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IN VITRO APPLICATION OF BIOCONTROL AGENTS TO IMPROVE THE SAFETY AND QUALITY OF GRAPEVINE PRODUCTS

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Dedication

At the end of my way of being a good scientist and at the primarily steps of being a useful person to helping living beings,

I need to dedicate my everything that I gained,

to my family:

To my father, Alireza, who is my forever hero, since I have the passion and motivation of being useful and being successful, just because of him.

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Abstract

One of the main horticultural sectors is represented by viticulture, with an area of considerable cultivation of 7.5 million ha in the world. Pathogenic fungi belonging to the genera *Botrytis*, *Phaeomoniella*, *Fusarium*, *Alternaria* and *Aspergillus* are responsible for vine diseases that affects, in its turn, the grapevine yield and the organoleptic quality of the final wine products. Among innovative strategies for in-field plant disease control, one of the most promising is represented by the use of biocontrol agents, including wild epiphytic yeast and bacterial strains. This approach can represent a valid, sustainable alternative to the use of synthetic fungicides whose intense use can have negative effects on the ecosystem with disruptive effects on the ecological relationship between the different species able to colonize grapevines and stimulating, also, the selection of resistant pathogen population to synthetic agents.

In this framework, the principal aim of my PhD thesis was the isolation, characterization and testing of new yeast strains isolated from Italian and Malaysian vineyards to be used as potential biocontrol agents and the comprehension of the mechanism of antimicrobial action of *Bacillus subtilis* SV108, previously isolated from the researchers of DISTAL, in order to be used as an additional biocontrol agent.

To realize the prefixed objectives, twenty wild yeast and one selected bacteria isolated among 62 samples, isolated from different Italian and Malaysian regions and molecularly identified, were evaluated in a preliminary screening test on agar to select isolates with inhibition against *Botrytis cinerea.* On the basis of the results, nine yeasts belonging to genera *Hanseniaspora*, *Starmerella*, *Metschnikowia*, *Candida* were selected and then tested against five grape berry pathogens: *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Fusarium oxysporum*, *Alternaria alternata* and *Phaeomoniella chlamydospore*. *Starmerella bacillaris* FE08.05, *Metschnikowia pulcherrima* GP8 and *Hanseniaspora uvarum* GM19 showed the highest effects on inhibiting mycelial growth, which ranged between 15.1 and 4.3 mm for the inhibition ring. On the other side, *Bacillus subtilis* SV108 was selected for further experiments on antimicrobial activity. It has been confirmed to have the ability of inhibit the mycelial growth of *Botrytis Cinerea*, *Aspergillus carbonarious* and *Phaeomoniella chlamydospora* by producing antimicrobial compounds on Malt Extract Broth medium recovered by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and identified by electrospray ionization (ESI) tandem mass spectrometer Triple TOF 5600. Moreover, in order to analyze the volatile fraction of compounds with antimicrobial effects, released during the growth of the biocontrol agents, the quantitative analysis of the VOCs profiles was performed by GC/MS/SPME. The

analysis highlighted the presence of isoamyl and phenylethyl alcohols and an overall higher presence of low-chain fatty acids and volatile ethyl esters.

All the data collected during my studies suggest that the tested yeasts, found among the epiphytic microbiota associated with grape berries, can be potentially effective for the biological control of pathogenic moulds, such as *Botrytis cinerea* and *Aspergillus carbonarious*. On the other hand, the proteomic study conducted on *B. subtilis* SV108 revealed that there are two cyclic antifungal peptides that correspond to the Iturin A synthesize B (ituB) and Mycosubtilin synthesize A (mycA) sequences which can explain the antimicrobial effect of *Bacillus subtilis* SV108 acting as biocontrol agent against fungal pathogens in grapevine.

Unfortunately, due to the Covid Pandemic situation, the final trial in field programmed at the Center of Tebano (Faenza, Italy), devoted to viticulture and oenology, was not conducted.

1. General introduction

Plants make up the living organisms with direct and indirect way. The sunlight energy conversion into stored, proteins, carbohydrates, and fats chemical energy utilization is only done by plants as they are the only higher organisms capable for this. Either the plants are cultivated or wild, all living organism, especially humans survival, depends on them.

More than 3000 species of edible plants have been used for food throughout our history, today only about 30 plant species make up 90% of our food supply. One of the most important source of food for human is fruit which has a wide range of use in everyday life. Grapevine (*Vitis spp*.) is one of the most considerable plant which has a wide spread cultivation all over the world with multitude products and different way of use. One of the main horticultural industries is described by viticulture, with the area of considerable cultivation of 7.5 million ha (OIV, 2019). Wine production is the most prepose of grapevine cultivation but, it has been used as fresh eating at the first time of its discovery. Nowadays, grapevine fruits are used in a wide range of products including wines, fresh fruits, juice, raisins, seed oil and preservers. Moreover, they are rich in vitamins, carbohydrates, fiber, phytochemicals and proteins (Dopico-García et al., 2008).

As well as other organisms, plants specially grapevine, also face to diseases. Whenever plants are infected, their production and growth may effected and reduced. Different kind of symptoms may appear and in worth case whole plant may ruined. The causal agents of plant disease are similar to those with the same effect in human or animal including whether viruses, bacteria, and fungi or nutrition deficiency and adverse environment (Creasy & Creasy, 2018).

In order to control the grapevine disease and pests, significant amounts of chemicals particularly fungicide alongside negative impact on the environment and health, have been consumed and are currently used. The negative impact of using chemical not only effect on the grapevine product, but also, it has a wide impact on environment including soil and underground water. In addition, it is cause of resistance to chemical on grapevine pathogen agents. It is not avoidable that this negative impact may include the toxicity of the chemical to nontarget organism, animal, birds or human.

To escape negative consequences and to guarantee product quality, chemicals and fungicides need to be replaced by other approaches including the engineering of disease-resistant plants, gene-silencing techniques, and use of biocontrol agents against grapevine pathogens. Selection of suitable yeasts or bacteria strains to be used as biocontrol agents against major fungal pathogens of grapevines may replace or reduce the use of synthetic fungicides in field and it may improve the safety, quality and functionality of resulting grapevine products (Li et al. 2021).

According to the increasing of the world population and decrease of the natural resource, the necessity of controlling plant pathogens in sustainable mode of action will be one of our most important need. Moreover, the use of biocontrol agents will fit with the FAO sustainable goal agenda foreseen by the 2030.

1.2 Geographical origin of grapevine

Different area of the world have the possibility of growing grapevine according to a great variety of emerging species (Figure 1). The real origin of *V. vinifera* is apparently in southern Caucasia which is inhabited by north-west Turkey, northern Iraq, Iran, Azerbaijan and Georgia (Creasy & Creasy, 2018). The relation between grapevine and oak could be because of the fact that vine using oak tree as support considering *Saccharomyces cerevisiae* (winemaking yeast). It is isolated for the first time from oak trees (Sniegowski et al., 2002).

Since different applications of grapevine were discovered by people living in these areas, they started to trade and export it to Palestine, Syria, Egypt, Mesopotamia and Mediterranean countries. Spread of the vines and the methods for its cultivation from Greece and Roma (Buxó, 2008) was started throughout Europe and Britain. The rout of exporting *V. vinifera* to North America was from Europe. Then it was transferred to Peru and Chile as the same time with Dutch people brought them to South Africa 1616. In 1788, packed grapevine was moved to Australia and New Zealand from England.

Figure 1. Red star showed the first domestication, stars in green show putative domestications. Center of admixture demonstrated by light green, Putative center illustrate by star in blue (Grassi & De Lorenzis, 2021). 1a) (Buono & Vallariello, 2002.,Marvelli et al., 2013), 1b) (Paschou et al., 2014), 2) (De Lorenzis et al., 2014), 3a) (Bouby et al., 2013), 3b and 4) (Buono & Vallariello, 2002), 5 and 6) (Grassi & De Lorenzis, 2021).

1.3 Taxonomy of grapevine

Vines are commonly woody plant with the ability of climbing up or growing as shrub. *Vitaceae* is the grapevine family include *Vitis*, *Ampelocissus*, *Clematicissus*, *Ampelopsis* along with *Cissus* (kangaroo vine) and *Parthenocissus* (Virginia creeper; Table 1). The most interested genus for grapevine industry is *Vitis* which is incorporate of two subgenera, *Vitis* and *Muscadinia*. The differences between these two subgenera are about the flower petals which are separated from the bottom in *Vitis*. Furthermore, these two subgenera have different chromosome number including *Vitis* and *Muscadinia* (38 and 40 chromosome respectively; Creasy & Creasy, 2018).

Table 1. Taxonomy Hierarchy of *V. vinifera* based on International Taxonomy Information System (ITIS)

Kingdom	Plantae	Plantes, Planta, Vegetal, Plants
Subkingdom	Viriplantae	Green plants
Infrakingdom	Streptophyta	Land plants
Superdivision	Embrophyta	
Division	Tracheophyta	Vascular plants, Tracheophyts
Subdivision	Spermatophyta	Spermatophytes, seed plants, phanerogames
Class	Magnoliopsida	
Superorder	*Rosanae*	
Order	Vitales	
Family	*Vitaceae*	Grapes
Genus	Vitis L.	Grape
Specious	*Vitis Vinifera*	Wine grape

1.4 History

Collecting vines from wild trees by people did not take long time to finding out the benefits of cultivation and industrial use. It is the fact that using grapevine by Egyptians was illustrated in pictures as growing vines from 1500 to 3000 bC (Janick, 2000). The first cultivation of vines (*V. vinifera*) was started probably by Chinese people in 2000 bC (Needham & Lu, 2000).

Fermentation of grapes in order to produce alcoholic beverages discovered at 7000 bC by Chinese people (P. E. McGovern et al., 2004) and the evidence in the Near East was around 6000 bc (P. McGovern et al., 2017). Grapes and the yeast relation, living inside and on the surface of berries, may cause natural fermentation on picked fruits resulted an alcoholic mixture that may interesting for some people, and that is the reason to having desire to repeat the procedure. The winemaking started probably from this point. Since wine can be naturally consider as long term storage form of grape and because of the large demands in this product, most of world production of grapes resulted in producing wine (OIV, 2019).

1.5 Use

As well as grapevine has highly adaptation capacity to different environments, the fruits also have high potential for different uses. Specially what makes the grapes unique is the amount of sugar and pectin production when ripening along with a wide range of aromatic compound. Wine production and associated fermented products takes 50% of global production. 36% of grape production consumed as fresh eating and table grapes in 2015. Raisins and juices as the other form of usage of grapes make up %8 to 5% (OIV, 2019). Further application of wine product can be described as sweetening, port and sherry as the result of making additional alcohol. Brandy, grappa and marc are the other form of wine by distillation.

1.6 World production

Since grapevine has different way of use all around the world, it has been cultivated in many countries even with unsuitable climate for its growing by using green houses and under control conditions. Near to 70% of grape production in the world are belong to top 10 producer countries (Figure 2).

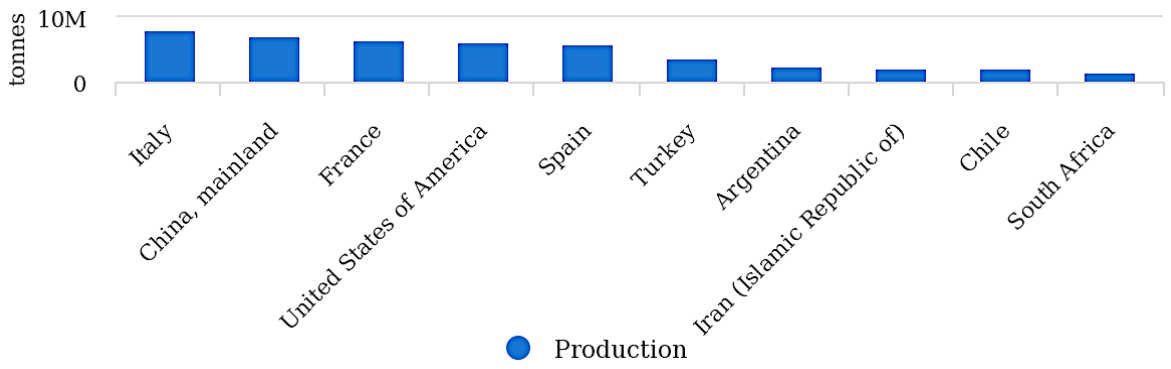

Figure 2. production of grape by top 10 producers (1994-2019)- FAOSTAT

The top producer is China with a significant margin. According to the wide range of soil type and diversity of climate in China, there is a large demand of investment on grape cultivation. There is a notable increase and decrease in the amount of vineyard area. This may related to its consistency and the social demands. Accordingly, Iran, Italy and Spain reduced their cultivation land and in contrast, China, India and New Zealand showed significant increase since 2005 and comes up to the top list. While changing in the amount of world production happened, there is a big change in cultivation method by up to date technologies and modern cultivars (Smart, 1996; Figure 3).

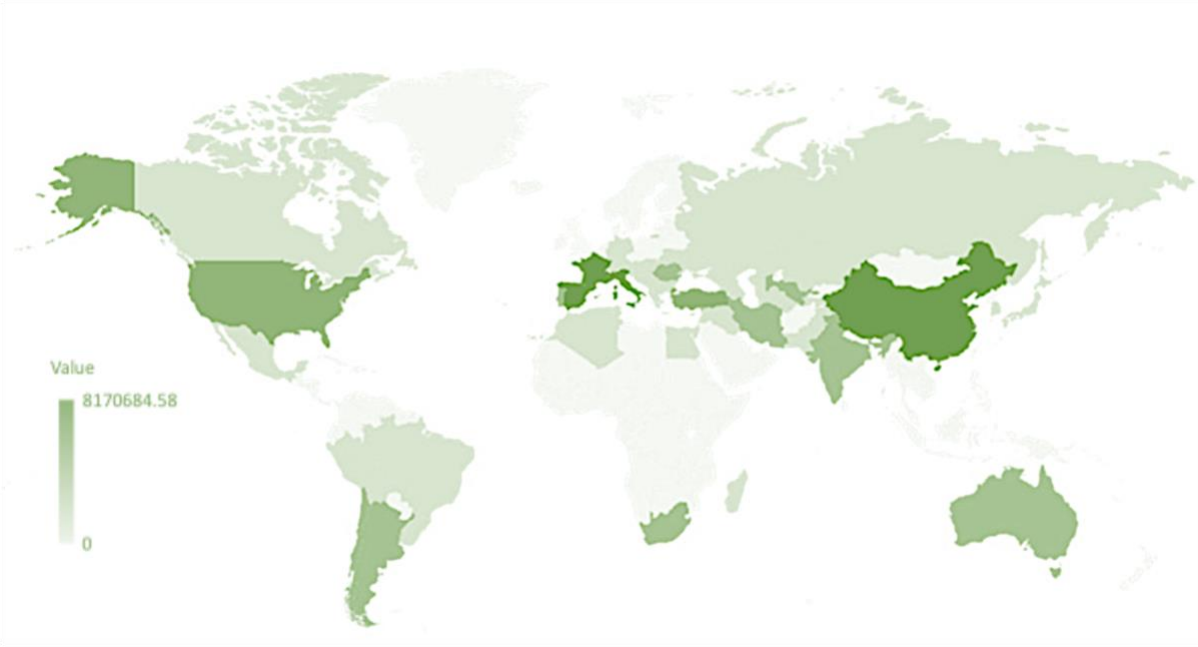

Figure 3. Production quantities of Grapes by country (tone)- (1994-2019)- FAOSTAT

China is the highest level investor in wine making production and table grape regarding to its wide range of climate and different type of the soil. Lower cost production and increasing rate

of quality make the vineyards still competitive in the USA. The international trading of wine and table grape is mostly present in South America (Figure 4 and 5).

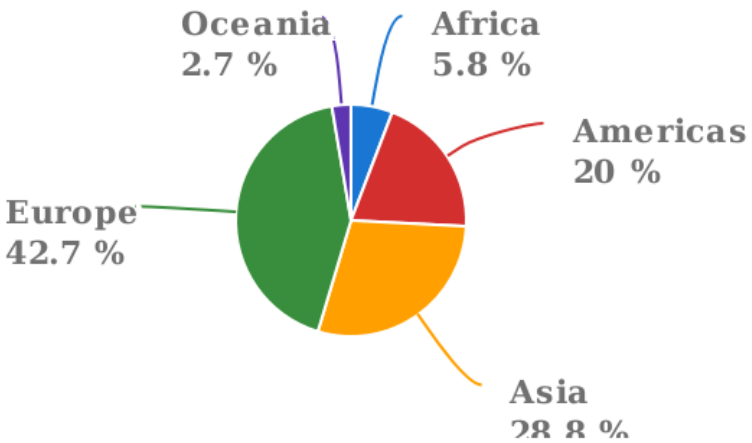

Figure 4. Production share of grapes by region-(1994-2019)- FAOSTAT

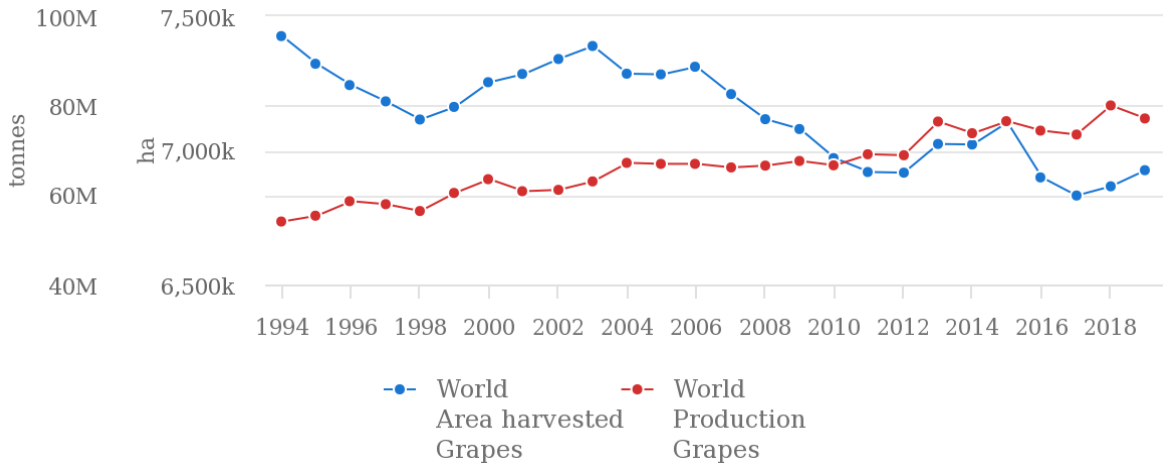

Figure 5. Production/Yield quantities of Grapes in World-(1994-2019)- FAOSTAT

1.7 Grapevine microbial ecology

Microbial ecology of grapevine with physiological characteristics which includes filamentous fungi, yeasts and bacteria, can affect the quality of wine production Some of parasitic fungi, endophyte bacteria and yeast are only occurred in grape berries.

However, there wide range of microorganisms are live and survive in wines with different microbial association. This microbial community contain yeast species, lactic acid bacteria and acetic acid bacteria. The availability of these specious is related to the time of ripening and nutrition disposal of grape (Figure 6).

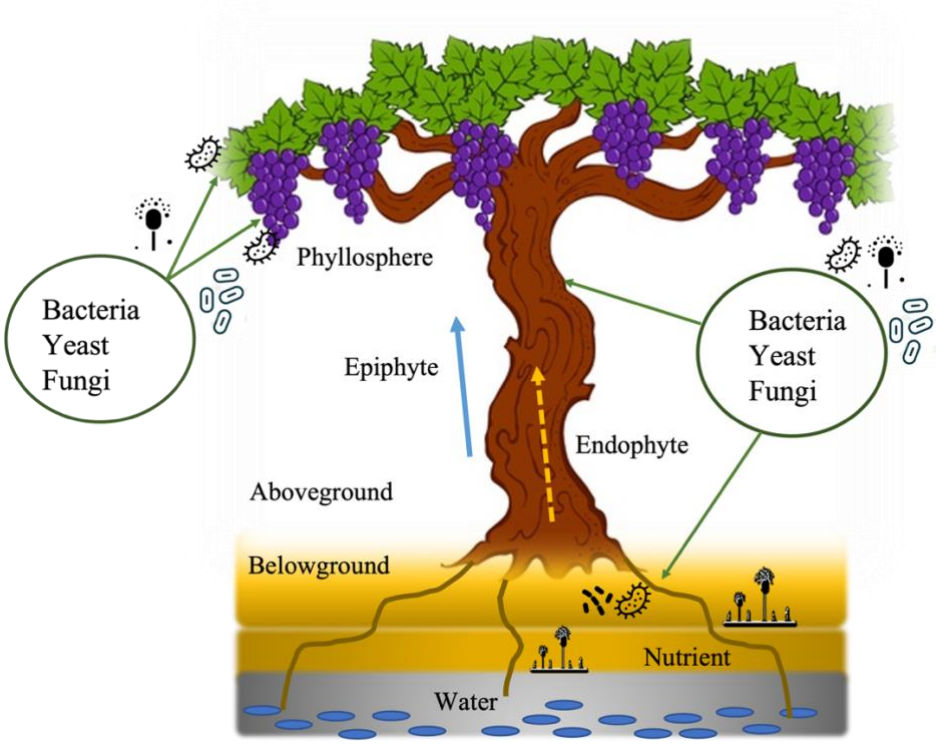

Figure 6. Microbial ecology of Grapevine (D. Liu et al., 2019)

1.8 Grapevine diseases

Grapevine infection with different kind of disease is the main reason of vine health problem and economic lose (Creasy & Creasy, 2018). The Management of grapevine disease has a significant contribution on vineyard costs and it may decrease or increase with considering climate situation. Grapevine appearance is important in local market as fresh fruit, whereas, in processing fruit, appearance is not so important. Grapevine disease caused by fungi, bacteria, viruses or nematodes can damage grapevine roots, the different part of the trunk, cordons, shoots, leaves, arms and berries.

1.8.1 Fugal disease

Most of the research and concerns are focused on fungal disease of grapevine including *B. cinerea* (Bunch rot), downy mildew, powdery mildew and Phomopsis (Figure 7). *B. cinerea* is one of the most important disease, consequently, any kind of management strategies for this pathogen can be applied for other fungal disease of grapevine as well (D. Liu et al., 2019).

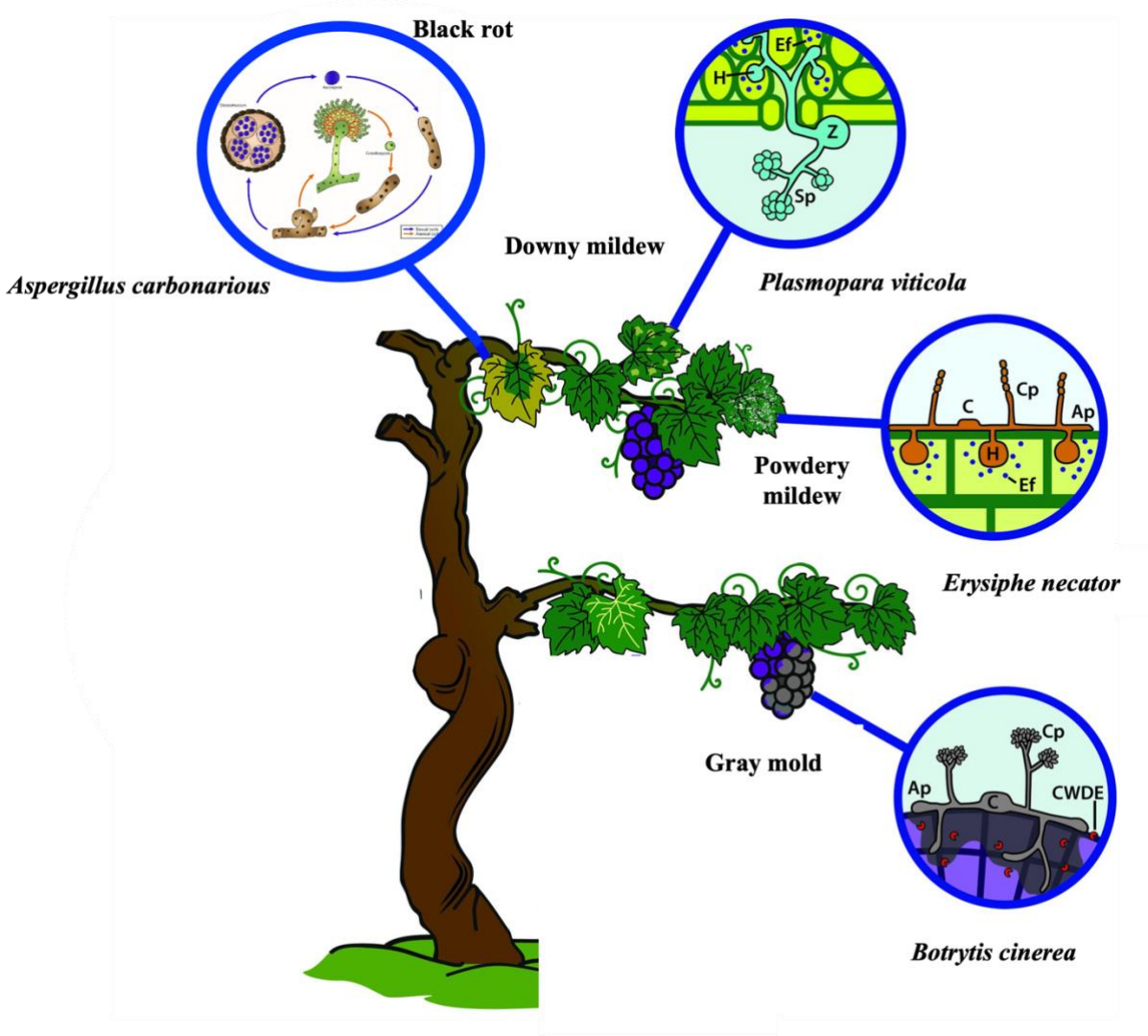

Figure 7. Major grapevine fungal pathogen and their infection strategies (D. Liu et al., 2019)

1.8.1.1 *Botrytis cinerea*

One of the most current and important fungal disease is *Botrytis* bunch rot which is famous for making gray mould on leaves and berries of the grapevine. The ubiquitous and widespread fungi infecting grapevine is *Botrytis cinerea.* This name is associated with grapevine which is made from Greek term in 18th century (Rosslenbroich & Stuebler, 2000a). The favorite climate situation for this mould is represented by the cool temperature. However, it develops rapidly in warm and humid environments (Creasy & Creasy, 2018).

Grapevine is not the only host for *B. cinerea.* It can infect a lot of wild or cultivated plants and is has the ability to grow as a saprophyte on live or dead tissue. Accordingly, it is unavoidable in vineyard or other cultivation places. Regarding to widespread and large growing capacity of *Botrytis*, single control method would not be enough to contain it needing of an integrated management of strategies (Liu et al. 2019).

Overwintering of *Botrytis* can be done as a sclerotia which is a dark-colored structures with high resistance in cold, hot and adverse conditions on dried grape. Moreover, it can survive better in moderate climate inside or under buds and barks as a dormant hyphae. Obviously, it will spread and grow again when the weather condition improve (Wilcox et al., 2015). *Botrytis* produces conidiophores in a wide range of environmental situation and it has the possibility of spreading by wind water. The superlative temperature for spread and infection described as 23.7°C and 20.8°C following by proper free water and humidity (Creasy & Creasy, 2018). The time requested for the infection is about one hour and it depends on temperature and humidity. The better situation makes the less time of infection.

Berries are protected by skin which makes them more resistance to be infected with *Botrytis* than leaves. However, natural open pores like stomata and coticule can be a way of infection (Coertze et al., 2001). Furthermore, the most common way of *Botrytis* infection is mechanical injury which can be done by insect or birds bites, storm and the infection of powdery mildew as well (Creasy & Creasy, 2018).

1.8.1.2 *Phaeomoniella chlamydospore*

Phaeomoniella chlamydospore can inhabit the woody tissue of the vines and for the most part their symptoms arise from the same causes – restriction of xylem capacity, resulting in water or nutrient-type symptoms, and through the presence of toxins that the fungi may make. The term esca may be the most appropriate to describe this situation. Externally, there may be stunted growth, shoot dieback and chlorotic leaves (the yellowing being in a distinct pattern than has been called tiger stripe or grapevine leaf stripe). In some cases, the vine may suddenly and catastrophically fail (called apoplexy), usually in response to increased water demand during early-season growth outstripping the ability of the compromised vascular system to deliver water. Fruit may develop blotchy darker patches, which earned it the name of 'black measles' in California. Internally, there would be darkening of the wood where the xylem has been affected by fungal development, and sometimes exudation of 'black goo' (an early name for the disease) from individual xylem vessels (Wilcox et al., 2015)

1.8.1.3 *Aspergillus* sp.

Another important post-harvest grapevine disease is *Aspergillus* rot (*Aspergillus* sp.) which contaminate grapes by producing a variety of mycotoxins (Madden et al., 2017; Serra et al., 2005; N G Allam et al., 2008; Nanis G Allam et al., 2012). Mycotoxins formed by rotten grape berries associated with several specious of fungi such as *Aspergillus* and *Penicillium*

Aspergillus sp. produced a potential carcinogenic nephrotoxic, named Ochratoxin A which is related to tumors and serious kidney disorders in human. Ochratoxin A quantity in wine production is significantly different in various regions and it depends on environmental and winemaking aspects of vineyards. Moreover, *Aspergillus carbonarius* generally produce Ochratoxin A more than *A. niger* (de Andrade Santiago et al. 2018). In comparison with bunch rot, *Aspergillus* spp. Need a warm and dry climate as ideal condition for growing, consequently, there is more concern about ochratoxin A in southern Europe.

1.8.1.4 *Alternaria alternata*

Alternaria alternata is a ubiquitous, cosmopolitan, saprophytes, endophytic, post-harvest pathogen and also infect animal and human as well. There is a significant diversity in morphology of Alternaria that is make it a challenging group for taxonomical studies.(Andrew et al., 2009). It is wide spread in soil and decayed organic materials (Pavón et al., 2012). As a case in point, it has been reported that about 380 plants specious hosting *Alternaria alternata* and grapevine is one of them (Bernadovičová & Ivanová, 2011).

1.9 Control strategies

Every year, significant amount (30-40 %) of table grapes are lost because of the softness made by fungal infection (Jiang et al., 2014; Williamson et al., 2007; Lappa et al. 2018)). In order to manage table grape fungal pathogens, chemical strategies have been suggested (Youssef et al., 2015). The control of the fungal disease with chemical fungicides is normal in vineyards. There are two kinds of fungicides (organic and synthetic) with different mechanism such as protecting from pre and post-harvest infection, preventing sporulation, and complete eradication. Chemical fungicides have frequently been using since the end of World War 2. However, it makes serious concern regarding consumer health, product quality and environmental issues (Waewthongrak et al., 2015).

On the other hand, using same chemical fungicide repeatedly, may induce resistance in pathogens and this is one of the most important factors for leading the management strategies to find a biodegradable and non-chemical alternatives in order to support sustainable agriculture strategies.

Despite the fact that fungicides are presently being avoided owing to human health and environmental concerns (Waewthongrak et al., 2015) and till now scientists are working to produce a natural, safe, and biodegradable fungicides (Grzegorczyk et al., 2017).

1.10 Biocontrol

Different kinds of microorganisms were investigated for their protentional ability to inhibit and eliminate pathogens in plants, in order to create an alternative from application of chemicals in management strategies for sustainable agriculture (Maachia et al. 2015). Furthermore, many researches have been conducted regarding to find plant base chemical compound for induction of resistance in host plants against invading pathogen.

There are many microorganisms originally coming from plants surface (epiphytes) or inside plants (endophytes). Epiphytic and endophytic bacteria, yeast and fungi have been taken attention to be use as biological control agent (BCA), to induce resistance and promote the growth.

There are confirmations from previous studies regarding the benefits of using biocontrol agents against fungal pathogens in grapevine (Droby et al., 2009; Furuya et al., 2011; Russi et al., 2020; Maachia et al., 2015). This has two major advantages compare to conventional chemical methods to protect grapevine from fungal pathogens: (I) biocontrol agents are derived from grapevine itself and they do not have not any effect on the safety and quality of related products (II) increase or development pathogen resistance would not be an issue (Otoguro & Suzuki, 2018b).

The use of synthetic fungicides is effective for the in-field management of grapevine diseases (Rosslenbroich & Stuebler, 2000b) but their intensely use had huge negative effects on ecosystems with disruptive effects on the ecological relationship between the different species able to colonize grapevines and stimulated the selection of resistant pathogen population to synthetic agents (Pinto et al., 2014).

The increased public concern on the harmful synthetic agents for the crop diseases management on the environment, in addition to the restrictions imposed by governmental organizations like European Union (UE; Directive 2009/128 /EC; Durel et al., 2015), stimulated researchers towards the development of innovative and sustainable systems for harvest crop diseases control (Pinto & Gomes, 2016).

Among these, innovative and eco-friendly solutions, the use of biopesticides is promising. Biopesticide could be defined as biocontrol agent inhabitant of the same ecological niche of crop pathogens and able to counteract their habit and growth (Maachia et al., 2015; Pertot et al., 2017). Grapevine represents a great source of microbial community including yeasts, responsible for safety, quality and the yield of product (Martins et al., 2013). Moreover, grapevine microbiome plays important role in plant growth, especially effects on resistance or struggle to various type of pathogens (Pinto & Gomes, 2016). Biodiversity of microorganisms

on grapevine berries has been widely studied (Barata et al., 2012; Patrignani et al., 2017; Morgan et al., 2017).

Natural occurring surface grape berry microbiota is constituted by a combination of wild yeasts, mainly belonging to *non-Saccharomyces* genera including *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*, *Zygoascus* and *Issatchenkia* (Vivier & Pretorius, 2000; Jolly et al., 2014; Patrignani et al., 2017; Patrignani et al. 2017; Sabaghian et al., 2021) that have significant effect on healthy and quality of fruit berries and may have great impact on wine making process as well (Martins et al., 2013).

Due to their ability to colonize grapevine wound sites, simple nutritional demand, and good rate of growth, epiphytic grape berry natural occurring non-*Saccharomyces* yeasts have been largely studied as prospective biocontrol agents (Ribes et al., 2018; Wisniewski et al., 2007; Droby et al., 2009; Maachia et al., 2015; Spadaro & Droby, 2016).

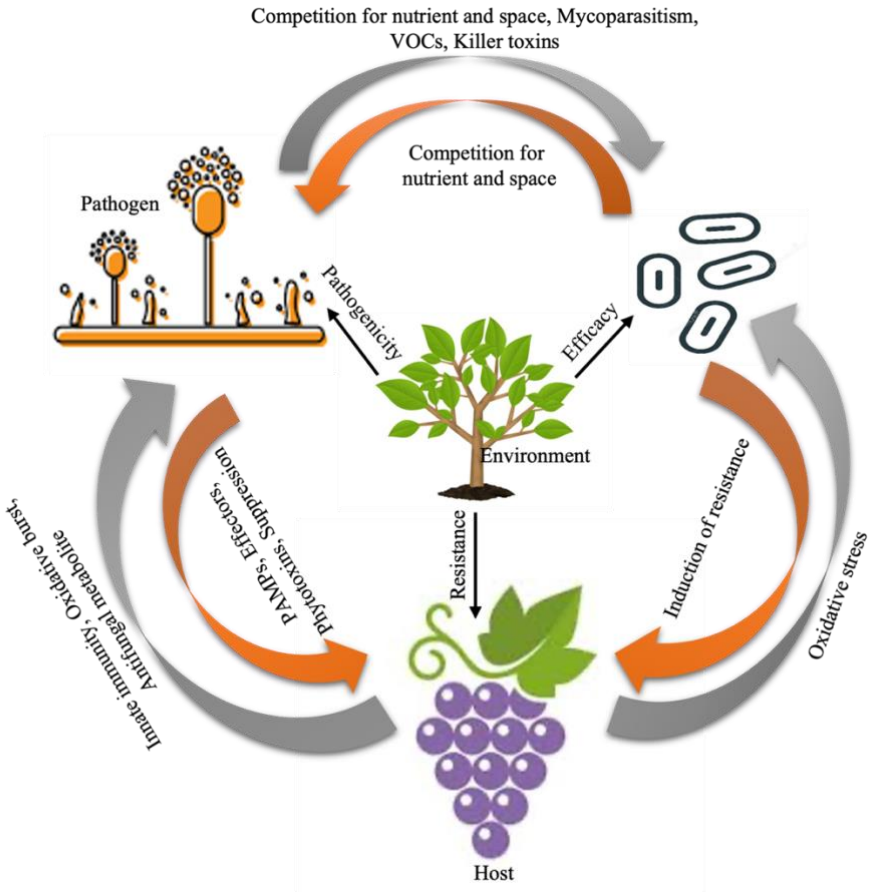

Figure 8. schematic diagram of probable interaction between elements of biocontrol procedure

The great amount of grapevine cultivation is related to wine production but nowadays it is also used as fresh fruit, preserver juice and raisins. For enhancing the quality of grapevine products, selective breeding has been used during recent years.

To guarantee product quality, fungicides need to be replaced by biocontrol agents which are a potential alternative to chemical fungicide application for fungal disease management as well as improve the safety, quality and functionality of resulting grapevine products in vineyards. Biocontrol agents are described as microorganisms isolated from nature to farm. Due to the safe application of microorganism based products to control the fungal disease in grapevine, a large number of biological control candidate have been introduced (Mochizuki et al., 2012).

1.10.1 Control by using yeast

Different non-*Saccharomyces*, including species belonging to genera *Aureobasidium*, *Candida*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Rhodotorula*, and *Wickerhamomyces* have been reported as reducer of fungal pathogen (i.e. *Botrytis cinerea*), impact on fruits through different mechanism including nutrient/space competition (Suzzi et al., 1995), iron deficiency (Freimoser et al., 2019; Parafati et al., 2015), enzyme related to cell wall degradation (Parafati et al., 2015), tolerance to reactive oxygen species (Parafati et al., 2015; Carmona-Hernandez et al., 2019), biofilm production (Freimoser et al., 2019) as well as host resistance induction against phytopathogen by phytoalexin production (Freimoser et al., 2019) or synthesis of pathogenesis-related proteins (Freimoser et al., 2019; Zanzotto & Morroni, 2016; Dukare et al., 2019). Grapevines represent a great source of microbial community, including yeasts, which are responsible for the safety, quality, and yield of products (Pertot et al., 2017). Moreover, the grapevine microbiome plays an important role in plant growth, especially in resistance to various types of pathogens (Pertot et al., 2017). The biodiversity of microorganisms on grapevine berries has been widely studied (Barata et al., 2012; Morgan et al., 2017).

The naturally occurring surface microbiota of grape berry is constituted by a combination of wild yeasts, mainly belonging to non-*Saccharomyces* genera, including *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*, *Zygoascus* and *Issatchenkia* (Patrignani et al., 2017; Vivier & Pretorius, 2000) that have a significant effect on the health and quality of fruit berries and may have a great impact on the wine-making process as well (Steel et al., 2013). Due to their ability to colonize grapevine wound sites, simple nutritional demand, and good rate of growth, epiphytic naturally occurring non-*Saccharomyces* yeasts on grape berries has been largely studied as potential biocontrol agents (Maachia et al., 2015; Ribes et al., 2018; Spadaro & Droby, 2016).

1.10.2 Control by using bacteria

Recently, different fungal disease control strategies by application of bacteria have been reviewed by Compant *et al*. (2013). There are three different mechanisms for bacteria to inhibit the pathogens. The first one is antibiosis and it is coming from the toxicity of bacteria for pathogen which is happening by producing secondary metabolites. Induction of resistance to plant against pathogens is the second mechanism for controlling plant disease by bacteria. Bacteria also can compete with pathogen on consuming nutrition and this is the third way of affecting pathogen's progressing (Wang et al., 2018).

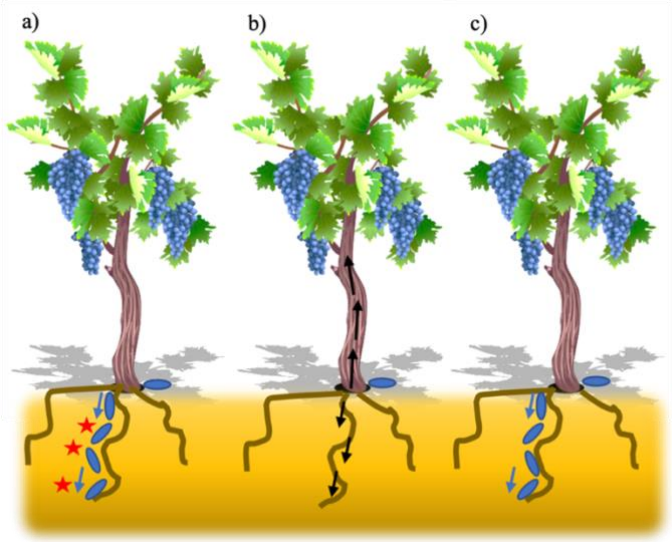

Figure 9. (a) Colonization of root by bacteria (antibiosis), induction of resistance by bacteria (ISR), (c) nutrient competition (X. Q. Wang et al., 2018).

1.10.2.1 *Bacillus* spp.

The majority of these biocontrol agents contain antibacterial, antifungal, and other secondary metabolites. These antimicrobial secondary metabolite production plays an important role against pathogens where they are complex compounds that appear only in stressing conditions (Keswani et al., 2020). One of the most prevalent microbial species that has been frequently utilized as a biocontrol agent is *Bacillus subtilis*, which is an endospore-forming and gram-positive bacterium (Ongena & Jacques, 2008). Since this bacterium has protentional antifungal activities following by potential capacity to produce lots of antimicrobial compounds, it is used as biocontrol agent to control and inhibit grapevine fungal pathogen in vitro and in the field, too (Ribes et al., 2018; Patrignani et al., 2017; Barata et al., 2012; Wisniewski et al., 2007). *Bacillus* genome is related to antimicrobial compound production approximately four to five percent, which are mainly antimicrobial peptides (Figure 10).

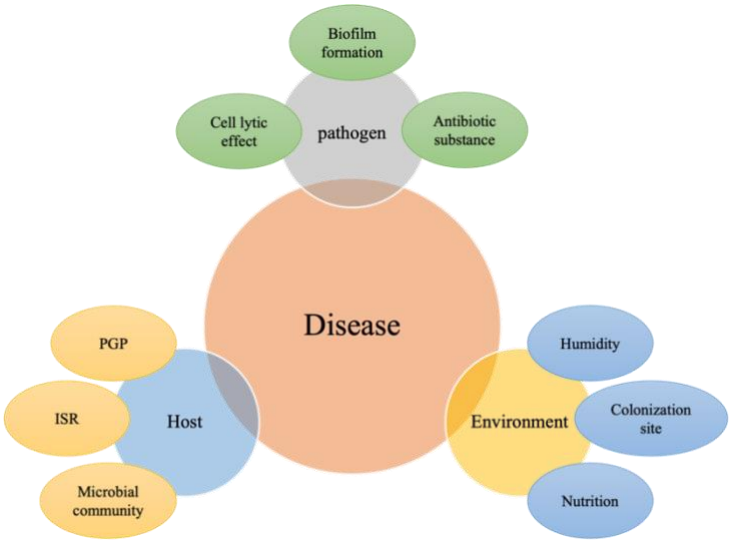

Figure 10. Theoretical model for interaction between *B. subtilis* and the triangle of disease

1.10.3 Antimicrobial compounds

Antimicrobial secondary metabolite production plays an important role against pathogens. The secondary metabolites produced from microorganisms are low molecular weight. They are complex compounds and they appear only in stressing condition (Keswani et al., 2020). One of the common microbial species that has been widely used as biocontrol agent is *Bacillus subtilis* (Ongena & Jacques, 2008).

Four to five percent of the genome of *Bacillus* spp. is associated with producing the antimicrobial compound which are mainly antimicrobial peptides (Stein, 2005). Moreover, antifungal protein production by this bacterium, containing ribosomal peptides, volatile compounds, polyketides molecules, non-ribosomal peptides, bacteriocins were approved (Alvarez et al., 2012; Caulier et al., 2019). Peptides produced by *B. subtilis* along with abundant prospective for biocontrol application was studied. The antimicrobial compounds included: the lipopeptide surfactin (Charles W Bacon & Hinton, 2002), fengycin and iturin (Bent, 1999) which includes iturin A, B and C (C W Bacon et al., 2004). The chemical structure of iturin A is a cyclic heptapeptide with an alkaline chain and its mechanism of action is making a pore formation in cell membrane (Figure 11).

Many other lipopeptides have close relation to iturin family including mycosubtilin which is iturinic lipopeptide multigene family (Dunlap et al., 2019). Iturin is cycle heptapeptide that is connected with β-amino acid residue of a fatty acid chain ((L)Asn-(D-)Tyr-(D-)Asn-(L-)Gln-(L-)Pro-(D)Asn-(L-)Ser) where amino acid changed, the iturin homologue name becomes

mycosubtilin ((L-)Asn-(D-)Tyr-(D-)Asn-(L-) Gln-(L-)Pro-(D)Ser-(L)Asn) (Nasir & Besson, 2012; (Figure 12).

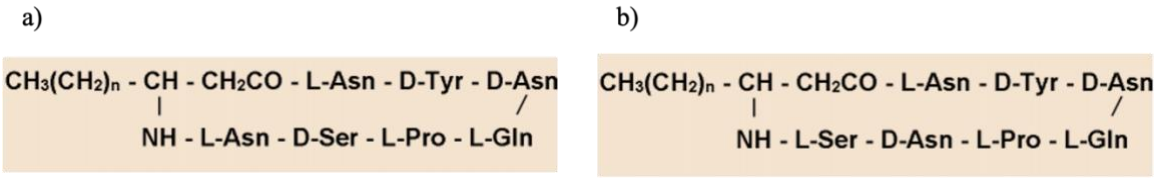

Figure 11. Schematic structure of a) mycosubtilin and b) Iturin A through carbon chain structure of fatty acid.

They are well known since having strong antifungal activity as well as inducing defence reaction in plant (Farace et al., 2015). Mycosubtilin is a biosurfactant and peptide-antibiotic with strong antimicrobial activity (Farace et al., 2015; Li et al., 2019).

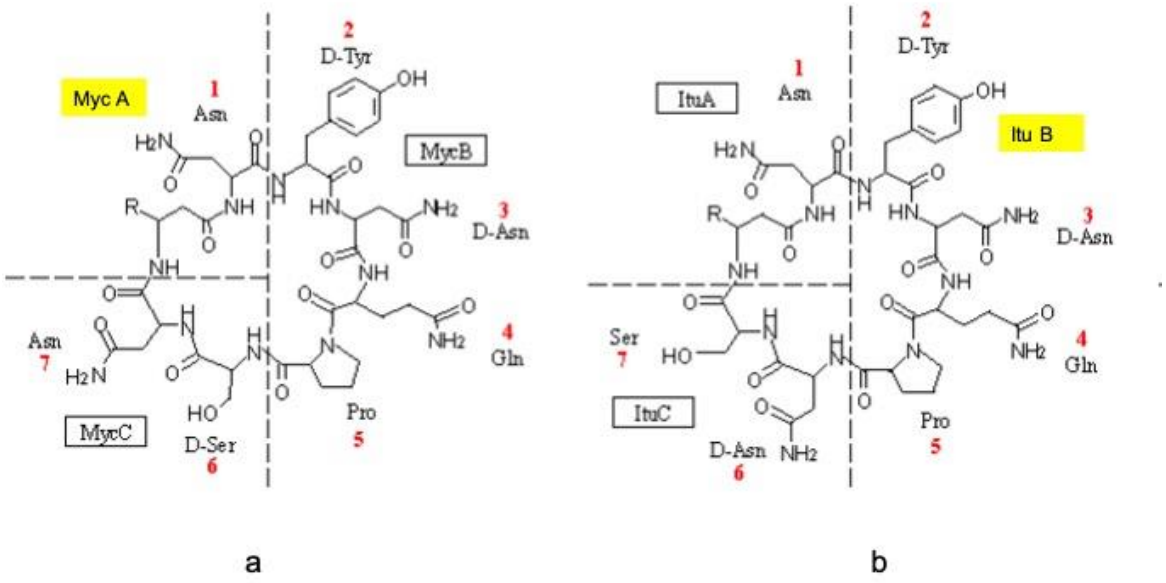

Figure 12. Non ribosomal lipopeptide structure (a) Mycosubtilin and (b) Iturin

The antimicrobial compounds produced by *B. subtilis* depend on strain and environmental factors. Consequently, in order to obtain antimicrobial substances, process situations optimization and genetic modification have been applied (X. Wang et al., 2015). Presently, effective methods to prepare peptides with a large amount of antimicrobials need to take into account strain proteolysis profile, proteomic and meta genomic patterns (Buddhika & Abeysinghe, 2021).

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2. General objectives

The rationale of my PhD project was the identification, characterization and comprehension of the key factors of the antimicrobial activity of selected yeasts and bacterial strains to be used as biocontrol agents against major fungal pathogens of grapevine, in order to replace or reduce the use of synthetic fungicides in field and to improve the safety, quality and functionality of resulting grapevine products.

In fact, Fungi are one of the most significant economic losses pre- and post-harvest in vineyards worldwide. *Botrytis cinerea* and *Aspergilus carbonarius* are two of important grapevine pathogens, causal agents of gray mold and black rot with the high level of toxic Ochratoxin which causes heavy losses in table and wine grapes (Creasy & Creasy, 2018). About 30–40% of post-harvest table grapes are drifted every year as a result of softening caused by fungal infection (Jiang et al., 2014). In order to manage table grape fungal pathogens, chemical strategies have been suggested (Youssef et al., 2015). fungicides are currently an avoided strategy due to the human health and environmental issues (Waewthongrak et al., 2015).

Accordingly, researchers are focusing to develop natural, safe and biodegradable substitutes to stand for chemicals fungicide (Grzegorczyk et al., 2017). There are confirmations from previous studies regarding the benefits of using biocontrol agents against fungal pathogens in grapevine (Droby et al., 2009; Furuya et al., 2011; Russi et al., 2020; Maachia et al., 2015). A wide range of peptides produced by *B. subtilis* strains along with abundant prospective for biocontrol application was studied. New ways must be found to control them since there is no direct control of these diseases. The use of microbial antagonists for the control of postharvest diseases received special attention, and has been extensively considered. Biological control of postharvest diseases of fruits and vegetables by antagonistic microorganisms seems hopeful in replacing or reducing the use of synthetic fungicides (Freimoser et al., 2019). Yeasts have been extensively studied because they possess many structures that make them suitable as biocontrol agents in fruits (Dukare et al., 2019). Yeasts are rarely related to occurrences of foodborne gastroenteritis, intoxications or other infections, unlike bacteria and viruses (Prakitchaiwattana et al., 2004).

Among these innovative and eco-friendly solutions, the use of biopesticides is promising. Biopesticides could be defined as biocontrol agent inhabitants of the same ecological niche as crop pathogens able to counteract their habits and growth (Maachia et al., 2015; Pertot et al., 2017). Grapevines represent a great source of the microbial community, including yeasts, which are responsible for the safety, quality, and yield of products (Pertot et al., 2017). Moreover, the grapevine microbiome plays an important role in plant growth, especially in

resistance to various types of pathogens (Pertot et al., 2017). The biodiversity of microorganisms on grapevine berries has been widely studied (Barata et al., 2012; Morgan et al., 2017; Dukare et al., 2019).

In this contest, the first part of this project was focused on the relations between plant fungus diseases and yeasts as the biocontrol agents, by relying on laboratory and molecular methods and investigating the role of yeast on increasing the safety and quality of grapevine products. For this, isolated indigenous yeasts, isolated in Malaysian and Italian vineyards, were characterized in order to assess their *in vitro* ability to counteract the growth of several grapevines phytopathogens, such as *Botrytis cinerea*, *Phaeomoniella chlamydospora*, *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus carbonarius* and *Aspergillus ochraceus.* In addition, to understanding the mechanisms of the yeast antifungal activities, the strains VOCs profiles were also investigated by using GC/MS/SPME approach.

Moreover, in the second part a selected biocontrol agent, *B. subtilis* SV108, isolated from grape berries by the DISTAL researchers and endowed with a high antimicrobial activity against grapevine major fungal pathogens, was studied by purification and identification of the antimicrobial compounds produced, by using a proteomic approach, as the key factor for inhibition of mycelial growth. In fact, the research explored the role of identified peptides in the proteomic response mechanisms.

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3. Chapter 1

Isolation and Identification of Wild Yeast from Italian and Malaysian vineyards and Evaluation of Their Potential Antimicrobial Activity against Grapevine Fungal Pathogens

Abstract

Pathogenic fungi belonging to the genera *Botrytis*, *Phaeomoniella*, *Fusarium*, *Alternaria* and *Aspergillus* are responsible for vines diseases that affect the growth, grapevine yield and organoleptic quality of final products. Among innovative strategies for in-field plant disease control, one of the most promising is represented by biocontrol agents, including wild epiphytic yeast strains of grapevine berries. Twenty wild yeasts, isolated and molecularly identified from three different Malaysian regions (Perlis, Perak and Pahang), were evaluated in a preliminary screening test to select *in vitro* isolates with inhibition against *Botrytis cinerea.* On the basis of the results, nine yeasts belonging to genera *Hanseniaspora*, *Starmerella*, *Metschnikowia*, *Candida* were selected and then tested against five grape berry pathogens: *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Fusarium oxysporum*, *Alternaria alternata* and *Phaeomoniella chlamydospora*. *Starmerella bacillaris* FE08.05 and *Metschnikowia pulcherrima* GP8 and *Hanseniaspora uvarum* GM19 showed the highest effect on inhibiting mycelial growth, which ranged between 15.1 and 4.3 mm for the inhibition ring. The quantitative analysis of the *VOCs* profiles highlighted the presence of isoamyl and phenylethyl alcohols and an overall higher presence of low-chain fatty acids and volatile ethyl esters. The results of this study suggest that antagonist yeasts, potentially effective for the biological control of pathogenic moulds, can be found among the epiphytic microbiota associated with grape berries.

3.1 Introduction

Grapevines (*Vitis vinifera*) are commonly associated with a temperate climate, but, over the past decades, a few varieties have been inbred or found to grow well in a tropical environment (Keller, 2020). In Malaysia, *V. vinifera* grapes for commercial use are produced mainly in Perlis, Perak and Pahang provinces. In 2013, the cultivation area with grapevines was estimated to be 6.6 ha. with 228.5 MT production (Gobilik & Enggihon, 2019). In wet tropical areas, however, a successful planting of grapevines will depend on several factors, including the use of greenhouse and pesticides, to protect the vines from rain and fungus outbreaks.

The uncontrolled proliferation of pathogenic fungi belonging to the genera *Botrytis*, *Phaeomoniella*, *Fusarium*, *Alternaria* and *Aspergillus* are responsible for vine diseases that affect plant growth, grapevine yield and organoleptic quality, consequently causing economic losses (Di Canito et al., 2021). The use of synthetic fungicides is effective for the in-field management of grapevine diseases (Steel et al., 2013), but their intense use has extensive negative effects on ecosystems. Disruptive effects can impact the ecological relationship between the different species able to colonize grapevines and stimulate the selection of resistant pathogen populations to synthetic agents (Pinto et al., 2014).

The increased public concern over the harmful effect of synthetic agents used for crop disease management on the environment, in addition to the restrictions imposed by governmental organizations like the European Union (UE; Directive 2009/128 /EC) (Durel et al., 2015), have stimulated researchers towards the development of innovative and sustainable systems for harvest crop disease control (Pinto & Gomes, 2016).

Among these innovative and eco-friendly solutions, the use of biopesticides is promising. Biopesticides could be defined as biocontrol agent inhabitants of the same ecological niche as crop pathogens able to counteract their habits and growth (Maachia et al., 2015; Pertot et al., 2017). Grapevines represent a great source of the microbial community, including yeasts, which are responsible for the safety, quality, and yield of products (Pertot et al., 2017). Moreover, the grapevine microbiome plays an important role in plant growth, especially in resistance to various types of pathogens (Pertot et al., 2017). The biodiversity of microorganisms on grapevine berries has been widely studied (Barata et al., 2012; Morgan et al., 2017).

The naturally occurring surface microbiota of grape berry is constituted by a combination of wild yeasts, mainly belonging to non-*Saccharomyces* genera, including *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*, *Zygoascus* and *Issatchenkia* (Patrignani et al., 2017; Vivier & Pretorius, 2000) that have a significant effect on the health and quality of fruit berries and

may have a great impact on the wine-making process as well (Steel et al., 2013). Due to their ability to colonize grapevine wound sites, simple nutritional demand, and good rate of growth, epiphytic naturally occurring non-*Saccharomyces* yeasts on grape berries has been largely studied as potential biocontrol agents (Maachia et al., 2015; Ribes et al., 2018; Spadaro & Droby, 2016).

Different non-*Saccharomyces* species, including those belonging to the genera *Aureobasidium*, *Candida*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Rhodotorula* and *Wickerhamomyces*, have been reported as reducers of fungal pathogens (i.e., *Botrytis cinerea*). Furthermore, they have an impact on fruits through different mechanisms, including nutrient/space competition (Tofalo et al., 2016), iron deficiency (Freimoser et al., 2019), enzymes related to cell wall degradation (Parafati et al., 2015), tolerance to reactive oxygen species (X. Cao et al., 2011), biofilm production (Freimoser et al., 2019) as well as host resistance induction against phytopathogen by phytoalexin production (Freimoser et al., 2019)or synthesis of pathogenesis-related proteins (Zanzotto & Morroni, 2016; Dukare et al., 2019). In this context, the major aim of the presented work was to perform an ecological study on the grape-berry yeast population associated with grape berries from three different regions of Malaysia: Perlis, Perak and Pahang in order to find grape-berry epiphytic yeasts to be used as potential in-field biocontrol agents. For this, isolated indigenous yeasts were characterized in order to assess their in vitro ability to counteract the growth of several grapevines phytopathogens, such as *Botrytis cinerea*, *Phaeomoniella chlamydospora*, *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus carbonarius* and *Aspergillus ochraceus.* In addition to understanding the mechanisms at the base of the yeast antifungal activities, the strains VOCs profiles were also investigated.

3.2. Materials and Methods

3.2.1 Epiphytic Yeast Strain Isolation and Taxonomic Classification

Epiphytic yeast strains were isolated from grape berries, collected in three different Malaysian regions (Perlis, Perak and Pahang) before harvest in March 2020.

Yeasts were collected by washing grape samples using NaCl saline solution (0.9% p/v). The resulting supernatants were serially diluted (1:10) in the same saline solution and plated on Malt extract Agar (Sigma-Aldrich, Milan, Italy), supplemented with 200 mg/L of chloramphenicol (Merck, Darmstadt, Germany).

Plates were incubated at 25 °C for 48 h, and the selection of colonies with different morphologies was randomly completed. To obtain pure isolates, single colonies were streaked

on MA plates. Purification was repeated at least three times or until all the colonies on the streaked isolate had the same morphology. Isolates were stored at −80 °C in YPD broth (yeast extract 10 g/L, bacteriological peptone 20 g/L and dextrose 20 g/L) added with 25% glycerol. Before each trial, the isolated yeast strains were cultured 2 times in YPD broth and aerobically incubated for 24 h at 25 °C (PH: 5.5).

Extraction of total DNA was conducted by a QIAquick® Genomic Extraction Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Determination of the DNA purity and yields were done by NanoDrop ND1000 UV–Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). For all the samples, the yields were approximately 130 ng/µL, and only samples with a ratio of $260_{nm}/280_{nm}$ between 1.9 and 2.1 were used for the polymerase chain reaction.

The total DNA extracted was then used to amplify the internal transcribed spacer region (ITS) that comprises the highly conserved genomic region of ribosomal 5.8S, among two variable zones ITS1 and ITS2. Amplification was carried out by polymerase chain reaction (PCR) using ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′- TCC TCC GCT TAT TGA TAT GC -3′) with primers as described by [22]. Each 25 µL PCR reaction mixture contained 2.5 µL of 10X reaction buffer, 0.75 mM $MgCl_2$ 0.5 mM of each primer, 0.2 mM deoxynucleotides triphosphates (dNTPs), 0.2 U/µL Amplibiotherm Taq DNA Polymerase and 1 µL of total genomic DNA. Primers were purchased from MWG Biotech (, Ebersberg, Germany), while all the PCR reagents were from AURA Biotechnologies Pvt Ltd., Chennai, India. The PCR conditions were as follows: 95 °C for 5 min (initial denaturation) followed by 35 cycles of 95 °C for 1 min (denaturing), 55.5 °C for 2 min (primers annealing), 72 °C for 2 min (elongation). After that, a post-elongation step was performed at 72 °C for 5 min. Amplicons were purified with a QIAquick PCR Purification kit (Qiagen) according to manufacturer specifications and sent to sequencing services at Beijing Genomics Institute (BGI, Shenzhen, China). Obtained sequences were edited with MEGA6 software v2013, and comparisons were made with already published sequences available at GenBank database in NCBI as a reference sequence (National Centre of Biotechnology Information, http://www.ncbi.nlm.nih.gov/ July 2020) using BLAST (Basic Local Alignment Search Tool). The consistent homologous sequences were aligned by the CLUSTALX 1.8 (Madeira et al., 2019). Multiple sequence alignments of nt sequences were used for the construction of phylogenetic trees using the neighbour-joining method (Kumar et al., 2016), p-distance method (Filatov, 2009) and bootstrap consisting of 1000 pseudo-replicates and finally evaluated using the interior branch test method with MEGA v.6.06 (Steel et al., 2013) software.

3.2.2 Pathogen Mould Strains and Growth Conditions

Grapevine pathogen moulds used in this experimentation were *Botrytis cinerea*, P*haeomoniella chlamydospora*, *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus carbonarius* and *Aspergillus ocharceus*. All the moulds tested were provided by the Department of Science IIUM, Kuantan, Malaysia. Before the experiments, to obtain sporulating colonies, they were cultured for two weeks on Malt extract agar (Oxoid, Thermofisher, Milan, Italy) at 25 °C. From each plate, after incubation, spores were collected using NaCl 0.9% saline solution (5 mL). To remove the mycelial mass, conidia suspensions were filtered on 0.45 µm cutoff diameter filtering membranes, and conidia suspension concentrations were adjusted to give approximately 10^6 spores/mL. Spore suspensions were stored at 4 °C until used for antifungal assays.

3.2.3 Antifungal In-Vitro Assays

The antifungal activity of grapevine yeast isolates against *Botrytis cinerea, Phaeomoniella chlamydospora*, *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus carbonarius* and *Aspergillus ocharceus* were evaluated in-vitro by the agar-well-diffusion method, as described by (Alfonzo et al., 2012), with some modifications. Briefly, for each mould, 1 mL of conidial suspension was transferred into an empty petri dish and then covered with 14 mL of sterile malt extract agar cooled at 40 °C. Plates were gently shaken in order to diffuse conidia inoculants, and when the media was solidified, in each plate, an inoculation well (approx. 6 mm ø) was aseptically punched with a tip. Each well was inoculated with 50 µL of the yeast isolate cell-suspension cultured as previously described. Plates were incubated at 25 °C for 7 days. At the end of incubation, yeast antifungal activities were expressed as millimeters of inhibition ring (mm IR). The inhibition ring was measured, using a caliper, from the edge of the inoculation well to the innermost mould growth perimeter, as shown in Figure 13. Antifungal activities were tested in triplicates using plates inoculated with NaCl saline solution (0.9% p/v) as a negative control.

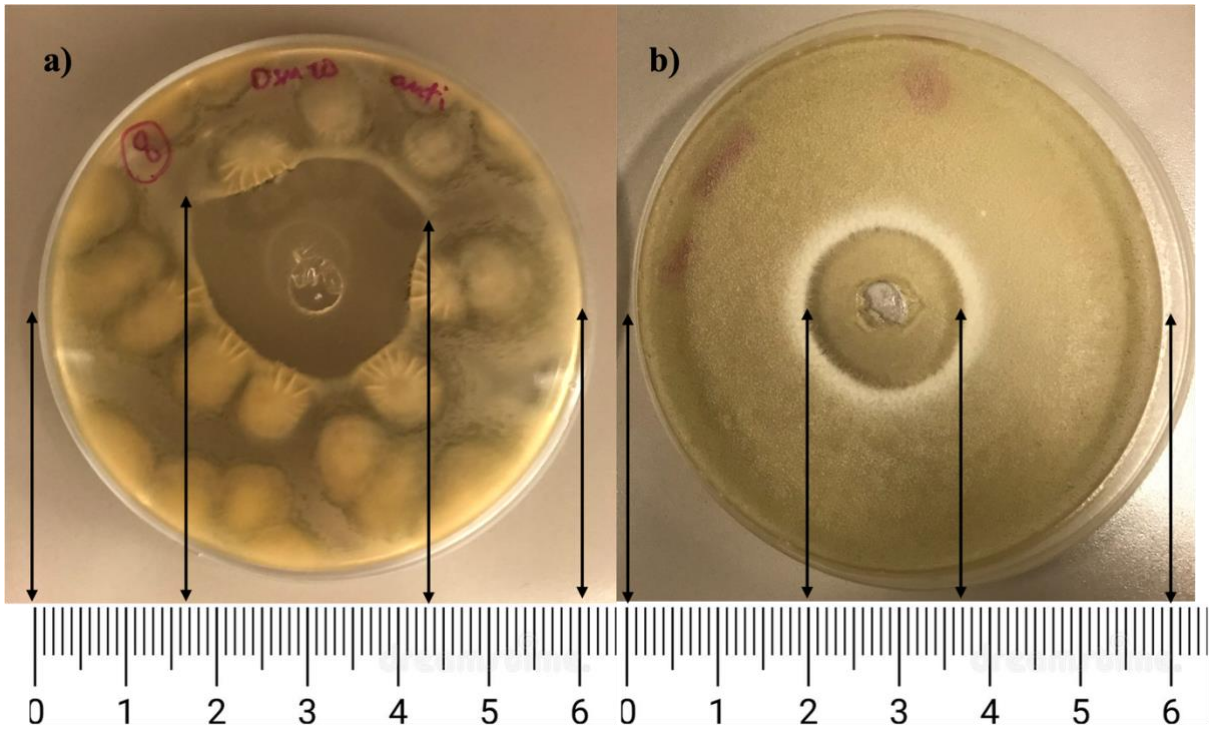

Figure 13. Schematic view of the measurement of inhibition of *B. cinerea* mycelial growth. Three different inhibition rings (mm IR) were measured for each plate considered, as illustrated with black double arrows. (**a**) *Starmerella bacillaris* FE08.05, (**b**) *Metschnikowia pulcherrima* GP8.

3.2.4 Yeast Volatile Organic Compound (VOC) Profiles

The yeast VOCs compositions were qualitatively and quantitatively evaluated with head space solid-phase microextraction using a gas chromatograph coupled with a mass spectrometer detector (GCMS-SPME). Analyses were performed after 6 days of growth at 25 °C in liquid media (malt extract broth) of *M. pulcherrima* GP8, *S. bacillaris* FE08.05, *H. opuntiae* GA22, *H. pseudoguilliermondii* GP14, *H. lanchancei* GM32, *H. guilliermondii* GA1, *H. uvarum* GM19, *H. opuntiae* GM10 and *C. awuaii GM3*. A CAR/PDMS 75µm fiber (SUPELCO, Bellafonte, PA, USA) was used to perform the solid-phase microextraction (SPME). The samples (5 mL) were placed in vials and incubated for 10 min at 45 °C. Then, the fiber was exposed to the headspace of the vial for 30 min at 45 °C. The volatile molecules adsorbed were desorbed in the gas chromatograph (GC) injector port in splitless mode at 250 °C for 10 min. The headspace of the VOCs was analyzed using chromatography (GC) 6890 N, Network GC System with mass spectrometry (MS) 5970 MSD (Aglient technologies, Milan, Italy). The column used was J&W CP-Wax 52 (50 m × 320 µm × 1.2 µm) (Aglient technologies, Milan, Italy) (Figure 14). The initial temperature was 40 °C for 1 min and then increased by 4.5 °C/min up to 65 °C. After that, the temperature increased by 10 °C/min up to 230 °C and remained at this temperature for 17 min. The gas-carrier was helium at 1.0 mL/min flow. Compounds were

identified by comparison based on the NIST 11 (National Institute of Standards and Technology) database, while the quantitative analysis was performed with the internal standard method using 4-methyl-2-pentanol (6 mg/L) and expressed as equivalent ppm (ppm eq.). For each compound detected, the ppm eq. represents the amount of compound present in the headspace in dynamic equilibrium with the aqueous phase. The chemical analyses were performed in triplicate and are expressed as means (Figure 14).

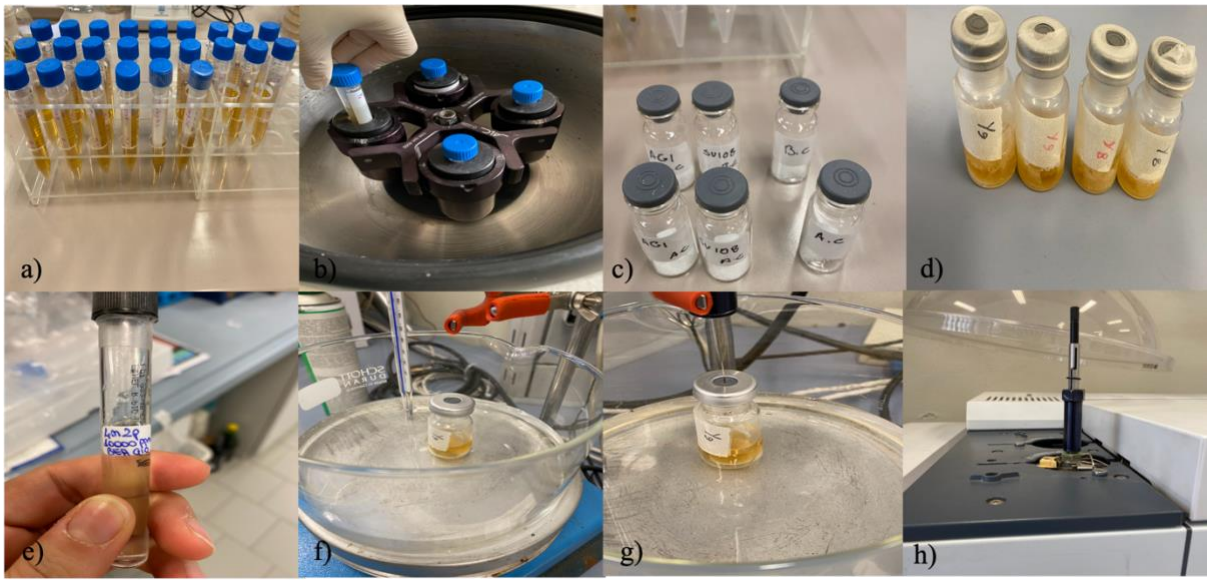

Figure 14. Evaluation of VOCs with head space solid-phase microextraction using a gas chromatograph coupled with a mass spectrometer detector (GCMS-SPME). a) Yeast isolates, b) collecting supernatant by using centrifuge 30 minutes, 4000 rpm, c) Preparing vials by adding NACL, d) Adding the supernatants into the vials, e) adding 4-methyl-2-pentanol (6 mg/L) as internal standard, f) incubation for 10 min at 45 °C, g) the fiber was exposed to the headspace of the vial for 30 min at 45 °C, and h) J&W CP-Wax 52 injected column.

3.2.5 Detached Berry Antifungal Assay

Yeast isolates were tested for antagonistic activity against *A. carbonarius* in a detached berry test as described previously by (Pantelides et al., 2015), with some modifications. Briefly, the selected yeast strains were grown in liquid culture of Malt extract broth (Oxoid, Thermofisher, Milan, Italy) without stirring for 48 h at 25 °C. Mature grape berries of the Red Globe variety detached from bunches were sanitized on the surface using 1% commercial sodium hypochlorite for 15 min and rinsed with sterile deionized water, and dipped inside yeast 48 h cultures. After 4 h of incubation at 25 °C, berries were air-dried, and a wound (about 2 mm diameter) was made on each berry with a sterile needle. The wound was spot-inoculated with 20 µL of *A. carbonarious* conidial suspension (approx. 10^6 conidia/mL). Berries were incubated at 25 °C for 10 days. The inhibition expressed as *A. carbonarius* diameter of growth (ø. mm) was monitored daily using a caliper.

3.2.6 Statistical Analysis

Data were processed using the SPSS software tool (Version 26). Yeast antifungal in vitro properties against the selected phytopathogens moulds, as well as mycelial growth inhibition on detached berries, were considered statistically different ($p < 0.05$) based on ANOVA and TUKEY HSD post-hoc tests.

To obtain a visual overview of the VOC of the selected yeast isolates, principal component analysis (PCA) was used. Quantitative data of VOC profiles were analyzed using ANOVA followed by DUNCAN's tests ($p < 0.05$).

3.3 Results

3.3.1 Molecular Analysis of Yeast Isolates

A total of 41 yeasts were isolated from grape samples obtained from Italian and three Malaysian sampling regions Perlis, Perak and Pahang. Isolated yeasts were identified according to the nucleotide sequences of the ITS region. Among them 20 isolates was selected. As shown in Figure 15 and Table 2, the identification based on the ITS region sequences revealed a dominant non-*Saccharomyces* indigenous population. Specifically, isolated yeast belonged to eight different genera, including *Hanseniaspora*, *Starmerella*, *Metschnikowia*, *Pichia*, *Candida*. All the sampling regions were characterized by the presence of *Metschnikowia pulcherrima* and *Starmerella bacillaris* and a strain belonging to the *Hanseniaspora* genus.

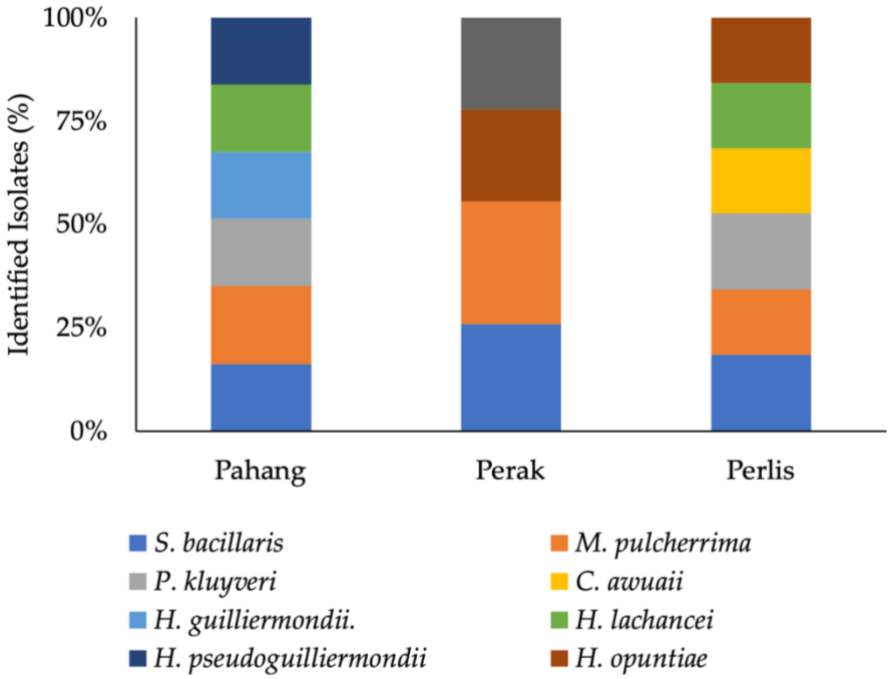

Figure 15. Occurrence of isolated yeast in three main regions of sampling (Pahang, Perak and Perlis). Vertical axis shows the occurrence percentage.

Within the *Hanseniaspora* genus, the species isolated were *H. lachancei*, *H. opuntiae*, *H. guilliermondii*, *H. pseudoguilliermondii* and *H. uvarum*. Yeast isolated from the Pahang and Perlis regions showed a higher variability compared to the ones isolated from Perak. In addition to the species already mentioned, grape berries from the Pahang and Perlis regions were characterized by the presence of *Pichia kluyveri* and *Candida awuaii* strains.

Table 2. Isolated yeast from grape berries from different sampling regions

Yeast isolates	Strain	Source	Country/region	Medium
Metschnikowia fructicola	3WLS(8)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Metschnikowia pulchelima	4UVAWL(9)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Metschnikowia pulchelima	1UVALM(10)	Grapevine berries	Italy/Palermo	MEA
Metschnikowia pulchelima	2LH2(7)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Metschnikowia pulchelima	2GLUYMA1	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Pichia membranifaciens	SIDRO1(3)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Candida sake	42B32(4)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Meyerozyma caribbica	DPRE 4(5)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Hanseniaspora uvarum	AUFORA1(2)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Quambalaria fabacearum	(22c)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Pichia kluyveri	(2)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
[Candida] awuaii	(3)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Pichia eremophila	(5)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Zygoascus hellenicus	(121)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Starmerella stellata	(621)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Hanseniaspora pseudoguilliermondii	(21a)	Grapevine berries	Italy/Cesena (DISTAL)	MEA
Hanseniaspora opuntiae	(21b)	Grapevine berries	Italy/Faenza	MEA
Hanseniaspora lachancei	(21c)	Grapevine berries	Italy/Faenza	MEA
Hanseniaspora uvarum	(22d)	Grapevine berries	Italy/Faenza	MEA
Metschnikowia pulcherrima	(611y)	Grapevine berries	Italy/Faenza	MEA
Starmerella bacillaris	F08.06.MY	Grapevine berries	Malaysia/ Pahang	MEA
Starmerella bacillaris	FH08.08.MY	Grapevine berries	Malaysia/ Pahang	MEA
Starmerella bacillaris	FA09.01.MY	Grapevine berries	Malaysia/ Pahang	MEA
Starmerella bacillaris	FE08.05.MY	Grapevine berries	Malaysia/ Pahang	MEA
Starmerella bascillaris	GP17.MY	Grapevine berries	Malaysia/ Pahang	MEA
Metschnikowia pulcherrima	H12.08.MY	Grapevine berries	Malaysia/ Pahang	MEA
Metschnikowia pulcherrima	A05.01.MY	Grapevine berries	Malaysia	MEA
Metschnikowia pulcherrima	B05.02.MY	Grapevine berries	Malaysia	MEA
Metschnikowia pulcherrima	F12.06.MY	Grapevine berries	Malaysia	MEA
Metschnikowia pulcherrima	G12.07.MY	Grapevine berries	Malaysia	MEA
Metschnikowia pulcherrima	GP8.MY	Grapevine berries	Malaysia	MEA
Pichia kluyveri	GP5.MY	Grapevine berries	Malaysia	MEA
Pichia kluyveri	GM7.MY	Grapevine berries	Malaysia	MEA
Pichi kluyveri	F1-B263-2B	Grapevine berries	Malaysia	MEA
Candida awuaii	GM3.MY	Grapevine berries	Malaysia	MEA
Hanseniaspora guilliermondii.	GA1.MY	Grapevine berries	Malaysia	MEA
Zygoascus hellenicus	GP11.MY	Grapevine berries	Malaysia	MEA

Hanseniaspora pseudoguilliermondii	GP14.MY	Grapevine berries	Malaysia	MEA
Hanseniaspora opuntiae	GM10.MY	Grapevine berries	Malaysia	MEA
Hanseniaspora opuntiae	GA22.MY	Grapevine berries	Malaysia	MEA
Hanseniaspora uvarum	GM19.MY	Grapevine berries	Malaysia	MEA

Twenty isolates obtained in this study grouped with representative type strains of known yeast species in the phylogenetic tree with high nucleotide similarity (Table 3), including previously described yeast species (Figure 16). The phylogenetic tree based on the complete nucleotide sequence of the ITS region generated two different groups, while the identified isolates scattered in both groups 1 and 2, close to isolates of different distances, indicating the variation and long-distance migration in Malaysian isolates and other countries (Figure 16).

Table 3. Identification of yeasts with potential for biological control. Values from pairwise sequence comparisons based on the highest sequence identity found in BLAST analysis. E-value (expectation value) represents the number of expected hits of similar quality (score) that could be found by chance.

Isolate	Species Designation	Accession Number/Country/Region	Identity (%)	E-Value
F08.06	*Starmerella bacillaris*	OK329946/Malaysia/Perak	99.28	0.00
FH08.08	*Starmerella bacillaris*	OK329947/Malaysia/Pahang	99.28	0.00
FA09.01	*Starmerella bacillaris*	OK329948/ Malaysia/Perak	98.8	0.00
FE08.05	*Starmerella bacillaris*	OK329949/Malaysia/Pahang	99.04	0.00
GP17	*Starmerella bascillaris*	OK329950/Malaysia/Perlis	99.75	0.00
PSWCC_137	*Starmerella bascillaris*	MW301555/ USA	Ref-isolate	-
H12.08	*Metschnikowia pulcherrima*	OK329951/Malaysia/Perak	97.93	2×10^{-162}
A05.01	*Metschnikowia pulcherrima*	OK329952/Malaysia/Perak	97.93	2×10^{-162}
B05.02	*Metschnikowia pulcherrima*	OK329953/Malaysia/Perak	97.12	2×10^{-162}
F12.06	*Metschnikowia pulcherrima*	OK329954/Malaysia/Pahang	97.12	2×10^{-162}
G12.07	*Metschnikowia pulcherrima*	OK329955/Malaysia/Perlis	97.93	2×10^{-162}
GP8	*Metschnikowia pulcherrima*	OK560819/Malaysia/Perlis	96.43	0.00
E20671	*Metschnikowia pulcherrima*	MK267584/USA	Ref-isolate	-
GM3	*Candida awuaii*	OK329958/Malaysia/Pahang	87.27	1×10^{-94}
CBS.11011	*Candida awuaii*	NR_151796/USA	Ref-isolate	-
GA1	*Hanseniaspora guilliermondii.*	OK329959/Malaysia/Perlis	99.57	0.00
CBS:6619	*Hanseniaspora guilliermondii.*	KY103526/Netherland	Ref-isolate	-
GM10	*Hanseniaspora opuntiae*	OK329962/Malaysia/Perak	100	0.00
GA22	*Hanseniaspora opuntiae*	OK329963/Malaysia/Pahang	99.85	0.00
EB2016-98	*Hanseniaspora opuntiae*	MN378465/USA	Ref-isolate	-
GP14	*Hanseniaspora pseudoguilliermondii*	OK329961/Malaysia/Perlis	99.41	0.00
CBS.8772	*Hanseniaspora pseudoguilliermondii*	NR_155181/ USA	Ref-isolate	-
GM19	*Hanseniaspora uvarum*	OK329964/Malaysia/Pahang	100	0.00
B-WHX	*Hanseniaspora uvarum*	KC544511/China	Ref-isolate	-
GP5	*Pichia kluyveri*	OK329956/Malaysia/Perlis	98.46	0.00
GM7	*Pichia kluyveri*	OK329957/Malaysia/Pahang	99.22	0.00
F1-B263-2B	*Pichi kluyveri*	MK329984/China	Ref-isolate	-
GP11	*Zygoascus hellenicus*	OK329960/Malaysia/Perlis	99.81	0.00
1KUT24	*Zygoascus hellenicus*	HE965021/Italy	Ref-isolate	-

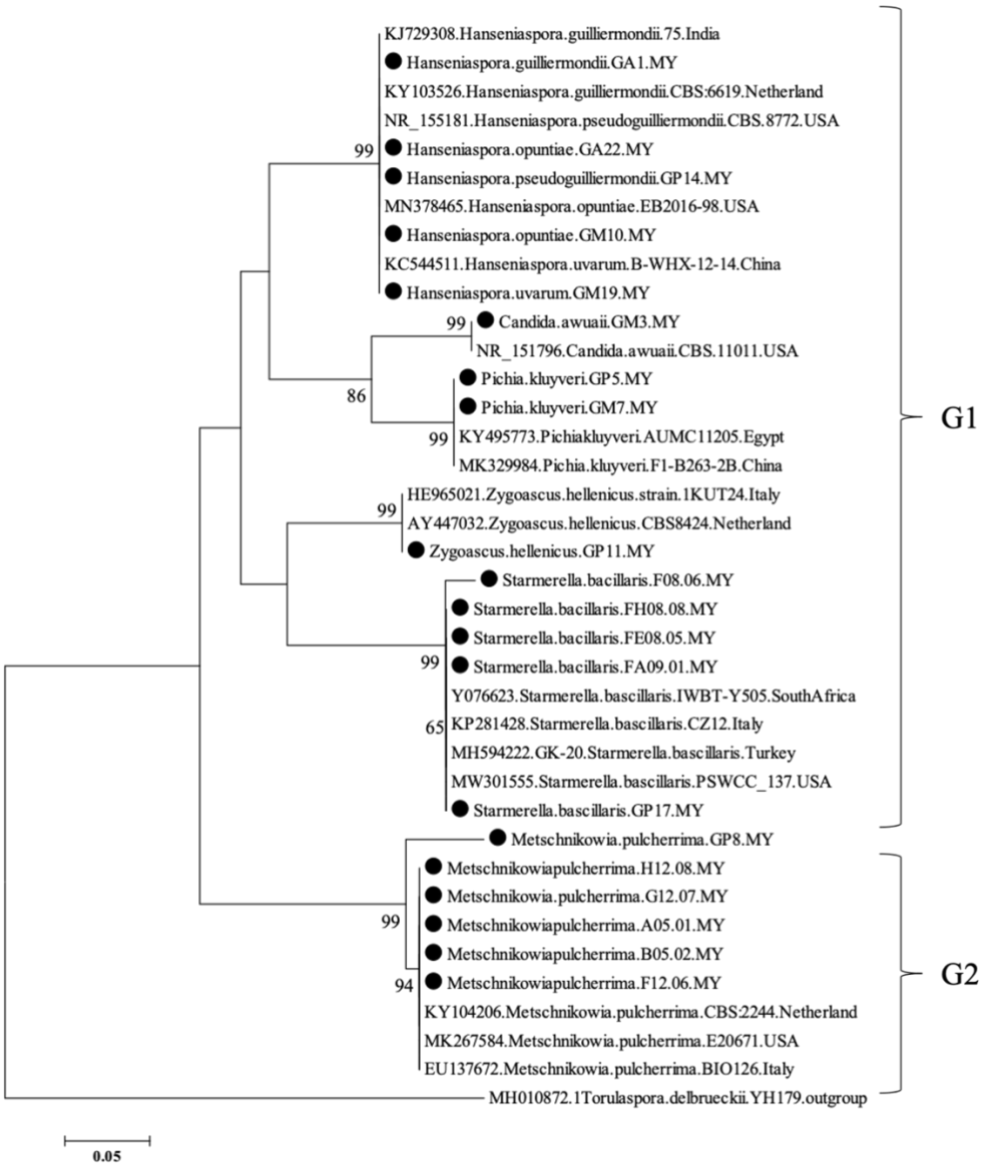

Figure 16. Phylogenetic tree constructed on the sequence alignment of ITS1 and ITS4 regions representing isolated yeasts of grapevine berry and their homologue-related species. Black circles represented the isolated yeast in this study. The phylogenetic tree is inferred from the "Neighbour joining method" and numbers on branches are derived from bootstrap resembled datasets, indicated as percentage of support from 1000 bootstrap replications. Branch lengths represent bootstrap values. Nodes with less than 70% bootstrap support were collapsed. *Torulaspora delbrueckii* was used as an outgroup species to root the tree. The bar represents 0.05 changes per site.

3.3.2 In-Vitro Antifungal Assays

Twenty yeast isolates were tested for their potential in vitro antifungal activity. First, a preliminary screening was performed against the phytopathogen *B. cinerea* (Figure 17) and only strains characterized by an inhibitory activity were also tested against other selected pathogens (*A. carbonarius*, *A. ochraceus*, *F. oxysporum*, *A. alternata* and *P. chlamydospora)* (Figure 18). Among 20 yeast strains tested, all *Pichia kluyveri* strains had no antagonistic activity against the selected moulds (data not shown), and only nine isolates had the ability to reduce *B. cinerea* mycelial growth (Table 4).

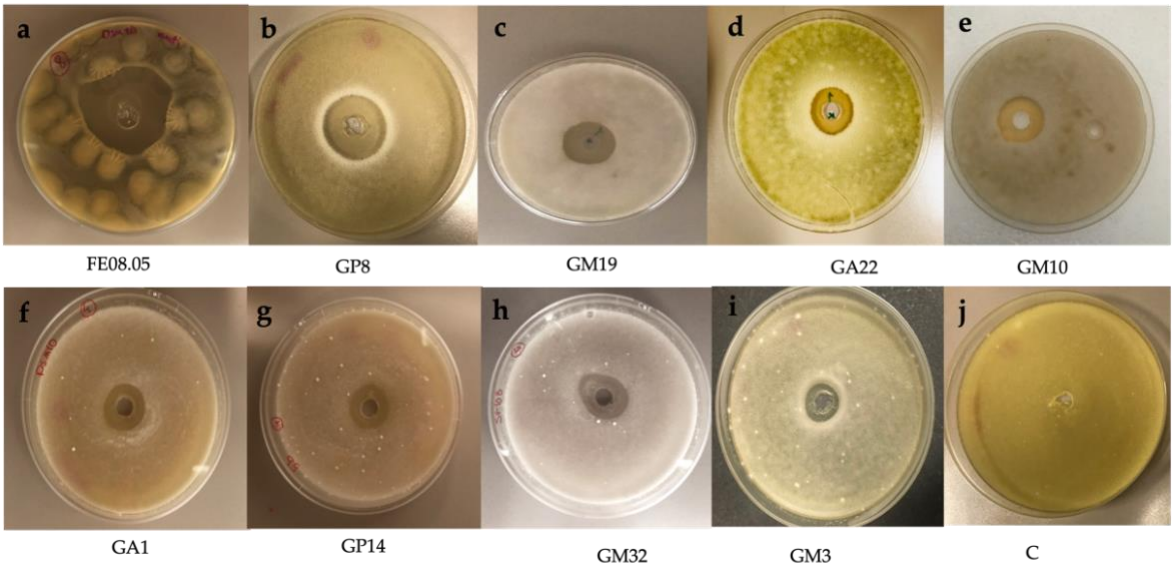

Figure 17. Mycelial growth inhibition of 9 different isolated yeast on *Botrytis cinerea*. (**a**) *Starmerella bacillaris* FE08.05, (**b**) *Metschnikowia pulcherrima* GP8, (**c**) *Hanseniaspora uvarum* GM19, (**d**) *Hanseniaspora opuntiae* GA22, (**e**) *Hanseniaspora opuntiae* GM10, (**f**) *Hanseniaspora guilliermondii* GA1, (**g**) *Hanseniaspora pseudoguilliermondii* GP14, (**h**) *Hanseniaspora lanchancei* GM32, (**i**) *Candida awuaii* GM3. (**j**) Negative control (C) filled with NaCl saline solution (0.9% p/v).

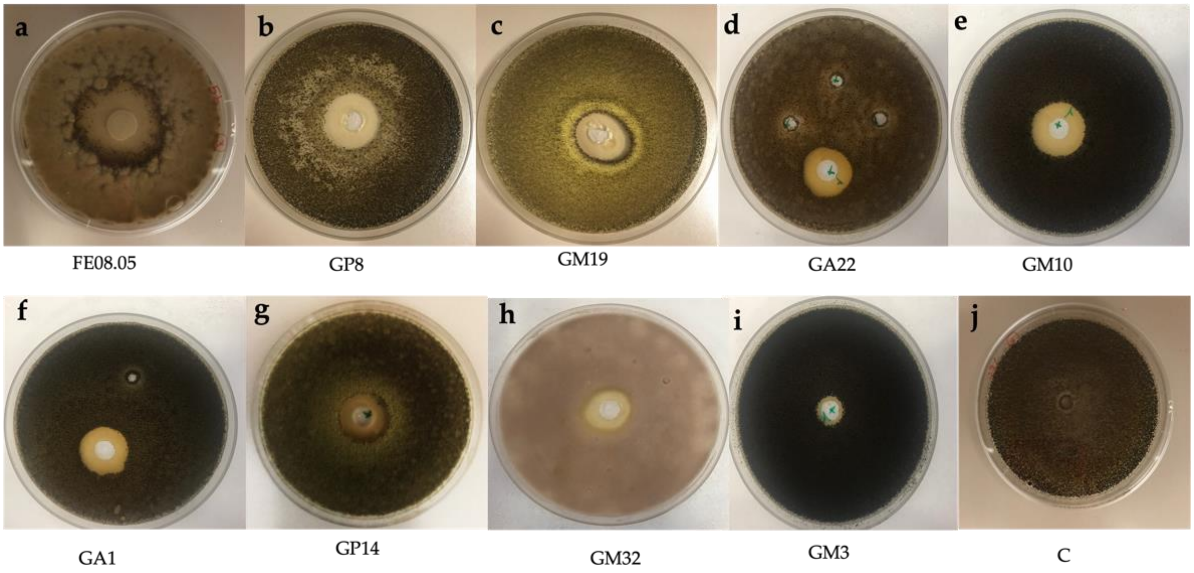

Figure 18. Mycelial growth inhibition of 9 different isolated yeast on *Aspergillus carbonarius*. (**a**) *Starmerella bacillaris* FE08.05, (**b**) *Metschnikowia pulcherrima* GP8, (**c**) *Hanseniaspora uvarum* GM19, (**d**) *Hanseniaspora opuntiae* GA22, (**e**) *Hanseniaspora opuntiae* GM10, (**f**) *Hanseniaspora guilliermondii* GA1, (**g**) *Hanseniaspora pseudoguilliermondii* GP14, (**h**) *Hanseniaspora lanchancei* GM32, (**i**) *Candida awuaii* GM3. (**j**) Negative control (C) filled with NaCl saline solution (0.9% p/v).

Table 4. Yeast strains isolated from grape berries and characterized by in vitro antifungal activity against *Botrytis cinerea.*

Isolate	Designated Species	*B. cinerea* Antifungal Activity
FE08.05	*Starmerella bacillaris*	+
GP8	*Metschnikowia pulcherrima*	+
GM19	*Hanseniaspora uvarum*	+
GA22	*Hanseniaspora opuntiae*	+
GM10	*Hanseniaspora opuntiae*	+
GA1	*Hanseniaspora guilliermondii*	+
GP14	*Hanseniaspora pseudoguilliermondii*	+
GM32	*Hanseniaspora lanchancei*	+
GM3	*Candida awuaii*	+

Yeast strains with anti-mycelial activities were *S. bacillaris* FE08.05, *M. pulcherrima* GP8, *H. uvarum* GM19, *H. opuntiae* GA22, *H. opuntiae* GM10, *H. guilliermondii* GA1, *H. lachancei* GM32, *H. pseudoguilliermondii* GP14 and *C. awuaii* GM3 (Table 3). *S. bacillaris* FE08.05 and *M. pulcherrima* GP8 showed the highest inhibitory effects against all the pathogens tested, except in the case of *P. chlamydospora*, which was not inhibited from any yeast tested (Table 3). The inhibitory effects of these strains were similar, and the yeast significantly ($p < 0.05$) affected the growth of *B. cinerea* (Figure 17) with inhibition rings of 15.1 mm, 12.4 mm and 10.8 mm, respectively.

S. bacillaris FE08.05 and *M. pulcherrima* GP8 also strongly inhibited the growth of *A. carbonarius* (14.2 and 10.2 mm IR) (Figure 18) and *A. ochraceus* (12.2 and 8.2 mm IR), while only *S. bacillaris* FE08.05 strongly reduced the growth of *A. alternata* (15.8 mm IR) and *F. oxysporum* (10.5 mm IR) (Table 5).

Table 5. In vitro inhibition of mycelial growth (diameter of inhibition mm) of *Botrytis cinerea*, *Aspergillus carbonarious*, *Aspergillus ochraceus*, *Alternaria alternata*, *Fusarium oxysporum*, *Phaeomoniella chlamydospora* by *Starmerella bacillaris* (FE08.05), *Metschnikowia pulcherrima* GP8, *Hanseniaspora, uvarum* GM19, *Hanseniaspora*, *opuntiae* GA22, *Hanseniaspora opuntiae* GM10, *Hanseniaspora guilliermondii* GA1, *Hanseniaspora lachancei* GM32, *Hansenaspora pseudoguilliermondii* GP14 (*H. peseudoguller* GP14) and *Candida awuaii* GM3. Results are the mean of three replicates. For each pathogen considered, different letters indicate significantly ($p < 0.05$) different inhibition on the basis of ANOVA and TUKEY HSD tests.

	Inhibition Ring (mm)								
	***S. bacillaris* FE08.05**	***M. pulcherrima* GP8**	***H. uvarum* GM19**	***H. opuntiae* GA22**	***H. opuntiae* GM10**	***H. guilliermondii* GA1**	***H. lachancei* GM32**	***H. pseudoguillier.* GP14**	***C. awuaii* GM3**
B. cinerea	$15.1^{a} \pm 0.4$	$12.4^{b} \pm 0.5$	$10.8^{c} \pm 0.3$	$8.1^{d} \pm 0.3$	$5.8^{e} \pm 0.3$	$3.0^{g} \pm 0.2$	$2.0^{h} \pm 0.2$	$4.4^{g} \pm 0.5$	$6.3^{e} \pm 0.3$
A. carbonarius	$14.2^{a} \pm 0.3$	$10.2^{b} \pm 0.3$	$8.2^{c} \pm 0.3$	$6.2^{d} \pm 0.3$	$3.2^{e} \pm 0.3$	$2.2^{f} \pm 0.3$	< 1 *	$2.1^{f} \pm 0.1$	$1.2^{g} \pm 0.3$
A. ochraceus	$12.2^{a} \pm 0.3$	$8.2^{b} \pm 0.3$	$3.2^{e} \pm 0.3$	$5.9^{c} \pm 0.1$	$3.2^{d} \pm 0.3$	$2.2^{f} \pm 0.3$	< 1	$2.1^{f} \pm 0.1$	$1.2^{g} \pm 0.3$
A. alternata	$15.8^{a} \pm 0.2$	$6.3^{b} \pm 0.5$	$8.2^{c} \pm 0.3$	$5.2^{d} \pm 0.3$	$2.1^{e} \pm 0.1$	$1.1^{f} \pm 0.1$	$1.2^{f} \pm 0.3$	$5.2^{d} \pm 0.3$	$2.2^{e} \pm 0.3$
F. oxysporum	$10.5^{a} \pm 0.5$	$5.4^{a} \pm 0.4$	$4.3^{c} \pm 0.2$	$1.2^{d} \pm 0.3$	< 1	< 1	< 1	< 1	< 1
P. chlamydospora	< 1	< 1	< 1	< 1	$1.2^{a} \pm 0.3$	< 1	< 1	< 1	< 1

* Under the detection limit (inhibition ring < 1mm). The letters (a-g) shows mean comparison analysis and same letters means no statistically significant difference between the yeast inhibition zone.

Among the *Hanseniaspora* species assessed, *H. uvarum* isolate GM19 showed a similar inhibition pattern to *S. bacillaris* FE08.05 and was more active against *B. cinerea* and *A. carbonarius* (Figures 17 and 18). The inhibitory ring against the moulds ranged between 6.2 and 10.8 mm. On the other hand, *H. opuntiae* GM10, *H. opuntiae* GA22, H. *guilliermondii GA1*, *H. lanchancei* GM32 and *H. pseudoguilliermondii* GP14 were less effective in inhibiting the mycelial growth of the selected pathogens. *C. awuaii* GM3 did not show a strong inhibitory effect against the selected phytopathogens (Table 5).

3.3.3 Volatile Organic Compound Profiles

In Figure 19 we show the principal component analysis (PCA) loading plots of yeast VOCs produced after 6 days of growth on malt extract broth. The principal component analysis allowed the discrimination of yeast in relation to their VOCs produced during the growth in relation to their species and genus (Figure 19).

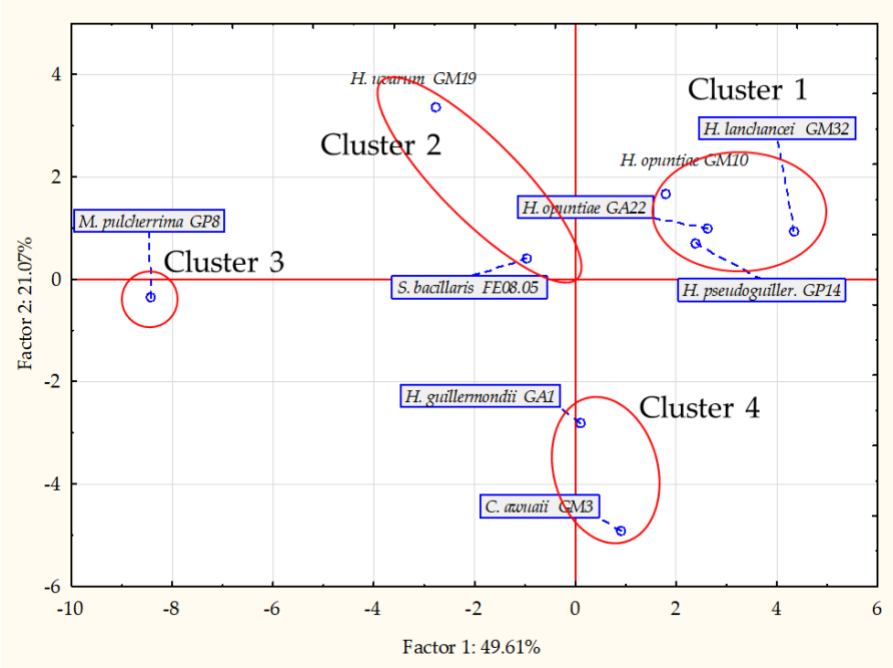

Figure 19. Principal component analysis loading plot of VOCs of the selected yeast strains with antifungal properties after 6−days of growth in malt extract broth at 25 °C.

Samples were mapped in the space spanned by the first two principal components, PC1 and PC2. The analysis allowed us to explain over 70% of the total variability observed (Figure 19). PC1 accounted for 49.61% of the total variability, and PC2 for 21.07%. Except for *H. guilliermondii* GA1 and *H. uvarum* GM19, *Hanseniaspora* genus strains grouped alongside the variable plane defined by PC1 and PC2 (Cluster 1). *M. pulcherrima* GP8 was clearly separated, along the PC1, from the other species (Cluster 3), while *C. awuaii* GM3 and *H.*

guilliermondii GA1 formed a cluster separated from the other species along the PC2. *S. bacillaris* FE08.05 separated along the PC1 from *M. pulcherrima* GP8 clustered next to *H. uvarum* GM19 (Cluster 2).

VOC profiles were mainly characterized by alcohols, organic acids and esters (Table 4). After 6 days of growth, *M. pulcherrima* GP8 and *S. bacillaris* FE08.05 produced the highest level of isoamyl (8.69 and 8.99 ppm) and phenylethyl alcohol (10.91 and 3.16 ppm) (Table 4). These strains also produced moderate amounts of other VOCs, including low-molecular-weight organic acids and esters ranging from 0.13 to 1.80 ppm (Table 4). Among *Hanseniaspora* strains, the production of VOCs belonging to *H. uvarum* GM19 was notable. This strain produced comparable amounts of isoamyl to *S. bacillaris* FE08.05 (8.07 ppm) and phenylethyl (2.51 ppm) alcohols. *C. awuaii* GM3 and *H. guilliermondii* GA1 showed an overall reduced production of VOCs (Table 4).

Table 6. The VOCs produced by 6-day-old cultures of the selected strains with antifungal properties. Data are the mean of three replicates. Standard deviation observed ranged between 5 and 7%. Results are the mean of three replicates. For each compound considered, different letters indicate significantly ($p < 0.05$) different amounts based on DUNCAN's tests

					ppm eq.						
	Alcohols				**Organic acids**					**Esters**	
Yeast Isolate	**Isobutyl Alcohol**	**Isoamyl Alcohol**	**Phenylethyl Alcohol**	**Isoprenyl Alcohol**	**Acetic Acid**	**Isovaleric Acid**	**n-Caprylic Acid**	**Pelargonic Acid**	**n-Capric Acid**	**Ethyl Propionate**	**Lauric Acid, Ethyl Ester**
M. pulcherrima GP8	0.66 [a]	8.69 [a]	10.91 [a]	1.04 [a]	0.49 [a]	1.30 [a]	0.89 [a]	0.34 [a]	0.77 [a]	-	1.80 [a]
S. bacillaris FE08.05	- *	8.99 [a]	3.16 [b]	0.95 [a]	0.22 [b]	0.17 [b]	0.76 [a]	0.19 [b]	0.88 [a]	0.18	0.26 [b]
H. opuntiae GA22	0.28 [b]	2.17 [b]	0.17 [c]	0.25 [b]	-	-	-	-	-	-	-
H. opuntiae GM10	0.16 [b]	3.33 [c]	1.85 [d]	0.21 [b]	-	-	-	-	0.30 [b]	-	-
H. uvarum GM19	1.60 [c]	8.07 [a]	2.51 [b]	0.58 [c]	0.23 [b]	0.49 [c]	0.23 [b]	0.50 [c]	-	-	0.86 [c]
H. lanchancei GM32	-	-	-	-	-	-	-	-	-	-	-
H. pseudoguiller. GP14	0.30 [a]	2.50 [b]	0.14 [c]	0.38 [b]	-	-	-	-	-	-	-
H. guilliermondii GA1	-	0.35 [d]		0.59 [c]	0.25 [b]	0.26 b	0.18 [b]	0.45 [c]	-	-	0.11 [b]
C. awuaii GM3	-	0.15 [d]	-	0.58 [c]	0.18 [b]	-	-	0.12 [b]	-	-	-

* Under the detection limit (<0.1 ppm eq). The letters (a-c) show mean comparison analysis and same letters means no statistically significant difference between the yeast inhibition zone.

3.3.4 Detached Berry Antifungal Assay

The nine yeast isolates showing in vitro antifungal activity were evaluated for their efficacy to inhibit the growth of *A. carbonarius* on detached berries (Figure 20). Among the tested strains, after 10 days of incubation, *M. pulcherrima* GP8, *S. bacillaris* FE08.05, *H. uvarum* GM19, *H. opuntiae* GA22 and *H. opuntiae* GM10 had a similar and significant ($p < 0.05$) inhibition against *A. carbonarius*.

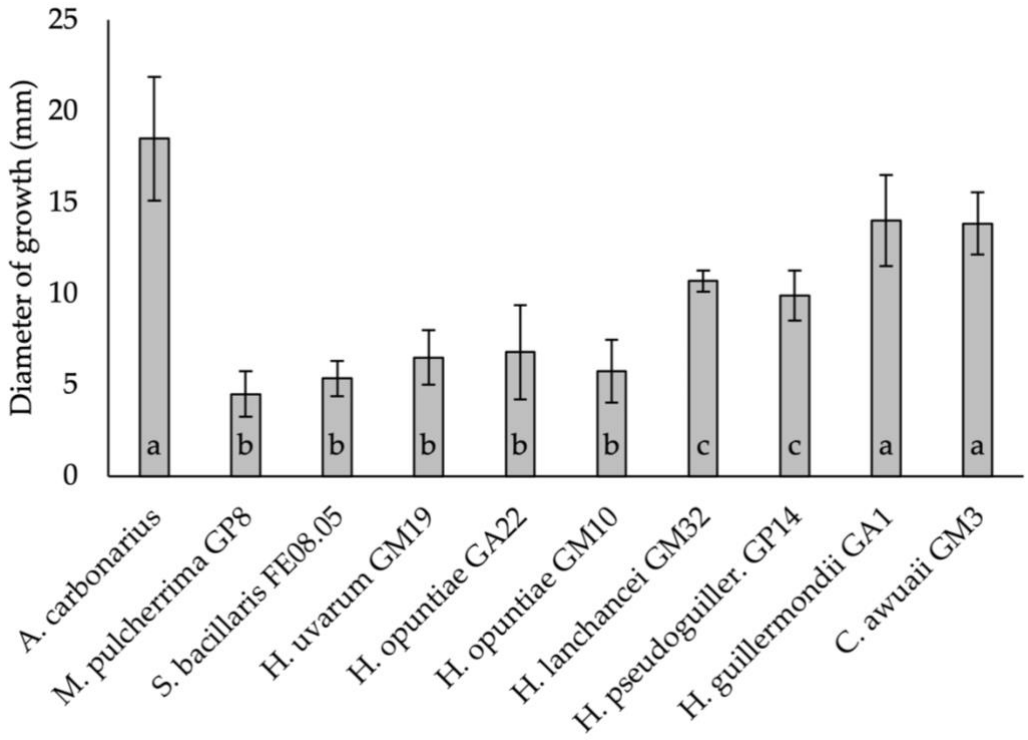

Figure 20. Evaluation of the antifungal activity (growth ø. mm) after 10 days of incubation at 25 °C of the selected yeast strains against *Aspergillus carbonarius* on the detached berry. Results are the mean of 3 replicates. Different letters indicate significantly ($p < 0.05$) different growth of *Aspergillus carbonarius* on the base of ANOVA and TUKEY HSD tests

In the control berry batch, *A. carbonarious* reached 18.5 mm of growth diameter (ø. mm), while in the presence of the yeast strains, the mycelial growth ranged between 4.5 and 6.8 mm. Moderate inhibitory effects, compared to the other stains, were also observed for *H. lachancei* GM32 (10.7 mm) and *H. pseudoguilliermondii* GP14 (9.9 mm), while *H. guilliermondii* GA1 and *C. awuaii* GM3 had no effects on the mycelial growth inhibition (Figure 20).

3.5 Discussion

The main aim of the presented work was to isolate epiphytic yeast from *V. vinifera* grape berries grown in different regions of Malaysia (Perlis, Perak and Pahang) and evaluate their ability to inhibit the mycelial growth of six selected grapevine phytopathogens: *Botrytis* cinerea, *A. carbonarius*, *A. ochraceus*, *F. oxysporum*, *A. alternata* and *P. chlamydospora*. The major component of the microbiota on the surface of plants, fruits and vegetables is represented by epiphytic yeasts (Bleve et al., 2006). Yeasts are evolutionarily adapted to such ecosystems and are able to colonize in many different environmental conditions, plants and grape surfaces or wounds (Bleve et al., 2006).

Many ecological studies have revealed that epiphytic yeasts present on grape berries belong to non-*Saccharomyces* genera, including *Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*,

Zygoascus and *Issatchenkia* (Barata et al., 2012; Vivier & Pretorius, 2000). According to the literature, yeast species isolated from the three different Malaysian regions belongs to the genera: *Hanseniaspora*, *Starmerella*, *Metschnikowia*, *Pichia*, *Candida.*

However, epiphytic yeast populations isolated from the Pahang and Perlis regions were characterized with a high variability compared to the Perak region.

Although yeasts were isolated simultaneously, the different grapevine plants physiological status could influence the indigenous epiphytic yeast population (Barata et al., 2012).

The in-field management of plant pathogens using naturally occurring epiphytic yeasts represent one promising and sustainable strategy to reduce chemicals and pesticides commonly used to achieve these purposes. The results presented in this study suggest that antagonist yeasts with the potential to control *B. cinerea*, *A. carbonarius*, *A. ochraceus*, *F. oxysporum*, *A. alternata* and *P. chlamydospora* on grapes can be found among the microflora associated with the berries. Generally, the selected yeasts have antagonistic activity against the selected pathogen fungi and the ability to inhibit mycelial growth was more frequently observed. The highest effect on inhibiting mycelial growth was shown by *S. bacillaris* FE08.05, which was able to strongly reduce mycelial growth in all tested fungi, while the next significant inhibition belongs to the *M. pulcherrima* GP8 and *H. uvarum* GM19 strains (Table 3).

In addition, these stains were characterized by the highest anti-mycelial growth activity against *A. carbonarius* when inoculated in detached grapevines berries (Figure 19).

S. bacillaris is available in oenological environments regarding its osmotolerant nature and is periodically detected on fruit surfaces, *Drosophila spp.* and soil (Solomon et al., 2019). Several surveys have largely demonstrated that its use, together with selected *Saccharomyces cerevisiae*, in mixed culture fermentations enhanced the analytical composition and aroma profile of wine (Solomon et al., 2019; Morgan et al., 2017). However, few investigations have analyzed the antifungal activity of *S. bacillaris* strains on post-harvest fruits. Some researchers have shown the inhibitory activity of *S. bacillaris* strains against *B. cinerea* on grapes in vineyards, in line with our results (Nadai et al., 2018; Prendes et al., 2018)

S. bacillaris has been introduced as a safe microorganism with the potential ability to be used as a biocontrol agent against different food pathogens (Lemos Junior et al., 2016). Junior et al. [33] reported that there is no pathogenicity factor for human health regarding *S. bacillaris* as a biocontrol agent. *S. bacillaris* FE08.05 also successfully controlled the growth of *A. alternata* (Table 3). The biocontrol of *A. alternata* could be the result of these yeast species colonizing wound sites, which implies competitive mechanisms (Prendes et al., 2018). A similar inhibition was also observed using *M. pulcherrima* GP8 and *H. uvarum* GM19.

Guinebretière et al. (Guinebretière et al., 2000) reported *M. pulcherrima* showing an inhibitory effect against *Botrytis cinerea* in grape and strawberry.

Mycelial growth of *A. alternata* was significantly inhibited by all tested yeasts; again, *S. bacillaris* strain FE08.05, *M. pulcherrima* strain GP8 and *H. uvarum* GM19 were the most effective among others. Stocco et al. (Stocco et al., 2019) indicated that *M. pulcherrim*a could be used as a biocontrol agent against *A. alternata* in table grape, which is in line with our results. Moreover, *Aspergillus ochraceus* mycelial growth was significantly inhibited by *S. bacillaris* FE08.05, *M. pulcherrima* GP8.

Furthermore, previous research confirms that yeast *Hanseniaspora opuntiae* HoFs can protect plants against *Botrytis cinerea* and *Corynespora cassiicola* (Ferreira-Saab et al., 2018). *Hanseniaspora uvarum* had an intermediate effect on *Phaeomoniella chlamydospora* mycelial growth, and this result is in accordance with Zhang et al. (X. Q. Wang et al., 2018), who reported considerable inhibition of the spore germination of *Penicillium digitatum by H. uvarum* Y3 in orange.

This study confirmed that *M. pulcherrima* is able to reduce the growth of *A. carbonarius*, *Aspergillus ochraceus* and *Fusarium oxysporum* growth, which is in line with previous research by Bleve et al. (Bleve et al., 2006)and Turkel et al.(Freimoser et al., 2019), who indicated that *M. pulcherrima* is able to reduce *A. niger*, *A. carbonarius* and *Fusarium* spp. growth on agar plates.

The PCA of VOCs produced during the yeast growth allowed the grouping of the selected strains into four different clusters. In agreement with the presented data, clusters 2 and 3 formed by *M. pulcherrima* GP8, *S. bacillaris* FE 08.05 and *H. uvarum* GM19 were capable of inhibiting the selected grapevine pathogenic moulds.

The quantitative analysis of their VOC profiles highlighted the presence compared to the other strains of higher levels of isoamyl and phenylethyl alcohols and an overall higher presence of other secondary metabolites, including low molecular weight organic acids (acetic, isovaleric, n-caprylic, pelargonic and n-capric acid) and volatile ethyl esters.

Phenylethyl ethanol and isoamyl alcohols successfully demonstrated inhibition of mycelial growth of *Aspergillus flavus* (Hua et al., 2014; Oro et al., 2018)and *Aspergillus brasiliensis* (Oro et al., 2018; Heyman et al., 2015). Although present in small amounts, short and medium-chain fatty acids and ethyl fatty acids esters can also synergize with higher alcohol antimicrobial activity (Pohl et al., 2011).

Even though further investigations are needed to assess whether these yeast isolates have practical value in the control of other fungi occurring on grapes, the data reported here indicate

that these yeasts originated from grapevine itself and can be described as “ecological fungicides” without any effect on the balance of the environment. This could be a motivation for industry and manufacturing sectors to produce biosafety products using those species in the near future. Our outcomes showed that the protentional biocontrol activity is related to the characterization of strain, as observed by Suzzi et al. (Suzzi et al., 1995) in a previous study on the antagonistic aptitudes of wine yeasts against plant pathogenic fungi.

3.6 Conclusions

In conclusion, the preliminary results presented in this work highlight the occurrence of epiphytic indigenous yeast on grapes isolated from three different Malaysian regions that can potentially counteract the mycelial growth of several grape berry pathogen moulds. Among the isolated strains, *M. pulcherrima* GP8, *S. bacillaris* FE 08.05 and *H. uvarum* GM19 seems to be the most promising, as highlighted by in vitro antifungals and in detached berry trials. VOCs revealed the production from these strains of different volatile antimicrobial compounds, including higher alcohols, low-chain fatty acids and esters. However, more trials are needed.

Since non-*saccharomyces* species, as well as those belonging to the *Metschnikowia* and *Hanseniaspora* genera, could have misidentifications based on ITS sequences, and for these reasons, other genetic identifications based on D1/D2 ribosomal subunits and 26S rRNA sequences will be performed.

Since biological control agent efficacy can vary according to the pathogen’s inoculum level and environmental conditions (Cordero-Bueso et al., 2017), in-field trials are necessary.

In addition, a deeper knowledge about yeast inhibitory mechanisms is essential for the development of tailor-made strategies that can be more effective and guarantee better performance in the field. For these reasons, non-VOCs produced by yeast strains during growth will be considered.

Finally, the presented research pinpointed the importance of studying and exploiting natural and indigenous microflora to find sustainable and wild microbial strains, alternatives to engineered ones, able to counteract the main crop pathogens.

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4. Chapter 2

Bacteria- fungal interaction

Investigation of Potential *Bacillus subtilis* SV108 antifungal activity against main grapevine pathogens

Abstract

Bacillus subtilis SV108 was isolated from grape berries from experimental vineyard of University of Bologna (Faenza, Italy). Throughout this experimental work it has been confirmed to have *in vitro* antimicrobial activity due to the production of antimicrobial compounds isolated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-page) and characterized by electrospray ionization (ESI) tandem mass spectrometer TripleTOF 5600 (ESI). In addition, the analysis of the VOCs produced during the interaction with *Botrytis cinerea* and *A. carbonarious* .confirmed the presence of a volatile fraction able to inhibit the mycelial growth. These results suggest the probable capacity of *Bacillus subtilis* SV108 as a biocontrol agent against fungal pathogens in grapevine.

4.1 Introduction

Grapevines (*Vitis vinifera* L.) are one of the leading fruit crops cultivation in the world, with approximately 7.5 million hectares worldwide (OIV, 2019). Grapevine has a wide range of products including raisin, wine, juice, vinegar, sweet spread and seed oil. Moreover, it is rich in vitamins, carbohydrates, fiber, phytochemicals and proteins (Dopico-García et al., 2008). Grape berries contain different plant secondary metabolites including organic acids, VOCs, polyphenols, and phenolic acids. Growing, reproduction, and defense responses are the important roles played by these compounds in plants (Šikuten et al., 2020).

Every year, approximately 30–40% of post-harvest table grapes are lost as the consequence of softness induced by fungal infection (Jiang et al., 2014; Steel et al., 2013). In order to manage table grape fungal pathogens, in filed chemical strategies have been suggested including treatment with thiophanate methyl (THM), iprodione (IPR), cyprodinil (CYP) (Youssef et al., 2015). Despite the fact that fungicides are presently being banned due to human health and environmental concerns (Waewthongrak et al., 2015) and till now scientists are working to produce natural, safe, and biodegradable fungicides (Grzegorczyk et al., 2017). There are confirmations from previous studies regarding the benefits of using biocontrol agents against fungal pathogens in grapevine (Carmona-Hernandez et al., 2019; Otoguro & Suzuki, 2018a; Elmer & Reglinski, 2006; Maachia et al., 2015). In comparison with traditional chemical approaches for protecting grapevines against fungal diseases, the biocontrol agent contains two main benefits: (I) biocontrol agents are derived from grapevine itself and they do not have any effect on the safety and quality of related products (II) the use of biocontrol agents can reduce the onset of resistant pathogen population to synthetic agents used for crop diseases management (Otoguro & Suzuki, 2018a). The majority of these biocontrol agents produce antibacterial, antifungal, and other secondary metabolites. These antimicrobial production plays an important role against pathogens where they are complex compounds that appear only in stressing conditions (Keswani et al., 2020). One of the most prevalent microbial species that has been frequently utilized as a biocontrol agent is *Bacillus subtilis*, a gram-positive endospore-forming bacterial species (Ongena & Jacques, 2008). *Bacillus subtilis* strains are known for their metabolic capability and environmental versatility as well as for their ability to manage bacterial and fungal pathogens infecting crop plants both in-vitro and in field trials (Baumgartner & Warnock, 2006; Maachia et al., 2015; Pertot et al., 2017). For these reasons, *B. subtilis* could represent a promising biocontrol agent for in-filed management of crop diseases (Gurupada & Subhash, 2011). *Bacillus* spp. genome related to antimicrobial compound productions range between four and five percent. These genes, are mainly related

to the production of antifungal properties compounds including bioactive peptides, VOCs, polyketides molecules and bacteriocins.

The antimicrobial compounds included: the lipopeptide surfactin, fengycin and iturin which include iturin A, B and C (Wubshet & Geneti, 2021). and they are well-known for having significant antifungal properties as well as generating plant defense responses (Farace et al., 2015). However, the antimicrobial substances found in *B. subtilis* are depending on the strain and process/environmental factors. Consequently, in order to obtain antimicrobial substances, process situations optimization and genetic modification have been applied (Wang et al., 2015). Presently, effective methods to prepare peptides with a wide range of antimicrobials need to take into account strain proteolysis profile, proteomic and meta genomic patterns (Buddhika & Abeysinghe, 2021).

In this study, a selected biocontrol agent, *B. subtilis* SV108, which was isolated from grape berries from experimental vineyard of University of Bologna (Faenza, Italy) with high antimicrobial activity against grapevine major fungal pathogens was used. Purification of *B. subtilis* SV108 supernatant with the aim of identification the essential component in inhibiting mycelial growth, and examining the VOCs emitted from bacteria, bacteria-fungal pathogen interactions , were the major goals of this study.

4.2 Material and methods

4.2.1 Strain and culture condition

Bacillus subtilis (Cohn 1872) strain SV108 belonging to the collection of the Department of Agricultural and Food Sciences (University of Bologna, Italy) was used in this study as considered the most suitable among different biocontrol agents tested against grapevine fungal pathogens (unpublished data). It was re-cultured in Malt extract agar (MEA) (Oxoid, Thermofisher, Milan, Italy) media overnight incubation at 37°C. *Bacillus amyloliquefaciens* AG1 granted by Dipartimento DEMETRA, Università degli Studi di Palermo, Italy, as a positive control.

Antagonism assays were conducted against *Botrytis cinerea* (Pers., 1794) and *Phaeomoniella chlamydospore* (Crous and Gams, 2000), granted by Department of Integrated Pest Management of Mediterranean fruit and vegetable crops, Istituto Agronomico Mediterraneo (Valenzano, Bari, Italy), *Fusarium oxysporum*, grapevine rhizosphere isolate, *Alternaria alternata* (Keissl., 1912), obtained from grapevine leaves and *Verticillium dahliae* (Klebahn, 1913), acquired from decayed grapevine, *Aspergillus carbonarius* (Thom, 1916), *Aspergillus*

ochraceus (Wilhelm, 1877) isolated from grapes. Maintained on yeast potato dextrose (YPD) (Oxoid, Thermofisher, Milan, Italy) and incubated at 25°C for 2 weeks.

4.2.2 Extraction of antimicrobial metabolites and Antifungal assay

The extraction of antimicrobial metabolites (ESM) produced by *B. subtilis* SV108 was carried out according to Leelasuphakul et al (2008) with some modifications. Pure bacterial colonies of *B. subtilis* SV108 grown on malt extract agar at 30°C for 24h were used to inoculate 300 mL of malt extract broth (20 g/L, pH 6 ± 0.2). After 24h at 30°C, *B. subtilis* liquid culture was filtered using 0.2 μm cut off filtering membrane and the collected supernatant was used for the extraction of antimicrobial secondary metabolites by liquid-liquid extraction.

Collected supernatants (200 mL), were diluted 1:1 with pure ethyl-acetate vigorously stirred for five minutes and then transferred into separating funnels. After separation phase, the upper organic phase was collected, and the extraction repeated again. Organic phases collected (approx. 400 mL) were filtered in presence of anhydrous $NaSO_4$ and the solvent removed by vacuum drying at 40 °C. Once the raw extract was obtained, few washes with hexane were done to eliminate the fat fraction. Finally, the crude extract was dissolved in dimethyl sulfoxide (DMSO) and stored in vials at -80 °C (Figure 21).

The antifungal properties of *B. subtilis* SV108 cell free supernatant was tested on dual culture methods (Zhang et al., 2017). According to this method, YPD agar plates were used for fungal colonies growth until full plate invasion was reached. Then, physiological solution (5 ml; 0.9% g/L) was added to sporulating colonies. Obtaining a concentration of about 110-120 spores per milliliter, the fungal suspensions were collected. Separation of these propagules was done by collecting fungal suspension by sterile filtered the conidia from the mycelial mass. The resulting suspension is limited by only mycelium. Subsequently, each fungal suspension (1mL) was aliquoted and placed in petri dishes and enclosed with 14 mL malt extract agar (MEA). Following the agar medium solidification, 50 μL *B. subtilis* SV108 supernatant was filled in a well (5 mm). In addition, a negative control was filled with physiological solution. An arbitrary unit was used to investigate the amount of inhibition on fungal pathogens. The arbitrary units (AU/mL) are utilized consistent with the following formula: AU/mL: radius of inhibition (mm)/[well capacity (mL) x concentration ESM (mg/mL)] (Alfonzo et al., 2012).

Figure 21. Steps of extraction of antimicrobial compound. a) bacterial colonies of *B. subtilis* SV108 grown on malt extract agar. b) dilution with ethyl-acetate, c and d) transferring to separation funnl, e and f) separation phases and removing organic phase. g) *B. subtilis* liquid culture was filtered using 0.2 µm cut off filtering membrane. I and j) solvent removed by vacuum drying. k) washed with Hexan.

4.2.3 Volatile Organic Compound (VOC)

The VOCs released by *B. subtilis* SV108 and *B. amyloliquefaciens* AG1, used as biocontrol agents, during their interaction with t *B. cinerea*, and *A. carbonarius were evauated by a* Gas chromatograph method in combination with a mass spectrometer detector and the solid phase micro extraction technique (GCMS-SPME). In order to accomplish solid-phase microextraction, a CAR/PDMS 75µm fiber (SUPELCO, Bellafonte, PA, USA) was used. Five mL of each sample incubated for 10 min at 45 °C following by exposing the fiber into the headspace of the vial for 30 min at 45 °C. Gas chromatograph (GC) injector port was set in splitless mode at 250 °C for 10 min. Mass spectrometry (MS) 5970 MSD (Aglient technologies, Milan, Italy) along with chromatography (GC) 6890 N, Network GC System analysed the VOCs. Evaluation of VOCs were implemented three replicates and showed as means.

4.2.4 Protein extraction and SDS PAGE

The identification of protein gel strips was done to separate the sample proteins by tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) (H. Cao et al., 2013).

First, 50μL of pure water used for gel soaking for 30 min, then it was taken out and cut (1-2 mm) and transferred to the corresponding microcentrifuge tube. Next, 1 mL decolorizing solution (50 mM NH_4HCO_3: acetonitrile (ACN) = 1:1, V: V) was added to it and was vortexed for ten seconds. Decolorization was done at 37 °C for 30 min before centrifuging and drying. To dehydrate the gel, 500 μl of acetonitrile was added until it completely whitens. Following this, centrifuge tube with open led was placed on a clean bench for 10 minutes to air dry acetonitrile and 10 mM dithiothreitol (DTT) (1M DTT: 25mM NH_4HCO_3=1:100) was added until the liquid covered the gel, then it was placed into water bath at 56 °C for 1 h. After cooling to room temperature. Next, it was soaked up and added 55 mM iodoacetamide (IAM) (0.55 M IAM: 25 mM NH_4HCO_3= 1:10) quickly until the liquid covered the gel and it was placed in room temperature for 45 min. After soaking, it was washed twice with 500 μl of decolorizing solution (50 mM NH4HCO3: (ACN) = 1:1, V: V) and 500 μl of CAN and then vortexed for five minutes. Centrifuge tube for 10 minutes to dry completely followed by enzyme concentration dilution to 0.01 μg/μL with 25 mM NH4HCO3 and stored at 4 °C or ice for 30 min. After the gel was swelled, corresponding buffer was added to it and incubated overnight at 37 °C. Then, five time volume of 50% ACN was added the next day and vortexed for 5 min and centrifuged at 5000 *g* for one min. Finally, the resulting supernatant, which is transferred to the new centrifuge tubes, was centrifuged at 25000 *g* for 5 min, and freeze drying applied on the super natant. Dried peptide samples were reconstituted using the mobile phase A (2% ACN, 0.1% Formic acid (FA)), centrifuged and the supernatant injected.

4.2.5 Protein gel strips identification

High Performance Liquid Chromatography (HPLC) was performed using Shimadzu LC-20AD model nanoliter liquid chromatography (Hsieh et al., 2008). The sample enriched in the trap column and desalted, then entered a tandem C18 column (75 micron internal diameter, 3 micron column size, 15 cm column length), with the flow rate of 300 nl/min was separated by the following effective gradient: 0 -6 minutes, 6% mobile phase B (98% ACN, 0.1% FA); 6-40 minutes, mobile phase B linearly increased from 6% to 25%; 40-48 minutes, mobile phase B rose from 25% to 40 %; 48-51 minutes, mobile phase B rose from 40% to 90%; 51-55 minutes, 90% mobile phase B; 55.5-60 minutes, 6% mobile phase B.

Liquid phase chromatography peptides were passed to TripleTOF 5600 (SCIEX, Framingham, MA, USA), an ESI (Electrospray ionization) tandem mass spectrometer, the ion source was Nano spray III source (SCIEX, Framingham, MA, USA) and the emitter drawn from quartz material. Setting up the mass spectrometer parameters were as follows: ion source spray voltage 2,300V, nitrogen pressure 30 psi, spray gas 15 and spray interface temperature 150 °C. Scanning in high sensitivity mode, the MS1 scan cumulative time was 250ms, and the scan quality range was 350-1,500Da. Based on the MS1 scanning information, 30 ions above 150 cp were selected for fragmentation following the MS2 data was scanned. The criteria for screening was as bellow:

(1) The m/z range was 350. -1250 Da; (2) Charges number was 2-5 charges; (3) Parent ion dynamic was set to: within half of the peak time (about 12 s), the fragmentation of the same parent ion did not exceed 2 times. The scan accumulation time of the MS2 mass spectrum was 100 ms. The fragmentation energy selection was adjusted according to the Isobaric tags for relative and absolute quantitation (iTRAQ) reagent for data collection. The second quadrupole Q2 at 100 Da of Ion transmission efficiency was 100.

4.2.6 Statistical and bioenformatic Analysis

The obtained data from bacteria antifungal properties against grapevine pathogens were managed by SPSS software (Version 26) tool with statistical differences ($p < 0.05$) based on ANOVA and TUKEY HSD post-hoc tests. Principal component analysis (PCA) was applied to make a visualization of data obtained from VOCs.

The protein identification used experimental (Tandem Mass Spectrometry) MS/MS data. Alignments was done with theoretical MS/MS data according to database. The procedure starts from raw MS data conversion into a peak list following by searching matches in the database (reference sequence in National Center for Biotechnology Information (NCBI)).

Exact filtering and quality control was operated on search results, and possible protein identifications are produced. Rescoring and preprocessing was done on results from search engine using Percolator 3.0 software (The et al., 2016) in order to make an improvement to the matching accuracy (Carrera et al., 2021) The output filtration was applied by false discovery rate index (FDR) 1% at spectral level (PSM-level FDR <= 0.01) in order to achieve identified spectrum and peptide list. According to parsimony principle, groups of proteins was generated using protein inference on peptides. Then, the candidate protein were proceed with functional annotation analysis such as GO, COG/KOG, and pathway analysis (KEGG).

These annotation of genes function was performed on the basis of following databases: NCBI non-redundant protein sequences (NR; ftp://ftp.ncbi.nih.gov/blast/db/) (Carrera et al., 2021); NCBI non-redundant nucleotide sequences (NT; ftp://ftp.ncbi.nih.gov/blast/db/FASTA/); Clusters of Orthologous Groups of proteins (KOG/COG; http://www.ncbi.nlm.nih.gov/KOG, http://www.ncbi.nlm.nih.gov/COG/) (Zuo et al., 2018), KEGG (http://www.genome.jp/kegg), GO (http://www.geneontology.org/) (Blake & Harris, 2003) (Figure 22).

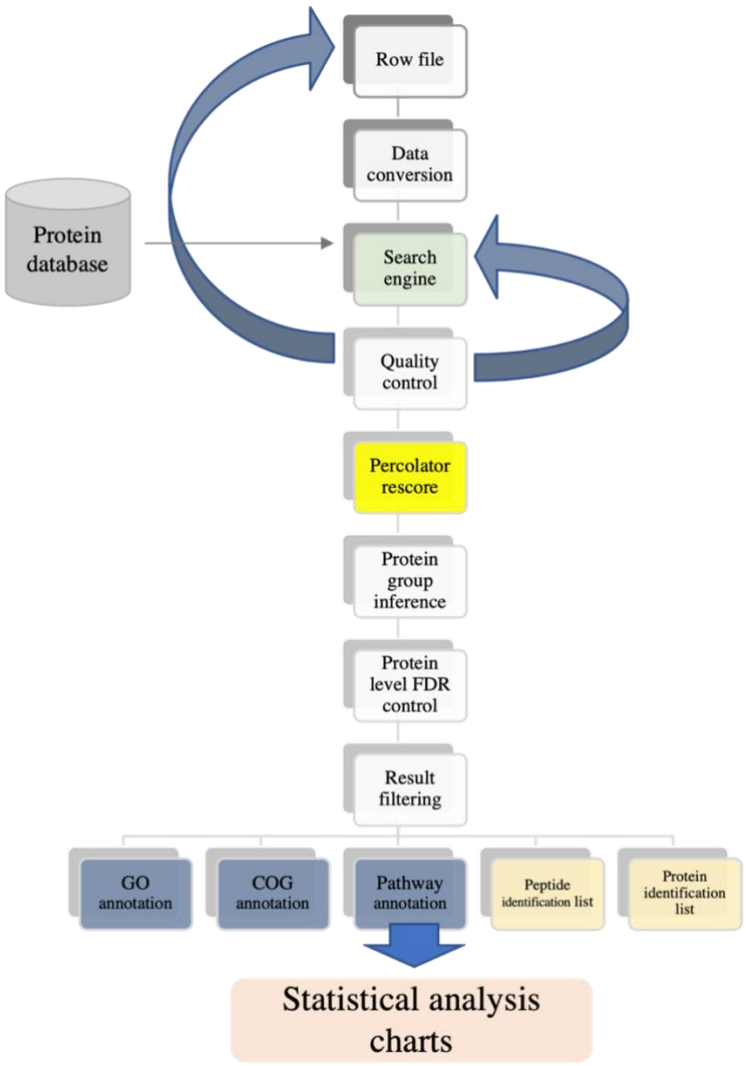

Figure 22. Protein Profiling of Bioinformatics Pipeline.

Since the identified protein sequences are from the selected database, database selection is the important part in MS based protein identification. The reference sequence (RefSeq) standard provides a basis for functional annotation of the human genome. It provides a stable reference

for mutation analysis, gene expression studies, and polymorphic discovery. Mascot is one of the most important and widely used protein identification software in the field of proteomics. The version used in the project is Mascot 2.3.02 (Z. Wang et al., 2018). In the operation, the Materials And Geometry Format (mgf) file was used as the original file, and the database that has been constructed was selected, and then the database search was performed. During the identification of this project, the parameters were configurated mentioned in Table 7.

Table 7. Mascot search parameters

Search Engine	Mascot v2.3
Enzyme	Trypsin
Peptide Mass Tolerance	0.05Da
Fragment Mass Tolerance	0.1Da
Fixed modifications	Carbamidomethyl (C)
Variable modifications	Oxidation (M); Gln->pyro-Glu (N-term Q); Deamidated (NQ)
Max Missed Cleavages	1
ccnnn	ESI-QUAD-TOF
Database	uniprot-taxonomy_1423.fasta (82874 sequences)

4.3 Results

4.3.*1 In vitro* antagonism assay

Among 21 samples (Table 8), *B. subtilis* SV108 was selected for further experiment. The antifungal activities of *B. subtilis* SV108 cell free supernatant was *in vitro* assessed against different grapevine fungal pathogens. Trials were performed using *B. subtilis* SV108. Each experiment was repeated three times. As shown in Figure 23 the highest inhibitory effect was observed against *B. cinerea* and *A. carbonarious.* For these microorganisms, the growth inhibition expressed as AU/mL was of 16536, 15808 respectively (Figure 23). Also there was a significant inhibition for *P. chlamydospore* which was 10920 AU/mL. By contrast, the extract was less effective against *F. oxysporum*, *A. alternata*, *V. dahliae* and *A. ochraceus*. Overall, the results demonstrated that, *B. subtilis* SV108 has broad antifungal activity in all tested fungal pathogens (Figure 23 and 24).

Table 8. isolated bacteria from grape berries from different vineyards

Bacterial isolates	Strain	Source	Country/region	Medium
Bacillus subtilis	DSM10	Grapevine berries	Italy/Cesena (DISTAL)	BHI
Bacillus subtilis	SV108	Grapevine berries	Italy/Cesena (DISTAL)	BHI
Bacillus amylofaciens	AG1	Grapevine berries	Italy/Palermo	BHI
Bacillus subtilis	FSPLG(46)	Rice milk isolate	Italy/Cesena (DISTAL)	BHI
Bacillus subtilis	FSPLP(47)	Rice milk isolate	Italy/Cesena (DISTAL)	BHI
Bacillus subtilis	NFL(15)	Rice milk isolate	Italy/Cesena (DISTAL)	BHI
Bacillus subtilis	NFST(12)	Rice milk isolate	Italy/Cesena (DISTAL)	BHI
Bacillus subtilis	FSP1(5b)	Rice milk isolate	Italy/Cesena (DISTAL)	BHI
Brevibacillus agri O brevis	(17)	Rice milk isolate	Italy/Cesena (DISTAL)	BHI
Brevibacillus agri	(19)	Rice milk isolate	Italy/Cesena (DISTAL)	BHI
Deinococcus deni	20	Grapevine berries	Italy/Cesena (DISTAL)	M17
pediococcus acidilactici	POU1	Grapevine berries	Italy/Cesena (DISTAL)	MRS
Lactobacillus brevis	LM6 6966	Grapevine berries	Italy/Cesena (DISTAL)	MRS
Lactobacillus plantarum	ATCC14197	Grapevine berries	Italy/Cesena (DISTAL)	MRS
Acetobacter malarum	DSM14337	Grapevine berries	Italy/Cesena (DISTAL)	YPM
Gluconobacter liquefaciens	DSM5603	Grapevine berries	Italy/Cesena (DISTAL)	YPM
Convivina intestini	(131-1)	Grapevine berries	Italy/Faenza	MRS
Convivina intestini	(6a)	Grapevine berries	Italy/Faenza	MRS
Leuconostoc mesenteroides	(3a)	Grapevine berries	Italy/Faenza	MRS
Leuconostoc fallax	(133-1)	Grapevine berries	Italy/Faenza	MRS
Acetobacter ghanensis	B60504	Grapevine berries	Malaysia/ Pahang	BHI
Acetobacter ghanensis	B50503	Grapevine berries	Malaysia/ Pahang	BHI
Acetobacter ghanensis	B30904	Grapevine berries	Malaysia/ Pahang	BHI
Acetobacter ghanensis	B10902	Grapevine berries	Malaysia/ Pahang	BHI
Acetobacter ghanensis	B20903	Grapevine berries	Malaysia/ Pahang	BHI
Acetobacter ghanensis	B40905	Grapevine berries	Malaysia/ Pahang	BHI
Acetobacter ghanensis	B50906	Grapevine berries	Malaysia/ Pahang	BHI

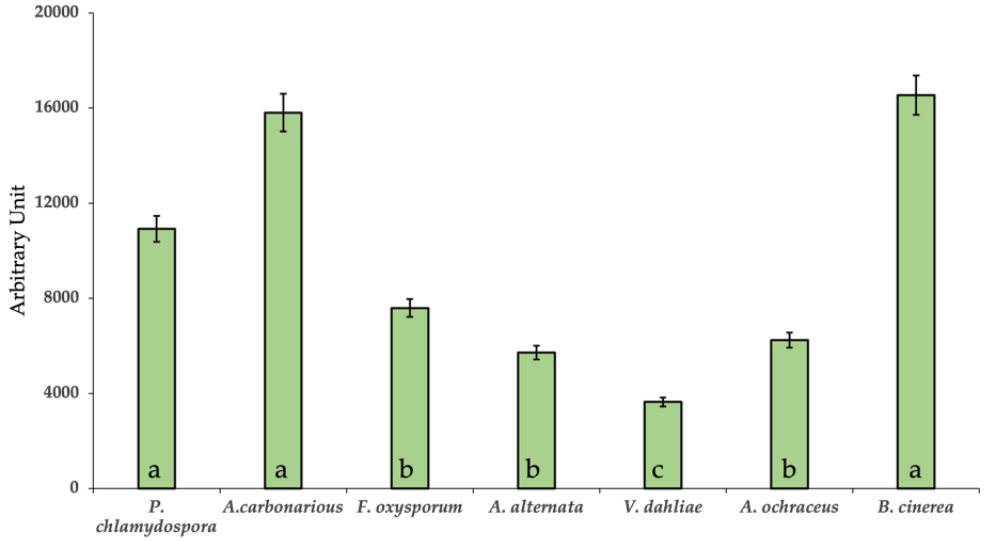

Figure 23. Sensitivity of fungal pathogens to *Bacillus subtilis* SV108. The X axis represent fungal pathogens and the Y axis showed the Arbitrary unit (AU/ml).

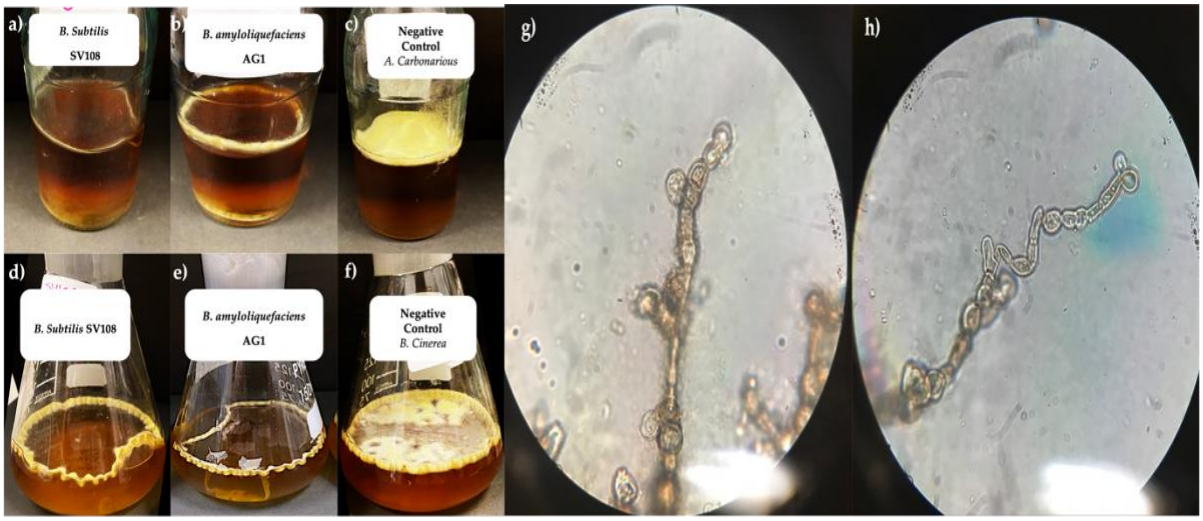

Figure 24. Mycelial growth inhibition of *B. subtilis* SV108 and *B. amyloliquefaciens* AG1 on *B. cinerea* and *A. carbonarius* in Malt extract broth. a) *B. subtilis* SV108 and *A. carbonarius*, b) *B. amyloliquefaciens* AG1 and *A. carbonarius*, c) *A. carbonarius* with MEB as a negative control. d) *B. subtilis* SV108 and *B. cinerea.* e) *B. amyloliquefaciens* AG1 and *B. cinerea.* f) *B. cinerea* with MEB as a negative control. g) mycelium of *A. carbonarius* before inoculation with *B. subtilis* SV108 h) mycelium of *A. carbonarius* after inoculation with *B. subtilis* SV108.

4.3.2 Volatile Organic Compound (VOC)

The yeast VOCs compositions were qualitatively and quantitatively evaluated with head space solid-phase microextraction using a gas chromatograph coupled with a mass spectrometer detector (GCMS-SPME). Analyses were performed after 6 days of growth at 25 °C in liquid media (malt extract broth) of *B. subtilis* A CAR/PDMS 75µm fibre (SUPELCO, Bellafonte, PA, USA) was used to perform the solid-phase microextraction (SPME). The samples (5 mL)

were placed in vials and incubated for 10 min at 45 °C. Then, the fiber was exposed to the headspace of the vial for 30 min at 45 °C. The volatile molecules adsorbed were desorbed in the gas chromatograph (GC) injector port in splitless mode at 250 °C for 10 min. The headspace of the VOCs was analyzed using chromatography (GC) 6890 N, Network GC System with mass spectrometry (MS) 5970 MSD (Aglient technologies, Milan, Italy) (Figure 14). The column used was J&W CP-Wax 52 (50 m × 320 μm × 1.2 μm) (Aglient technologies, Milan, Italy). The initial temperature was 40 °C for 1 min and then increased by 4.5 °C/min up to 65 °C. After that, the temperature increased by 10 °C/min up to 230 °C and remained at this temperature for 17 min. The gas-carrier was helium at 1.0 mL/min flow. Compounds were identified by comparison based on the NIST 11 (National Institute of Standards and Technology) database, while the quantitative analysis was performed with the internal standard method using 4-methyl-2-pentanol (6 mg/L) and expressed as equivalent ppm (ppm eq.). For each compound detected, the ppm eq. represents the amount of compound present in the headspace in dynamic equilibrium with the aqueous phase. The chemical analyses were performed in triplicate and are expressed as means.

B. subtilis SV108, *B. amyloliquefaciens* AG1, *B. cinerea*, *A. carbonarius*, interaction between *B. cinerea* and *B. subtilis* SV108, *B. cinerea and B. amyloliquefaciens* AG1, *A. carbonarius and B. subtilis* SV108, and *A. carbonarius* with *B. amyloliquefaciens* AG1, was analysed. Two factors of principal components were plotted in the space coverd by the samples (Factor 1 and Factor 2). Over %53 of total variability explained the analysis (Figure 25).

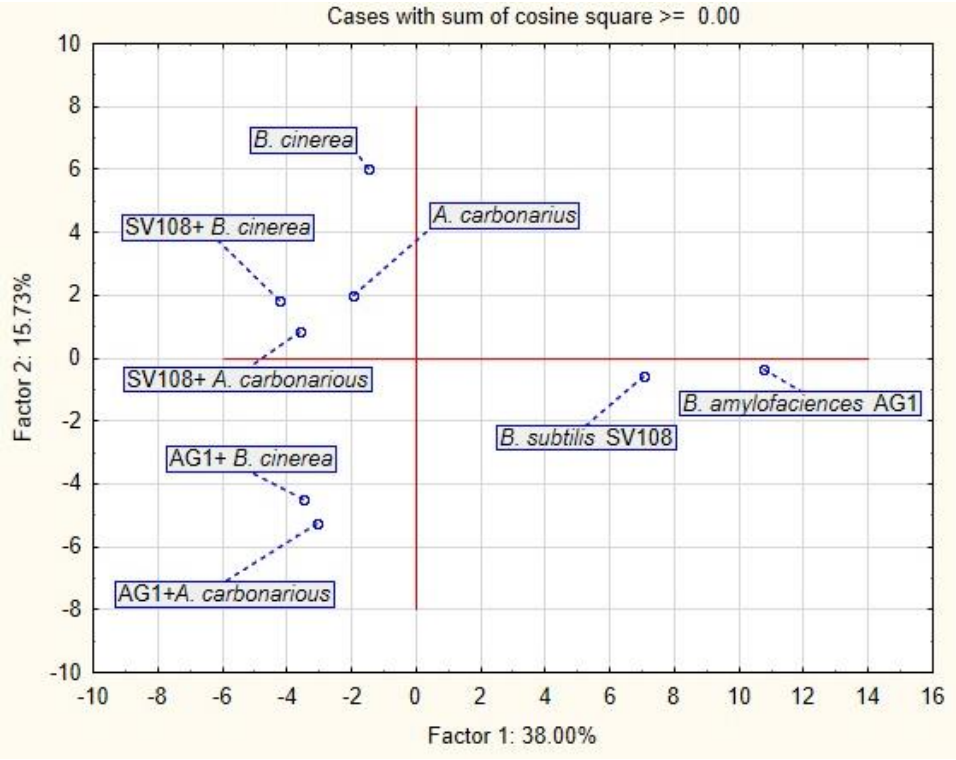

Figure 25. Principal component analysis loading plot of VOCs of the bacteria strains and their interactions with fungal pathogens.

The characterization of VOC profiles principally included alcohols, aldehydes, ketones, esters, acids, phenol and organic compounds. As shown in Table 2, *B. subtilis* SV108 produced a high level of Alcohols including Ethanol, 1-Butanol and Phenylethyl Alcohol. Specifically ,the level of Ethanol production (1.15 ppm), increased in interaction with *A. carbonarious* (2.35ppm) and *B. cinerea* (3.42 ppm). *B. amylofaciences* AG1 as positive control also produced a good level of Ethanol (1.22 ppm) which increased slightly in interaction with *A. carbonarious* (1.25ppm) and *B. cinerea* (3.42 ppm). Regarding the production of aldehydes, both *B. subtilis* SV108 and *B. amylofaciences* AG1 produced a good level of aldehyde (Nonanal and Benzaldehyde) which are well-known to effectively inhibit the fungal mycelial growth (Li et al. 2021). Moreover, ketones production in *B. subtilis* SV108 and the positive control decreed in interaction with two tested pathogens.

Table 9. The VOCs classified as alcohol, aldehyde and ketone emitted by bacterial and fungal strains.

	Alcohol (ppm*)			**Aldehyde (ppm)**		**Ketone (ppm)**	
	Ethanol	1-Butanol	Phenylethyl Alcohol	Nonanal	Benzaldehyde	2-Butanone	4-Heptanone
B. subtilis SV108	1.15	0.49	0.17	0.2	0.29	0.15	0.29
SV108 and *A. carbonarious*	2.35	0.16	0.92	0.14	0.1	0.1	0.1
SV108 *B. cinerea*	3.42	0.1	0.28	0.1	0.13	-	0.1
B. amylofaciences AG1	1.17	0.35	-**	-	0.1	0.33	0.65
AG1 and *A. carbonarious*	1.25	0.1	0.11	0.1	0.14	-	0.1
AG1 and *B. cinerea*	1.22	0.1	0.12	0.1	0.11	-	0.1
B. cinerea	4.09	0.13	3.04	-	-	0.12	0.14
A. carbonarius	3.14	0.12	0.69	0.11	0.14	-	0.21

** Parts per million equivalent*
*** Under the detection limit (<0.1 ppm eq).*

According to Table 3, acid production by two tested biocontrol agents was increased in interaction with *A. carbonarious* and *B. cinerea* including Acetic acid (0.21), Propanoic acid, 2-methyl- (0.19) and Hexanoic acid (0.75). Furthermore, there was a good level of Ester and phenol production as well.

Table 10. The VOCs classified as ester, acid and phenol emitted by bacterial and fungal strains.

	Ester (ppm*)		**Acid (ppm)**		**Phenol (ppm)**
	Acetic acid ethenyl ester	Acetic acid	Propanoic acid, 2-methyl-	Hexanoic acid	Phenol, 2-methoxy-
B. subtilis SV108	0.39	0.14	0.16	0.11	0.17
SV108 and *A. carbonarious*	-**	0.21	0.19	0.75	0.1
SV108 *B. cinerea*	-	-	0.06	-	-
B. amylofaciences AG1	0.15	0.12	0.1	-	0.1
AG1 and *A. carbonarious*	-	-	0.11	-	0.12
AG1 and *B. cinerea*	-	-	0.22	-	-
B. cinerea	-	-	0.28	-	-
A. carbonarius	-	-	-	-	-

** Parts per million equivalent*
*** Under the detection limit (<0.1 ppm eq).*

As stated in Table 4, there was a high amount of different kind of Pyrazine (Pyrazine, 2,dimethyl-, Pyrazine, trimethyl- and Pyrazine, 3-ethyl-) produced by *B. subtilis* SV108 which may act as one of the main factors enrolled in fungal mycelium inhibition (Guevara-Avendaño et al. 2020) as far as pyrazines act as antifungal compound produce by *Bacillus* spp. species. Specially, Pyrazine, 2,dimethyl- was the most significant VOC produced by both *B. subtilis* SV108 (6.77 ppm) and *B. amylofaciences* AG1 (7.38 ppm).

Acetoin was another considerable compound which was increased during the *B. subtilis* SV108 interaction with fungal pathogens (0.79 and 0.61) compare to *B. amylofaciences* AG1 (1.84

and 1.82). However, two tested bacteria produced specific organic compounds known for antifungal properties, such as 1H-Imidazole, 1H-Pyrrole.

Table 11. The VOCs classified as Organic compound emitted by bacterial and fungal strains.

	Organic compound (ppm*)									
	2,3-Butanedione	Acetoin	Pyrazine, 2,dimethyl-	Pyrazine, trimethyl-	Pyrazine, 3-ethyl-	1H-Imidazole	1H-Pyrrole	2-Methylisoborneol	2-Acetylthiazole	Mequinol
B. subtilis SV108	-**	0.51	6.77	1.04	1.22	0.21	-	-	0.19	-
SV108 and *A. carbonarious*	0.12	0.79	-	-	-	0.13	-	0.31	-	-
SV108 *B. cinerea*	0.1	0.61	-	-	-	-	1.53	0.11	-	0.32
B. amylofaciences AG1	-	0.26	7.38	1.37	1.61	0.37	-	-	0.17	-
AG1 and *A. carbonarious*	0.37	1.84	-	-	-	-	-	0.41	-	-
AG1 and *B. cinerea*	0.48	1.82	-	-	-	-	1.91	0.1	-	-
B. cinerea	-	-	-	-	-	-	-	-	-	-
A. carbonarius	-	-	-	-	-	-	-	-	-	-

** Parts per million equivalent*
*** Under the detection limit (<0.1 ppm eq).*

4.3.3 Basic protein identification information

The mass spectrometer Triple TOF 5600 was utilized to analyze and identify proteins and peptides associated with *Bacillus subtilis* SV108's antifungal activity. Total number of 38034 spectra were obtained from the sample group. According to identification by search engine (Mascot 2.3.02), among 38034 detected spectra, 108 spectra were matched, 49 proteins and 53 peptides were identified (Figure 26).

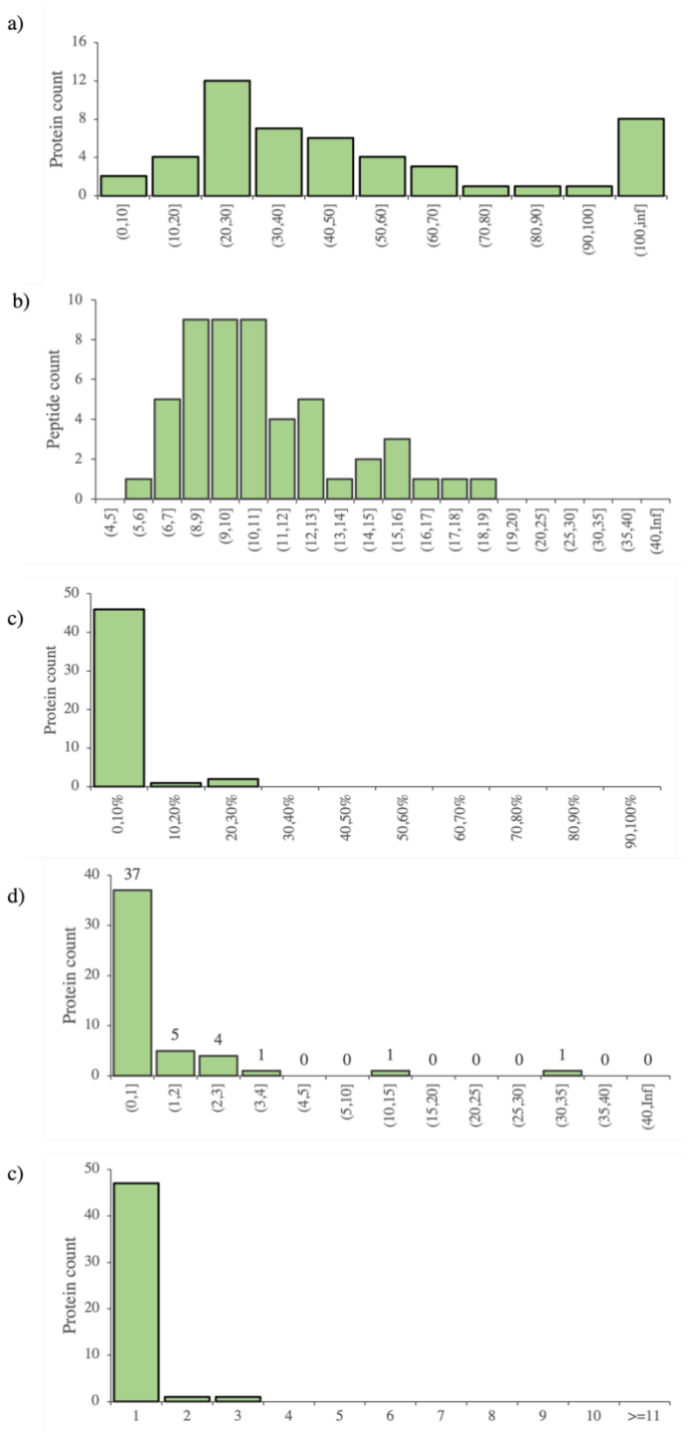

Figure 26. A) Protein mass distribution of the 49 proteins identified from ESMs; B) Unique peptide number, the X axis represent the number of unique peptide match to each protein; C) Peptide length distribution of 53 peptide identified from ESMs; D) Unique spectrum number, the X axis represent the number of unique spectra matched to each protein; E) Protein coverage of identified proteins from ESMs, The X-axis represents protein coverage percentage interval

The molecular weights were used as reference for statistical analyses for all the proteins and showed that the proteins' molecular weight distributions were relatively broad and covered the sizes of the different proteins. The analysis of the peptides mass distribution showed that most of the lengths were 5-19 kDa, among which the highest area of distribution was 6–10kDa. The

distribution of the peptide sequence coverage was analyzed and the results showed that most of the expressed proteins showed better peptide coverage.

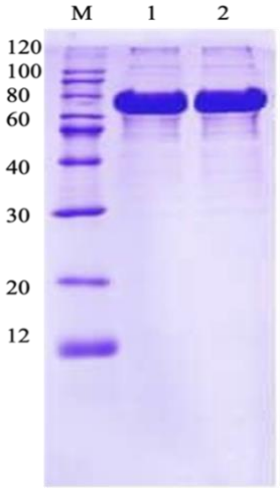

Figure 27. Tricine-SDS-PAGE and the associated antifungal bioassay corresponding zone of inhibition. (M: protein molecular weight marker, 1: active substances 2: corresponding zone of inhibition

The zone of inhibition was used to determine the inhibitory effect of *B. subtilis* SV108 with the single band in the gel stained (Figure 27). A single band in the gel stained with Comassin blue corresponded with the zone of inhibition alongside an approximate molecular weight in the range 70-80 kDa. The peptides were identified by reference sequence (RefSeq) of NCBI based on the Mascot searching results. Sorting total Mascot score suggested some different substances based on the identified peptides. The results showed the high presence of two peptides sequence i.e. **lnmmtk** and **sstldhk** (Figure 28).

>tr|B3TLA2|B3TLA2_BACIU Iturin A synthetase B OS=Bacillus subtilis OX=1423 GN=ituB PE=3 SV=1

```
4160        4170        4180        4190        4200
HADLEPIIGM FVNTLAMRNY PEKGKTFSQY LSEVKENALK AYEHQDYPFE
4210        4220        4230        4240        4250
VLIDQLNIAR DLSRNPLFDT MFVLQNTEQE QLEINDVTFK PYPNGHTMAK
4260        4270        4280        4290        4300
FDLTLTAVEE GAGIQFTLEY LTALFKPETI ERMMGHFEQL VDSIIKQPEA
4310        4320        4330        4340        4350
ELARLNMMTK EEERDIQQLF NDTAVAEKRI PTTIHQLFEQ QAERNPDHEA
4360        4370        4380        4390        4400
VMFGNQTLTY RQLNERSNQL ARVLQDKGAC TDQVVAVLTD RSAHMIIGIL
4410        4420        4430        4440        4450
AILKAGAAFL PIDPELPEKR RAFMLKDSGA DVLLTCAGHA IPPLFEGEVL
4460        4470        4480        4490        4500
```

>sp|Q9R9J1|MYCA_BACIU Mycosubtilin synthase subunit A OS=Bacillus subtilis OX=1423 GN=mycA PE=1 SV=1

```
2210        2220        2230        2240        2250
QLTKQAGLVI GIPTAGQLHM KQPMLVGNCV NMVPVKNTAS SESTLADYLG
2260        2270        2280        2290        2300
HMKENMDQVM RHQDVPMTLV ASQLPHDQMP DMRIIFNLDR PFRKLHFGQM
2310        2320        2330        2340        2350
EAELIAYPIK CISYDLFLNV TEFDQEYVLD FDFNTSVISS EIMNKWGTGF
2360        2370        2380        2390        2400
VNLLKKMVEG DSASLDSLKM FSKEDQHDLL ELYADHQLRI SSTLDHKGVR
2410        2420        2430        2440        2450
AVYEEPENET ELQIAQIWAE LLGLEKVGRS DHFLSLGGNS LKATLMLSKI
2460        2470        2480        2490        2500
QQTFNQKVSI GQFFSHQTVK ELANFIRGEK NVKYPPMKPV EQKAFYRTSP
2510        2520        2530        2540        2550
AQQRVYFLHQ MEPNQVSQNM FGQISIIGKY DEKALIASLQ QVMQRHEAFR
```

Figure 28. Amino acid sequence of Iturin A synthetase B and Mycosubtilin synthetase subunit A. the two peptides identified in this research are highlighted in yellow.

These two peptides were found related to the amino acid sequence of Iturin A synthesize B (ituB) and Mycosubtilin synthesize A (mycA), that belong to *B. subtilis* antibiotic biosynthetic process system.

Table 12. Description of Identified proteins in *Bacillus subtilis* SV108

Protein_ID	PeptideSeqs	Protein_Qs	Protein_Mass	Abundance	iBAQ	Description	Coverage
tr|A0A5D4NCP8|A0A5D4NCP8_BACIU	QLNDFVKTNR	2.063	611075.74	4438.418739	17.54315707	Amino acid adenylation domain-containing protein	0.0019
sp|P80859|6PGD_BACSU	NLALNIESR	3.369	51742.50	40868.3528	1277.136025	6-phosphogluconate dehydrogenase, NADP	0.0192
tr|D4G3T0|D4G3T0_BACNB	QLPHIPDK	3.369	113935.58	12324.65595	208.8924737	Uncharacterized protein	0.0082
tr|A0A5F2KKD3|A0A5F2KKD3_BACIU	VAVATVGAVLPGNFK	3.369	87747.96	4602.747336	104.607894	Phenylalanine--tRNA ligase beta subunit	0.0187
tr|A0A4Q1E496|A0A4Q1E496_BACIU	ETIYSMVRNK	3.369	7367.77	1070.035173	267.5087933	DNA-binding protein OS=*Bacillus subtilis*	0.1587
tr|A0A5F2KK46|A0A5F2KK46_BACIU	IINEPTAAALAYGLDK	3.369	65917.92	10655.53179	273.2187639	Chaperone protein DnaK OS=*Bacillus subtilis*	0.0262
tr|A0A5D4N2S6|A0A5D4N2S6_BACIU	KASIMFVR	3.369	12704.18	1011.570012	126.4462516	L-rhamnose mutarotase	0.0769
tr|A0A5D4PBR2|A0A5D4PBR2_BACIU	AVIIAATGTGK	3.369	121723.60	874.4475325	13.24920504	DUF3427 domain-containing protein	0.0105
tr|A0A162SAG3|A0A162SAG3_BACIU	AVSIPVLR	3.369	27723.92	11835.43065	739.7144155	Indole-3-glycerol phosphate synthase	0.0321
tr|A0A5F2KIM9|A0A5F2KIM9_BACIU	VIHIITK	3.369	23054.82	270.8544869	16.92840543	HD domain-containing protein OS=*Bacillus subtilis*	0.0341
tr|E0TZG9|E0TZG9_BACPZ	GFLLDGFPR	3.369	24090.12	4119.713969	257.4821231	Adenylate kinase OS=*Bacillus subtilis*	0.0415
tr|A0A140G068|A0A140G068_BACIU	LNMMTK	2.063	608023.24	596.9150772	2.227295064	ItuB OS=*Bacillus subtilis* OX=1423 GN=ituB PE=4	0.0011
tr|A0A3A5I5J1|A0A3A5I5J1_BACIU	IPLIGNLVR	3.369	39911.79	0	0	Competence protein ComG OS=*Bacillus subtilis*	0.0261
tr|G4NWC0|G4NWC0_BACPT	VMNTILK	3.369	29619.35	1412.028633	94.13524223	YdfB OS=*Bacillus subtilis* subsp. spizizenii	0.027
tr|A0A4V3C0M1|A0A4V3C0M1_BACIU	NIAIIAHVDHGK	3.369	68360.06	7373.575986	216.8698819	GTP-binding protein TypA/BipA OS=Bacillus	0.0196
tr|G4ER08|G4ER08_BACIU	VIRELINAR	3.369	22469.09	69100.08547	5758.340456	PglD_N domain-containing protein	0.0417
tr|A0A4V1MDP5|A0A4V1MDP5_BACIU	IALPLHPEYR	3.369	55463.99	16666.43966	574.7048158	Nitrate reductase subunit beta OS=*Bacillus subtilis*	0.0205
tr|G3GBT6|G3GBT6_BACIU	DIYDAQNGTQLPGKPVR	3.369	37684.77	2468.251298	137.1250721	Neutral metalloproteinase OS=*Bacillus subtilis*	0.0499
tr|A0A3A5I702|A0A3A5I702_BACIU	LPVDEIIK	3.369	46093.42	35673.72414	1426.948966	Aminoacetone oxidase family FAD-binding enzyme	0.019
tr|A0A5D4NAV7|A0A5D4NAV7_BACIU	QSAQEKAEELLR	3.369	27047.19	4669.295552	291.830972	Arginine ABC transporter ATP-binding protein ArtR	0.05
tr|A0A0D1KVK5|A0A0D1KVK5_BACIU	NNPVLIGEPGVGK	3.369	90063.39	9687.452302	206.1160064	Class III stress response-related ATPase OS=Bacillus	0.016
tr|E0TUA1|E0TUA1_BACPZ	VVFNEITK	3.369	78929.90	9662.245607	247.7498874	DNA topoisomerase 1	0.0116
tr|A7L755|A7L755_BACIU	AGENVGVLLR	10.108	21969.56	60358.85459	4311.346757	Translation elongation factor Tu (Fragment)	0.2893
tr|A0A165ATL5|A0A165ATL5_BACIU	CDMVDDEELLELVEMEVR	3.369	43542.83	237.2617589	11.298179	Elongation factor Tu OS=*Bacillus subtilis*	0.0833
tr|E0TYM0|E0TYM0_BACPZ	ATTTEKLGFTGR	3.369	17114.93	4048.826395	368.0751268	2-C-methyl-D-erythritol 2,4-cyclodiphosphate	0.0759
tr|A0A0D1IX15|A0A0D1IX15_BACIU	LSIIDEKATFTR	3.369	16869.54	21186.02538	1926.002308	Uncharacterized protein OS=*Bacillus subtilis*	0.0845
tr|A0A4T2HPY9|A0A4T2HPY9_BACIU	QVEAKLILK	3.369	16706.52	545.2876163	60.58751292	Uncharacterized protein OS=*Bacillus subtilis*	0.0629
tr|M4KV88|M4KV88_BACIU	ILNLLGLK	3.369	31007.86	1157.137028	96.42808565	Undecaprenyl-diphosphatase OS=*Bacillus subtilis*	0.0284
tr|A0A5F2KQ95|A0A5F2KQ95_BACIU	AFGVTVAQLR	3.369	31973.95	2817.589963	234.7991636	LysM peptidoglycan-binding domain	0.0337
tr|A0A5C8KUC6|A0A5C8KUC6_BACIU	AEINTIVR	3.369	40274.42	25249.43467	901.7655238	Uncharacterized protein OS=*Bacillus subtilis*	0.0218
tr|A0A4R6HRQ6|A0A4R6HRQ6_BACIU	TLLPSIVIK	3.369	134489.36	608.2578627	9.357813272	ATP-dependent helicase/deoxyribonuclease	0.0077
sp|P20458|IF1_BACSU	VELENGHTVLAHVSGK	3.369	8208.35	9089.4844	2272.3711	Translation initiation factor IF-1 OS=*Bacillus subtilis*	0.2222
tr|A0A4R6HI95|A0A4R6HI95_BACIU	QMAISQAGELR	3.369	40642.19	9858.705517	410.7793965	AraC family two component transcriptional regulator	0.0298
tr|A0A3A5I0M6|A0A3A5I0M6_BACIU	NRIQPISER	3.369	64103.44	3978.23283	117.0068479	Nitroreductase domain-containing protein	0.0157
tr|A0A5D4PEX5|A0A5D4PEX5_BACIU	VAPLNLEEAK	3.369	24747.41	12893.65937	920.9756691	GntR family transcriptional regulator	0.0457
tr|A0A5D4N2V6|A0A5D4N2V6_BACIU	QDILQALLAR	3.369	56389.30	2384.199341	95.36797363	Urate oxidase OS=*Bacillus subtilis*	0.0202

Protein_ID	PeptideSeqs	Protein_Qs	Protein_Mass	Abundance	iBAQ	Description	Coverage
tr|A0A3A5I7B7|A0A3A5I7B7_BACIU	VTAIISQNGNVYR	2.063	32459.33	10130.50834	633.1567712	Hydrolase OS=*Bacillus subtilis*	0.0463
tr|A0A5D4NB47|A0A5D4NB47_BACIU	GPLTTPVGGGIR	3.369	46420.89	31784.74631	1222.490243	Isocitrate dehydrogenase [NADP]	0.0284
tr|A0A0D1JCZ7|A0A0D1JCZ7_BACIU	FATSDLNDLYR	6.739	134181.61	10674.65854	128.6103438	DNA-directed RNA polymerase subunit beta'	0.0209
tr|A0A3A5I6R6|A0A3A5I6R6_BACIU	VNQMYPNK	3.369	22062.19	1368.543307	97.75309333	Histidine phosphatase family protein	0.0415
tr|A0A5D4P9R9|A0A5D4P9R9_BACIU	IVLDDPK	3.369	263415.94	2120.008947	18.2759392	Nonribosomal peptide synthetase DhbF	0.0029
tr|A0A5D4NBW0|A0A5D4NBW0_BACIU	EKLHQEIELLK	3.369	28676.64	32559.82691	1479.992132	Uncharacterized protein OS=*Bacillus subtilis*	0.0442
tr|A0A5Q2Y5U8|A0A5Q2Y5U8_BACIU	LAGGVAVIK	3.369	57317.88	16899.34809	545.140261	Chaperonin GroEL OS=*Bacillus subtilis*	0.0166
sp|Q9R9J1|MYCA_BACIU	ISSTLDHK	3.369	448985.38	4406.037684	19.58238971	Mycosubtilin synthase subunit A	0.002
tr|E0U3Q1|E0U3Q1_BACPZ	ISEMAIKAFK	3.369	39813.07	3242.920667	202.6825417	D-alanine--D-alanine ligase OS=*Bacillus subtilis*	0.0277
tr|A0A1J0AKL2|A0A1J0AKL2_BACIU	GYKESGK	3.369	25084.95	136.2493615	15.13881795	Uncharacterized protein OS=*Bacillus subtilis*	0.0307
tr|A0A0D1JGU5|A0A0D1JGU5_BACIU	YVNLLNFRK	3.369	21105.65	9973.134971	997.3134971	DUF1851 domain-containing protein	0.05
tr|G4P1F4|G4P1F4_BACPT	INPIVTR	3.369	49688.19	4545.440681	239.23372	Teichuronic acid biosynthesis protein TuaB	0.0157
tr|A0A3A5I3T8|A0A3A5I3T8_BACIU	LVLDGIQVVGSLVGTR	656.43433001	28.540623044	3.36933012663	36006.7046747	Alcohol dehydrogenase AdhP OS=*Bacillus subtilis*	0.0473

The other activating pathways included the biosynthesis of secondary metabolite pathway, biosynthesis of antibiotic, biosynthesis of amino acid and lysine biosynthesis (figure 29) which played an important role in antimicrobial activity of *Bacillus subtillis* (Liu et al., 2016).

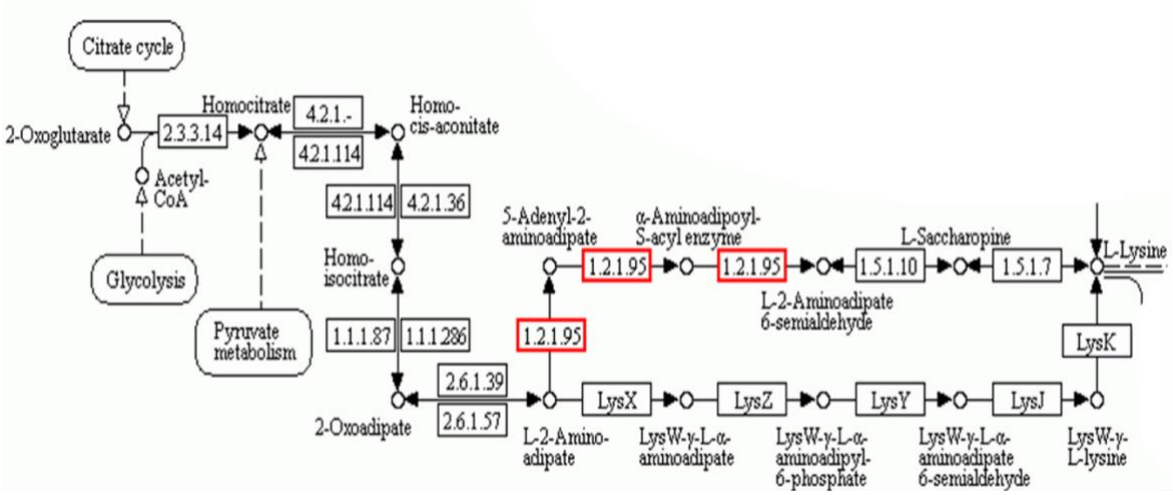

Figure 29. pathway of lysine biosynthesizes generated by KEGG enrichment analysis of identified proteins. Mycosubtilin subgroup A and Iturin B involved in this pathway highlighted in red boxes.

4.3.4 Gene ontology (GO) enrichment analysis and KOG annotation

Gene Ontology (GO) provides a timely updated standard vocabulary to comprehensively describe the properties of genes and gene products in organisms. GO functional annotation analysis was carried out to all identified proteins to reveal the function of *B. subtilis* supernatant, and the results include two parts: protein2go (for each protein, a list of IDs and all corresponding GO functions are given) (Yan et al., 2013) and go2protein.

GO annotation study was done on the 49 proteins using Blast2go v2.5 software to further investigate their subcellular localization in molecular function, and biological processes group. The results showed that proteins had been annotated into three functional group including biological processes, cellular components and molecular functions (Figure 30).

In biological processes class, the proteins were mainly involved in metabolic process (23 proteins and cellular process (19 proteins. Other downregulated proteins were involved in biological regulation (4 proteins), cellular component organization or biogenesis (3 proteins), developmental process (2 proteins), localization (1 protein), multi-organism process (1 protein), response to stimulus (5 proteins) and regulation of biological process (4 proteins) and signaling (1 protein). In the cellular components class, the proteins were mainly focused on the cells (14 proteins), and membranes (5 proteins) which are significantly enriched in GO

analysis. Macromolecular complex, membrane part and organelle were the low enriched. In molecular functions class, the proteins were mainly involved in catalytic activities (32 proteins) and binding (24 proteins). These results indicated that the key functional proteins of the inhibitory function, may happen by upregulating metabolic activities, cellular process, cell, catalytic and binding activity.

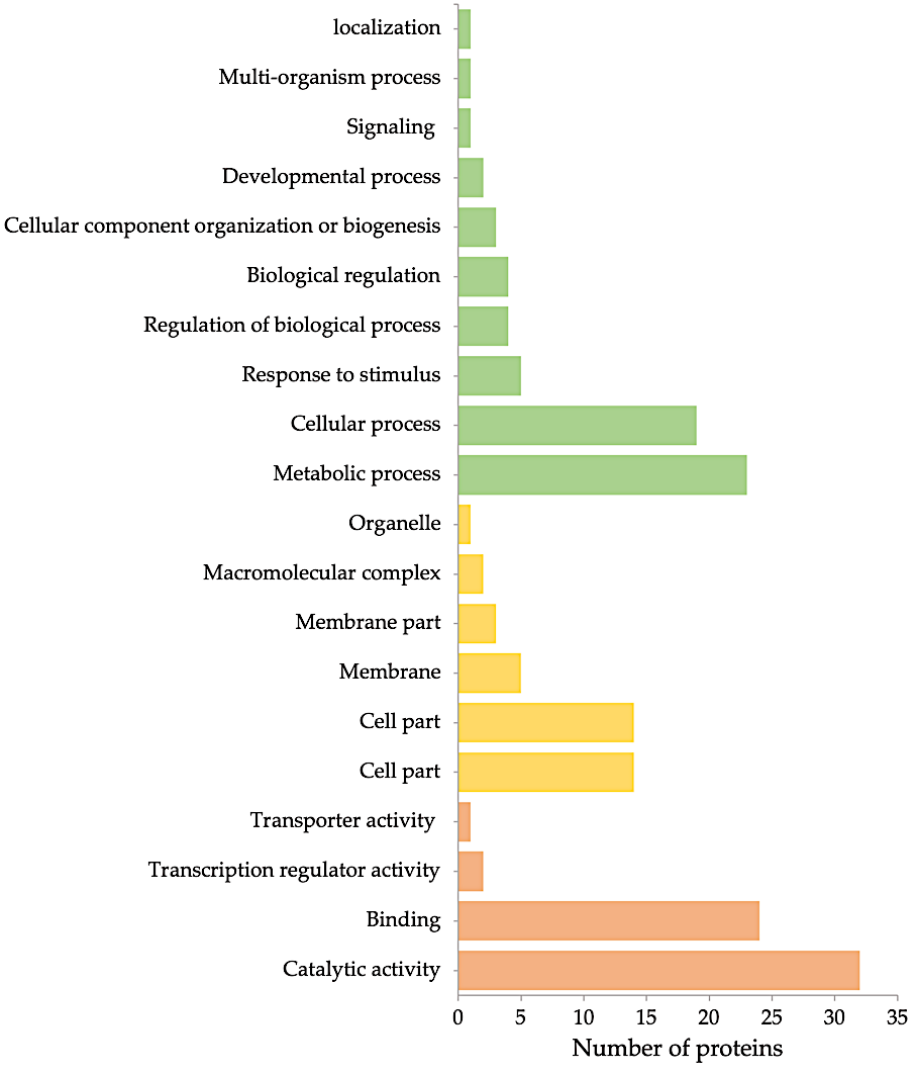

Figure 30. GO functional annotation

The Clusters of Orthologous Groups (COGs) tool, in eukaryote-specific version (KOGs), was used to identify ortholog and paralog proteins (Figure 31). Each KOG entry contains a series of orthologous or paralogous proteins (ref). The blast analysis of the identified proteins against the KOG database, predicted the possible functions of these proteins and performs functional classification statistical analysis (Tatusov et al., 2003). The results showed proteins were annotated to four KOG categories. Most of proteins were mainly concentrated in three functional categories including metabolism, information storage and processing, cellular

processes and signaling. Five family involved in metabolism category including amino acid transport and metabolism (4 proteins), Nucleotide transport and metabolism (1 protein), lipid transport and metabolism (6 proteins), Inorganic ion transport and metabolism (1 protein), Secondary metabolites biosynthesis, transport and catabolism (7 proteins). Information storage and processing category includes, RNA processing and modification (1 protein), translation, ribosomal structure and biogenesis (4 proteins), transcription (1 protein), replication, recombination and repair (2 proteins). Cellular processes and signaling category include posttranslational modification, protein turnover, chaperones (3 proteins) and Intracellular trafficking, secretion, and vesicular transport (1 protein). These results indicated that most of the proteins are associated with the secondary metabolite biosynthesis that is the key function of antimicrobial activity of *B. subtilis* SV108.

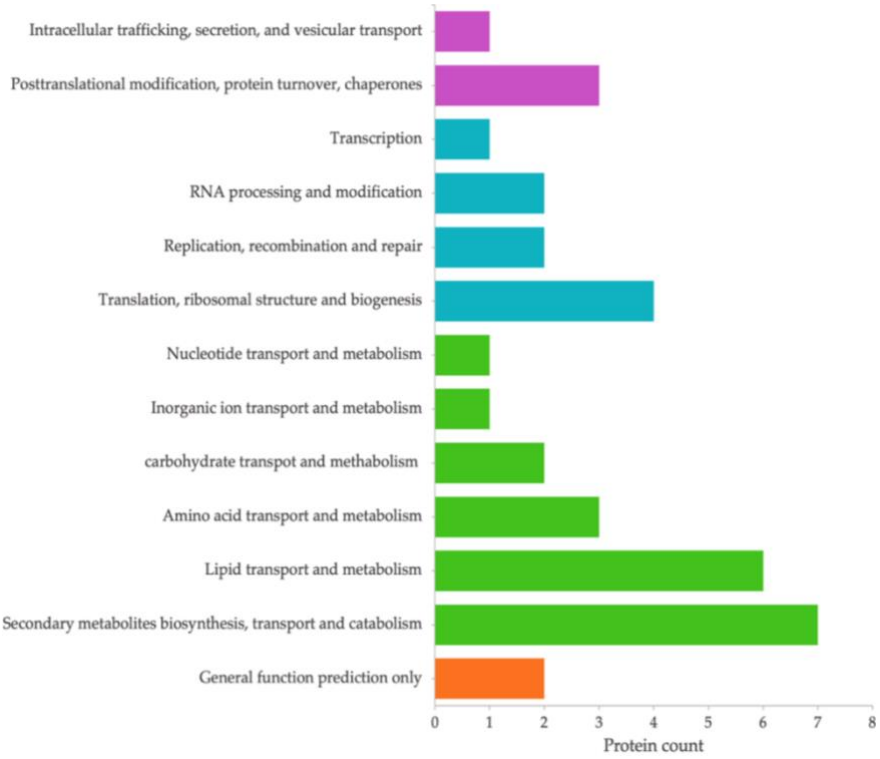

Figure 31. KOG analysis. KOG classification map show the distribution of the entries.

4.3.5 Kyoto Encyclopaedia of genes and genomes (KEGG)

Different proteins coordinate and carry out their biological behavior in vivo, and pathway-based analysis helps to further understand their biological functions. KEGG is the main public database of pathway study (Okuda et al., 2008). Pathway analysis can determine which primary biochemical metabolomic pathways and signal transduction pathways a protein is involved in. The KEGG pathway enrichment analysis was carried out in a KEGG pathway database using Blast_v2.2.26 software. The results showed that identified proteins were annotated to 29

KEGG pathways. All the pathways divided into two levels include three pathways in level one and 14 pathways in level two. The metabolic pathways (ko01100, 14 proteins) were the primary pathway enrichment and the second was the biosynthesis of secondary metabolites (ko01110, 12 proteins; Otoguro & Suzuki, 2018a).

The other activating pathways included the biosynthesis of secondary metabolite pathway, biosynthesis of antibiotic, biosynthesis of amino acid and lysine biosynthesis which had an important role in the antimicrobial activity of *Bacillus subtillis* (Liu et al., 2016; Figure 32).

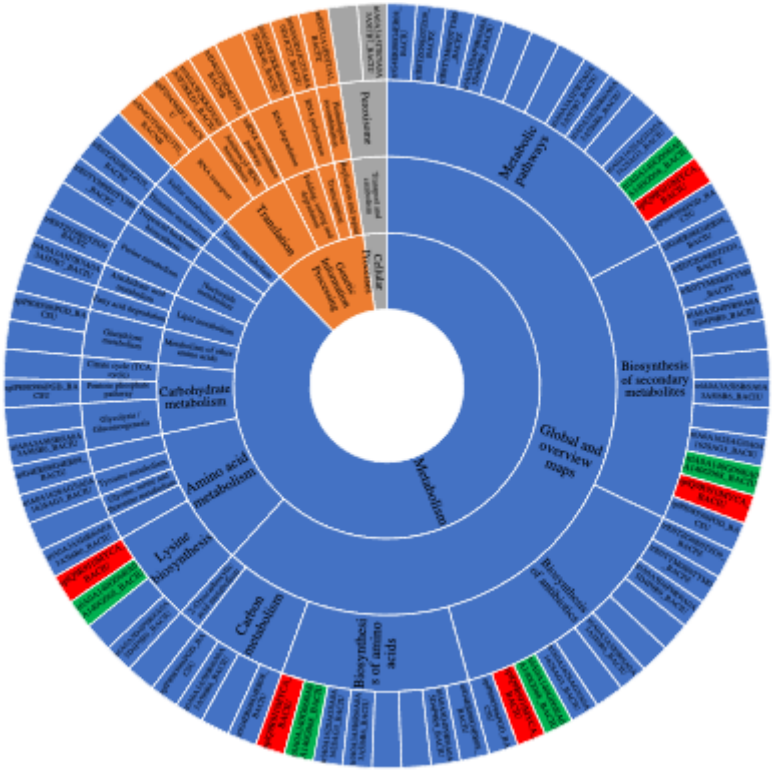

Figure 32. Sun burst chart of the KEGG analysis. Protein related to Iturin B (highlighted in red) and mycosubtilin subgroup A, highlighted in green.

4.4 Discussion

B. subtilis is one of the most studied biocontrol agent and it is widely used in the manufacture of biological control products in agriculture (Liu et al., 2016). For many years *Bacillus spp.* species have been widely used in control of plant disease, industrial enzyme and antibiotic production.

The mechanism of action in *Bacillus* spp. species includes a variety of enzyme synthesis, antibiotic and plant growth hormones production, VOC emitting and inducing the systemic resistance to plants (Chowdhury et al., 2015). Previous researches confirmed the association of VOCs with biocontrol activity of *Bacillus* spp. species (Carmona-Hernandez et al., 2019).

Results of this study demonstrated that *B. subtilis* SV108, an endophyte derived from berries of grapevine, has a potential antimicrobial activity against fungal pathogen inhibiting mycelial growth, especially *A. carbonarious* and *B. cinerea*. The studies performed by Alfonso et al. (2009) are in agreement with this research, reporting the inhibition of a strain of *B. amyloliquefaciens* AG1 on in-vitro growth of four phytopathogenic fungi (*Phaeoacremonium aleophilum*, *Phaemoniella chlamydospora, Fomitiporia mediterranea and Lasiodiplodia theobromae*). For this reason , this isolate was used as a positive control in this study.

A total of 21 VOCs were produced by *B. subtilis* SV108 and *B. amyloliquefaciens* AG1, including mainly alcohols, aldehydes, ketones, esters, acids, Phenol and organic compounds. Most of the identified compounds were known for their antifungal activity. For example, derivatives of Pyrazine, which was the most produced VOC in this study, have proven evidences regarding their role to inhibit the growth of fungal mycelium (Janssens et al., 2019). Yuan et al. (2012) showed the significant impact of Pyrazine on *B. cinerea* mycelial growth inhibition (Yuan et al., 2012). Additionally, Edgar Guevara-Avendaño et al. (2021) revealed that Pyrazine bioactive compound produced by rhizobacteria had high antifungal activity against *Fusarium kuroshium* (Guevara-Avendaño et al., 2020). Another VOC produced by two tested bacteria was acetoin which was increased during the interaction with fungal pathogens. Acetoin has limit impact on inhibit the mycelium growth according to other studies (Wu et al., 2019) but, it has been shown its important role in promoting plant growth and induce systemic resistance and it can describe the reason of increasing the amount of its production in interaction with *B. cinerea* and *A. carbonarious*. Beside this, 1H-Pyrrole and 1H-Imidazole, known for their antimicrobial activity, were produced by *B. subtilis* SV108 and *B. amyloliquefaciens* AG1. 1H-Pyrrole is described as an active antifungal agent (Bhardwaj et al., 2015) and as it shown in Table 3. There is a considerable production of 1H-Pyrrole in interaction with *B. cinerea* in both bacteria (1.53, 1.91 ppm). Furthermore, 1H-Imidazole which is a carbazole-based azole derivation has been reported to have antimicrobial activity and due to have the six carbon chain spacer, it can be a better bioactive compound (Zhang et al., 2010). The production of antifungal antibiotics is the potential mechanisms for the inhibition of fungal pathogen growth, showing a strong wide-ranging scale in antifungal activity (Cho et al., 2003). Previous research showed that, in grapevines, Mycosubtilin is an efficient activator of the innate response, activating the plant immune system and generating varying levels of local resistance to the fungus (Farace et al., 2015). Iturins also can induce cell death in most of fungal pathogen by generating ROS and making the level of NADPH oxidase gene increased (Cao et al., 2011). The mechanism of action of these two homologs is linked to amphiphilic nature.

Lipopeptides such as Iturin A have the power of decreasing surface, tension of biofilms and disrupting the membrane organization (Zhao et al., 2017). The isoform of Iturin A is Mycosubtilin which is able to interrelate with membranes through its sterol alcohol group. Fungi membrane ergosterol is the main target of mycosubtilin letting *Bacillus* spp. to demonstrate resistance to different fungi (Nasir & Besson, 2012). The identified peptides of *Bacillus* spp. species also have been reported in *B subtilis*, *B. clausii*, *B. cereus* and, *B. antracis* and *B. amyloliquefaciens* (Gohar et al., 2002; Eymann et al., 2004; Jeong & Son, 2021; H. Cao et al., 2013).

According to GC/MS7SPME analysis, the identification of the antimicrobial compounds of *B. subtilis* isolate SV108 demonstrated two cyclic non-ribosomal peptides synthetase (NRPS) that are part of the N-terminal sequence of Iturin A synthetase B (ituB) which is one of the four (ituA, ituB and ituC) open reading frame of Iturin A. ItuB is responsible for biosynthesize of Iturin A (Ongena et al., 2005) and enclosed by amino acid activator unit encoded the bacillomycin D's peptide and subunits. Peptides involved in the antibiosis was purified by Alfonso et al. in 2012 and reported two cyclic peptides that related to the N-terminal sequence of Subtilisin BPN (Alfonzo et al., 2012). The two identified peptides may be related to biosynthesize of Iturin family and suggesting the association of *B. subtilis* SV108 antifungal activity.

One of the most frequent used tool for identifying proteins is MALDI-TOF-TOF MS (Nakkeeran et al., 2019), which was employed for identifying of proteins in *B. subtilis* SV108 cell free supernatant (Baslam & Mitsui, 2020). Most of the identified peptides presented in GO and KOG annotation derived from metabolic and catalytic metabolism and secondary metabolite synthetic process. For this, It was suggested to design the experiment for scaling the number of gene expressed associated with inhibition in fungal pathogens for further experiments. The antagonistic compound of biocontrol agents such as secondary metabolite may have different mechanisms (Chen et al., 2010). Cao et al. (2013) suggested that energy metabolism has an important role in antagonism exhibited by strain *Bacillus subtilis* strain G1 (Cao et al., 2013). In this present study, over 50% of identified proteins were perceived to be concerned in glycolysis, citrate cycle and other metabolic pathways correlated with carbohydrate metabolism. It is proposed that the production of high energy is associated with antagonism mechanism (Lushchak, 2011).

Zhang et al. (2017) revealed in a similar study that proteins engaged in basic metabolism were linked to antagonistic activity, which is in agreement with the findings of this

investigation, which showed that the majority of the identified proteins were found in basic metabolic pathways as described also by Zhang et al. (2017).

The majority of *Bacillus* spp. lipopeptides limited the plant disease progression by interfering with certain fungal disease processes (Palanisamy, 2008). As shown in the results (Figure 23), there is a wide range of activating pathways with *B. subtilis* SV108 which suggested significant potential for antifungal action. Some of the identified proteins have been documented to be required for folding, sorting, degradation, replication, repair and transcription. Furthermore, some proteins expressed significantly involved in amino acid and nucleotide metabolism, lipid, cellular and genetic information processing as well as peroxisome metabolism. This may propose that, an improvement in the capacity of antagonistic activity of *B. subtilis* by upgrading in stress resistance and reactions to oxidative stress. Competition for space and nutrient as well as increasing in protein synthesis ability may effect in the production of metabolites and enzymes related to cell wall-degradation (Cao et al., 2013).

More research is needed, including inquiry into the mechanism of the entire interaction between host, biocontrol agent, bacterial, and fungal pathogen interaction, in order to better understand the use of other *B. subtilis* secondary metabolites as biocontrol agents.

4.5 Conclusion

In conclusion, the presented study highlighted the potential *B. subtilis* isolate SV108 to be a biocontrol agent against certain grapevine fungal pathogens. In vineyards, the use of *B. subtilis* SV108 might help to expand integrated pest management strategies to cope with grapevine diseases while reducing application of chemical fungicides. In order to have a full view of the potential of *B. subtilis* SV108 as a biological control agent, more tests of biological activities in the field are needed. Since the secondary metabolites produced by potential biocontrol bacteria demonstrate wide range of biological compounds, it can be used for biotechnological or bio active pharmaceutical prospects. These lipopeptide with natural source can be applied in synthetizing recombinant peptide as well. The *Bacillus* spp. which produce these lipopeptide naturally may utilize as a genetic source for designing novel bioactive peptides. Furthermore, the range of inhibition occurred by *Bacillus* spp. attract the attention of industrial sectors who intend to shift in sustainable production (Fira et al. 2018) . In order to have a complete view of the potential of *B. subtilis* SV108 strain, gene knockout strategies suggested the presence of genetic structure and molecular modifications related to invading fungal pathogens.

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5. General Conclusion of the PhD project

In this study, wild yeasts and bacteria isolated from different Italian and Malaysian regions and molecularly identified, were evaluated for their antimicrobial activity against main grapevine fungal pathogens. On the basis of the obtained results, nine yeasts belonging to genera *Hanseniaspora*, *Starmerella*, *Metschnikowia* and *Candida* were selected and then tested against five grape berry pathogens: *Botrytis cinerea*, *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Fusarium oxysporum*, *Alternaria alternata* and *Phaeomoniella chlamydospore. Starmerella bacillaris* FE08.05 and *Metschnikowia pulcherrima* GP8 and *Hanseniaspora uvarum* GM19 showed the highest effects on inhibiting mycelial growth. Most of the antimicrobial activity was explained, in this case, by the volatile compound produced even if deeper investigations are necessary to study potential antimicrobial peptides produced.

Among bacterial isolates, *Bacillus subtilis* SV108 was selected from isolated strains for further experiments on antimicrobial activity. It has been confirmed to have the ability of inhibit the mycelial growth of *Botrytis cinerea*, *Aspergillus carbonarious* and *Phaeomoniella chlamydospora* by producing antimicrobial compounds. In particular, the VOCs profiles performed by GC/MS/SPME highlighted the presence of Aldehydes, Alcohols and Pyrazines and volatile ethyl esters. In order to have a full view of the potential *B. subtilis* SV108, as a biological control agent, more tests of biological activities in the field are needed. In order to have a complete view of the potential of *B. subtilis* SV108 strain, gene knockout strategies suggested the presence of genetic structure and molecular modifications related to invading fungal pathogens. The proteomic approach used showed the presence of two peptides related to the amino acid sequence of Iturin A synthesize B (ituB) and Mycosubtilin synthesize A (mycA), that belong to *B. subtilis* antibiotic biosynthetic process system.

All the data collected during these studies suggest that antagonist yeasts and bacteria, potentially effective for the biological control of pathogenic moulds, can be found among the epiphytic microbiota associated with grape berries. Consequently, making a consortium of potential antimicrobial bacteria and yeasts could be realized in order to improve the action mechanism of biocontrol the grapevine pathogens.

Unfortunately, due to the Covid Pandemic situation, the final trial in field programmed at the Center of Tebano (Faenza, Italy), devoted to field experiments, was limited to lab trials with table grape berries.