi: 10.2135/cropsci2004.1920.

[114] C. M. J. Pieterse, C. Zamioudis, R. L. Berendsen, D. M. Weller, S. C. M. Van Wees, e P. A. H. M. Bakker, «Induced Systemic Resistance by Beneficial Microbes», *Annu. Rev. Phytopathol.*, vol. 52, fasc. 1, pp. 347–375, ago. 2014, doi: 10.1146/annurev-phyto-082712-102340.

[115] D. K. Choudhary, A. Prakash, e B. N. Johri, «Induced systemic resistance (ISR) in plants: mechanism of action», *Indian J Microbiol*, vol. 47, fasc. 4, pp. 289–297, dic. 2007, doi: 10.1007/s12088-007-0054-2.

[116] U. Conrath, O. Thulke, V. Katz, S. Schwindling, e A. Kohler, «Priming as a Mechanism in Induced Systemic Resistance of Plants».

[117] A. Varnier *et al.*, «Bacterial rhamnolipids are novel MAMPs conferring resistance to *Botrytis cinerea* in grapevine», *Plant Cell & Environment*, vol. 32, fasc. 2, pp. 178–193, feb. 2009, doi: 10.1111/j.1365-3040.2008.01911.x.

[118] S. Lakkis *et al.*, «Strengthening Grapevine Resistance by Pseudomonas fluorescens PTA-CT2 Relies on Distinct Defense Pathways in Susceptible and Partially Resistant Genotypes to Downy Mildew and Gray Mold Diseases», *Front. Plant Sci.*, vol. 10, p. 1112, set. 2019, doi: 10.3389/fpls.2019.01112.

[119] S. Loqman, E. A. Barka, C. Clément, e Y. Ouhdouch, «Antagonistic actinomycetes from Moroccan soil to control the grapevine gray mold», *World J Microbiol Biotechnol*, vol. 25, fasc. 1, pp. 81–91, gen. 2009, doi: 10.1007/s11274-008-9864-6.

[120] P. Vatsa-Portugal *et al.*, «How Streptomyces anulatus Primes Grapevine Defenses to Cope with Gray Mold: A Study of the Early Responses of Cell Suspensions», *Front. Plant Sci.*, vol. 8, p. 1043, giu. 2017, doi: 10.3389/fpls.2017.01043.

[121] P. Trotel-Aziz *et al.*, «Bacillus subtilis PTA-271 Counteracts Botryosphaeria Dieback in Grapevine, Triggering Immune Responses and Detoxification of Fungal Phytotoxins», *Front. Plant Sci.*, vol. 10, p. 25, gen. 2019, doi: 10.3389/fpls.2019.00025.

[122] I. Pertot *et al.*, «A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implementation of IPM in viticulture», *Crop Protection*, vol. 97, pp. 70–84, lug. 2017, doi: 10.1016/j.cropro.2016.11.025.

[123] G. D. C. Silva, I. T. Kitano, I. A. D. F. Ribeiro, e P. T. Lacava, «The Potential Use of Actinomycetes as Microbial Inoculants and Biopesticides in Agriculture», *Front. Soil Sci.*, vol. 2, p. 833181, feb. 2022, doi: 10.3389/fsoil.2022.833181.

[124] J. Köhl, R. Kolnaar, e W. J. Ravensberg, «Mode of Action of Microbial Biological Control Agents Against Plant Diseases: Relevance Beyond Efficacy», *Front. Plant Sci.*, vol. 10, p. 845, lug. 2019, doi: 10.3389/fpls.2019.00845.

[125] D. Spadaro e S. Droby, «Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists», *Trends in Food Science & Technology*, vol. 47, pp. 39–49, gen. 2016, doi: 10.1016/j.tifs.2015.11.003.

[126] L. C. Van Loon, «Helping Plants To Defend Themselves: Biocontrol By Disease-Suppressing Rhizobacteria», in *Developments in Plant Genetics and Breeding*, vol. 6, Elsevier, 2000, pp. 203–213. doi: 10.1016/S0168-7972(00)80123-1.

[127] J. M. Raaijmakers, L. V. D. Sluis, P. A. H. M. Bakker, B. Schippers, M. Koster, e P. J. Weisbeek, «Utilization of heterologous siderophores and rhizosphere competence of fluorescent *Pseudomonas* spp.», *Can. J. Microbiol.*, vol. 41, fasc. 2, pp. 126–135, feb. 1995, doi: 10.1139/m95-017.

[128] D. Saravanakumar, A. Ciavorella, D. Spadaro, A. Garibaldi, e M. L. Gullino, «Metschnikowia pulcherrima strain MACH1 outcompetes Botrytis cinerea, Alternaria alternata and Penicillium expansum in apples through iron depletion», *Postharvest Biology and Technology*, vol. 49, fasc. 1, pp. 121–128, lug. 2008, doi: 10.1016/j.postharvbio.2007.11.006.

[129] P. Jeffries, «Biology and ecology of mycoparasitism», *Can. J. Bot.*, vol. 73, fasc. S1, pp. 1284–1290, dic. 1995, doi: 10.1139/b95-389.

[130] J. Bérdy, Bioactive Microbial Metabolites. *J Antibiot* **58**, 1–26 (2005). https://doi.org/10.1038/ja.2005.1

[131] Y. Deng *et al.*, «Antifungal activities of *Bacillus velezensis* FJAT-52631 and its lipopeptides against anthracnose pathogen *Colletotrichum acutatum*», *J Basic Microbiol*, vol. 63, fasc. 6, pp. 594–603, giu. 2023, doi: 10.1002/jobm.202200489.

[132] D. Fira, I. Dimkić, T. Berić, J. Lozo, e S. Stanković, «Biological control of plant pathogens by Bacillus species», *Journal of Biotechnology*, vol. 285, pp. 44–55, nov. 2018, doi: 10.1016/j.jbiotec.2018.07.044.

[133] K. Jindo *et al.*, «Application of biostimulant products and biological control agents in sustainable viticulture: A review», *Front. Plant Sci.*, vol. 13, p. 932311, ott. 2022, doi: 10.3389/fpls.2022.932311.

[134] A. Bonaterra, E. Badosa, N. Daranas, J. Francés, G. Roselló, e E. Montesinos, «Bacteria as Biological Control Agents of Plant Diseases», *Microorganisms*, vol. 10, fasc. 9, p. 1759, ago. 2022, doi: 10.3390/microorganisms10091759.

[135] S. Compant, G. Brader, S. Muzammil, A. Sessitsch, A. Lebrihi, e F. Mathieu, «Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases», *BioControl*, vol. 58, fasc. 4, pp. 435–455, ago. 2013, doi: 10.1007/s10526-012-9479-6.

[136] O. Mesguida *et al.*, «Microbial Biological Control of Fungi Associated with Grapevine Trunk Diseases: A Review of Strain Diversity, Modes of Action, and Advantages and Limits of Current Strategies», *JoF*, vol. 9, fasc. 6, p. 638, mag. 2023, doi: 10.3390/jof9060638.

[137] A. Nogales, J. Aguirreolea, E. Santa María, A. Camprubí, e C. Calvet, «Response of mycorrhizal grapevine to Armillaria mellea inoculation: disease development and polyamines», *Plant Soil*, vol. 317, fasc. 1–2, pp. 177–187, apr. 2009, doi: 10.1007/s11104-008-9799-6.

[138] A. Salerno *et al.*, «On the Way to the Technological Development of Newly Selected Non-Saccharomyces Yeasts Selected as Innovative Biocontrol Agents in Table Grapes», *Microorganisms*, vol. 12, fasc. 2, p. 340, feb. 2024, doi: 10.3390/microorganisms12020340.

[139] M. V. Kamble, S. M. Joshi, S. Hadimani, e S. Jogaiah, «Biopriming with rhizosphere Trichoderma harzianum elicit protection against grapevine downy mildew disease by triggering histopathological and biochemical defense responses», *Rhizosphere*, vol. 19, p. 100398, set. 2021, doi: 10.1016/j.rhisph.2021.100398.

[140] C. Zang, Q. Lin, J. Xie, Y. Lin, K. Zhao, e C. Liang, «The biological control of the grapevine downy mildew disease using Ochrobactrum sp.», *Plant Prot. Sci.*, vol. 56, fasc. 1, pp. 52–61, mar. 2020, doi: 10.17221/87/2019-PPS.

[141] X. Zhang, Y. Zhou, Y. Li, X. Fu, e Q. Wang, «Screening and characterization of endophytic Bacillus for biocontrol of grapevine downy mildew», *Crop Protection*, vol. 96, pp. 173–179, giu. 2017, doi: 10.1016/j.cropro.2017.02.018.

[142] Q. Zeng, J. Xie, Y. Li, T. Gao, X. Zhang, e Q. Wang, «Comprehensive Genomic Analysis of the Endophytic Bacillus altitudinis Strain GLB197, a Potential Biocontrol Agent of Grape Downy Mildew», *Front. Genet.*, vol. 12, p. 729603, set. 2021, doi: 10.3389/fgene.2021.729603.

[143] G. Assante, S. Dallavalle, L. Malpezzi, G. Nasini, S. Burruano, e L. Torta, «Acremines A–F, novel secondary metabolites produced by a strain of an endophytic Acremonium, isolated from sporangiophores of Plasmopara viticola in grapevine leaves», *Tetrahedron*, vol. 61, fasc. 32, pp. 7686–7692, ago. 2005, doi: 10.1016/j.tet.2005.05.094.

[144] S. Lo Piccolo, A. Alfonzo, S. Giambra, G. Conigliaro, L. V. Lopez-Llorca, e S. Burruano, «Identification of Acremonium isolates from grapevines and evaluation of their antagonism towards Plasmopara viticola», *Ann Microbiol*, vol. 65, fasc. 4, pp. 2393–2403, dic. 2015, doi: 10.1007/s13213-015-1082-5.

[145] S. Burruano, «The life-cycle of Plasmopara viticola, cause of downy mildew of vine», *Mycologist*, vol. 14, fasc. 4, pp. 179–182, nov. 2000, doi: 10.1016/S0269-915X(00)80040-3.

[146] A. Kortekamp *et al.*, «Identification, isolation and characterization of a CC-NBS-LRR candidate disease resistance gene family in grapevine», *Mol Breeding*, vol. 22, fasc. 3, pp. 421–432, ott. 2008, doi: 10.1007/s11032-008-9186-2.

[147] K. Koledenkova, Q. Esmaeel, C. Jacquard, J. Nowak, C. Clément, e E. Ait Barka, «Plasmopara viticola the Causal Agent of Downy Mildew of Grapevine: From Its Taxonomy to Disease Management», *Front. Microbiol.*, vol. 13, p. 889472, mag. 2022, doi: 10.3389/fmicb.2022.889472.

[148] P. Tiwari, S. Kang, e H. Bae, «Plant-endophyte associations: Rich yet under-explored sources of novel bioactive molecules and applications», *Microbiological Research*, vol. 266, p. 127241, gen. 2023, doi: 10.1016/j.micres.2022.127241.

[149] F. De Paula Nogueira Cruz, A. Cristina Bogas, e C. Paiva De Sousa, «Plant-Associated Microorganisms as a Potent Bio-Factory of Active Molecules against

Multiresistant Pathogens», in *Antimicrobial Resistance - A One Health Perspective*, M. Mareș, S. Hua Erin Lim, K.-S. Lai, e R.-T. Cristina, A c. di, IntechOpen, 2021. doi: 10.5772/intechopen.93598.

[150] M. Arachevaleta, C. W. Bacon, C. S. Hoveland, e D. E. Radcliffe, «Effect of the Tall Fescue Endophyte on Plant Response to Environmental Stress», Agronomy Journal, vol. 81, fasc. 1, pp. 83–90, gen. 1989, doi: 10.2134/agronj1989.00021962008100010015x.

[151] P.-J. Zhao, L.-M. Fan, G.-H. Li, N. Zhu, e Y.-M. Shen, «Antibacterial and antitumor macrolides fromstreptomyces sp. Is9131», *Arch Pharm Res*, vol. 28, fasc. 11, pp. 1228–1232, nov. 2005, doi: 10.1007/BF02978203.

[152] N. Kim *et al.*, «Cytotoxic 6-Alkylsalicylic Acids from the Endophytic».

[153] T. Taechowisan, S. Chaisaeng, e W. S. Phutdhawong, «Antibacterial, antioxidant and anticancer activities of biphenyls from *Streptomyces* sp. BO-07: an endophyte in *Boesenbergia rotunda* (L.) Mansf A.», *Food and Agricultural Immunology*, vol. 28, fasc. 6, pp. 1330–1346, nov. 2017, doi: 10.1080/09540105.2017.1339669.

[154] H.-N. T. Vu *et al.*, «Antimicrobial and Cytotoxic Properties of Bioactive Metabolites Produced by Streptomyces cavourensis YBQ59 Isolated from Cinnamomum cassia Prels in Yen Bai Province of Vietnam», *Curr Microbiol*, vol. 75, fasc. 10, pp. 1247–1255, ott. 2018, doi: 10.1007/s00284-018-1517-x.

[155] Y.-T. Chen, Q. Yuan, L.-T. Shan, M.-A. Lin, D.-Q. Cheng, e C.-Y. Li, «Antitumor activity of bacterial exopolysaccharides from the endophyte Bacillus amyloliquefaciens sp. isolated from Ophiopogon japonicus», *Oncology Letters*, vol. 5, fasc. 6, pp. 1787–1792, giu. 2013, doi: 10.3892/ol.2013.1284.

[156] M. J. Ek-Ramos, R. Gomez-Flores, A. A. Orozco-Flores, C. Rodríguez-Padilla, G. González-Ochoa, e P. Tamez-Guerra, «Bioactive Products From Plant-Endophytic Gram-Positive Bacteria», *Front. Microbiol.*, vol. 10, p. 463, mar. 2019, doi: 10.3389/fmicb.2019.00463.

[157] Y. Ma, «Editorial: Biotechnological Potential of Plant-Microbe Interactions in Environmental Decontamination», *Front. Plant Sci.*, vol. 10, p. 1519, nov. 2019, doi: 10.3389/fpls.2019.01519.

[158] T. Wu *et al.*, «Characterization and Initial Application of Endophytic Bacillus safensis Strain ZY16 for Improving Phytoremediation of Oil-Contaminated Saline Soils», *Front. Microbiol.*, vol. 10, p. 991, mag. 2019, doi: 10.3389/fmicb.2019.00991.

[159] S. Bisht *et al.*, «Utilization of endophytic strain Bacillus sp. SBER3 for biodegradation of polyaromatic hydrocarbons (PAH) in soil model system», *European Journal of Soil Biology*, vol. 60, pp. 67–76, gen. 2014, doi: 10.1016/j.ejsobi.2013.10.009.

[160] Y. Ma, RS. Oliveira, F. Nai, M. Rajkumar, Y. Luo, I. Rocha, H. Freitas. The hyperaccumulator Sedum plumbizincicola harbors metal-resistant endophytic bacteria that improve its phytoextraction capacity in multi-metal contaminated soil.

J Environ Manage. 2015 Jun 1;156:62-9. doi: 10.1016/j.jenvman.2015.03.024. Epub 2015 Mar 19. PMID: 25796039.

[161] A. G. Babu, J.-D. Kim, e B.-T. Oh, «Enhancement of heavy metal phytoremediation by Alnus firma with endophytic Bacillus thuringiensis GDB-1», *Journal of Hazardous Materials*, vol. 250–251, pp. 477–483, apr. 2013, doi: 10.1016/j.jhazmat.2013.02.014.

[162] Natalia C. De Oliveira, «Endophytic bacteria with potential for bioremediation of petroleum hydrocarbons and derivatives», Afr. J. Biotechnol., vol. 11, fasc. 12, feb. 2012, doi: 10.5897/AJB10.2623.

[163] X.-F. Sheng, J.-J. Xia, C.-Y. Jiang, L.-Y. He, e M. Qian, «Characterization of heavy metal-resistant endophytic bacteria from rape (Brassica napus) roots and their potential in promoting the growth and lead accumulation of rape», *Environmental Pollution*, vol. 156, fasc. 3, pp. 1164–1170, dic. 2008, doi: 10.1016/j.envpol.2008.04.007.

[164] T. Wu *et al.*, «Pseudomonas aeruginosa L10: A Hydrocarbon-Degrading, Biosurfactant-Producing, and Plant-Growth-Promoting Endophytic Bacterium Isolated From a Reed (Phragmites australis)», *Front. Microbiol.*, vol. 9, p. 1087, mag. 2018, doi: 10.3389/fmicb.2018.01087.

[165] N. Weyens *et al.*, «Bioaugmentation with Engineered Endophytic Bacteria Improves Contaminant Fate in Phytoremediation», *Environ. Sci. Technol.*, vol. 43, fasc. 24, pp. 9413–9418, dic. 2009, doi: 10.1021/es901997z.

[166] L. Hnamte, Vanlallawmzuali, A. Kumar, M. K. Yadav, Zothanpuia, e P. K. Singh, «An updated view of bacterial endophytes as antimicrobial agents against plant and human pathogens», *Current Research in Microbial Sciences*, vol. 7, p. 100241, 2024, doi: 10.1016/j.crmicr.2024.100241.

[167] U. F. Castillo *et al.*, «Munumbicins, wide-spectrum antibiotics produced by Streptomyces NRRL 30562, endophytic on Kennedia nigriscans».

[168] U. Castillo *et al.*, «Kakadumycins, novel antibiotics from *Streptomyces* sp. NRRL 30566, an endophyte of *Grevillea pteridifolia*», *FEMS Microbiology Letters*, vol. 224, fasc. 2, pp. 183–190, lug. 2003, doi: 10.1016/S0378-1097(03)00426-9.

[169] S. Guan, I. Sattler, W. Lin, D. Guo, e S. Grabley, «*p* -Aminoacetophenonic Acids Produced by a Mangrove Endophyte: *Streptomyces g riseus* subsp.», *J. Nat. Prod.*, vol. 68, fasc. 8, pp. 1198–1200, ago. 2005, doi: 10.1021/np0500777.

[170] M. Singh, A. Kumar, R. Singh, e K. D. Pandey, «Endophytic bacteria: a new source of bioactive compounds», *3 Biotech*, vol. 7, fasc. 5, p. 315, ott. 2017, doi: 10.1007/s13205-017-0942-z.

[171] M. Numan, M. Shah, S. Asaf, N. Ur Rehman, e A. Al-Harrasi, «Bioactive Compounds from Endophytic Bacteria Bacillus subtilis Strain EP1 with Their Antibacterial Activities», *Metabolites*, vol. 12, fasc. 12, p. 1228, dic. 2022, doi: 10.3390/metabo12121228

[172] S. Nisa *et al.*, «Therapeutic prospects of endophytic Bacillus species from Berberis lycium against oxidative stress and microbial pathogens», *Saudi Journal of Biological Sciences*, vol. 29, fasc. 1, pp. 287–295, gen. 2022, doi: 10.1016/j.sjbs.2021.08.099.

[173] Y. Liu, W. Liu, e Z. Liang, «Endophytic bacteria from *Pinellia ternata* , a new source of purine alkaloids and bacterial manure», *Pharmaceutical Biology*, vol. 53, fasc. 10, pp. 1545–1548, ott. 2015, doi: 10.3109/13880209.2015.1016580.

[174] A. D. Marsico *et al.*, «Native Vineyard Non-Saccharomyces Yeasts Used for Biological Control of Botrytis cinerea in Stored Table Grape», *Microorganisms*, vol. 9, fasc. 2, p. 457, feb. 2021, doi: 10.3390/microorganisms9020457.

[175] H.H. McKinney. Influence of Soil Temperature and Moisture on Infection of Wheat Seedlings by Helmin-thosporium Sativum. J Agric Res (1923), 26, 195–217.

[176] W. S. Abbott, «A Method of Computing the Effectiveness of an Insecticide», *Journal of Economic Entomology*, vol. 18, fasc. 2, pp. 265–267, apr. 1925, doi: 10.1093/jee/18.2.265a.

[177] A. Cruz-Silva, A. Figueiredo, e M. Sebastiana, «First Insights into the Effect of Mycorrhizae on the Expression of Pathogen Effectors during the Infection of Grapevine with Plasmopara viticola», *Sustainability*, vol. 13, fasc. 3, p. 1226, gen. 2021, doi: 10.3390/su13031226

[178] K. J. Livak e T. D. Schmittgen, «Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method», *Methods*, vol. 25, fasc. 4, pp. 402–408, dic. 2001, doi: 10.1006/meth.2001.1262.

[179] P. J. A. Cock, C. J. Fields, N. Goto, M. L. Heuer, e P. M. Rice, «The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants», *Nucleic Acids Research*, vol. 38, fasc. 6, pp. 1767–1771, apr. 2010, doi: 10.1093/nar/gkp1137.

[180] H. Li e R. Durbin, «Fast and accurate short read alignment with Burrows–Wheeler transform», Bioinformatics, vol. 25, fasc. 14, pp. 1754–1760, lug. 2009, doi: 10.1093/bioinformatics/btp324.

[181] H. Li *et al.*, «The Sequence Alignment/Map format and SAMtools», *Bioinformatics*, vol. 25, fasc. 16, pp. 2078–2079, ago. 2009, doi: 10.1093/bioinformatics/btp352.

[182] M. A. DePristo *et al.*, «A framework for variation discovery and genotyping using next-generation DNA sequencing data», *Nat Genet*, vol. 43, fasc. 5, pp. 491–498, mag. 2011, doi: 10.1038/ng.806.

[183] V. Boeva *et al.*, «Control-FREEC: a tool for assessing copy number and allelic content using next-generation sequencing data», *Bioinformatics*, vol. 28, fasc. 3, pp. 423–425, feb. 2012, doi: 10.1093/bioinformatics/btr670.

[184] K. Wang, M. Li, e H. Hakonarson, «ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data», *Nucleic Acids Research*, vol. 38, fasc. 16, pp. e164–e164, set. 2010, doi: 10.1093/nar/gkq603.

[185] G. Luo, L. P. Samaranayake, e J. Y. Y. Yau, «*Candida* Species Exhibit Differential In Vitro Hemolytic Activities», *J Clin Microbiol*, vol. 39, fasc. 8, pp. 2971–2974, ago. 2001, doi: 10.1128/JCM.39.8.2971-2974.2001.

[186] R. Buxton, Blood Agar Plates and Hemolysis Protocols. American Society for Microbiology (2016), 1–9

[187] R. G. Lawson e P. C. Jurs, «New index for clustering tendency and its application to chemical problems», *J. Chem. Inf. Comput. Sci.*, vol. 30, fasc. 1, pp. 36–41, feb. 1990, doi: 10.1021/ci00065a010.

[188] D.S. Katz The Streak Plate Protocol. *Am Soc Microbiol* **2016**, 1–14.

[189] S. Kaundal, A. Deep, G. Kaur, e K. G. Thakur, «Molecular and Biochemical Characterization of YeeF/YezG, a Polymorphic Toxin-Immunity Protein Pair From Bacillus subtilis», Front. Microbiol., vol. 11, p. 95, feb. 2020, doi: 10.3389/fmicb.2020.00095.

[190] L. E. Holberger, F. Garza-Sánchez, J. Lamoureux, D. A. Low, e C. S. Hayes, «A novel family of toxin/antitoxin proteins in *Bacillus* species», *FEBS Letters*, vol. 586, fasc. 2, pp. 132–136, gen. 2012, doi: 10.1016/j.febslet.2011.12.020.

[191] A. Ahmad, S. Majaz, e F. Nouroz, «Two-component systems regulate ABC transporters in antimicrobial peptide production, immunity and resistance», *Microbiology*, vol. 166, fasc. 1, pp. 4–20, gen. 2020, doi: 10.1099/mic.0.000823.

[192] A. Staroń, D. E. Finkeisen, e T. Mascher, «Peptide Antibiotic Sensing and Detoxification Modules of *Bacillus subtilis*», *Antimicrob Agents Chemother*, vol. 55, fasc. 2, pp. 515–525, feb. 2011, doi: 10.1128/AAC.00352-10.

[193] M. Hahn, «The rising threat of fungicide resistance in plant pathogenic fungi: Botrytis as a case study», *J Chem Biol*, vol. 7, fasc. 4, pp. 133–141, ott. 2014, doi: 10.1007/s12154-014-0113-1.

[194] A. Verma *et al.*, «Fungal Endophytes to Combat Biotic and Abiotic Stresses for Climate-Smart and Sustainable Agriculture», *Front. Plant Sci.*, vol. 13, p. 953836, lug. 2022, doi: 10.3389/fpls.2022.953836.

[195] M. P. de-Assis, R. C. Barcella, J. C. Padilha, H. H. Pohl, e S. B. F. Krug, «Health problems in agricultural workers occupationally exposed to pesticides», *Rev Bras Med Trab*, vol. 18, fasc. 03, pp. 352–363, 2020, doi: 10.47626/1679-4435-2020-532.

[196] W. J. Janisiewicz e L. Korsten, «B IOLOGICAL C ONTROL OF P OSTHARVEST D ISEASES OF F RUITS», *Annu. Rev. Phytopathol.*, vol. 40, fasc. 1, pp. 411–441, set. 2002, doi: 10.1146/annurev.phyto.40.120401.130158.

[197] K. Koledenkova, Q. Esmaeel, C. Jacquard, J. Nowak, C. Clément, e E. Ait Barka, «Plasmopara viticola the Causal Agent of Downy Mildew of Grapevine: From Its Taxonomy to Disease Management», *Front. Microbiol.*, vol. 13, p. 889472, mag. 2022, doi: 10.3389/fmicb.2022.889472.

[198] F. Faretra, S. Pollastro, Genetic basis of resistance to benzimidazole and dicarboximide fungicides in Botryotinia fuckeliana (Botrytis cinerea), Mycological Research, Volume

95, Issue 8, 1991, Pages 943-951, ISSN 0953-7562, https://doi.org/10.1016/S0953-7562(09)80091-9.

[199] S. Boso, V. Alonso-Villaverde, P. Gago, J. L. Santiago, e M. C. Martínez, «Susceptibility to downy mildew (Plasmopara viticola) of different Vitis varieties», *Crop Protection*, vol. 63, pp. 26–35, set. 2014, doi: 10.1016/j.cropro.2014.04.018.

[200] S. Furuya *et al.*, «Isolation and characterization of *Bacillus subtilis* KS1 for the biocontrol of grapevine fungal diseases», *Biocontrol Science and Technology*, vol. 21, fasc. 6, pp. 705–720, giu. 2011, doi: 10.1080/09583157.2011.574208.

[201] K. Hamaoka; Y. Aoki; Suzuki, S. Isolation and Characterization of Endophyte *Bacillus velezensis* KOF112 from Grapevine Shoot Xylem as Biological Control Agent for Fungal Diseases. *Plants* **2021**, *10*, 1815. https://doi.org/10.3390/plants10091815.

[202] G. Berg, Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* **84**, 11–18 (2009). https://doi.org/10.1007/s00253-009-2092-7

[203] S. Duchateau, J. Crouzet, S. Dorey, e A. Aziz, «The plant-associated Pantoea spp. as biocontrol agents: Mechanisms and diversity of bacteria-produced metabolites as a prospective tool for plant protection», *Biological Control*, vol. 188, p. 105441, gen. 2024, doi: 10.1016/j.biocontrol.2024.105441.

[204] A. M. Walterson e J. Stavrinides, «*Pantoea:* insights into a highly versatile and diverse genus within the Enterobacteriaceae», *FEMS Microbiology Reviews*, vol. 39, fasc. 6, pp. 968–984, nov. 2015, doi: 10.1093/femsre/fuv027.

[205] A. Baukova *et al.*, «New Insights into Pseudomonas spp.-Produced Antibiotics: Genetic Regulation of Biosynthesis and Implementation in Biotechnology», *Antibiotics*, vol. 13, fasc. 7, p. 597, giu. 2024, doi: 10.3390/antibiotics13070597.

[206] F. Rabiço *et al.*, «Novel Pseudomonas Species Prevent the Growth of the Phytopathogenic Fungus Aspergillus flavus», *BioTech*, vol. 13, fasc. 2, p. 8, mar. 2024, doi: 10.3390/biotech13020008.

[207] T. Nürnberger, F. Brunner, B. Kemmerling, L. Piater. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev. 2004 Apr;198:249-66. doi: 10.1111/j.0105-2896.2004.0119.x. PMID: 15199967.

[208] J.D. Jones, J.L. Dangl. The plant immune system. Nature. 2006 Nov 16;444(7117):323-9. doi: 10.1038/nature05286. PMID: 17108957.

[209] M. Ramos, N. Daranas, M. Llugany, R. Tolrà, E. Montesinos, E. Badosa. Grapevine response to a *Dittrichia viscosa* extract and a *Bacillus velezensis* strain. Front Plant Sci. 2022 Dec 16;13:1075231. doi: 10.3389/fpls.2022.1075231. PMID: 36589113; PMCID: PMC9803176.

[210] Y. Xu, L. Wang, W. Liang, M. Liu, Biocontrol potential of endophytic Bacillus velezensis strain QSE-21 against postharvest grey mould of fruit, Biological Control, Volume 161, 2021, 104711, ISSN 1049-9644, https://doi.org/10.1016/j.biocontrol.2021.104711.

[211] J. Köhl, J. Postma, P. Nicot, M. Ruocco, B. Blum, Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria, Biological Control, Volume 57, Issue 1, 2011, Pages 1-12, ISSN 1049-9644, https://doi.org/10.1016/j.biocontrol.2010.12.004.

[212] M.R. Farag, M. Alagawany, Erythrocytes as a biological model for screening of xenobiotics toxicity. Chem Biol Interact. 2018 Jan 5;279:73-83. doi: 10.1016/j.cbi.2017.11.007. Epub 2017 Nov 8. PMID: 29128605.

[213] H.Wang, J. Guo, X. Chen, H. He, The Metabolomics Changes in Luria-Bertani Broth Medium under Different Sterilization Methods and Their Effects on *Bacillus* Growth. Metabolites. 2023 Aug 18;13(8):958. doi: 10.3390/metabo13080958. PMID: 37623901; PMCID: PMC10456909.

[214] Strasser, Hermann, and Martin Kirchmair. "Potential health problems due to exposure in handling and using biological control agents. " *An ecological and societal approach to biological control*. Dordrecht: Springer Netherlands, 2006. 275-293.

[215] M. M. A. Baharudin *et al.*, «Antimicrobial activities of Bacillus velezensis strains isolated from stingless bee products against methicillin-resistant Staphylococcus aureus», *PLoS ONE*, vol. 16, fasc. 5, p. e0251514, mag. 2021, doi: 10.1371/journal.pone.0251514.

[216] J. A. Priyanto, M. E. Prastya, E. N. W. Hening, E. Suryanti, e R. Kristiana, «Two Strains of Endophytic Bacillus velezensis Carrying Antibiotic-Biosynthetic Genes Show Antibacterial and Antibiofilm Activities Against Methicillin-Resistant Staphylococcus aureus (MRSA)», *Indian J Microbiol*, vol. 64, fasc. 4, pp. 1884–1893, dic. 2024, doi: 10.1007/s12088-024-01262-1.

[217] G. R. Williams, «Haemolytic Material from Aerobic Sporing Bacilli», *Journal of General Microbiology*, vol. 16, fasc. 1, pp. 16–21, feb. 1957, doi: 10.1099/00221287-16-1-16.

[218] A. W. Bernheimer e P. Grushoff, «Extracellular Hemolysins of Aerobic Sporogenic Bacilli», *J Bacteriol*, vol. 93, fasc. 5, pp. 1541–1543, mag. 1967, doi: 10.1128/jb.93.5.1541-1543.1967.

[219] T. Huang, H. Geng, V. R. Miyyapuram, C. S. Sit, J. C. Vederas, e M. M. Nakano, «Isolation of a Variant of Subtilosin A with Hemolytic Activity», *J Bacteriol*, vol. 191, fasc. 18, pp. 5690–5696, set. 2009, doi: 10.1128/JB.00541-09.

[220] T. S. Manyatsi, Y.-H. Lin, e Y.-T. Jou, «The isolation and identification of Bacillus velezensis ZN-S10 from vanilla (V. planifolia), and the microbial distribution after the

curing process», *Sci Rep*, vol. 14, fasc. 1, p. 16339, lug. 2024, doi: 10.1038/s41598-024-66753-z.

[221] L. M. Brutscher, S. Gebrechristos, S. M. Garvey, e J. L. Spears, «Genetic and Phenotypic Characterization of Bacillus velezensis Strain BV379 for Human Probiotic Applications», *Microorganisms*, vol. 12, fasc. 3, p. 436, feb. 2024, doi: 10.3390/microorganisms12030436.

[222] X. Pan, X. Chen, X. Su, Y. Feng, Y. Tao, e Z. Dong, «Involvement of SpoVG in hemolysis caused by Bacillus subtilis», *Biochemical and Biophysical Research Communications*, vol. 443, fasc. 3, pp. 899–904, gen. 2014, doi: 10.1016/j.bbrc.2013.12.069.

[223] Department of Plant Protection, Faculty of Agriculture, Omdurman Islamic University, Omdurman, Sudan e O. Sidahmed, «Possibilities of Biological Control of Bactroceradorsalis(Hendel) (Diptera: Tephritidae) Using Bacillus subtilis/amyloliquefaciensand Bacillus sphaericus in Sudan», *J. Agric. Food Dev.*, vol. 3, fasc. 1, pp. 27–33, feb. 2018, doi: 10.30635/2415-0142.2017.03.5.

[224] F. Bove, L. Bavaresco, T. Caffi, e V. Rossi, «Assessment of Resistance Components for Improved Phenotyping of Grapevine Varieties Resistant to Downy Mildew», *Front. Plant Sci.*, vol. 10, p. 1559, nov. 2019, doi: 10.3389/fpls.2019.01559.

[225] A. Bejarano e G. Puopolo, «Bioformulation of Microbial Biocontrol Agents for a Sustainable Agriculture», in How Research Can Stimulate the Development of Commercial Biological Control Against Plant Diseases, vol. 21, A. De Cal, P. Melgarejo, e N. Magan, A c. di, in Progress in Biological Control, vol. 21. , Cham: Springer International Publishing, 2020, pp. 275–293. doi: 10.1007/978-3-030-53238-3_16.

[226] Z. Qiu, E. Egidi, H. Liu, S. Kaur, e B. K. Singh, «New frontiers in agriculture productivity: Optimised microbial inoculants and in situ microbiome engineering», Biotechnology Advances, vol. 37, fasc. 6, p. 107371, nov. 2019, doi: 10.1016/j.biotechadv.2019.03.010.

[227] P. Trivedi, P. M. Schenk, M. D. Wallenstein, e B. K. Singh, «Tiny Microbes, Big Yields: enhancing food crop production with biological solutions», Microbial Biotechnology, vol. 10, fasc. 5, pp. 999–1003, set. 2017, doi: 10.1111/1751-7915.12804.

[228] J. I. Clippinger, E. P. Dobry, I. Laffan, N. Zorbas, B. Hed, e M. A. Campbell, «Traditional and Emerging Approaches for Disease Management of Plasmopara viticola, Causal Agent of Downy Mildew of Grape», *Agriculture*, vol. 14, fasc. 3, p. 406, mar. 2024, doi: 10.3390/agriculture14030406.