Antagonistic yeasts for biocontrol of non-biotrophic grape fungi: an integrative selection approach

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Ebenézer!

Preface

The work presented in this thesis was performed at Lab Bugworkers | M&B-BioISI of Faculty of Sciences of the University of Lisbon (Lisbon, Portugal), during the period from February 2019 to March 2020, under the supervision of Professor Rogério Tenreiro. The thesis was co-supervised at Instituto Superior Técnico (Lisbon, Portugal) by Professor Miguel Teixeira.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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Abstract

In this work, 85 wine non-*Saccharomyces* yeast strains were evaluated by different *in vitro* assays namely, killer phenotype, production of volatile organic compounds (VOC) and inhibition of mycelial growth which was performed with three procedures. Additionally, *Aspergillus* strain (TLS001) was evaluated by the inhibition of spore germination (ISG) on solid and liquid medium. From a total of 85 yeasts screened, nine exhibited killer phenotype. The results distribution obtained with yeasts of the five genera against TLS001 and FSC040 growth using three approaches were compared. In the three experimental confrontation methods, *Candida* yeasts presented the highest values of antagonism against TLS001. In VOC assay, *Lachancea* displayed the best antagonistic yeasts against TLS001 while *Candida* was most efficient in FSC040 growth inhibition. When all the results were integrated, 27 yeasts were selected. In all methods, Y678 was the yeast more included. From the 27 best- ranked yeasts, a subset of 6 yeasts were selected according to antagonism action displayed. Regarding this subset, further *in vivo* screenings aiming a selection of an efficient biocontrol agent should be applied.

Keywords: Biological control; antagonistic yeasts; Selection; *Aspergillus* rot; Gray mold

Resumo

Neste trabalho, 85 leveduras não-*Saccharomyces* foram avaliadas usando diferentes ensaios *in vitro* nomeadamente factor killer, produção de compostos orgânicos voláteis (COV), inibição do crescimento micelial do fungo, que foi dividido em três métodos. Adicionalmente, foi avaliada a capacidade antagonista da coleção de leveduras contra o fungo TLS001, em inibir a germinação de esporos (ISG) em meio sólido e líquido. Das 85 leveduras testadas, nove exibiram fenótipo killer. A distribuição dos resultados obtidos nos ensaios de confronto contra TLS001 e FSC040 foi avaliada usando três abordagens. Nos três métodos experimentais de confronto, as leveduras *Candida* apresentaram os maiores valores de antagonismo contra TLS001. No ensaio VOC, *Lachancea* apresentou as melhores leveduras antagonistas contra TLS001, enquanto que as de *Candida* foram mais eficientes na inibição do crescimento de FSC040. Quando todos os resultados dos ensaios foram integrados, um total de 27 leveduras foram selecionadas. Nos diferentes métodos, o Y678 foi a levedura mais incluída. Das 27 leveduras mais bem cotadas, um subconjunto de 6 leveduras foi selecionado de acordo com a ação de antagonismo exibida. Em relação a este subconjunto, deverão ser aplicadas novas triagens *in vivo* visando a seleção de um agente de biocontrolo eficiente.

Palavras-chave: Controlo biológico; Leveduras antagonistas; Selecção; Podridão por *Aspergillus*; Podridão Cinzenta

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List of Abbreviations

IER - Inhibition of the target fungal growth rate

IAC – Inhibition of the target fungal growth

IRG - Inhibition of the radial growth of the fungal target

CTY method - target and yeast are inoculated simultaneously

CY method- yeast strains were inoculated and incubated before target inoculation

CT method - yeast strains were inoculated and incubated after target inoculation

VOC – Volatile Organic Compounds

Introduction

1.1. Horticultural commodities and their socio-economic importance

Horticultural crops are essential for a healthy and balanced diet. They have a pivotal role in the agricultural sector, covering almost 21.2 million hectares, with an annual production of 234.5 million tons (Singh *et al.,* 2011). Fruits and vegetables have an important role in human nutrition since they are rich sources of vitamins, minerals, proteins, carbohydrates, and fiber. Moreover, they have components that provide health benefits, including prevention of certain diseases such as cardiovascular diseases, diabetes, obesity, cancer, and respiratory conditions (Vincente *et al.*, 2014; Yahia E., 2019).

From 2000 to 2018, the global production of fresh fruit has increased by 291.44 million tons (Shahbandeh M., 2020). However, about 33% of the total production is lost because of physiological and mechanical factors that occur in the field and the postharvest cycle (FAO, 2011; Dukare *et al.*, 2019). As a result of inadequate storage and transportation facilities, fungal spoilage is one of the most severe causes of these losses, representing up to 25% in developed countries and 50% in non-developed countries (Sharma *et al.*, 2009; Cordero-Bueso *et al.*, 2017; Dukare *et al.*, 2019). The use of synthetic fungicides has been the primary source for controlling these postharvest diseases. However, they are not safe for humans and the environment, being a global concern. Moreover, postharvest chemical treatments can increase the cost of fruits and vegetables several folds from field to consumer (Kasfi *et al.*, 2018).

It is estimated that the global population could grow around 8.5 billion in 2030, 9.7 billion in 2050, reaching 10.9 billion in 2100 (United Nations, 2019; Yahia *et al.*, 2019). Considering the production losses and the exponential growth of the world population, it is essential to counteract this trend of production losses. Thus, greater efforts should focus on research and technologies to reduce postharvest losses and thus to enhance food security and availability through biological, ecological, and safe alternatives.

Viticulture

Grapes along with olives, figs, and dates are considered the oldest domesticated crops (This *et al.*, 2006; Maia *et al.*, 2015; Keller M., 2020). With the archaeological evidence of grapevine domestication dating back from 6 000 to 8 000 years ago in Near East (Myles *et al.*, 2011; Pirrello *et al.*, 2019; Keller, 2020). Grapevines (order Vitales, family Vitaceae) comprise 10 genus however, *Vitis* species are distinguished for their agroeconomic importance. This genus comprises 60 to 70 species and is divided into two major groups: the American and the Eurasian groups (This *et al.*, 2006; Myles *et al.*, 2011; Keller M., 2020). Eurasian grapevine *Vitis vinifera* L. is the most cultivated and prized fruit crop worldwide (Pirrello *et al.*, 2019). According to the report of the International Organization of Vine and Wine (OIV), it is estimated that in 2019, 7,4 million ha of the global area was dedicated to grape production, and this trend has been stable since 2016 (OIV, 2020). Grapes were considered, during 2018, the third major fresh fruit produced worldwide with a yield of about 77,8 million tons (OIV, 2019; Statista, 2019).

Grapes and their products are recognized to have anthocyanins, tannins, and polyphenols, insomuch that their consumption avoids the incidence of degenerative diseases, cancer and prevents inflammations and the aging process (Xia *et al.*, 2010). Grape production was intended for various purposes: mostly for wine production (57%), fresh consumption as table grapes (36%), dried into raisins (7%), and juices, jams, and jellies (Alabi *et al.*, 2016; Solairaj *et al.*, 2020). Wine production during 2019 was estimated at 260 million hl. In the ranking of wine-producing countries were Italy, France, and Spain, jointly liable approximately half of world production. However, Portugal was the only country in the European Union with an increase of 10% of wine production in 2018 (OIV, 2020). In 2019, Portugal dedicated a vineyard area of 195 000 ha and produced about 6.7 million hl of wine being the 11th wine producer and the 9th exporter in the world (OIV, 2019). Portugal is worldwide recognized for its production of the famous Porto Wine and for its vineyards in the terraced landscape that are considered by UNESCO since 2001 a World Heritage (Fraga H., 2019; Santos *et al.*, 2019).

Table grape production needs to be transported over long distances, and it is necessary to store them for several days until it reaches the consumer. Table grapes are a high perishable non-climacteric fruit and unprocessed fresh produce (Vishwakarma *et al.*, 2019). Thus, during the postharvest chain is estimated that 10-40% of total grape production is lost due to fungal attack, mainly during transportation and storage processes. To extend the shelf-life of table grapes during postharvest while maintaining its high-quality in the market with better prices and lower losses has been the focus of the industry (Myles *et al.*, 2011; Kasfi *et al.* 2018; Stocco *et al.*, 2019).

1.2. From the field to the consumer

In 1976, David Spurgeon depicted an idyllic postharvest system, which “encompasses the delivery of a crop from the time and place of harvest to the time and place of consumption, with a minimum loss, maximum efficiency and maximum return for all involved”. Until products reach the market, there is a dynamic chain of food supply that is composed of different interconnected stages (Figure 1), namely harvesting, packaging, storage, and transport. Understanding this dynamic chain is essential to maintaining the quality and quantity of the products.

Harvesting is considered the bridge between pre-harvest and the beginning of the postharvest system (Spurgeon, D., 1976). The time of harvesting is determined by the maturity degree of a horticultural commodity and the weather conditions. Harvest can be manual, semi-mechanical, or mechanical, and should be performed by trained operators (Yahia E., 2019). During the process, it is essential to avoid rough handling to reduce deterioration and quantitative losses since wounds and injuries during handling can be the gateway for pathogens infection on the following stages, especially storage (Eckert J., 1978; Fourie J., 2008; Yahia E., 2019). Packing operation can be done in the field where products are picked, sorted, and packed directly into the shipping boxes, or in a packing house, under controlled temperature, where products are placed onto a packing belt, cleaned (some of them are disinfected to minimize microbiological contamination on produce surface), sorted, and clustered according to quality grade (Zoffoli *et al.*, 2011; Yahia *et al.*, 2019).

During transport and storage, according to the type of transport used (air, land, sea) and the type of products, there are environmental factors that affect the "life" of the products, such as temperature, water loss, and atmosphere gas composition (Vigneault *et al.*, 2009; FAO, 2011; Yahia E., 2019). Temperature is the most important factor affecting the quality of the product since it accelerates physiological processes. For example, fresh fruits and vegetables, even after harvest, remain metabolically active, maintaining respiration. Respiratory activity is the chemical process by which carbohydrates are converted to energy, it consumes oxygen and produces heat, carbon dioxide, and water. Cooling is used to reduce temperature and slow down its metabolic activity, minimizing respiratory activity, which in consequence reduces water loss by transpiration, and decreases ethylene production. Cooling is a fundamental practice that begins with the rapid removal of field heat by precooling and should be maintained by refrigerated transportation and cold storage throughout the chain (Vigneault *et al.*, 2009; Yahia E., 2019). Fresh fruits and vegetables weight are mostly composed of water. As referred, after harvest, products lose water through transpiration. Thus, water loss is the main cause of deterioration and dehydration which results in weight and nutritional quality losses, colour changes, and softening (Palou *et al.*, 2010). The deterioration rate is minimized through the management of relative humidity (90-95%), low temperature, and modified atmosphere gas composition (Ku *et al.*, 2000). Changes in the atmosphere composition are achieved by replacing the air surrounding, reducing oxygen content, and increasing other gaseous mixture (e.g. N_2 an inert gas, or CO_2) (Ben-Yehoshua *et al.*, 2005; Bodbodak *et al.*, 2016). The package has an important role to the quality maintenance of the product since it is a mechanical barrier between the product and the atmosphere surrounding it. Some are designed to allow the creation of a modified atmosphere. Modified atmosphere packaging (MAP) is semi-permeable and allows the accumulation of high humidity inside the package by reducing gas exchanges, insomuch that oxygen content decreases and in turn carbon dioxide content increases, slowing down the respiration process (Tano *et al.*, 2005).

All the processes and factors referred are important and will have a huge impact on the quality on the products until they reach the market and the consumer. When one of these factors fail (e.g., the cold chain be broken or improper handling during the postharvest chain) (Figure 1) besides quality and quantity losses, it will also facilitate the fungal infection and, consequently damage of the product, which will imply economic losses.

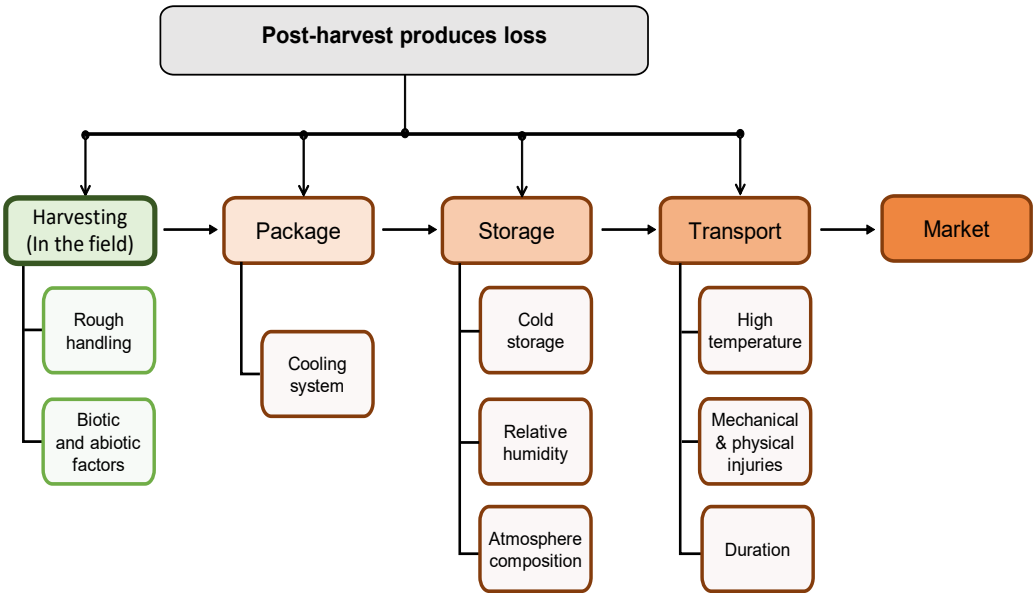

Figure 1- Schematic overview of produces loss during the postharvest system: The main stages of the postharvest system and the respective factors responsible for losses within the chain.

1.3. Postharvest diseases

1.3.1. Postharvest pathogens: a global concern

The postharvest diseases are determined by the interaction between host, pathogen, and environmental factors. Usually, the development of the disease results from an imbalance in this interaction, in which the pathogens have the ability to overcome the complexities of the host's defence responses in favorable conditions for germination (Prusky *et al.*, 1997; Alkan *et al.*, 2015).

Regarding the postharvest fruit decay, the main postharvest pathogenic fungal spoilage reported in produces include species of *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Geotrichum*, *Gloeosporium*, *Mucor*, *Monilinia*, *Penicillium* and *Rhizopus* (Barkai-Goland R., 2001). The fungal infection process can develop in different ways. It can breach the host cuticle during the fruit growth, or they can penetrate taking advantage of a wound. In the first case, the infection can occur with the entrance of fungi in the fruit through natural openings such as lenticels (openings in wood tissue), pedicel-fruit interface, stem ends, and sometimes they live endophytically in the stem ends. In another hand, the infection can occur when fungal pathogen germinates and penetrate the host tissue cuticle through wounds and injuries during the fruit development by biotic and abiotic factors or during handling and throughout the postharvest chain (Prusky *et al.*, 2009; Nunes *et al.*, 2012; Alkan *et al.*, 2015; Dukare *et al.*, 2019). Some pathogenic fungi such as *Alternaria*, *Botrytis*, and *Monilinia* stay in the quiescent stage during fruit development wherefore fungal infection is inactive and unidentified by a visual assessment during harvest. When in favourable conditions, fungal germinate and fruit rot develops. In another hand, atmospheric factors including hail, rain, hydrous imbalances, biotic agents (e.g., insects), ripening, harvesting, and mechanical injuries favors wounds are gateways for fungal infection (Dukare *et al.*, 2019). In addition, after harvesting and during the postharvest chain, fresh fruits and vegetables once detached from the plant lose their intrinsic resistance. Moreover, fruits and vegetables have a high

content in water and nutrients availability and their organic acid content is sufficient to produce low pH values. These factors make fruits susceptible to fungal infections (Droby *et al.*, 1992; Prusky *et al.*, 2009; Stocco *et al.*, 2019).

Besides the quality deterioration and economic losses, some fungi are responsible for mycotoxin produce contamination which is a global health concern. Mycotoxins are secondary metabolites that under specific environmental factors (such as temperature and moisture, water activity, and low pH values) may have ecological advantages enhancing fungi fitness in the ecosystem (Mandappa *et al.*, 2018; Dukare *et al.*, 2019; Kagot *et al.*, 2019). Once fungi are present in the host, mycotoxins can be produced before or at any stage during the postharvest cycle (Stein and Bulboacă, 2017; WHO, 2018). Among several secondary metabolites produced by fungi about 30 have been identified to be a health risk on livestock and in humans. Some potential health issues include liver and kidney diseases, nervous system damage, immunosuppression, and/or carcinogenicity (Pitt J., 2013; WHO, 2018; Kagot *et al.*, 2019). Aflatoxins, produced by *Aspergillus* (mostly *Aspergillus flavus* and *Aspergillus parasiticus)* are the most poison mycotoxins found in food production and feed supplies. *Fusarium* species are responsible for the production of several classes of toxins such as fumonisins, trichothecenes, and zearalenones. Ochratoxins and patulin mycotoxins are produced by *Aspergillus* and *Penicillium* species, being *Penicillium expansum* the main cause of patulin in apples (Pitt J., 2013; Stein R., 2017; Mandappa *et al.*, 2018; WHO, 2018). Once contaminated, products containing mycotoxins are discarded since some toxins are resilient and stable to thermal, physical, and chemical treatments during food processing (Kagot *et al.*, 2019).

1.3.2. Grapevine pathogens that affect grape yield and postharvest quality

Grapevine is susceptible to a wide range of fungal pathogens throughout its lifecycle (Armijo *et al.*, 2016; Kassemeyer *et al.*, 2017). Some of these pathogens are necrotrophic meaning that they feed on dead tissue, secreting lytic enzymes and phytotoxins into the host cells to promote cellular death (i.e., *Botrytis cinerea*). Others are biotrophic and hemibiotrophic pathogens that feed on living tissue, developing structures to invade the hosts cells and obtain metabolism products (i.e., *Erysiphe necator*, *Plasmopara viticola*, *Agrobacterium vitis*, *Xylella fastidiosa*) (Armijo *et al.* 2016). These pathogens are responsible to affect the stems, leaves, inflorescence, and grape berries, triggering disease symptoms in the pre-harvest period or remain quiescent until the postharvest period (Kassemeyer *et al.*, 2017). During the postharvest life of table grapes, *Botrytis cinerea* is the most important pathogen and causal agent of gray mold rot, followed by other saprophytic fungi, namely *Alternaria alternata*, *Aspergillus* spp. (mainly *A. niger*), *Cladosporium* spp., *Mucor* spp., *Penicillium* spp. (mainly *P. expansum*) and *Rhizopus stolonifera*, which causes Alternaria rot, black rot, Cladosporium rot, Mucor rot, blue or green mold rot, and Rhizopus rot, respectively (Lichter *et al.*, 2002; Romanazzi *et al.*, 2012; Sonker *et al.*, 2016; Kassemeyer *et al.*, 2017).

1.3.2.1. *Botrytis cinerea*

Botrytis cinerea, described in 1801 by Persoon (Hennebert G., 1973), is a ubiquitous filamentous fungi and has a large host range causing disease in more than 1000 species of agronomically important crops and harvested commodities such as grape, tomato, strawberry, onion, and ornamental plants (rose, lily and tulip) *(Williamson et al.*, *2007;* Hyde *et al.,* 2014; Aktaruzzaman *et al.,* 2017; Veloso *et al.,* 2018) which makes it as the second most important fungal pathogen (Dean *et al., 2012)*. For these reasons*, B cinerea* has been extensively studied and considered as a model pathogen, to search strategies for gray mold rot management *(*Hua *et al.,* 2018).

The genus *Botrytis* was one of the first genera described of fungi (Rosslenbroich and Stuebler, 2000) and is classified in the family Sclerotiniaceae, order Helotiales and class Leotiomycetes (Pezet *et al.*, 2005; Hyde *et al.*, 2014; Rodenburg *et al.*, 2018). Described by Pier Antonio Michelli in 1729, the genus name is derived from the structure of the macroconidia, which resemble clustered grapes, botryose. The *Botrytis* community in a meeting in Italy (June 2013) discussed the project "One Fungus = One name" and accorded for the exclusive use of the asexual name *Botrytis* over *Botryotinia* the name of the teleomorph, since *Botrytis* is the oldest name (Hyde *et al.*, 2014). *Botrytis* genus comprises 35 described species and *Botrytis cinerea* is the most studied (Valero-Jiménez *et al.*, 2019).

Botrytis cinerea is commonly found in the conidial stage (anamorph) and its characterization has relied on morphological traits such as mycelium, conidia, structure of the conidiophores, and size and form of sclerotia (Figure 2). The formation of ascospores in an apothecium as the sexual stage is rare (Kassemeyer *et al.*, 2017). *Botrytis cinerea* produces abundant greyish-brown mycelium and long, branched conidiophores that have rounded apical cells bearing clusters of colourless or dark-brown, one-celled, ellipsoid, or ovoid conidia. Conidiophores arise singly or brunching in botryose clusters, light brown, and range in size. Sclerotia, the survival structures, are black, flat, convex, and irregular with sizes ranging between 1 and 10 mm (Ellis *et al.*, 1974; Agrios *et al.*, 2005; Terhem *et al.*, 2015).

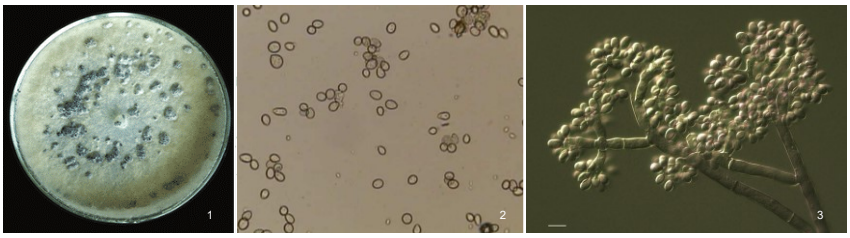

Figure 2 – *Botrytis cinerea* - (1) Cultural characteristics of the colony on PDA, 12 days, 20 °C, with grey-white aerial mycelia and abundant grey to black sclerotia randomly distributed (Source: Zhang ***et al.***, 2010); (2) Conidia cells solitary, hyaline to pale brown with ellipsoidal and globose forms (Source: Miclea ***et al.***, 2012); (3) Conidiophores in clusters. Scale bar = 15 µm (Source: Hocking A., 2014)

Morphological features present limitations in distinguishing *Botrytis* species, since some are morphologically identical, and molecular techniques have been used to overcome these problems (Aktaruzzaman *et al.*, 2017). *Botrytis cinerea* presents a significant genetic diversity and an ability to adapt to various environmental conditions. In order to study this genetic diversity molecular tools have been used such as Restriction Fragment Length Polymorphism (RFLP), presence or absence of transposable elements (*boty* and *flipper*), fingerprinting of repetitive sequences, microsatellite typing,

and phylogenetic positioning. For the phylogenetic analyses are used three nuclear genes sequences of G3PDH encoding glyceraldehyde-3-phosphate dehydrogenase, HSP60 encoding heat-shock protein 60, and RPB2 encoding DNA-dependent RNA polymerase subunit II (Zhang *et al.*, 2010; Kumari *et al.*, 2014; Zhang *et al.*, 2018).

Life cycle and infection strategy (Figure 3)

B.cinerea remains saprophytically overwintering in the vineyard in the form of sclerotia on the vine branches and mycelium on the vine bark or in the soil in organic debris. Infection on grapes can initiate in spring during blossom time, under favourable weather conditions with high relative humidity (> 94 %) and temperatures ranging from 15 to 25 ºC. *B. cinerea* initiates the primary infection through the germination of spores and its dissemination occurs by conidiophores, producing airborne conidia (asexual spores) abundantly. These spores are easily dispersed by biotic (grape moth, powdery mildew infections, fruit fly) or abiotic agents (wind, rain, hail). On the host plant surface, the conidia adhere and germinate 1–3 h after inoculation forming penetration structures. When sugar and nutrients are available, the germ tubes of *B.cinerea* form a multilobed appressorium and colonizes different floral organs killing host tissue. The receptacle constitutes natural openings, being susceptible to infection. The fungus can remain in a quiescent or latent state until the berries ripening without causing disease symptoms (Keller *et al.*, 2003; Viret *et al.*, 2004; Elad *et al.*, 2004; Choquer *et al.*, 2007; Latorre *et al.*, 2016; Kassemeyer *et al.*, 2017; Abbey *et al.*, 2019). The susceptibility of grape berries increases from veraison to ripening (i.e., the sugar concentration increases, and antifungal plant compounds decrease), and the fungus can often grow vigorously and infect all berries in a cluster causing huge losses before harvest (González- Domínguez *et al.*, 2015; Pertot *et al.*, 2017b).

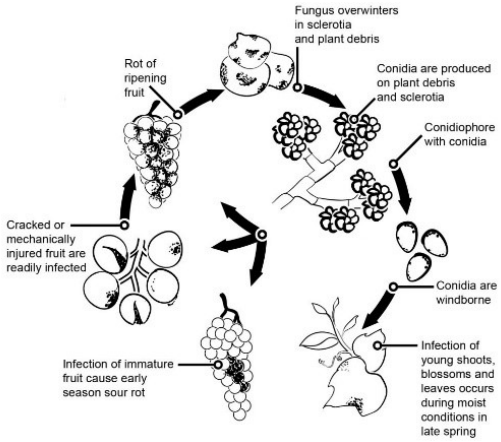

Figure 3 – Infection strategy of *Botrytis cinerea* in grapes: Penetration in the host surface, death of the host tissue (primary lesion formation), lesion expansion/ tissue maceration, and sporulation. Source: METOS, Grapevine disease models: Biology of *B. cinerea* http://metos.at/disease-models-grapevine/#biology-and-life-cycle, accessed on 28 December 2020.

After harvest, particularly during transit and storage, *B. cinerea* is able to grow effectively at low temperatures (0-10 °C) and infect grapes through direct mycelium penetration through surface injuries (Nally *et al.*, 2012; AbuQamar *et al.*, 2017). As a necrotrophic and polyphagous pathogen, the fungus

uses an array of hydrolytic enzymes and metabolites, namely cell wall degrading enzymes (CWDEs) which facilitate infection, colonization, and degradation of host tissues. Through the secretion of enzymes such as endopolygalacturonases, pectin methylesterases, cellulases, and hemicellulases, the fungus cause tissue necrosis (Prins *et al.*, 2000; Elmer *et al.*, 2006; Armijo *et al.*, 2016). Along with this class of enzymes, *B. cinerea* secretes toxins and oxalic acid during infection; the latter acidifies locally the infected region, allowing the activation of pectinases and laccases secreted by the fungus, favouring hyphal growth, and inducing morphogenic signalling of infectious structures (Armijo *et al.*, 2016).

Berry symptoms can delay several weeks until turned visible. The first symptoms begin with water-soaked spots, due to the enzymes produced by the fungus in the cuticle and epidermal cells, and the skin easily slips away from the berry pulp (slip skin). After, the berry changes colour from beige to dark colour, and grape surface lesions are covered with brown rot, along with small, round, reddish- brown necrotic spots. In the last stage of decay, berries may shrivel and lose their juice becoming mummified (Figure 8) . Aside from this, mycelium growing from the infected berry can spread to adjacent berries contaminating other clusters or also, other crops on storage (Raspor *et al.*, 2010; Gubler *et al.*, 2013; Latorre *et al.*, 2016).

1.3.2.2. *Aspergillus* spp.

Aspergillus species are cosmopolitan, ubiquitous, and have a worldwide distribution. Along with its diverse range of ecological habitats and abiotic growth conditions, many species have raised the interest of the biotechnology industry by the production of a wide variety of secondary metabolites. Some species of the genus have been reported by the production of mycotoxins, food spoilage, and as human and animal pathogens (Krijgsheld *et al.*,2013; Samson *et al.*, 2014; Tsang *et al.*, 2018).

The genus *Aspergillus* resides in the family Trichocomaceae, order Eurotiales and, class Eurotiomycetes. Erected by Pier Antonio Michelli in 1729 (Bennett J., 2010) the genus name is derived by on resembling between its conidiophores of aspergillum and a device used to sprinkle holy water. The genus was validated in 1768 by Haller and latter in 1832, Fries sanctioned the generic name (Samson *et al.*, 2014). The classification has relied on morphological traits such as the conidium colour, conidiophore morphology, the growth rate in different media, and physiological characteristics (Raper and Fennell, 1965; Samson *et al.*, 2014; Tsang *et al.*, 2018). In 1965, Raper and Fennell, according to these morphological traits, divided the genus into 18 groups. This infrageneric classification of the genus does not have any nomenclatural status. Then, in 1985 Gams *et al.* introduced the use of *Aspergillus* subgenera and sections. Since then, the infrageneric classification of the genus was subject to several changes that have been overcome with a polyphasic approach. Thus, taxonomic identification incorporates the inference of molecular phylogenetics along with macro and micromorphological, physiological, molecular, and ecological characterization. Currently, in the *Aspergillus* genus are recognized about 400 species which are distributed by into six subgenera *Aspergillus*, *Circumdanti*, *Cremei*, *Fumigati*, *Nidulantes,* and *Polypaecilum* and assigned into 24 sections (*Aspergillus*, *Restricti*; *Candidi*, *Circumdati*, *Flavi*, *Flavipedes*, *Jani*, *Nigri*, *Petersonii*, *Robust*i, *Tanneri*, *Terrei*; *Cervini*, *Clavati*, *Fumigati*; *Aenei*, *Bispori*, *Cavernicolus*, *Ochraceorosei*, *Nidulantes*, *Raperi*, *Silvati*, *Sparsi*, *Usti*) (Krijgsheld *et al.*, 2013; Frisvad *et al.*, 2015; Tsang *et al.*, 2018; Visagie *et al.*, 2020).

Aspergillus rot may be caused by different species of the *Aspergillus* genus. In grapes, they are part of the epiphytic flora and may be present on grape berries (Krijgsheld *et al.*, 2013; Rousseaux *et al.*, 2014; Visagie *et al.*, 2020). Besides food spoilage and yield losses associated, *Aspergillus* rot has been an important concern as *Aspergillus* species produces several toxins (Serra *et al.*, 2005; Allam *et al.*,2008; 2012; Madden *et al.*, 2017). *Aspergillus niger* is reported as one of the species responsible for ochratoxin A (OTA) contamination in grapes and derived products (Abarca, *et al.*, 2019). Although *Aspergillus niger* has been used for industrial applications and certified as being worthy of the GRAS (Generally Regarded as Safe) status followed by *Botrytis cinerea*, the fungi are the primary cause of rot in grapes before harvest (Varga *et al.*, 2011). Infection of the berries with *Botrytis cinerea* leads to the opening of wounds in the skin of the berries and favours the secondary infection with this opportunistic fungi (Kassemeyer *et al.*, 2009; Kasfi *et al.*, 2018).

Aspergillus niger sporulates asexually by forming conidia, conidiophores, and sclerotia. Conidia develop on conidiophores which are sometimes aggregated and visible as a black powdery pad. The unbranched conidiophores terminate in vesicle on which phialides arise (Figure 4). At the tip of the flask-shaped phialides, conidia develop in chains and are airborne. The spores of the *Nigri* section are strongly pigmented and have a high resistance to solar radiation and UV radiation (Kassemeyer *et al.*, 2009).

Symptoms begin when berries become tan to pale brown. The infected tissue becomes water-soaked following loose and slippery of berry skin, and soon covered with masses of brown or black spores. Progressively, the berry is covered with masses of brown or black spores which can scatter for other berries and bunch (Figure 8) (Somma *et al.*, 2012).

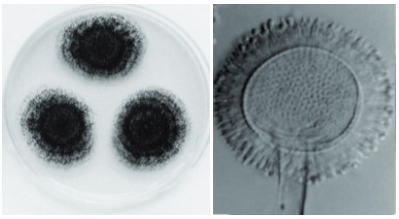

Figure 4 -*Aspergillus niger* - Cultural characteristics of the colony on MEA, 7 days, 25 °C (left) and microscopic features of fungi head with the vesicle, phialides and conidia. Scale bar = 15 µm (Source: Wild *et al.*, 2012).

1.3.2.3. *Cladosporium* spp.

The genus *Cladosporium* was erected by Link in 1816 (Ogórek *et al.*, 2012) and resides in the family Cladosporiaceae, order Capnodiales, and class Dothideomycetes. The genus is constituted by ubiquitous species, the majority are saprotrophs of plant debris and are airborne dispersed. The genus assembles a huge diversity of hyphomycetous ascomycete species and morphologically are characterized to present brown septate hyphae, conidiophores erected, branched, floccose, and pigmented (olive-colored), and conidia globose and ovate. Macroscopically, colonies texture is velvety to powdery, gray-green to olivaceous-green on the front and black from the reverse (Figure 5) (Zalar *et al.*, 2007; Bensch *et al.*, 2012; Ogórek *et al.*, 2012). Within the genus, the species *Cladosporium*

cladosporioides and *Cladosporium herbarum* (the type species) are selected as the main cause of *Cladosporium* rot in grapes. The grape spoilage can begin in the vineyard due to a delay of harvested grapes, but also occurs wound-associated in grapes after a long period at cold storage (Briceño *et al.*, 2007; Kassemeyer *et al.*, 2009).

Symptoms are characterized by the development of dark-green necrotic lesions with a velvety appearance that affects the surface of the berries reaching up two-thirds (Figure 8). Mycelial growth of C.*cladosporioides* and C.*herbarum* is favored by moist conditions. In cold storage, their growth can be delayed, but not inhibited since they have a broad temperature range (4-30 ºC) (Briceño *et al.*, 2007; Kassemeyer *et al.*, 2009).

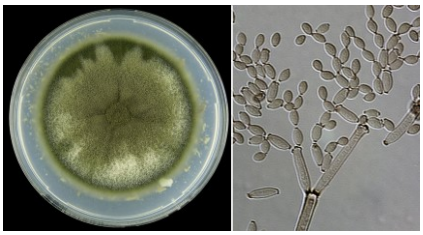

Figure 5 - *Cladosporium cladosporioides*. Cultural traits of colony on PDA, 14 days, 25 °C (left) and microscopic traits of conidiophores and conidial chains (right). Scale bar = 10 µm (Source: Bensch *et al.,* 2018)

1.3.2.4. *Alternaria* spp.

The genus *Alternaria* was first described by Nees von Esenbeck in 1816 (Woudenberg *et al.*, 2013) and resides in the family Pleosporaceae, order Pleosporales and, class Dothideomycetes (Tweedy *et al.*, 1963; Jayawardena *et al.*, 2019). The genus comprises about 280 species which are distributed worldwide as saprophytes and endophytes in several habitats, or plant pathogens with the ability to spoilage a wide range of hosts (Woudenberg *et al.*, 2014; Somma *et al.*, 2019; Stocco *et al.*, 2019). *Alternaria* species are also recognized to produce a variety of secondary metabolites during their active growth and reproduction, in many crops including grape (Woudenberg *et al.*, 2014). Production of phytotoxins makes this genus as an important phytopathogen concern, especially during the storage which leads to a huge yield loss (Mikušová *et al.*, 2014; Dalinova *et al.*, 2020). *Alternaria* rot in table grapes is mainly caused by *Alternaria alternata* (Swart *et al.*, 1994; Kassemeyer *et al.*, 2009; Lorenzini *et al.*, 2014). Morphologically (Figure 6), *Alternaria alternata* (the type species) is characterized to have brown septate hyphae and conidia often in branched chains. The conidia and conidiophore are typically ovoid in shape, with the development of short conical or cylindrical beak at the tip (Kassemeyer *et al.*, 2009; Troncoso-Rojas *et al.*, 2014; Basım *et al.*, 2018). Small conidia, one important characteristic of this species, are ovate and divided by septate and vertical walls. In potato dextrose agar, typical colonies are brown to olive-green with a white margin (Troncoso-Rojas *et al.*, 2014). Alternaria rot can be initiated in the field, when in later summer and autumn with frequent rain, in which fungus enters the berry through capstem until berry ripening and remain latent until storage. During storage, the fungal infection is associated with wounded and injured berry epidermis in high relative humidity conditions (Kassemeyer *et al.*, 2009; Stocco *et al.*, 2019). Symptoms are characterized by dark brown to black located lesions

on the berry surface, commonly named black spots (Swart *et al.*, 1994; Lorenzini *et al.*, 2014) (Figure 8).

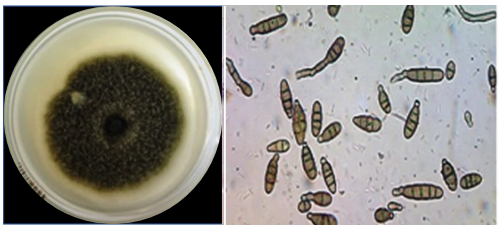

Figure 6 – ***Alternaria* sp.** Cultural traits of the colony on PDA with 12 h photoperiod, 6days, 28 °C (left) and microscopic traits of conidiophores and conidial chains (right). Scale bar = 10 µm (Source: Meena *et al.*, 2017)

1.3.2.5. *Penicillium* spp.

The genus *Penicillium* resides in the family Trichocomaceae, order Euratiales and, class Eurotiomycetes. Erected by John H.F. Link in 1809 (Errampalli *et al.*, 2014), the genus name is derived from the Latine word “Penicillus” which means “little brush” and was given by the resemblance of the conidiophores (Houbraken *et al.*, 2011; Visagie *et al.*, 2014; Phookamsak *et al.*, 2019). The species identification in the genus, before the genomic era, relied only on morphological characteristics namely: colour and texture of colonies, the branching of conidiophores (characteristically simple or branched and terminated by clusters of flask-shaped phialides), shape and ornamentation of conidia (generally dry chains from the tips of the phialides), the growth rate of the colony on standardized media and production of certain extrolites (Errampalli *et al.*, 2014; Yin *et al.*, 2017; Sawant *et al.*, 2019). However, using only these characteristics is often hard. Currently, to an unequivocal identification, is used a polyphasic approach which comprises morphological and genomic data, extrolite and metabolic profiling of the species (Visagie *et al.*, 2014; Yin *et al.*, 2017; Sawant *et al.*, 2019). *Penicillium* genus assembles about 400 species that are distributed worldwide in a diverse range of habitats and substrates (Visagie *et al.*, 2014; Yin *et al.*, 2017; Sawant *et al.*, 2019). Some species have been important in food (production of cheeses) and pharmaceutical industries (penicillin) (Errampalli *et al.*; 2014; Sawant *et al.*, 2019). On the other hand, species such as *Penicillium expansum*, *Penicillium digitatum*, and *Penicillium italicum*, among others, are important plant pathogens that cause food spoilage and economic losses in a wide range of fruits, vegetables, and cereal crops. Besides this, some species are producers of mycotoxins (Serra *et al.*,2007; Díaz *et al.*, 2011; Assaf *et al.*, 2020). *Penicillium expansum* in addition to being one of the major species of *Penicillium* found on grapevines, is the main causal agent of green mould, one of the responsible for grape spoilage in storage. This fungus is known as a wound pathogen, so wounds or injuries provide opportunities for grape spoilage. Furthermore, it was reported that *Penicillium expansum* can germinate at 0 °C and grow at -2 °C which permits production of mycotoxin patulin and contamination (Kassemeyer *et al.*, 2009; Díaz *et al.*, 2011; Errampalli *et al.*, 2014; Hocking A., 2014; Sardella *et al.*, 2018; Mincuzzi *et al.*, 2020). Symptoms are characterized by the observation of white pads in berries wounds. Berries begin softening and change colour from olive-green to light-brown. In a later stage, berries can shrink (Figure 8) (Kassemeyer *et al.*, 2009). Morphologically (Figure 7), *Penicillium expansum* (the type species) is characterized by presenting colonies initially white becoming dull yellow-green to blue-green powdery pad. The plate reverse is usually pale to yellowish. *P.*

expansum feature conidiophores smooth, long, and typically terverticillate. The conidial heads are asymmetric and once or twice branched, presenting a cluster of flask-shaped phialides at the tip of each branch. They bear dull green conidia in chains (Pitt *et al.*, 1997; Kassemeyer *et al.*, 2009; Errampalli *et al.*, 2014). Since this pathogen is dispersed by airborne and presents a mass of spores, even with carefully removing infected berries, conidia can be released for other berries (Kassemeyer *et al.*, 2009).

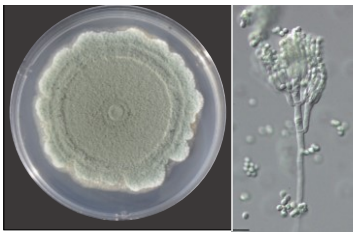

Figure 7 – *Penicillium expansum*. Cultural traits of the colony on PDA, 4 days, 25 °C (left) and microscopic traits of conidiophore with conidial heads, phialides and conidial chains (right). Scale bar = 10 µm (provided by Keith Seifert to Errampalli D., 2014)

1.3.2.6. *Rhizopus* spp. and *Mucor* spp.

The phylum Mucoromycota comprises about 1000 species and is characterized to produce sexual spores named zygospores, asexual reproduction by sporangiospores produced within specialized cells (sporangia), absence of multicellular sporocarps and, production of coenocytic hyphae. In the subphylum Mucoromycotina, the order Mucorales presents 55 genera, including *Mucor* and *Rhizopus*, the more studied genera due to the fact that they cause food spoilage on a wide range of fruits and vegetables. Species of these genera are characterized to be ubiquitous and widespread saprophytes occurring in soil, plant debris, and moist habitats. Indeed, they are classified as important postharvest pathogens due to fast-growing, taking advantage of wounds on fruits and vegetables, causing soft rot in few days (Pitt and Hocking, 2009; Levetin *et al.*, 2016; Money P., 2016; Spatafora *et al.*, 2016; Walther *et al.*, 2019). Morphologically, colonies of *Mucor* and *Rhizopus* grow rapidly and quickly reach the edge of the Petri dish. Their texture resembles a fluffy appearance. Colonies are white initially and become greyish brown (*Rhizopus* colonies may become also yellowish-brown) and the plate reverse is white. *Rhizopus* species are distinguished by the formation of rhizoids (Pitt and Hocking, 2009).

On grapes, the rot begins when a sporangiophore takes advantage of a wound or an injury. Once a berry is infected, mycelia rapidly spread around the site of infection. The fungus produces cellulase and pectinase enzymes and the affected tissue begins to appear water-soaked, followed to softening and white to dark-brown discolouration. White-grey mycelia are formed on infection sites and rapidly the whole fruit and bunch are covered with tufted grey sporangiophores and sporangia (Figure 8) (Gould *et al.*, 2009; Bautista-Baños *et al.*, 2014; Ghuffar *et al.*, 2018).

Figure 8- Symptoms of diseased grape caused by different fungi pathogenic, including ***Botrytis cinerea*** **-1**, **2** (source: Steel, C., 2016) (Taylor A., 2020)**;** ***Aspergillus niger*** – **3, 4** and **5** (source: Mondani *et al.*, 2020), (source: Somma *et al.*, 2012) and (source: Steel, C., 2016); ***Mucor*** **sp**.- **6**,**7** and **8** (source: Sawant *et al.*, 2008); ***Penicillium*** **sp.** – **9** and **10** (source: Lindsay G., 2016) and (source: Kathy E., 2010); ***Alternaria alternata*** - **11** (source: Lindsay G., 2016); ***Cladosporium*** **sp.** - **12** and **13** (source: Steel, C., 2016) and (source: Taylor A., 2020)**;** and **mixed infection** by *Botrytis cinerea* and *Penicillium* sp. - **14**,**15** and **16** (photo: Clark J., source: https://www.lodigrowers.com/bunch-rot-part-ii-sour-rot/, accessed on 28 December 2020).

1.3.3. Disease management: from preventive measures to safe alternatives

Table grapes are one of the major fruit crops worldwide and are susceptible to a wide spectrum of pathogens, which are mainly controlled by the intensive application of chemical fungicides to reach production standards (Perazzolli *et al.*, 2014; Pertot *et al.*, 2017; Bouagga *et al.*, 2019). The control of fungal infections consists in applications of synthetic fungicides on-field during the different phenological growth stages of the grape, and fumigation with sulphur dioxide during storage (Harvey *et al.*, 1978; Parafati *et al.*, 2015). Synthetic fungicides are grouped according to their biochemical action namely fungal respiration, microtube function, osmoregulation, methionine biosynthesis and ergosterol biosynthesis. The most common fungicides applied are cyprodinil and pyrimethanil, which are responsible inhibition of methionine biosynthesis and secretion of hydrolytic enzymes; boscalid which are involved in inhibition of spore germination, germ tube elongation and mycelial growth, and also can block DNA and RNA synthesis and cell division in fungi. The fenhexamid is responsible for ergosterol biosynthesis inhibition, and iprodione can affect the DNA and RNA synthesis and cell division in fungi and consequently the fungi growth. They are more effective as a preventive approach, thus the bunches are sprayed before the appearance of disease symptoms, namely at the end of flowering, bunch closure, at *véraison*, and before harvest (Cabras *et al.*, 2001; Romanazzi *et al.*, 2012; Romanazzi and Feliziani,

2014; Rupp *et al.*, 2017; Kasfi *et al.*, 2018). However, it has been estimated that only about 1% of a pesticide applied reaches the target pest, resulting in accumulation and contamination of the surrounding environment (soil, air, and water) (Pimentel and Levitan,1986; Marinho *et al.*, 2020). Regarding this, fungicides have many active molecules with a wide spectrum of activity thus regular applications might affect nontarget microorganisms, being an important driver of microbial communities including the plant microbiota (Komárek *et al.*, 2010; Perazzolli *et al.*, 2014; Marinho *et al.*, 2020). Indeed, the intensive application is correlated with the stepwise accumulation of resistances overtime leading to pathogens strains with multiple fungicide resistance (Rupp *et al.*, 2017). Table grape postharvest profitability, besides the coordination with relative humidity (90-95%), low temperature (- 0.5 ºC), packaging material, and modified atmosphere gas composition, is principally ensured by the SO_2 application (Lichter *et al.*, 2016; Stocco *et al.*, 2019). Sulphur compounds have been the primary means to control postharvest diseases since the Roman times and they are used due to their effectiveness against a broad spectrum of pathogens and affordable cost (Giraud *et al.*, 2012; Chen *et al.*, 2017; Ahmed *et al.*, 2018). Grape spoilage is avoided through the application of SO_2 by fumigation or generating pads. In the latter, SO_2-generating pads are inside the carton boxes and the gaseous SO_2 is released in the proportion of the reaction of sodium metabisulphite ($Na_2S_2O_5$) with environmental moisture. The rate of gas release may be constantly in low doses or periodically with higher doses (Palou *et al.*, 2010; Ahmed *et al.*, 2018). Nonetheless, it has been reported grape physiological disorders caused by the SO_2 high doses application. When in high concentration, the gas penetrates the berry stem end, through lenticels or skin wounds causing mainly berry bleaching and early browning of the rachis. It may also cause changes in the aftertaste, berry injuries, and health problems in the consumers (Palou *et al.*, 2010; Ahmed *et al.*, 2018). Due to these, SO_2 was removed from the list of compounds classified as safe (GRAS) by the United States Food and Drug Administration (US FDA) and is classified as an adjuvant with a tolerance of 10 μL/L (ppm) for sulphite residues. However, table grapes are one of the products that most exceed the pesticide residues maximum (EFSA *et al.*, 2020). This may impose toxicological problems in human health causing severe diseases. Consumers through life can be exposed to residues cumulatively directly, from ingestion or indirectly through water or air (Giraud *et al.*, 2012; Bouagga *et al.*, 2019). The use of synthetic fungicides has been an important tool for controlling postharvest diseases and keeping food supply networks but is imperative the application of sustainable alternatives (Rebollar-Alviter *et al.*, 2011). A great effort has been done into research and applies these alternatives. Among several, are outlined the physical (with the use of ultraviolet irradiation (UV-C)) (Nigro *et al.*, 1998), the pressure treatments (using hypobaric and hyperbaric pressures) (Romanazzi *et al.*, 2001; 2008), chemicals (the use of gaseous ozone) (Cravero *et al.*, 2016), coatings (with chitosan, aloe vera gel) (Serrano *et al.*, 2006; Freitas *et al.*, 2015), use of weak acids salts (e.g., sodium benzoate, potassium sorbate), essentials oils from the aromatic and medicinal plants. However, some of these approaches need to be applied in higher concentrations to be effective which change the organoleptic conditions of produces (Junior *et al.*, 2016). Thereby biological control is proposed as a better strategy, has the advantage that can be combined with alternatives mentioned to enhance their effectiveness, and also offers a safe alternative while simultaneously safeguarding the health of associated ecosystems and reducing dependency on natural resources (Perazzolli *et al.*, 2014).

1.4. Biological Control: an emergent eco-safe alternative

Biological control or its abbreviated synonym Biocontrol is defined as the use of living organisms or their by-products (i.e metabolites or botanicals) which suppress and/or control the population of plant pathogens through antagonistic activity, keeping the population at the threshold level to be non-harmful avoiding symptoms development (Grønvold *et al.*, 1996; Thomas *et al.*, 1998; Pretscher *et al.*, 2018; Köhl *et al.*, 2019; Raymaekers *et al.*, 2020). The organism that displays antagonistic activity is referred to as Biological Control Agent (BCA). These organisms have been found from diverse taxonomic groups including insects (Laceny *et al.*, 2015), nematodes, protozoans (Grewal *et al.*, 2005; Grønvold *et al.*, 1996), filamentous fungi (e.g. *Thricoderma* spp., *Gliocladium* spp., *Chlonostachys* spp., and *Ulocladium* spp), bacteria (e.g., *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp.), yeasts (e.g., *Aureobasidium* spp., *Candida* spp., *Metschnikowia* spp., and *Cryptococcus* spp.) and virus (Pretscher *et al.*, 2018; Köhl *et al.*, 2019; Raymaekers *et al.*, 2020). Among them, microbial organisms have been identified as biocontrol agents against different pre-and postharvest fungal pathogens in agricultural commodities, and their effectiveness is shaped by a complex network of variables, namely interaction with the host, pathogen, and the environment.

Research of biological control for plant pathogens is not a recent thematic, it was started in 1963 after the International Symposium at Berkel accompanied with the mission of compile knowledge of different disciplines and the assemblage of a multidisciplinary team (plant pathologists, microbiologists, soil science researchers, plant physiologist, plant anatomists, and so one) to better comprehension and further advances on biocontrol (Baker *et al.*, 1965; Spadaro *et al.*, 2005). After almost 60 years of extensive research invested in studying BCA, biopesticides represent a small niche on the global pesticide market (Spadaro *et al.*, 2005; Carmona-Hernandez *et al.*, 2019). Currently, there are some commercial products on the market based on active ingredients of yeast and antagonistic bacteria. They were registered and commercialized as first-generation biocontrol products, i.e., products composed of single antagonists or their by-products. Nowadays, the products available on the market to control postharvest pathogens (Supplementary Table I), include Nexy® (*Candida oleophila*) which was developed in Belgium and received registration approval throughout the European Union in 2013, Boniprotect® (*Aureobasidium pullulans*) for preharvest application, Amylo-X® (*Bacillus amyloliquefaciens*), Biosave® (*Pseudomonas syringae*) which can only be used in US, and Shemer® (*Metschnikowia fructicola*) registered in Israel for both pre and postharvest application on a broad range of fruits and vegetables, representing a successful example of biocontrol product. However, some products were not so successful namely Aspire® (*Candida oleophila*), Yieldplus® (*Cryptococcus albidus*), and Candifruit® (*Candida sake*) which were even commercialized for some years, but discontinued due to business and marketing-related shortcomings, Avogreen® (*Bacillus subtilis*) and Pantovital (*Pantoea agglomerans*) that were formulated but not reached the market (Janisiewicz and Lise, 2002., Droby *et al.*, 2009; Sharma *et al.*, 2009; Teixidó *et al.*, 2011; Droby *et al.,*2016; Usall *et al.*, 2016; Carmona-Hernandez *et al.*, 2019; Dukare *et al.*, 2019).

Research for a biocontrol product should not only analyse the single action of the potential antagonist but also evaluate their performance based on all the network of variables where it is integrated. Therefore, the development of a biocontrol product undergoes through a long drawn and

expensive process that includes several steps (Figure 9), the isolation and screening of *Candida*tes, evaluation of their antagonistic potential by its action and efficacy through bioassays, holistic exploitation of their mode of action, study how to enhance their efficacy, toxicological studies, mass production and formulation, registration and commercialization (Droby *et al.*, 2009; Teixidó *et al.*, 2011; Droby *et al.*, 2015; Parafati *et al.*, 2015).

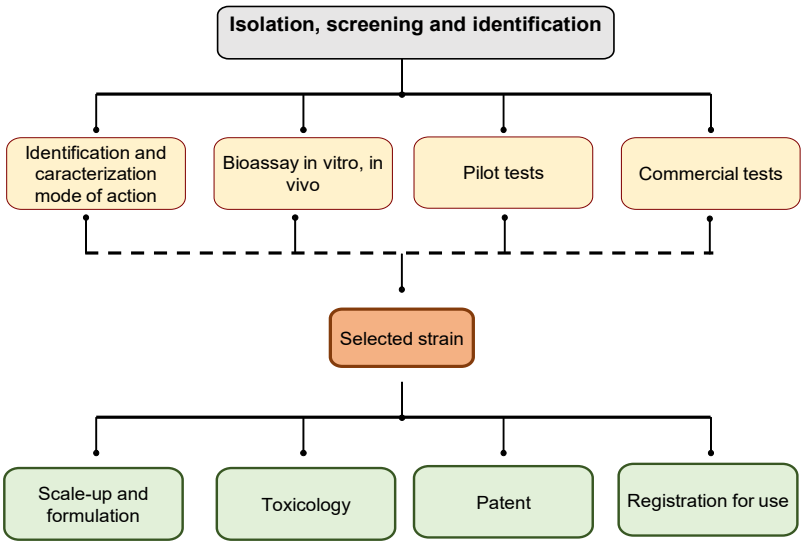

Figure 9 – The main steps involved in the development of a biocontrol product (source: Droby *et al.*, 2009)

1.4.1. Isolation and screening of potential antagonists

The selection of a biocontrol agent begins with the isolation and construction of a collection of isolates. To be a representative sample, isolates should be collected from different geographical regions or from vineyards managed at very different levels of intensity. Then, it should also be considered the isolation procedure and the growth conditions of the collected microorganisms since they have the potential of pre-selection of microorganisms (Köhl *et al.*, 2011; Pliego *et al.*, 2011). To avoid biased results, it is recommended that growth conditions of a potential antagonist should be the same or similar to those that favour the pathogen growth (Köhl *et al.*, 2011; Parafati *et al.*, 2015).

The screening procedure is a very complex process and depends on the aim of the research, number, and type of factors in the study (antagonists, pathogens, host, and environmental conditions), mechanisms of action, costs, and time available (Pliego *et al.*, 2011; Bouaoud *et al.*, 2017). Generally, screening methods are designed based on an increasing level of complexity. First, isolates are screened in rapid-throughput tests allowing to test a huge number of isolates at a low cost. This screening is mainly performed by *in vitro* assays and can be performed with only an isolate to evaluate the production of enzymes (chitinases, cellulases, glucanases, proteases, and siderophores) or with two different microorganisms (in liquid media or semi(solid) medium), to study their interaction (Pliego *et al.*, 2011; Teixidó *et al.*, 2011; Besset-Manzoni *et al.*, 2019; Raymaekers *et al.*, 2020). Potential antagonists expressing the ability to inhibit the growth of pathogens are chosen to the next screen where they are carried throughout complex, costly, and time-consuming assays. These assays integrate a higher number of factors besides the interaction between antagonist and pathogen and mimic the field situation.

This screening may be performed *in vitro*, using the host under controlled environmental conditions, but further goes through field trials (Köhl *et al.*, 2011; Pliego *et al.*, 2011; Besset-Manzoni *et al.*, 2019).

An antagonist microorganism to be selected must also meet biosafety and registration requirements namely genetic stability, not be fastidious and have complex nutritional requirements, the capacity to survive in adverse environmental conditions, be effective at low concentrations, and with a broad range of action against different pathogens in different commodities, amenable to production on inexpensive growth media, amenable to formulation with a long shelf-life, easy to dispense, unable to grow or with impaired growth at 37 °C, and not pathogenic for the host and harmless for plants, animals and humans health, resistant to chemicals applied on the postharvest environment, compatibility with chemical and physical treatments and commercial processing procedures (Wilson and Wisniewski, 1994; Spadaro and Gullino, 2004; Droby *et al.*, 2009; Teixidó *et al.*, 2011; Dukare *et al.*, 2019). Yeasts gather these features and also have advantageous properties for scale-up production (e.g., growth rapidly on inexpensive substrates bioreactors, formulatability, applicability, stress resistance), since they are widely used in the food industry and bioethanol production at industrial scale. Moreover, yeasts have an advantage over bacteria and filamentous fungi since they do not produce allergenic spores or mycotoxins and secondary metabolites (Droby and Chalutz, 1992; Suzzi *et al.*, 1995; Parafati *et al.*, 2015; Fremoiser *et al.*, 2019; Mukherjee *et al.*, 2020).

Microorganisms can be collected from their natural niches where it is known they act as natural antagonists and disease is present (existence of the produce itself) or can be artificially introduced from other produces or ecosystems (Sharma *et al.*, 2009; Devi *et al.*, 2015; Bouaoud *et al.*, 2018). Nevertheless, several studies reported that the best sources are their own natural environments in which they compete with epiphytic pathogens. Thus, using yeasts is an advantage, not only because they are the major component of the microbial community on the surface of the fruits and vegetables, but also are phenotypically well adapted to these niches (Suzzi *et al.*, 1995; Parafati *et al.*, 2015; Grzegorczyk *et al.*, 2017). On grape microbiota, yeasts are also the major component of the microbial communities, since yeast populations are higher than those of bacteria in sound grapes. On immature grape berries, populations range between 10 to 10^3 CFU g^{-1} but when reaching harvest, there is an increase of the populations reaching about 10^6 CFU g^{-1} on mature grapes (Fleet, 2003; Barata *et al.*, 2012; Pantelides *et al.*, 2015; Junior *et al.*, 2016). Also, yeasts belong to the vineyard phylloplane community which is shaped by drought stress, direct UV radiation, wide fluctuations in temperature, low water availability, and limited access to nutrients which makes them remarkable agents of biocontrol grape pathogens (Suzzi *et al.*, 1995; Ippolito and Nigro, 2000).

1.4.2. Mechanisms of action

After isolation, the following step is the exploitation of the pathways and mechanisms underlying action modes of BCA. For yeasts, several possible biocontrol modes of action have been suggested, including antibiosis by release of antimicrobial enzymes, parasitism, and competition for nutrients and space which directly control the pathogen. Indirect antagonism acts through the induction of host resistance (Wisniewski *et al.*, 1992; Droby S., 1994; Janisiewicz *et al.*, 2000; Liu *et al.*, 2003; 2004;

Heydari and Pessarakli, 2010; Spadaro and Droby, 2016; Bubici *et al.*, 2019). The diverse mechanisms of action rely on host, pathogen, and environment (Nunes C., 2012; Droby *et al.*, 2016). Also, more than one mechanism is generally involved, acting synergistically. Thus, it is difficult to discriminate the mechanism responsible for the specific antifungal action (Parafati *et al.*, 2015; Di Francesco *et al.*, 2016; Pretsher *et al.*, 2018).

1.4.2.1. Competition for nutrients and space

The main infection pathway used by pathogens is through fruit injuries and wounds, which are sources of high content of water and nutrients availability (e.g., nitrogen, oxygen, amino acids, vitamins) (Liu *et al.*, 2013; Di Francesco *et al.*, 2016; Köhl *et al.*, 2019). For the pathogens germination and growth in wound sites, nutrients uptake is required.

Competition can be defined as two or more microorganisms requesting the same nutrients (e.g., sugars, vitamins, minerals) in the same niche, preventing access to these resources by other microorganisms. By excluding other microorganisms, nutrients availability is higher for their own needs (Di Francesco *et al.*, 2016; Boynton P., 2019). To be effective the microbial antagonist should be highly competitive, managing to rapidly increase its population, consume nutrient sources quickly, and to be adapted to the ecological niche where it is competing. These abilities will suppress the pathogen population while keeping it in the threshold (Sharma *et al.*, 2009; Di Francesco *et al.*, 2016; Köhl *et al.*, 2019). Competition for nutrients and space is the main mechanism of microbial antagonists against pathogens population (Sharma *et al.*, 2009). Bouaoud *et al.* (2018) demonstrated that P*seudomonas extremorientalis*, *Pseudomonas azotoformans,* and *Pseudomonas helmanticens*is inhibited spore germination of *Botrytis cinerea*, which consequently reduced the development of lesions on tomato. The same mode of action had already been observed in *Enterobacter cloacae* against *Rhizopus stolonifer* on peaches (Wisniewski *et al.*, 1989). On grapes, two epiphytic bacteria were effective in reducing the decay incidence caused by *Aspergillus carbonarius* and *B. cinerea*. Kasfi *et al.* (2018) also identified an epiphytic *Bacillus* sp. strain that was effective in the reduction of the development of *B. cinerea* in wounded berries. Yeasts also act mainly by competing for space and nutrients. These microorganisms have the advantage of high colonization ability, rapid growth, quick depletion of the available nutrients, and are part of epiphytic grape microbiota with whom are natural competitors (Spadaro and Gullino, 2004; Di Francesco *et al.*, 2016). Regarding this aspect, Bleeve *et al.* (2006), reported that epiphytic yeasts *Issatchenkia orientalis, Issatchenkia terricola, Metschnikowia* pulcherrima, and *Candida incommunis,* when inoculated at 10^9 CFU/wound presented fungistatic activity, by inhibiting *A. carbonarius* and *A. niger* colonization on grape berry and consequently their mycotoxin production. However, this mode of action is only efficient when nutrients are scarce. Droby *et al.* (1989) inferred this when they observed a reduction of the antagonistic efficiency with a nutrient supplementation of exogenous nutrients when co-cultivated *Pichia guilliermondii* against *P digitatum* on synthetic media.

Iron depletion in fruit wounds is also a nutrient competition mechanism against postharvest fungal pathogens (Sipiczki M., 2006). Fe^{+3} is an essential nutrient in several metabolic processes and is required at a minimal concentration of 10^{-6} M (Cairo *et al.*, 2006; Shanmugaiah *et al.*, 2015; Di Francesco *et al.*, 2016) The depletion of this nutrient may cause changes in glucose metabolism,

amino acid and lipid biosynthesis (Shakoury-Elizeh *et al.*, 2010). Sequestration of iron is a biocontrol mechanism employed by different taxonomic groups of microorganisms (Sipiczki M., 2006; Spadaro *et al.*, 2011). Bacteria produce siderophores (iron carriers), a low-molecular-weight molecule with a high affinity for ferric ion. At low availability, siderophores scavenge Fe^{+3} of the environment, forming a Fe- siderophore complex, and transport it into the cell making it available (Shanmugaiah *et al.*, 2015). Indeed, *Pseudomonas aureofaciens* inhibited *in vitro* the mycelial growth of rice pathogens *Alternaria* sp., *Fusarium oxysporum,* and *Pyricularia oryzae* through siderophore production (Chaiharn *et al.*, 2009) The same mechanism was also observed in *B. subtilis against Fusarium oxysporum growth (*Yu *et al.*, 2011). Among yeasts, *Metschnikowia* species are the main biocontrol agents reported, controlling phytopathogens through iron depletion. Indeed, Sipiczki (2006) reported for the first time this mechanism in *Metschnikowia* species against *Botrytis cinerea*, Saravanakumar *et al.* (2008) has observed the same in *Metschnikowia pulcherrima* but also against *Alternaria alternata* and *Penicillium expansum* on apple. Yeasts release a water-soluble and diffusible pulcherriminic acid into the environment, which binds non-enzymatically Fe^{3+} and forms an insoluble and non-diffusible iron chelate complex, known as the pulcherrimin (Sipiczki M., 2006; 2020). When pathogen mycelium reaches near this marron-red pigment, surrounding the yeast colony, the iron immobilization leads to physiological changes in fungal cells, including hyphae crack and inhibition of mycelium growth and conidial germination. Although yeasts can biocontrol the pathogen, this inhibitory effect is suppressed when the medium is supplemented with ferric ions (Sipiczki M., 2006; Spadaro *et al.*, 2015; Sipiczki M., 2020).

Besides adherence, attachment, and colonization strategies for space competition, yeasts may also form biofilms in fruit wounds. They create an extracellular matrix that acts as a mechanical barrier that stands between the wound and pathogen (Carmona-Hernandez *et al.*, 2019). Biofilm also confers higher tolerance to heat and oxidative stress which ensure biocontrol efficacy (Pu *et al.*, 2014; Chi *et al.*, 2015). This mechanism was shown in *M. pulcherrima* and *Wickerhamomyces anomalus* strains against gray mould decay (Parafati *et al.*, 2015) and by a *Pichia fermentans* strain which controlled brown rot on apple fruit. However, when applied in peach fruit, *Pichia fermentans* strain changed the morphology from budding growth to pseudohyphal growth causing disease on the fruit (Giobbe *et al.*, 2007).

1.4.2.2. Release of antimicrobial enzymes, metabolites and toxins

Another mode of action of many antagonists is the release of metabolites or their by-products which will interferes with pathogen growth, either by inhibiting or killing it (Heydari and Pessarakli, 2010; Di Francesco *et al.*, 2016).

Fungal cell wall is constituted mainly by chitin, chitosan, glucans, and glycosylated proteins which are involved in the cell wall maintenance and protection against biotic and abiotic stresses (Spadaro *et al.*, 2011; Garcia-Rubio *et al.*, 2020). Biocontrol agents producers of lytic enzymes, (including chitinases, glucanases, lipases, and proteases), that can breakdown the fungal cellular components, leading to cell wall modification and disintegration. This mechanism was observed by an epiphytic yeast of citrus, *Pseudozyma antarctica*, which degraded the cell wall of *Penicillium digitatum* and *Penicillium italicum* through the secretion of lytic enzymes (Liu *et al.*, 2019). Castoria *et al.* (2001)

also observed the suppression of *B. cinerea* and *P. expansum* growth in apple wounds through exo chitinase and β-1-3-glucanase released by *Aureobasidium pullulans*. Secretion of β-1,3 glucanases by *P. membranifaciens* also suppressed the *B. cinerea* growth *in vitro* (Masih and Paul, 2002). This result was also supported by *W. anomalus* strain K against *B. cinerea* in apple (Jijakli and Lepoivre, 1998).

Production of volatile organic compounds (VOCs) is also a biocontrol mechanism used by several microorganisms. VOCs are small lipophilic molecules with low molecular weight, low polarity, and high vapour pressure which are produced during microorganisms metabolism (Korpi *et al.*, 2009; Schulz-Bohm *et al.*, 2017; Tilocca *et al.*, 2020) to be used during cross-talk interactions or antimicrobial antagonism (Tilocca *et al.*, 2020). Since the description of this mechanism in *Muscodor albus* (an endophytic ascomycete from cinnamon tree) and their antagonist effect against a wide range of microorganisms (Strobel *et al.*, 2001), increased effort has been dedicated in their exploitation, mainly for biotechnological purposes (Schulz-Bohm *et al.*, 2017). Indeed, VOCs have the advantage to be effective at low concentrations and diffusible (by water and air), which permits the spread of the activity to long-distances from where they were applied. Also do not require physical contact between the VOC-producer, target pathogen, and commodity. In this regard, VOCs are biodegradable and do not leave residues on commodity, making it an environmental-friendly alternative for replacing fumigant chemicals used during postharvest storage and transport (Kanchiswamy *et al.*, 2015; Spadaro *et al.*, 2016; Freimoser *et al.*, 2019; Tilocca *et al.*, 2020). VOCs are a blend of heterogeneous metabolites, termed as volatilome, which comprises different molecular classes, namely hydrocarbons, alcohols, thioalcohols, aldehydes, ketones, thioesters, cyclohexanes, heterocyclic compounds, phenols and benzene derivatives (Freimoser *et al.*, 2019; Tilocca *et al.*, 2020). Yeasts can produce a wide range of VOCs including alcohols, aldehydes, and esters (Fialho *et al.*, 2011) which, besides spore germination and mycelial growth inhibition, also control the expression of genes involved in toxins biosynthesis (Hua *et al.*, 2014; Farbo *et al.*, 2018). Since the antifungal activity of VOCs does not seems to be dependent only on single compound release, as they are produced in low concentrations, a synergic effect of a mixture of compounds is needed to enhance their antagonism (Fialho *et al.*, 2011; Don *et al.*, 2020).

Some yeasts can also modulate the community through the killer phenotype. Killer toxins, also referred to as mycocins, zymocins or zymocides, are proteins or glycoproteins of low molecular weight produced by killer yeasts, and described by Bevan and Makover (Bevan and Makover, 1963) in brewing strains of *S. cerevisiae*. Yeasts can be categorized into three possible phenotypes, killer, neutral or sensitive. The killer phenotype has the ability to kill sensitive strains from the same or different species and genus but immune to the activity of owner toxins. Neutral phenotype does not secrete but is resistant to killer toxins and, sensitives yeasts are affected by the killer toxins action (Rogers *et al.*, 1978; Mannazzu *et al.*, 2019). This phenomenon is not restricted to the genus *Saccharomyces* as it is taxonomically widespread (El-Banna *et al.*, 2011). In fact, it can be observed in more than 100 yeast species belonging to ascomycetous and basidiomycetous genera isolated from different geographic regions including Antarctica and diverse ecological niches (Buzzini *et al.*, 2007; Liu *et al.*, 2015; Bajaj and Singh, 2017; Boynton, 2019). Killer toxins can be encoded by chromosomal genes (mainly

observed in *S. cerevisiae* strains), extrachromosomal double-stranded RNA virus-like particles, or by extrachromosomal double-stranded DNA virus-like elements in the cytoplasm (Liu *et al.*, 2015; Bajaj and Singh, 2017; Boynton, 2019). The killer phenomenon seems to be mediated by a two-step mode of action. The killer toxin recognizes and binds to a primary receptor usually located on the cell wall of the sensitive strain. Depending on the target microorganism, the cell wall receptor can be a glucan, mannoprotein, chitin, or mannan which have an essential role for the killer action efficacy (Liu *et al.*, 2015). Then, the killer toxin is translocated to the secondary receptors in the plasma membrane. Once inside the target-cell, this killer toxin acts through various mechanisms namely changes in membrane permeability, inhibition of DNA replication, fragmentation of RNA, and inhibition of cell wall synthesis. The efficacy of killer toxins relies on pH values (between 3 to 5.5), salt concentrations (NaCl), and temperature (Liu *et al.*, 2015; Bajaj and Singh, 2017; Boynton P., 2019).

1.4.2.3. Mycoparasitism

Mycoparasitism is a direct physical competition between microorganisms in which antagonists recognize the pathogen, attack, and wreck cell wall and fungal structures. To kill the pathogen, BCA generally releases hydrolytic enzymes. In predatory mechanism, BCA physically attacks, kill, and consumes the pathogen (Junker *et al.*, 2019; Köhl *et al.*, 2019). The antagonists can prey the host with the aid of other mechanisms, including nutrient competition, the release of hydrolytic enzymes and lectins, morphological changes (coiling around the pathogen hypha), and development of appressorium-like structures (infection pegs) which leads to penetration and death of the pathogen. The efficacy of antagonism seems to be correlated to the presence of organic sulphur (Lachance and Pang, 1997; Zeilinger and Omann, 2007; Lachance *et al.*, 2012; Junker *et al.*, 2019). Necrotrophic parasitism is a biocontrol mechanism characteristic of yeasts belonging to the genus *Saccharomycopsis* (Lachance *et al.*, 2012; Junker *et al.*, 2019).

1.4.2.4. Induced resistance

The fruit can also respond against pathogens infection with the activation of different metabolic and physiologic pathways. Induction of resistance is initially mediated by systemic acquired resistance (SAR) where is activated the salicylic acid (SA) signalling pathway, following the expression of pathogenesis-related (PR)-proteins (Heydari and Pessarakli, 2010). Then is activated the induced systemic resistance (ISR) pathway, mediated by jasmonic acid (JA) and or ethylene (ET) signalling. Thus, a variety of defense-related genes are expressed including inhibitors of cell wall-degrading enzymes of the pathogen, catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD) (Pétriacq *et al.*, 2018); activation of the antioxidant machinery (reactive oxygen species (ROS)), and reinforcement of their cell wall (Trotel-Aziz *et al.*, 2008; Spadaro and Droby, 2016; Zhang *et al.*, 2020). BCA can induce or enhance the activation of resistance mechanisms in fruit against pathogens infection. Zhang *et al.* (2020) described this in *Pichia membranefaciens* and observed their indirect effect in the activation of resistance mechanisms against Rhizopus rot in peach fruit. The yeast

triggered the MAPK cascade signalling pathway and mediate the expression of defense-related genes which resulted in the regulation of 25 proteins namely antioxidants and PR-proteins in the fruit.

1.5. Taxonomic review of some yeast genera

Yeasts were classified in 1755 for the Dictionary of the English Language by Samuel Johnson as "the ferment put into the drink to make it work, and into bread to lighten and swell it" (Johnson S, 1755). However, between 1836 and 1838, Charles Cagniard-Latour, Friedrich Traugott Kutzing, and Theodor Schwann through independent studies, concluded that yeast is a living organism and Schwann assigned the first descriptions of the asexual reproduction of the cell (budding) considering it as a fungus (Barnett J., 1998; 2004; Alba-Lois, 2010). Yeasts are a group of eukaryotic microfungi defined as fungi belonging to *Ascomycota* or *Basidiomycota* phyla, whose sexual state has no fruiting bodies and vegetative growth occurs by budding or fission (Deák *et al.*, 2013; Kurtzman C., 2017; Rosa L., 2019). The term “yeast” is not considered a taxonomic category neither a monophyletic group since they are distributed all over Basidiomycota and Ascomycota phyla. The phylum Basidiomycota comprises 32 000 described species distributed in 1589 genera and is characterized by the presence of basidia and basidiospores (Zhao *et al.*, 2017). Basidiomycetous yeasts are a polyphyletic group and are distributed by three major subphyla, namely Agaricomycotina, Pucciniomycotina, and Ustilaginomycotina (Kurtzman *et al.*, 2015). The asexual reproduction is generally characterized by enteroblastic conidia, arthroconidia or ballistoconidia, the presence of dikaryotic hyphae with clamp connections, and the sexual production of teliospores and/or basidia (Deák *et al.*, 2013). Cell wall structures such as pseudonigeran are also common in species of basidiomycetous yeasts (e.g. *Cryptococcus*) (Lachance *et al.*, 2018). Some basidiomycetous yeasts have been used for biological control of fungal plant pathogens, such as *Rhodotorula*, *Rhodosporium*, and *Cryptococcus* species (Ferraz *et al.*, 2019). The phylum Ascomycota comprises the majority of all described species which are distributed by Taphrinomycotina and Saccharomycotina subphyla (Naranjo-Ortiz *et al.*, 2019). They are characterized to have naked asci and reproduce asexually by budding (Gabaldón *et al.*, 2016). Several yeasts from the Saccharomycotina subphylum have been referred to as biocontrol agents of fungal pathogens (Kurtzman and Sugiyama, 2015). Regarding this, are outlined the ecological, taxonomic, and biological control features of the five genera of the subphylum that encompass the yeast strains used in the present work:

Metschnikowiaceae *family: Metschnikowia;*

Saccharomycopsidaceae family: *Saccharomycopsis*;

Saccharomycetaceae family: Candida, *Lachancea* and *Torulaspora.*

Metschnikowia genus comprises 79 species (Lachance *et al.*, 2016; Vicente *et al.*, 2020) which are widely distributed. Some clades are ecologically adapted to the phyllosphere, nectar insects, marine animals, and fruit-feeding insects (Lachance *et al.*, 2016; Freimoser *et al.*, 2019). Reproduction is characterized by multilateral budding and the formation of large needle-shaped spores in elongated asci (Vicente *et al.*, 2020). *Metschnikowia* species have been attractive as biocontrol agents, especially *M. fruticola* which was recently registered as a biocontrol product and *M. pulcherrima* (Freimoser *et*

al., 2019). As referred in section 4.2.1, species of *M. pulcherrima* clade are known by the production of pulcherreminic acid (Lachance *et al.*, 2016; Freimoser *et al.*, 2019). Besides this, *Metschnikowia* species presents several other ways of action namely competition for nutrients and space, stress tolerance, secretion of glucanases, chitinases, glucosidases, and the production of volatile organic compounds (Freimoser *et al.*, 2019; Pawlikowska *et al.*, 2019).

Saccharomycopsis genus comprises 12 recognized species *S. capsularis* (type species), *S. crataegensis*, *S. fermentans*, *S. fibuligera*, *S. fodiens*, *S. javanensis*; *S. malanga*, *S. microspora*, *S. schoenii*, *S. selenospora*, *S. synnaedendra*, *S. vini*; three new combinations *S. amapae*, *S. babjevae, S. lassenensis* and two new species *S. guyanensis* and *S. olivae* (Jacques *et al.*, 2014). Morphologically, they are characterized by multilateral budding and septate hyphae. Ascospores shape may be hat-shaped, spheroidal to elongate, and with or without equatorial ledges or short polar appendages (Jacques *et al.*, 2014). *Saccharomycopsis* species are known as necrotrophic mycoparasites and their antifungal action involves invading fungal prey cells with small haustoria-like penetration pegs, which leads to prey death (Lachance *et al.*, 2012; Junker *et al.*, 2019). Indeed, was reported *S. schoenii* ability through predation, attack, and kill of a range of pathogenic *Candida* species including multi-drug resistant isolates of *C. auris* (Junker *et al.*, 2018). Also, it has anti-fungal activity against *Penicillium italicum*, *P. digitatum,* and *P. expansum* on oranges (Pimenta *et al.*, 2008). *S. crataegensis* also present antifungal action against *P. digitatum* on oranges and presents tolerance to different sodium bicarbonate concentrations (Pimenta *et al.*, 2010). *S. fibuligera* display antagonistic activity against *Aspergillus ochraceus* and *Penicillium nordicum* on fresh meat cuts for speck production (Iacumin *et al.*, 2017).

Candida is a highly polyphyletic genus which assembles about a quarter of all yeast species, namely 314 species, with *C. vulgaris* as the type species (Schauer and Hanschke, 1999; Lachance *et al.*, 2011; García *et al.*, 2018). Species of the genus are widely distributed (Kieliszek *et al.*, 2017). There are several species with antagonist features including *C. diversa*, *C. ernobii*, *C. guillermonidi*, *C. intermedia, C. oleophila*, *C. saitoana*, *C. sake*, *C. subhashii* which have been envisioned as biocontrol agents against postharvest pathogens of pome, stone, and citrus fruit. Also, *C. oleophila* was the first yeast to be developed to a biocontrol product. The antifungal activity of *Candida* species is widespread and among the several biocontrol mechanism that includes, competition for nutrients and space, production of lytic enzymes and volatile organic compounds, biofilm formation, induction of host resistance and direct parasitism (Huang *et al.*, 2011; *Freimoser et al.*, 2019; Tilocca *et al.*, 2019).

Lachancea genus comprises 11 species, *L. thermotolerans* (type species), *L. cidri, L. dasiensis, L. fermentati, L. kluyveri, L. lazarotensis , L. meyersii, L. mirantina, L. nothofagi, L. quebecensis* and *L. waltii*. *Lachancea* species are ubiquitous and are widespread in diverse ecological niches, including plant products or association with plants, plant-associated insects, food, and beverages (Morata *et al.*, 2018; Porter *et al.*, 2019). *L. thermotolerans* has several anti-fungal modes of action that involve the production of volatile compounds (mainly 2-phenylethanol) against *Aspergillus carbonarius* and *A. ochraceus* inhibiting of both mycelial growth and ochratoxin A accumulation (Ponsone *et al.*, 2011; Fiori *et al.*, 2014; Ponsone *et al.*, 2016; Pawlikowska *et al.*, 2019). It has also

reported effectiveness against *Aspergillus* section *Nigri* on grape berries and in field trials through competition for space and nutrients (Ponsone *et al.*, 2011; Fiori *et al.*, 2014; Ponsone *et al.*, 2016). It also showed killer activity against *S.cerevisae* strains (Aponte *et al.*, 2016). This mode of action was also reported in *L. waltii* which presented killer activity against *Schizosaccharomyces pombe* (Kono *et al.*, 1997).

Torulaspora genus comprises six recognized species *T. delbrueckii* (type species), *T. franciscae, T. pretoriensis, T. microellipsoides, T. globosa, T. maleeae (*Benito *et al.*, 2018)*, and* two proposed species*, T. indica (*Saluja, *et al.*, 2012) *and T. quercuum* which occupy a myriad of natural and anthropic habitats (Wang *et al.*, 2009). Morphological features that permit to differentiate *Torulaspora spp.* from *Saccharomyces, Zygosaccharomyces,* and *Debaryomyces* species, are the cells being predominantly haploid and the ascospores usually roughened (Kurtzman *et al.*, 2011). Antifungal activity of *T.delbrueckii* is achieved by the production of a killer toxin against wine spoilage species (Ramírez *et al.*, 2015) and the release of glucanase and chitinase enzymes (Villalba *et al.*, 2016), it is also resistant to SO_2 treatment and tolerant to stressful conditions (Simonin *et al.*, 2018). Also, for *T. globose* it was reported killer activity and biocontrol action against phytopathogenic mold *Colletotrichum sublineolum* through antagonism by competition for space and nutrients (Rosa *et al.*, 2010).

1.6. Dissertation purpose and outline

Presently, one of the main challenges for the wine industry is to limit the use of chemicals in its production chain to control microbiological agents responsible for vine disease and wine spoilage, while ensuring the yield and quality of grapes and wines produced. To address this issue, a R&D project – ABCyeasts (Project nº 039/93 Norte 2020) - promoted by a consortium constituted Proenol Indústria Biotecnológica Lda, Universidade de Trás-os-Montes e Alto Douro (UTAD), Associação Desenvolvimento da Viticultura Duriense (ADVID) and Sogrape was established fostering the research and the development of yeasts-based products having an antagonistic action against phytopathogenic agents in the vineyard as well as against postharvest contamination microorganisms.

In this line, this dissertation developed in the frame of this project, aims the evaluation of the potential biocontrol activity of a collection of a wild non-*Saccharomyces* yeasts against *Aspergillus* spp. and *Botrytis cinerea* strains, two common contaminating agents found in viticultural sector. For this purpose, 85 wine yeast strains were evaluated. In addition, and in order to anticipate the potential underlying mechanism of fungal inhibition by these yeasts, different *in vitro* antagonism assays were used.

2. Materials and methods

2.1 Fungal strains

2.1.1 Isolation and identification at the genus level

In the context of ABCyeasts project, shoots, buds, leaves, and wine grapes from grapevines were collected from several vineyards of Douro region. Samples were collected in 2019 by ADVID team and sent to Lab Bugworkers (M&B BioISI | FCUL) for isolation of potential phytopathogenic agents. Furthermore, convenience samples of diseased table grapes from supermarket and symptomatic vine leaves were additionally collected in Lisbon district.

The isolations were made from conidia with stereomicroscope or by directly plating out pieces of diseased tissue (about 2 mm^2) on half-strength Potato Dextrose Agar (1/2 PDA) (BIOKAR Diagnostics, France). Cultures were incubated at 25 ºC in the dark for 3-5 d and sub-cultured for purification purpose. After purification, isolates were identified to the genus level based on cultural and phenotypic traits. Fungal cultures were stored on slants of half-strength PDA at 4 °C.

Two reference strains belonging to the Lab Bugworkers collection, one of *Aspergillus* section *Nigri* (TLS001; isolated from cork) and one of *Botrytis cinerea* (BCF1; isolated from spoiled food), were also used.

Fungal spore suspensions were prepared by collecting spores from 15 d old colonies (grown on PDA at 25 ºC in the dark) in PBS buffer 1X with 0.01% Tween 20, added to assist the dispersal of conidia. The colonies surface was scraped with glass beads, followed by filtration through sterile glass wool. The spore concentration was determined using a haemocytometer and the suspension was stored in 50% (v/v) glycerol at – 80 ºC.

2.1.2 DNA extraction

Genomic DNA (gDNA) was extracted from a pure culture, with 3-5 d growth on PDA at 25 °C in the dark, following a modified version of the guanidium thiocyanate method described by Pitcher *et al.* (1989). The mycelial culture was scrapped and collected in a 2mL microtube with 250 µL of lysis buffer (50 mM Tris, 250 mM NaCl, 50 mM EDTA, 0.3% (w/v) SDS, pH 8.0) and 100 µL of autoclaved glass microspheres. To facilitate cell disruption, microtubes were placed on ice for 10 min followed by 2 min of homogenization using a vortex with maximum velocity. This step was repeated after incubation at 65 °C for 30 min. Hereupon, 250 µL of GES reagent were added (5 M guanidium thiocyanate, 100 mM EDTA, 0.5% (v/v) Sarkosyl, pH 8.0) and, after a mix by inversion, microtubes were kept on ice for 10 min. This last step was repeated after the addition of 125 µL of 10 M ammonium acetate. To DNA extraction 1 mL of chloroform/isoamyl alcohol (24:1) (v/v) was added followed by inversion and centrifugation at 14 000 rpm for 10 min. The supernatant was recovered into a new 1.5 mL microtube to which was added an equal volume of cold absolute isopropanol, mixed by inversion followed by

centrifugation at 14 000 rpm for 10 min. For DNA precipitation, the supernatant was discarded, and the DNA pellet was washed with 1 mL of cold 70% (v/v) ethanol followed by centrifugation at 14 000 rpm for 10 min. The supernatant was discarded, and the pellets dried at 60°C for 5 min. DNA extracts were dissolved in 100 µL TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0) and stored at 4°C.

2.1.3 Molecular fingerprinting

Microsatellite and minisatellite markers are tandem repeats of DNA with 1-5 and 10-60 bp size, respectively. These regions are highly polymorphic and widely distributed throughout the genome, allowing the characterization of fungal population structure (Alves *et al.*, 2007; Kumari *et al.*, 2014; Vieira *et al.*, 2016; Varady *et al.*, 2019). The microsatellite-primed (MSP) -PCR fingerprinting technique, which relies on the use of oligonucleotides as single primers that are complementary to highly conserved and repeated sequences present in the fungal genomes, was used to assess the genomic diversity of *Aspergillus* and *Botrytis* isolates. These variable number of repetitions generate specific patterns which allow the inter-and/or intraspecific levels of fungi genus (Meyer *et al.*, 2001; Alves *et al.*, 2007). Regarding this, MSP-PCR fingerprinting can also be used as a preliminary clustering procedure in which representative isolates of the cluster are further sequenced (Ramirez-Castrillón *et al.*, 2014). Based on this, the reference strains of *Aspergillus* (TSL001) and *Botrytis cinerea* (BCF1) were also included.

PCR fingerprinting was carried out using csM13 (5' GAGGGTGGCGGTTCT 3'), $(GTG)_5$ (5' GTGGTGGTGGTGGTG 3') and $(GACA)_4$ (5′ GACAGACAGACAGACA 3′) as single primers (Meyer *et al.*, 1993). Each amplification reaction was performed in a final volume of 25 µL, containing 1× PCR reaction buffer (Invitrogen, UK), 3 mM $MgCl_2$, 25 µM of the respective primer, 0.2 mM of each four dNTP, 1 U of Taq DNA Polymerase (Invitrogen, UK) and 1 µL (50-100 ng) of DNA template. Amplification reactions were performed in a TGradient thermocycler (Biometra, Germany). For csM13 and $(GTG)_5$ the following amplification conditions were used: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 ℃ for 1 min, annealing at 60 ℃ for 2 min, and elongation at 72 ℃ for 2 min, and a final elongation step at 72 ℃ for 5 min. For $(GACA)_4$ the amplification conditions were: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. After amplification, 4.5 µL of each PCR product were separated by electrophoresis in a 1% (w/v) agarose gel, with 0.5x TBE buffer (40 mM Tris, 45 mM Boric acid, 1mM EDTA, pH 8.3) and 1kb Plus DNA Ladder (Invitrogen) at both sides of each gel and a constant voltage of 3.4 V/cm for 5 h. The gel was stained with 2.5 µg/mL ethidium bromide solution and visualized with an Alliance 4.7 UV transilluminator (UVITEC, Cambridge, UK). Fingerprinting profiles were analysed with BioNumerics software version 6.6 (Applied Maths, Belgium). A consensus dendrogram was created based on the genomic profiles obtained with the primers csM13, $(GACA)_4$, and $(GTG)_5$ using Pearson's correlation coefficient to generate the similarity matrix and the Unweighted Pair Group Method with Arithmetic Average (UPGMA) as the clustering algorithm. The reproducibility cut-off level was calculated as the mean value of the reproducibility obtained for each primer independently, using at least 10% of randomly

selected strains. A dendrogram constructed based on these duplicates was used to estimate the reproducibility cut-off level and to calculate the optimization and curve smoothing parameters (1% and 1.25%), that better paired the repeats for each primer. A conservative estimate of the reproducibility cut-off level was established at 99%.

2.2 Yeasts strains and phenotypic assay

A total of 85 wild non-*Saccharomyces* wine yeasts from the ICONE collection provided by Sogrape-Proenol-FCUL were used. These yeasts were isolated from fermenting grape musts, collected from Portuguese wine producer regions (Alentejo, Dão, Porto/Douro, and Bairrada). Taxonomically, these strains belonged to five different genera, namely *Candida* (n = 26), *Lachancea* (n = 29), *Metschnikowia* (n = 21), *Saccharomycopsis* (n = 1), and *Torulaspora* (n = 8).

Yeasts strains were recovered from cryopreserved cultures maintained in 20% (v/v) glycerol at - 80 ºC and streaked onto plates containing Yeast Peptone Dextrose medium (YPD) [1% (w/v) yeast extract (Biokar diagnostics), 2% (w/v) peptone (Biokar diagnostics) and 2% (w/v) glucose (MERCK)] supplemented with agar 2% (w/v) (Biokar diagnostics) -YEPDA medium], incubated for 72 h at 28 ºC and maintained at 4 ºC for follow-up experiments, and sub-cultured periodically.

2.2.1 Preparation of the inoculum

For the inoculum preparation, yeasts were activated from stored stock cultures and transferred with a loop onto 50 mL falcon tubes with 25 mL YPD broth and incubated on a rotatory shaker (160 rpm) at 28 ºC for 24 h. Cell concentration was determined spectrophotometrically by measuring their optical density (OD) at 600 nm and was initially adjusted to 0.1 OD. Yeast cultures were incubated overnight in 100 mL Erlenmeyer flasks with 50 ml YPD broth at 28 ºC at 160 rpm.

2.2.2 Screening for killer phenotype

In total, 85 yeasts were screened for killer activity. This experiment was performed according to Stumm *et al.* (1977) using the seeded-agar-plate technique in YEPDA-MB plates [YPDA buffered with 0.1 M citrate-phosphate (pH 4.5), supplemented with 0.003% (w/v) methylene blue]. An aliquot of 100 µL of a sensitive reference strain (10^6 cells/mL) was spread on the surface. After drying, 3 µL of each tested strain were inoculated and incubated at 25 ºC for 48 h. Methylene blue dye was used since can enter into live and dead cells. Living cells can reduce enzymatically the dye and remain colourless, while dead cells are unable to do it and are stained blue (Stewart, G. 2017). Regarding this, yeast strains were classified with killer phenotype if they were surrounded by a region of bluish coloured cells or by a clear zone of inhibition surrounded by coloured cells, the sensitive or neutral yeasts do not show an inhibition zone (Figure 10). Two *Saccharomyces cerevisiae* strains were used as a control, one with the killer phenotype and one with the sensitive phenotype. This experiment was carried out in duplicate.

Screening results were analysed using a qualitative experimental approach, recording as positives the strains with killer phenotype and negatives the strains with sensitive or neutral phenotype (Lopes and Sangorrín, 2010).

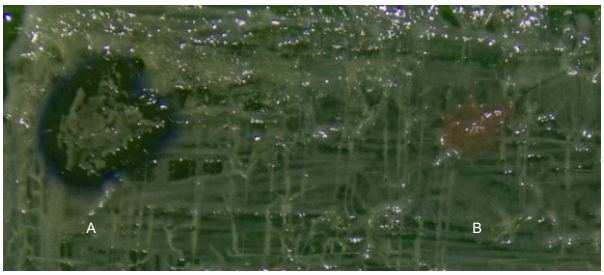

Figure 10 - Killer phenotype screening in YEPDA-MB seeded-agar-plate. A yeast scored with the killer phenotype (A) surrounded by a zone of inhibition and bluish coloured cells and, a yeast without killer phenotype (B) which did not inhibit the lawn cells after 48h of incubation at 25 °C.

2.3 Dual culture assays for mycelial growth inhibition

For the following assays, the FSC040 isolate of *Botrytis* sp. and TLS001 strain of *Aspergillus* section *Nigri* were used as fungal targets. Since it was only possible to obtain a spore suspension for TLS001 strain ($7.9x10^5$ spores/mL), in the assay of mycelial growth inhibition, TLS001 spore suspension and a mycelial plug of FSC040 culture with 3-5 d old were used.

2.3.1 Production of volatile organic compounds

The antagonism of yeast volatile compounds was evaluated using four-part Petri dishes (90 mm diam) with separated compartments, that were filled with 3.5 mL of YPD in each. In two of these compartments, an aliquot of 100 µL of yeast cell suspension was seeded and incubated at 28 ºC for 24 h. Then, 3 µL of spore suspension or mycelial plug (6 mm diam) of the target was inoculated in the corner of the other two compartments of each Petri dish (Figure 11, A). Petri dishes without the inoculation of yeasts were used as control (Figure 11, B). Each one was wrapped with two layers of parafilm around the edges to prevent air leakage and incubated at 25 ºC in dark. Mycelium growth was daily measured until it reached the Petri dish edge. Measurements were drawn at the time of assessment. The experiment was carried out in duplicate.

2.3.2 Inhibition of mycelial growth on agar plates

The biocontrol activity of yeast strains was evaluated using a dual culture assay in Petri dishes (90 mm diam) and three experimental confrontation approaches were used. In the first procedure, designed as the CTY method, target and yeast are inoculated simultaneously. In the second procedure, designated as the CY method, yeast strains were inoculated and incubated for 48 h at 28 ºC before

target inoculation. Lastly, in the CT method, the target was inoculated and incubated for 48 h at 28 ºC before yeast inoculation.

In a Petri dish divided into eight radius, 3 µL of yeast cell suspension were positioned at seven sites at about 30 mm from the centre of each Petri dish. TLS001 spore suspension (3 µL) or a plug of FSC040 taken from the edge of an actively growing FSC040 culture was inoculated upside down in the centre of the Petri dish at 30 mm distance from the yeast strain (Figure 11, C). The effect of yeasts on target growth was compared with a control, a Petri dish only with target inoculation (Figure 11, D). Three replicates were prepared for each strain experiment and the radius of mycelial growth was daily measured in the direction toward the yeast strain point of inoculation. Measurements were made utilizing pre-drawn lines before placement of the inoculating fungal plug.

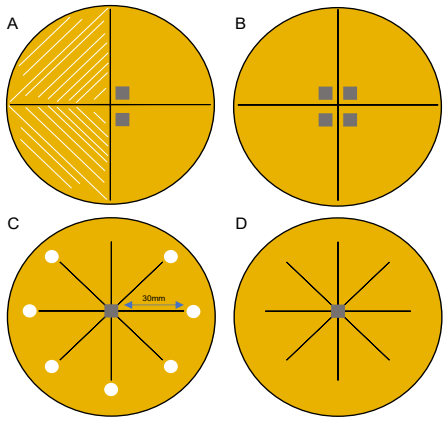

Figure 11 – Schematic overview of dual culture plates for mycelial growth inhibition: To screen for yeasts producing antimicrobial volatiles (A) Petri dishes were used with separated compartments. The yeast strain (white lines) was seeded in two compartments and the target (grey square) was inoculated at the corner of the other two compartments of the Petri dish. In mycelial growth inhibition assay (C), both the yeast strain (white circle) and the target (grey square) are spotted on the solid agar medium. The target was spotted at a 30 mm distance from the yeast strain. As a control (B, D) Petri dishes without the inoculation of yeasts were used.

2.3.3 Data analysis

To quantify fungal growth there are several methods, including the measure of colony diameter which according to Brancato and Golding (1953) is a reliable measure. In this regard, in the last two assays, the fungal growth was quantified using the measure of the colony radius since *Botrytis* and *Aspergillus* species have well-formed and circular colonies (Prosser and Tough, 1991). Colony radius was measured daily from the reverse side in millimeters with a ruler (± 0.5 mm). Fungal extension rate (ER; $mm.day^{-1}$) was calculated through these measurements plotted against time (Taniwaki *et al.*, 2006), by fitting a linear regression model. The absence of relevant deviation of linearity was assumed whenever the coefficient of determination (R-squared) was equal or greater than 0.95.

The inhibition (%) of the target fungal growth rate (IER) in the presence of yeasts strains was calculated considering the equation 1.1

$$[1 - \left(\frac{\text{ER Tested}}{\text{ER Control}}\right)] \text{ x } 100 \quad (1.1)$$

Fungal growth was also quantified through the total area under the curve (AUC), using the linear trapezoid rule. It was calculated the area under the fungal growth measure in each day. The sum gave the integrated fungal growth through time. The computation of the AUC allows the incorporation of the data value information obtained throughout all the measurements of the colony radius (Allgoewer *et al.*, 2018).

To calculate the target fungal growth inhibition (IAC; %) in the presence of yeasts strains, it was compared with the control according to the equation 1.2

$$[1 - \left(\frac{\text{AUC Tested}}{\text{AUC Control}}\right)] \text{ x } 100 \quad (1.2)$$

Lastly, the inhibition of the radial growth (IRG) of the fungal target in the presence of yeast strains was calculated as described by Lemos Junior *et al.* (2016) (equation 1.3). At the end of the incubation period (at day 5 for *Aspergillus* target and day 4 for *Botrytis* target), the radial growth of the fungal target was measured and compared with the control growth measure.

$$[1 - \left(\frac{\text{Tested}}{\text{Control}}\right)] \, x \, 100 \quad (1.3)$$

2.4 Dual culture assays for inhibition of Spore Germination

As described above, it was not possible to obtain a spore suspension for FSC040 isolate of *Botrytis* sp. and so the inhibition of spore germination was only tested with the TLS001 strain.

2.4.1 Assay on agar medium

A top agar was prepared by mixing 9 mL of YPD with 0.8% agar at 50 °C and 1 mL of spore suspension containing concentrations from 10^2 to 10^4 spores/mL in Petri dish. The agar-spore suspension was poured into Petri dishes that contained 15 mL of the YPD agar medium. Once the top agar had set, 3 µL of each yeast cell suspension was spotted on each Petri dish and incubated at 25 ºC for 72 h. Petri dish inoculated only with TLS001 suspension were used as control (Figure 12). A clear zone around the yeast colonies was interpreted as inhibition of spore germination, a positive result. Yeasts did not surrounded by an inhibition zone, were interpreted as a negative result. Two replicates experiments for each yeast strain were performed.

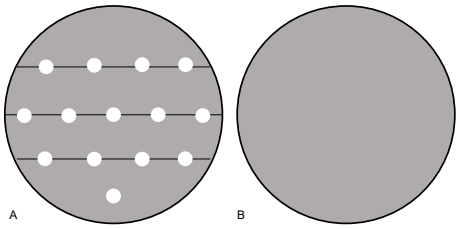

Figure 12 - Schematic overview of dual culture assay for inhibition of spore germination on agar medium: The spore suspension of the target was distributed in top agar as a lawn (grey) and yeast strains were subsequently spot-inoculated (white circle). They were spotted at the same distance from each other. If a yeast strain growth on the target cells lawn formed an inhibition halo, is scored as positive. As a control Petri dishes without the inoculation of yeasts were used.

2.4.2 Assay on liquid medium

Each of the well of microplate was filled with 1.5 mL of yeast suspension and incubated at 28 °C for 48 h. Non-inoculated wells were filled with 1.5 mL of YPD. Then, to each well was added 100 µL of different spore concentrations (10^6 – 10^2 spores/mL)) of TLS001 target (Figure 13). The microplates were incubated at 25 °C. Control was handled in an identical way except that no yeast suspensions were added. After 7 days, growth inhibition was evaluated with a 4-values scale (0 = no growth; 1 = growth without sporulation; 2 = growth less than the control; 3 = growth equal to the control).

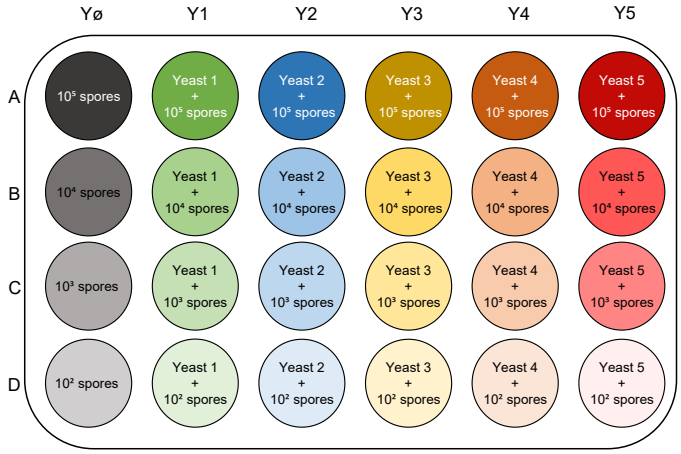

Figure 13 - **Schematic overview of dual culture assay for inhibition of spore germination on liquid medium.** Different yeasts suspensions were inoculated from Y1 to Y5 column wells. After incubation, the fungal suspension was inoculated to each well, according to the spore concentration. As a control, no was inoculated yeast suspension in Yø raw.

2.5 Global data analysis

The data set obtained by the three approaches (IER, IAC, and IRG) in the dual assays was analysed. Firstly, the antagonistic activity data dispersion of yeast genera was evaluated in the different methods VOC, CTY, CY, and CT by the three approaches application, against TLS001 and FSC040 target. Following, it was also evaluated the overall antagonistic activity according to the method used. To select

the yeasts with the best antagonism activity an arbitrary threshold was applied, in which yeasts with an antagonism activity equal or more than 25% were selected. Also, it was evaluated the potential antagonism of yeast collection according to the approach applied. As an integrative selection approach, the best-ranked yeasts were selected and compared using Venn diagrams (Venny 2.1 - Oliveros, J.C. (2007-2015)) It was included the three approaches and the methods applied. Additionally, results of spore gemination assay and killer phenotype assay were integrated to select the best biocontrol yeasts.

2.6 Workflow of the study

A workflow of the work developed in this study is represented in Figure 14

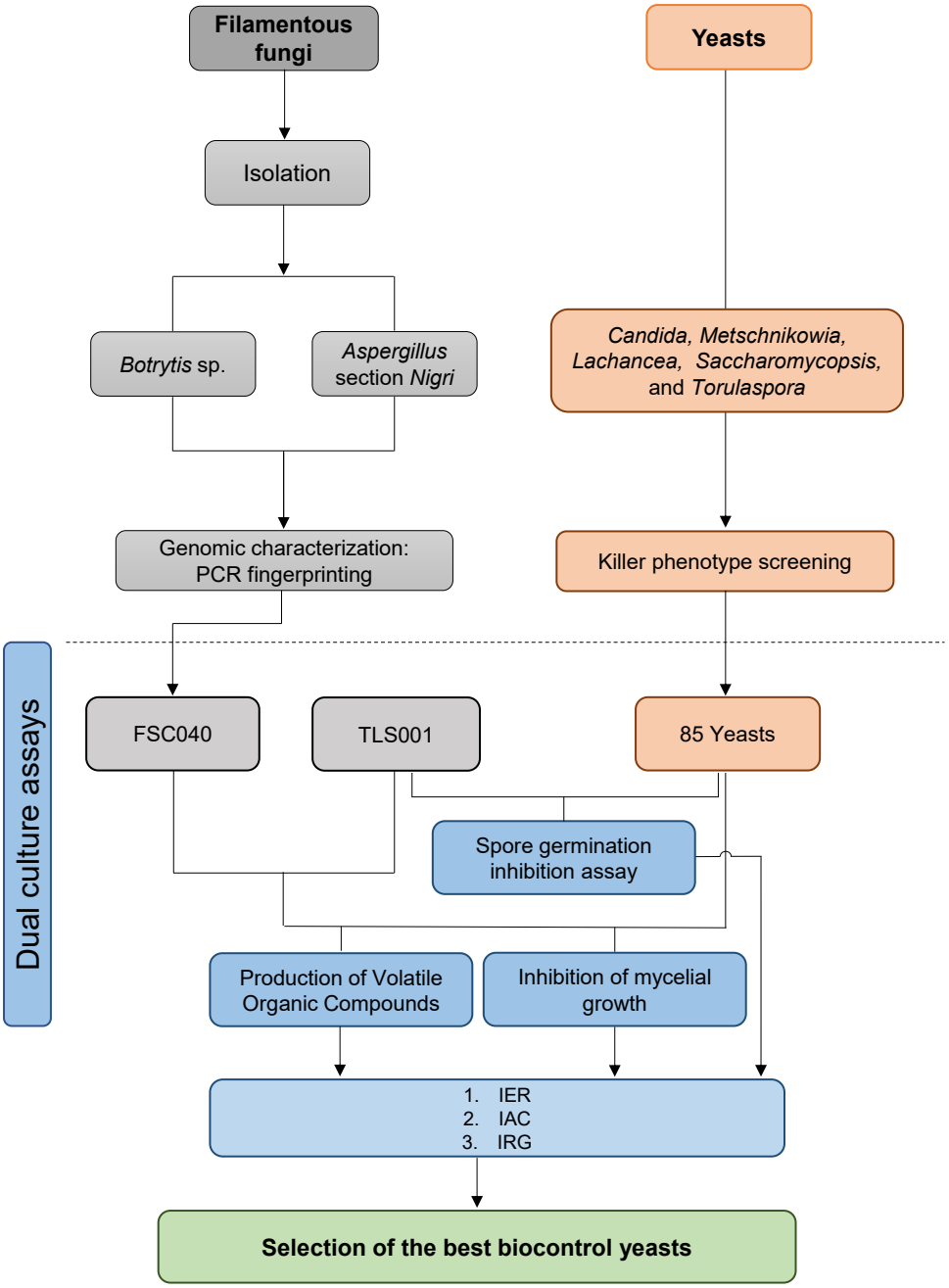

Figure 14- Workflow of the study

3. Results and Discussion

3.1. Genomic fingerprinting of fungal targets

3.1.1. *Aspergillus* isolates

From the samples collected by the ADVID team on the Douro region, a total of six isolates were identified as belonging to the *Aspergillus* genus, considering the following microscopic characteristics: septate mycelium and asexual reproduction through conidia, a non-septate-enlarged conidiophore at the tip forming a vesicle with phialides which form a basipetal spores chain.

Also, it was registered the macroscopic features of the isolates ABC063, ABC070, ABC071, ABC072, and ABC076 (Figure 15). On PDA, the colonies were initially white to yellow and became black with abundant conidial production. Also, colonies were encircled by a white to yellow border. The surface of the colonies seemed initially as cottony but becomes a velvety appearance. The colony reverse was white to yellow. Based on these characteristics, the five isolates were identified as members of *Aspergillus* section *Nigri*. Regarding the macroscopic characteristics of the ABC064 isolate was similar to the other isolates, but it presented a cinnamon-coloured colony with a yellow reverse (Figure 15, C, D). Based on these characteristics the isolate was considered as a member of *Aspergillus* section *Circumdati* (probably *A. ochraceus*).

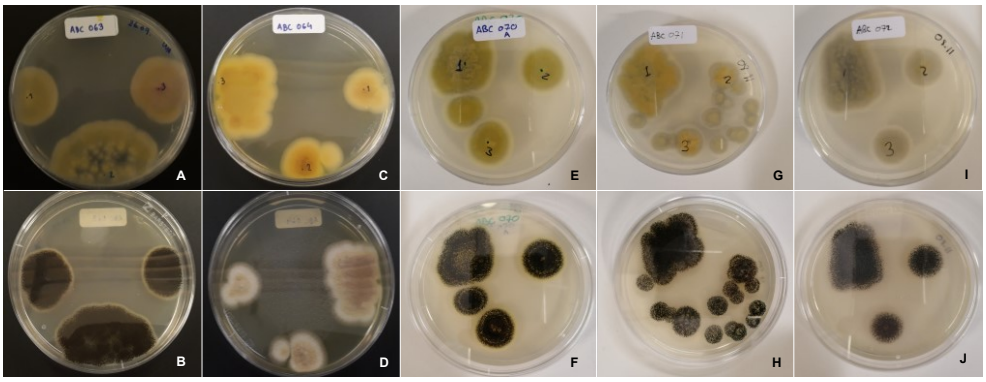

Figure 15 – **Cultural characteristics of *Aspergillus* isolates** (Reverse and Verse): **A**, **B**-ABC063; **C**, **D**-ABC064;**E**,**F**-ABC070; **G,H** - ABC071; **I,J** - ABC072

For the characterization of the genomic diversity of *Aspergillus* isolates, csM13 and $(GTG)_5$ primers were used. Based on these genomic fingerprints a composite dendrogram was constructed (figure 16). This approach yielded highly reproducible genomic fingerprints, with several bands ranging from 300 to 2000 bp.

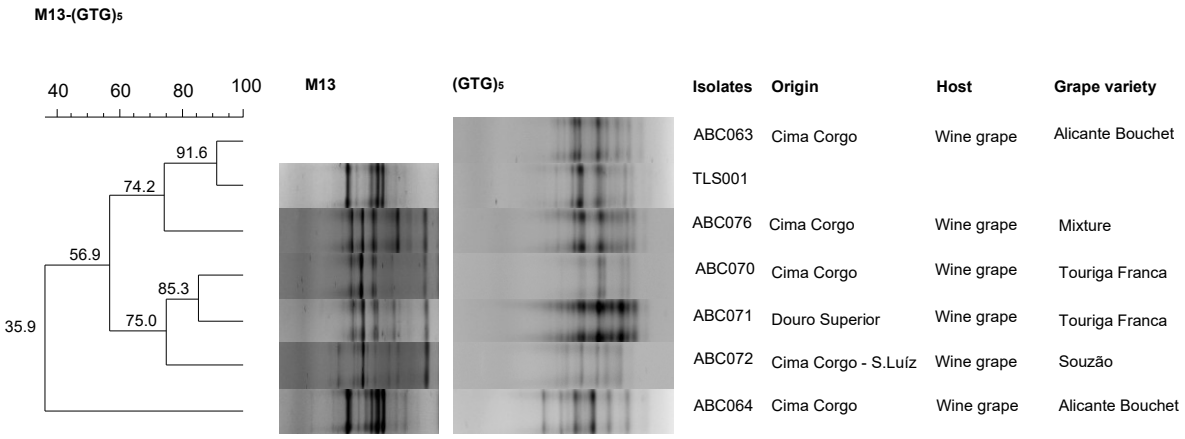

Figure 16 - **Composite dendrogram of *Aspergillus* isolates**. The composite dendrogram based on csM13 and $(GTG)_5$ profiles. Dendrogram was constructed using Pearson's correlation coefficient and UPGMA algorithm. The similarity percentage values are reported at each node.

The reproducibility level was calculated based on the percentage similarity mean between the duplicates. Concerning this, a conservative cut-off level of 99% was established, above which it was assumed that the isolates cannot be discriminated using this technique. The fingerprinting profiles obtained with csM13 and $(GTG)_5$ primers presented multiple bands with identical patterns among the isolates. In general, the csM13 primer generated the most discriminatory and informative fingerprinting profiles. A combined analysis with both primers was used to allow a higher discriminatory power, which was not achieved with only one primer. It was not possible to generate the csM13 fingerprint profile for ABC063 isolate due to the production of a dark pigment which is common in *Aspergillus* section *Nigri* (Jørgensen *et al.*, 2011). This pigment interfered in the MSP-PCR reaction, inhibiting the amplification reaction. However, this issue did not occur for the $(GTG)_5$ primer.

Applying an arbitrary threshold of 70% similarity, *Aspergillus* isolates were clustered into three groups, two with three isolates each and another with only one isolate. Reference strain TLS001 was included in the first group jointly with isolates ABC063 and ABC076, presenting a higher similarity with the former. Except for the reference strain (collected from cork), all isolates were sampled from diseased wine grapes. Nonetheless, clustering association according to the host did not seem to occur. The isolates ABC070 e ABC071 were clustered according to the Touriga Franca grape variety, sampled from different regions. The combined analysis of genomic fingerprints and phenotypic traits (Figure 15) allowed to confirm the distinction between the isolate ABC064 from the others.
Even though isolate ABC064 does not belong to the *Aspergillus* section *Nigri*, it was integrated in this analysis due to its food safety impact. When applying a lower cut-off value of 70%, the method was also able to detect intraspecific variation. Although the fingerprinting profiles were very similar within the clusters formed, it was possible to identify polymorphic bands between strains of the same cluster.

The MSP-PCR fingerprinting was used as a tool in order to get insights on the variability and population structure of *Aspergillus* isolates and it proved to be a method that generates highly reproducible results. This high reproducibility relies on the use of longer primers which allow PCR reactions to be carried out at higher annealing temperatures (Alves *et al.*, 2007). The

Aspergillus section *Nigri* is a difficult group to taxonomically study. Regarding this, there are some other fingerprinting methods applied which have enabled isolates differentiation, namely RFLPs analysis of *A. niger* and *A. tubingensis* isolates, or of the analysis of ITS region using specific restriction enzymes. Also, RAPD and AFLP approaches have been applied for reliable identification of species from the *Aspergillus* section *Nigri* (Samson *et al.*, 2007)

Above all, studies on the diversity of the *Aspergillus* populations are important to understand the ecological context of different species as well as their distinct adaptation to environmental and geographical conditions, and consequently, the toxigenic potential which they entail. The *Aspergillus* section *Nigri* distribution seems to be influenced by geographic conditions, which is particularly prevalent in warm and arid regions. Some of these species are ochratoxin A (OTA) producers namely *A. ochraceus* from section *Circumdati* and *A. niger* from section *Nigri* (Perrone *et al.*, 2007). The incidence of OTA-producing strains seems to be correlated not only with these geographic and climatic conditions but also with low altitudes. Additionally, OTA-producers are also dependent on terroir as well, i.e., the grape cultivation practices in conjunction with the microclimate of the vineyard (Serra *et al.*, 2003; 2005; Kizis *et al.*, 2014).

3.1.2. *Botrytis* isolates

From the samples collected by the ADVID team on the Douro region, only two isolates (ABC074 and ABC075) were identified as belonging to the *Botrytis* genus, considering the following characteristics. On PDA, the colonies initially presented different colours ranging from white, dirty white to greyish white and became dark grey. Also, it presented a fluffy, long, and abundant mycelium with randomly distributed black, flat, and convex sclerotia. Microscopically, it presented branched conidiophores with rounded apical cells, the conidiophores and conidia clusters resembled a bunch of grapes. Based on this set of characteristics, five additional *Botrytis* spp. isolates (FSC040, FSC069, FSC088, FSC117, and FSC262) were also identified among the collection of 227 fungal isolates obtained from the set of convenience samples collected in Lisbon.

For the characterization of genomic diversity of *Botrytis* isolates the primers $(GTG)_5$, csM13, and $(GACA)_4$, were used. The genomic fingerprints showed to be highly reproducible with a cut-off value of 99% and displayed several bands ranging from 500 to 2000 bp in csM13 and $(GTG)_5$, and from 400 to 1000 pb in $(GACA)_4$ primer. Similar to *Aspergillus* isolates, a composite dendrogram was constructed for the *Botrytis* isolates (Figure17).

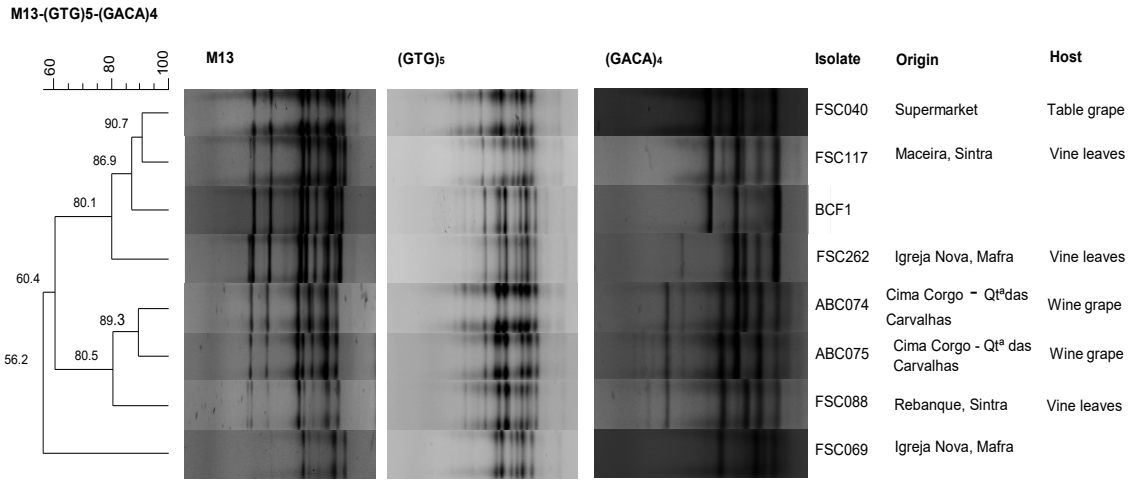

Figure 17 – **Composite dendrogram of *Botrytis* isolates**. The composite dendrogram based on csM13, $(GTG)_5$ and $(GACA)_4$ profiles. Dendrogram was constructed using the Pearson's coefficient correlation and UPGMA algorithm. The similarity percentage values are reported at each node.

Although high levels of similarity among isolates were observed in particular for csM13 and $(GTG)_5$ primers, the higher resolution power of the combined approach was confirmed. The fingerprints obtained with $(GACA)_4$ presented simple and superimposable bands. Regarding this, the fingerprint profiles were generated with the most discriminatory power and resolution. The primers M13 and $(GACA)_4$ generated the most discriminatory and informative fingerprint profiles. Furthermore, through literature review, it is suggested that this is the first study in which $(GTG)_5$ fingerprint used to evaluate the genetic diversity of *Botrytis* isolates. The objective of this combined analysis was to allow a higher discriminatory and resolution power than when the primers are used individually.

At the arbitrary threshold of 70% similarity, *Botrytis* isolates were clustered into three groups with one, three, and four isolates. The single-member cluster includes the isolate FSC069, which showed unique band patterns. Considering the reference strain, clustered with the isolates FSC040 and FSC117, isolate FSC040 was selected to be the fungal target used in the different biocontrol dual assays since it was isolated from table grapes. Excepting the isolates ABC074 and ABC075, there was no clustering association according to host or origin. The characterization of *Botrytis* isolates using MSP-PCR fingerprinting with the microsatellite $(GACA)_4$ and csM13 primers has been previously reported by Ma and Michailides (2005), where these results are supported. They showed that *Botrytis* isolates collected from several crops (grape, kiwifruit, pea, and squash) were not clustered based on their hosts. Also, they did not observe genetic differences between grape population samples from different locations. However, some reports showed the opposite, reporting that the isolates sampled from different hosts of different origins (France and Chile) presented genetic differences among them (Diolez *et al.*, 1995; Giraud *et al.*, 1997; Muňoz *et al.*, 2002). Additionally, Munoz *et al.*(2002) reported genetic differences in *Botrytis cinerea* isolates sampled from different hosts (grapes and tomatoes) using RAPD and PCR-RFLP analyses.

Indeed, *Botrytis cinerea* is a fungus that attacks a wide range of hosts and tissues, leading to the conclusion that the pathogen has no host specificity. Giraud *et al.* (1999), challenged this perspective

when reported that the annual prevalence and the association with asymptomatic/diseased host tissue of the two sympatric *B. cinerea* sibling species *transposa* and *vacuma* were significantly different, concluding that they have host specialization.

Genetic diversity of *Botrytis cinerea* species is widely reported in literature. It is estimated that 1.3% of its genome is composed of repetitive sequences. This genetic variation is a result of the presence of transposable elements (like the Boty and Flipper elements of *transposa*) and extrachromosomal genetic elements(e.g., mitochondrial DNA, plasmids, and mycoviruses) (Giraud *et al.*, 1997; Martinez *et al.*, 2003; Angelini *et al.*, 2016). They are associated with specialization with plant organs, virulence on grape berries, and acquired resistance against fungicides (Angelini *et al.*, 2016).

3.2. Screening for yeast killer phenotype

From a total of 85 yeasts screened, only nine strains exhibited killer phenotype, meaning that the collection is constituted by 10.6% of killer yeasts and 89.4% of sensitive or neutral yeasts. The tested yeasts of *Lachancea*, *Torulaspora,* and *Saccharomycopsis* genera did not display a killer phenotype. The killer yeasts represent 30.7% and 4.7% of the tested strains of *Candida* and *Metschnikowia* genera, the strongest killer activity being found in the strain from this last genus (Figure 18). Since the sensitive phenotype was not screened in this assay, no-killer yeasts cannot be discriminated as sensitive or neutral.

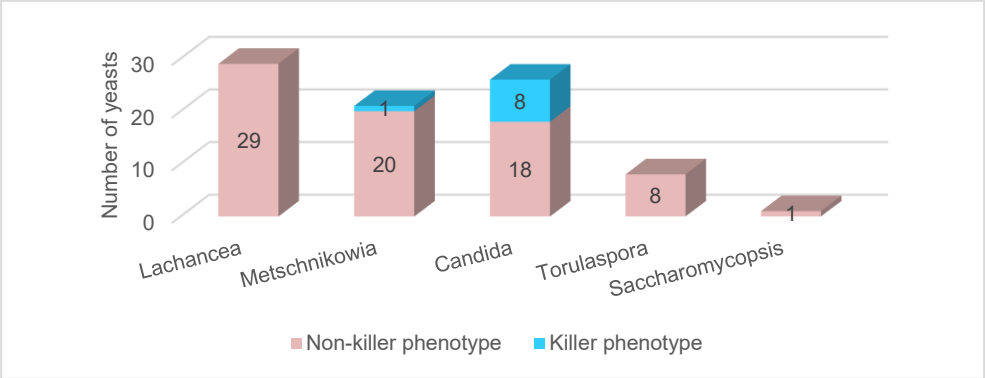

Figure 18 – **Killer phenotype screening**. Number of yeasts with Non-killer phenotype and killer phenotype

The killer phenotype does not seem to rely on the genus, since it is distributed among yeast genera, but dependent on strain function (Antunes and Aguiar, 2012; Bajaj and Singh, 2017). In this regard, it was expected killer phenotype expression by *Lachancea* and *Torulaspora* tested strains, since previous works have described it (Starmer *et al.*, 1987; Michalčáková *et al.*, 1993; Ramírez *et al.*, 2015; Aponte and Blaiotta, 2016). The killer phenotype was not observed in the tested strain of *Saccharomycopsis*, in agreement with the lack of reports about killer activity in this genus.

The non-killer phenotype was the main one found among the strains tested. This frequency was also observed in previous studies describing killer activity against *Saccharomyces cerevisiae*. Perez *et al.* (2016) show that from 437 native yeast strains isolated from citrus leaves and fruits, 30 strains (6.9%) displayed a killer phenotype. The frequency increased to 8.5% when the medium was supplemented with 2% NaCl, and 7 more yeast strains showed to be of the killer phenotype. González-Arenzana *et al.* (2017) tested 71 wine non-*Saccharomyces* yeasts strains belonging to *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Lachancea thermotolerans*, *Zygosaccharomyces bailii,* and *Williopsis pratensis* species. They reported 3 strains (4.2%) with killer phenotype belonging to the last species. Farris *et al.* (1991) revealed a low frequency (2.3%) of *Metschnikowia pulcherrima* strains with killer phenotype. From 260 strains isolated from grapes and musts also tested at pH 4.5, only 6 strains showed killer activity. According to Mannazzu *et al.* (2019), the frequency of the killer phenotype can range from 5 to 30%, reaching 50%. Regarding this, Antunes and Aguiar (2012), described an incidence of 34.3% of killer phenotype in a culture collection of 108 yeast strains from clinical and industrial origin. The same authors also highlighted the potential of killer phenotype for biocontrol application. Indeed,

several killer yeasts have been reported to display interference competition against filamentous fungi in laboratory (Walker *et al.*, 1995). Santos *et al.* (2004) showed that the halotolerant yeast *Pichia membranifaciens* suppressed *Botrytis cinerea* growth in apples through killer toxin production. Additionally, Ferraz *et al.* (2016) reported the same mode of action by *Candida azyma in controlling Geotrichum citri-aurantii*, responsible for causing citrus fruit spoilage. Concerning this, it has been hypothesized killer phenotype integration as a biocontrol strategy in pre-and postharvest disease, acting as a both preventatively and curatively approach (Antunes and Aguiar, 2011; Ferraz *et al.*, 2016; Belda *et al.*, 2017). The pH of grape berries wounds can present values lower than 4.6 and most killer toxins are effective at acidic pH levels, which can represent an advantage in grape pathogens biocontrol (Perez *et al.*, 2016; Stocco *et al.*, 2019).

The research on killer yeasts and their toxins is not recent and since its discovery in strains of *Saccharomyces cerevisiae* by Makower and Bevan (1963), researchers have been enhancing efforts to explore underlying mechanisms and their applications. Despite the knowledge about the biochemistry, genetics, and molecular biology of several killer yeasts and their toxins there are still concerning questions that need to be explored, namely the ecological impact of toxin production in natural environments and how it shapes the community structure. Regarding this, several studies suggested that killer yeasts are more abundant in natural habitats than in laboratory collections (Buzzini *et al.*, 2007; Boynton, P., 2019). This could be explained by a trade-off between growth and killer toxin expression. The latter implies a metabolic cost that can lead to a fitness reduction of the producer by around 4%. It can also be explained by the loss of extra-chromosomal DNA or RNA plasmids coding the killer toxin, during the cycles of cell proliferation in the laboratory environment (Buzzini *et al.*, 2007; Mannazzu *et al.*, 2019)

The seeded-agar-plate technique, where methylene blue was used as the dye, is the method more applied to screen yeast killer phenotype. Using this method affords a screen of a large number of strains, it is easy to use, fast, and cheap. If the size of the zone of dead cells is measured, it can be used as a semiquantitative method to assess killing activity (Sangorrín *et al.*, 2002; Lopes *et al.*, 2010). However, this method is not fully accurate and can be limited namely by pH, salt concentration, temperature, the reference strain inoculated, or intensity of relative killing activity interpretation, leading to misidentification of killer activity. Therefore, to avoid biased results and improve the method reproducibility complemented methods should be applied, namely search of killer-encoding genetic elements, using gene sequencing, northern blot, whole-genome or metagenomic sequencing (Buzzini *et al.*, 2007; Bajaj and Singh, 2017; Boynton, P., 2019; Mannazzu *et al.*, 2019). The killer phenotype pattern can also be used as a biotyping technique, serving as a fingerprinting tool to intraspecific differentiation (Buzzini *et al.*, 2007; Cihlar and Calderone, 2009). However, a combination with molecular techniques is recommended. Lopes *et al.* (2015) showed that the combined use of the mtDNA-RFLP analysis with the killer biotype method was an efficient tool in the fingerprinting of the oenological *S. cerevisiae* strains.

3.3. Does antagonistic activity against *Aspergillus* rely on the yeast genus?

The results distribution obtained with yeasts of the five genera against TLS001 growth in the three approaches IER, IAC, and IRG were compared using boxplots (Figure 19). Because the mean value is influenced by external values, the median value was used as a central trend measure since it is more indicated when the data, such as these, have an asymmetric distribution and small sample size (Manikandan, 2011). In this regard, the *Saccharomycopsis* yeast was not included in the genera comparison.

3.3.1 Production of volatile organic compounds

In the three approaches, the ***Lachancea* boxplot** displayed the data set widely dispersed which suggests a wide range of responses against TLS001 through VOC production. Considering the dispersion of antagonist activity data, in the IER (Figure 19 A) ranged from -8 to 42%, in the IAC from - 7% to 47% whereas in the IRG ranged from -20% to 35%. In IAC and IRG approaches (Figure 19 B and C), the ***Lachancea* boxplot** data set was left-skewed, and the median values were 25% and 14%, respectively. It suggests that according to the IAC approach, half of *Lachancea* yeasts presented more than 25% of antagonism activity. In the IER approach (Figure 19 A), the data set was right-skewed and presented the median value of 17%. ***Lachancea* boxplot** data presented the highest values of antagonism in IER and IAC approaches.

Followed by *Lachancea*, *Candida* assembled the highest number of yeasts with antagonism activity. In the three approaches, ***Candida* boxplot** displayed one outlier with the best antagonism activity, of 44% in IER and IRG approaches (Figure 19 A and C) and 51% in the IAC approach (Figure 19 B). Also, in the three approaches, ***Candida* boxplot** presented a dispersed data set right-skewed, slightly right-skewed in the IAC approach. Considering the dispersion of antagonist activity data, in the IER ranged from -4 to 32%, in the IAC from - 5% to 35% whereas in the IRG ranged from 6% to 34% but with a negative outlier of -8%. The median values were 13%, 16%, and 18%, respectively.

In the three approaches, ***Torulaspora* boxplot** was the more symmetric with a high level of data set agreement. This can be explained by the small sample size since the boxplot incorporates the data from only eight strains. Nonetheless, in the three approaches, *Torulaspora* was the genus with less antagonistic activity displayed. Considering the dispersion of antagonist activity data was quite similar in the three approaches. In the IER (Figure A) ranged from 9 to 15%, in the IAC (Figure 19 B) from 13% to 20% whereas in the IRG (Figure 19 C) ranged from 10% to 19%. The median values were 11%, 19%, and 15%, respectively.

The *Metschnikowia*, followed by the *Torulaspora* was the genus with lesser yeasts with antagonistic activity against TLS001 growth. The ***Metschnikowia* boxplot** data set presented an antagonism activity range below 30%. Considering the dispersion of antagonist activity data was also quite similar in the three approaches. In the IER (Figure 19 A), it displayed a long right tail and the right-skewed data set, bounded -4% to 25%, and with a median value of 15%. In IAC and IRG approaches (Figure 19 B and C) ***Metschnikowia* boxplot** presented a slightly left-skewed data bounded 6% to 28%

and 5% to 23%, and with median values of 19% and 17%, respectively. Also, they presented one negative outlier of -3% and -6%, respectively.

3.3.2 Yeast and target inoculated simultaneously (CTY)

Regarding the three approaches, the genera antagonistic activity data set ranged from 0% to 40%.

Considering ***Candida*** **boxplot** data set dispersion, in the IER (Figure 19 D) ranged from 21% to 34%, in the IAC from 22% to 43% whereas in the IRG ranged from 24% to 39%. It suggests a distribution of antagonistic activity less variable and relatively similar between the *Candida* yeasts in inhibition of mycelial TLS001 growth. In the three approaches, the ***Candida*** **boxplot** displayed left-skewed data and, all median values above 30%. It suggests that, according to the tree approaches, half of *Candida* yeasts presented more than 30% of antagonism activity against mycelial TLS001 growth.

Torulaspora was the second genus with the most yeasts with antagonistic activity. Considering ***Torulaspora*** **boxplot** data set dispersion, in the IER (Figure 19 D) ranged from 21% to 26%, in the IAC from 23% to 35% whereas in the IRG ranged from 24% to 30%. The median values were quite similar in the three approaches, ranging between 24% and 28%. The ***Torulaspora*** **boxplot** presented a high level of data set agreement, comparatively short and symmetrical. Similarly, to the ***Candida*** **boxplot** data, it suggests a less variable and relatively similar response to TLS001 mycelial growth inhibition.

According to IER and IRG approaches (Figure 19 D and F), *Metschnikowia* presented the lowest yeasts with antagonistic activity against TLS001 growth. Considering ***Metschnikowia*** **boxplot** data set dispersion, in the IER (Figure 19 D) ranged from 15% to 26%, in the IAC from 1% to 32% whereas in the IRG approach ranged from 6% to 29%. In the three approaches, ***Metschnikowia*** **boxplot** displayed a right-skewed data and left tail relatively longer in the IAUC and IER approaches. Also, it presented comparatively low median values of 11% (Figure 19 D), 7% (Figure 19 E), and 13% (Figure 19 F).

Of all genus, the ***Lachancea*** **boxplot** data set was the most dispersed which suggests a wide range of responses against TLS001 mycelial growth. In the three approaches, the ***Lachancea*** **boxplot** dispersion was quite similar. In the IER (Figure 19 D) ranged from 4% to 27%, in the IAC from -2% to 31% whereas in the IRG approach ranged from 5% to 31%. It also presented a left-skewed data and median values of 18% (Figure 19 D), 22% (Figure 19 E), and 24% (Figure 19 F).

3.3.3 Yeast inoculated before target (CY)

In the three approaches, the ***Candida*** **boxplot** displayed the data set widely dispersed which suggests a wide range of responses against TLS001 growth. Considering the dispersion of antagonist activity data, in the IER (Figure 19 G) ranged from -2 to 36%, in the IAC from -16% to 49% whereas in the IRG ranged from 2% to 41%. In the IER, IAC, and IRG approaches, the ***Candida*** **boxplot** data set was left-skewed, and the median values were 22%, 15%, and 18%, respectively. ***Candida*** **boxplot** data

presented the highest values of antagonism of the assay. It was the only one that presented more than 25% of antagonistic activity.

Followed the *Candida* data set, the boxplot data set of the other genera are quite similar.

In the IRG approach, the data dispersion of ***Metschnikowia* boxplot** ranged from 7% to 36%. It also presented a left-skewed data set with a left tail much longer and the median value of antagonism at 21%. In the IAC approach (Figure 19 H), ***Metschnikowia boxplot*** displayed right-skewed data ranging from -1% to 18%, with a median value at 7%. Also, it presented the best activity result defined by the outlier of 54%.it presented the best activity result defined by the outlier of 54%. In the IER approach (Figure 19 G), ***Metschnikowia* boxplot** was comparatively short and symmetrical, ranging antagonistic action from 6% to 18%, which suggests that antagonistic activity against TLS001 mycelial inhibition was less variable.

Similarly, ***Torulaspora* boxplot** also displayed comparatively short and symmetrical data with a right tail slightly longer. In ER and AUC approach (Figure 19 G and H), it presented a slightly left-skewed data with a range of action below 18% in both approaches.

The ***Lachancea* boxplot** data set presented the lowest antagonism values. Considering the dispersion of antagonist activity data, in the IER (Figure 19 G) ranged from -3 to 16%, in the IAC from -6% to 17% whereas in the IRG ranged from -4% to 15%. It presented median values of 10% (Figure 19 G) and 8% (Figure H and I).

3.3.4 Yeast inoculated after target (CT)

The genera antagonistic activity data set was quite similar in the three approaches.

***Candida* boxplot** data presented the highest values of antagonism of the assay. Considering the dispersion of antagonist activity data, in the IER (Figure 19 J) ranged from 11% to 30%, in the IAC from 5% to 39% whereas in the IRG ranged from 11% to 34%. In the three approaches, the ***Candida* boxplot** data set was left-skewed and with the right tail relatively long. It displayed the median values of 24% (Figure 19 J), 31% (Figure 19 K), and 30% (Figure 19 L). It suggests that according to the three approaches, half of *Candida* yeasts presented more than 25% of antagonism activity.

Followed the *Candida* data set, the antagonistic activity data set of the other genera are quite similar.

Considering the dispersion of ***Torulaspora* boxplot** data in the IER (Figure 19 J) ranged from 13% to 22%, in the IAC from -2% to 25% whereas in the IRG ranged from 6% to 24%. It displayed a left-skewed data and right tail relatively long in IAC and IRG approaches (Figure 19 K and L) and one outlier of 4% in the IER approach.

The *Lachancea* genus presented the lowest antagonism values. The ***Lachancea* boxplot** was comparatively short and symmetrical. With exception for the IER approach which presented right-skewed data, suggesting that its antagonistic activity was less variable. The data dispersion in the three approaches was quite similar. In the IER approach (Figure 19 J), ***Lachancea* boxplot** presented an antagonistic range of 8% to 18% with an outlier at -4%; in the IAC approach (Figure 19 K) it displayed

a range of 4% to 15% with an outlier of -10% while in IRG (Figure 19 L) showed an antagonist range of 10% to 21% with an outlier at 3%.

Following *Lachancea*, *Metschnikowia* was the genus which less antagonistic efficacy. ***Metschnikowia* boxplot** data set was the most dispersed displayed, which suggests that the genus displayed a variability of responses against mycelial growth of TLS001. Considering the dispersion of ***Metschnikowia* boxplot** data in the IER (Figure 19 J) ranged from -9% to 20%, in the IAC from -19% to 20% whereas in the IRG ranged from -2% to 23%. It displayed a left-skewed data and left tail relatively long in the three approaches. Also, the ***Metschnikowia* boxplot** presented the lowest median values of 6% (Figure 19 J), 1% (Figure 19 K), and 10% (Figure 19 L).

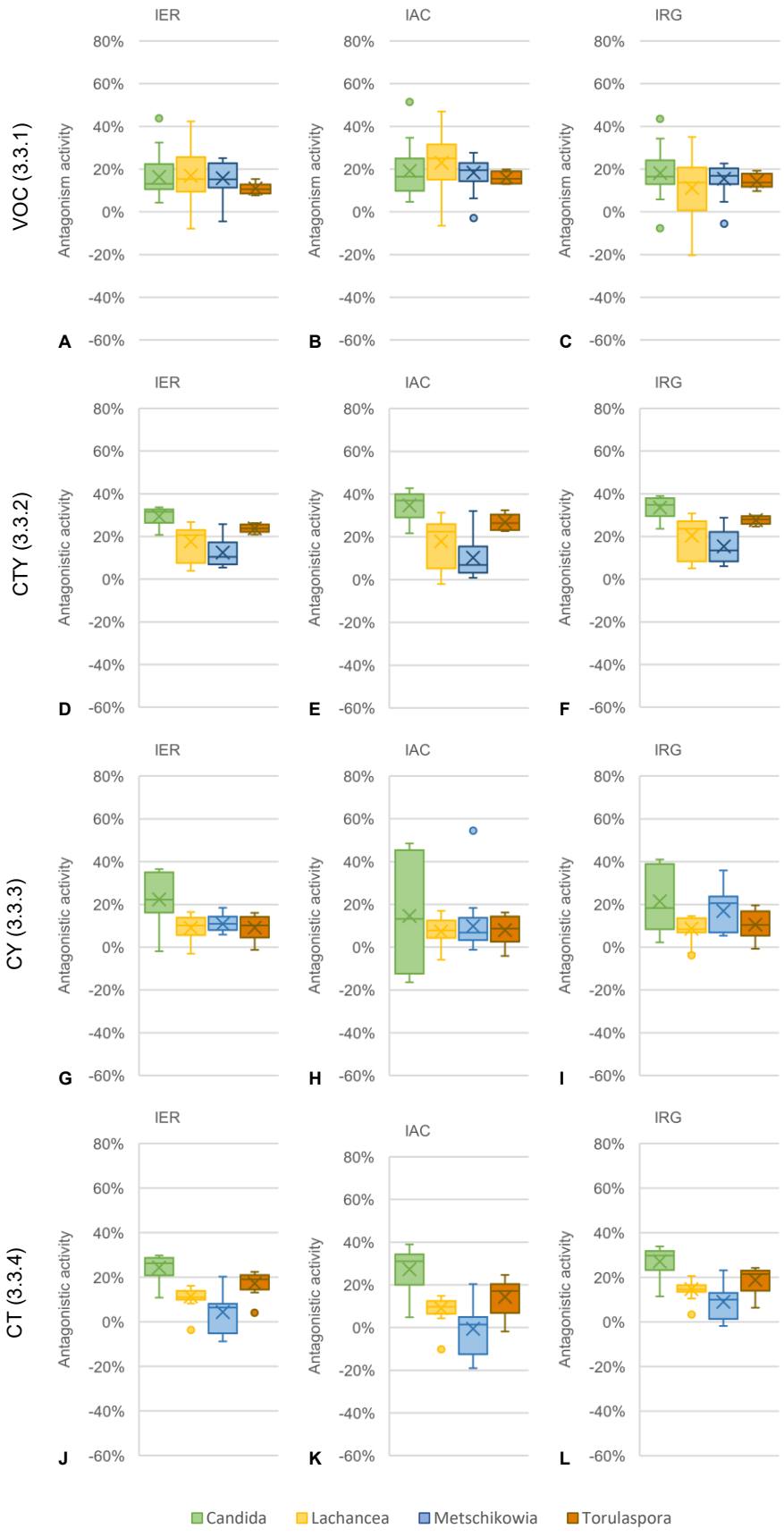

Figure 19 – Antagonistic activity data dispersion of yeast genera in the different methods VOC, CTY, CY, and CT by the three approaches application against TLS001

3.3.5 Global analysis

Production of volatile organic compounds

According to the **IER Overall boxplot** (Figure 20A), it was evident the dispersion of the genera antagonism data. It presented a right-skewed data set, with the median value of antagonism at 13.3%. The best antagonism activity results were defined by the three positive outliers: 41.4%, 42.4%, and 43.8%. The data dispersion was also evident in **IAC** and **IRG Overall boxplot** (Figure 20B and C) with values ranging from 13% to 26% and 11% to 35%, respectively. The **IAC Overall boxplot** (Figure 21B) displayed a median value of antagonism at 20%. The best antagonism results were defined by the three positive outliers: 46%, 47%, and 51%. The **IRG Overall boxplot** (Figure 20C) had the median value of antagonism at 15%, and the best antagonism results were defined by the two positive outliers, with 35% and 44%. In a general, the **IAC** approach presented the highest values of antagonism. In comparison with other assays, the VOC boxplot was the tallest of the three approaches.

Concerning the three approaches, to select the yeasts with the best antagonism activity a threshold was applied, in which yeasts with an antagonism activity equal or more than 25% were selected (Figure 21). Regarding the three approaches, 28 yeasts were selected (Figure 21A), namely 17 *Lachancea*, 8 *Candida*, and 3 *Metschnikowia* yeasts. A total of 27 yeasts including 17 *Lachancea*, 7 *Candida*, and 3 *Metschnikowia* yeasts were selected using the **IAC analysis**. It was the approach where more yeasts were selected. In the **IER approach**, 15 yeasts were selected, namely 7 *Lachancea*, 6 *Candida*, and 2 *Metschnikowia* yeasts. The **IRG** was the approach in which fewer yeasts were selected. The selection included 12 yeasts namely 6 *Candida* and 6 *Lachancea* yeasts. In the latter approach, one *Candida* yeast was selected, which was not in the IER and IAC approaches. Regarding the threshold, yeasts belonging to *Torulaspora* and *Saccharomycopsis* genus were not selected in any of the approaches.

The potential antagonism of yeast collection varied according to the approach. Indeed, 33% of the yeast collection has antagonist potential against TLS001 growth through VOC production. To each approach, the ratio of potential antagonism yeasts within the genus was analysed. In the **IER approach**, 24% of the yeasts within *Lachancea* were considered as potential antagonists, followed by 23% of the *Candida* and 10% of *Metschnikowia* yeasts. According to the **IAC approach**, 59% of yeasts within *Lachancea* were inserted as potential antagonists followed by 27% of the *Candida* and 14% of *Metschnikowia* yeasts. However, according to the **IRG approach**, only 23% of *Candida* and 21% of *Lachancea* yeasts were considered as potential antagonistic yeasts. Overall, *Lachancea* yeasts were represented in the three approaches as genus assembling the higher number of potential antagonist yeasts.

Yeast and target inoculated simultaneously (CTY)

Analysing the **Overall boxplot IER** (Figure 20A) a variability of the genera antagonism data was evident, which were bounded between 4% to 34%. Also, the data set was left-skewed, with the median value of antagonism sited at 22%. The data dispersion also was showed in **Overall boxplot IAC** and **IRG** (Figure 20B and C) in which were bounded between 11% to 32% and 14% to 31%, respectively.

Both data set distributions were also left-skewed. **Overall boxplot IAC** (Figure 20B) median value of antagonism was 24% while the **Overall boxplot IRG** (Figure 20C) approach was 26%. In a general, the IAC approach presented the highest values of antagonism. CTY boxplot data set presented the highest median values. It suggests that 50% of tested yeasts have more than 22% of antagonism activity, in this assay.

Concerning the three approaches, to select the yeasts with the best antagonism activity a threshold was applied, in which yeasts with an antagonism activity equal or more than 25%. In the three approaches 52 yeasts were selected (Figure 21B) namely 25 *Candida*, 14 *Lachancea*, 8 *Torulaspora*, 4 *Metschnikowia,* and 1 *Saccharomycopsis* yeasts. The same yeasts were selected by the **IRG approach**. The five yeasts genus were represented in this set, also the *Saccharomycopsis* yeast was selected in the three approaches. In **IAC analysis** a total of 40 yeasts was selected, including 24 *Candida*, 8 *Lachancea*, 5 *Torulaspora,* and 2 *Metschnikowia* yeasts. In the **IER approach**, 29 yeasts were selected, namely 20 *Candida*, 3 *Lachancea*, 4 *Torulaspora,* and 1 *Metschnikowia* yeasts.

Once again, the potential antagonism of yeast collection varied according to the approach. The yeast collection presented an antagonist potential value of 61% of TLS001 growth inhibition. In the **IER approach**, 77% of *Candida*, 50% of *Torulaspora*, 10% of *Lachancea,* and 5% of *Metschnikowia* yeasts were considered. According to the **IAC approach**, 92% of *Candida*, 63% of *Torulaspora*, 28% of *Lachancea,* and 10% of *Metschnikowia* yeasts were inserted as potential antagonists. The **IRG** was the approach in which more yeasts were selected. In this approach, all the *Torulaspora* yeasts, followed by 96% of the *Candida,* 48% of *Lachancea,* and 19% of *Metschnikowia* yeasts were considered as potential antagonists yeasts. Overall, *Candida* and *Torulaspora* yeasts were represented in the three approaches as the major genus assembling potential antagonist yeasts.

Yeast inoculated before target (CY)

According to **IER Overall boxplot** (Figure 20A), it displayed an antagonism data dispersion ranging from - 3% to 36%. It also presented a slightly right-skewed data set, with the median value of antagonism at 14%. The best antagonism activity was defined by the two positive outliers, 35% and 36%. The data dispersion was also evident in **IAC** and **IRG Overall boxplot** (Figure 20B and C) with values ranging from -16% to 54% and -4% to 41%, respectively. The **IAC Overall boxplot** (Figure 20B) displayed a median value of antagonism at 8%. The best antagonism results were defined by the four positive outliers, 45%, 47%, 48%, and 54%. The **IRG Overall boxplot** (Figure 20C) had the median value of antagonism at 12%, and the best antagonism results were defined by the only one outlier with 41% of antagonistic activity. In a general, the IAC approach presented the highest values of antagonism. Also, the CY boxplot data set presented the lowest median values.

Concerning the three approaches, to select the yeasts with the best antagonism activity a threshold was applied, in which yeasts with an antagonism activity equal or more than 25%. In the three approaches, 15 yeasts were selected, namely 11 *Candida*, 3 *Metschnikowia,* and 1 *Saccharomycopsis* yeasts (Figure 21 C). In **IAC analysis** a total of 9 yeasts were selected, including 7 *Candida* and 1

Metschnikowia yeast. In the **IER approach**, 10 yeasts were selected including 9 *Candida* yeasts. **IRG** was the approach where more yeasts were selected. The set included 15 yeasts namely 11 *Candida* and 3 *Metschnikowia* yeasts.

Concerning yeast collection, it presented an antagonist potential value of 18% of TLS001 mycelial growth inhibition. The *Saccharomycopsis* yeast was included in the three approaches. In the **IER approach**, 35% of *Candida* yeasts were considered. According to the **IAC approach**, 27% of *Candida*, and 5% of *Metschnikowia* yeasts were inserted as potential antagonists. Concerning the **IRG**, included 42% of the *Candida* and 14% of *Metschnikowia* yeasts. Overall, *Candida* yeasts were presented in the three approaches, which represent the main genus assembling potential antagonist yeasts.

Yeast inoculated before target (CT)

The three **Overall boxplot** data set presented a wide dispersion and right-skewed data. IER approach (Figure 20A) displayed a variability of antagonism data ranging from -9% to 30% while IRG displayed a data dispersion ranging from -2% to 34%, with median values sited at 14% and 16%, respectively. IAC was the approach with the higher data dispersion, ranging from -19% to 39% antagonistic activity but with a lower median value, 13%. AUC was also the approach with the highest values of antagonism.

Concerning the three approaches, to select the yeasts with the best antagonism activity a threshold was applied, in which yeasts with an antagonism activity equal or more than 25%. In the three approaches 21 yeasts were selected, namely 19 *Candida*, 1 *Saccharomycopsis,* and 1 *Torulaspora* yeasts (Figure 21D). The same yeasts were selected by **IAC analysis**. Regarding the **IER approach**, 19 yeasts were selected, namely 19 *Candida* and 1 *Saccharomycopsis* yeasts. The **IER and IRG approaches** selected the same yeasts.

Concerning yeast collection, presented an antagonist potential value of 25%. To each approach, the ratio of potential antagonism yeasts within each genus was analysed. The 19 *Candida* yeasts were selected by the three approaches, which means that 73% of *Candida* yeasts were considered as potential antagonists. In the **IAC approach**, one *Torulaspora* yeast was also included which suggests that according to this approach, 13% of *Torulaspora* could be inserted as potential antagonists.

The relations between the three approaches applied in the four methods are shown in Figure 20. In the VOC method, a strong relationship between IER and IAC was presented, with a correlation coefficient value of 0.91 (Figure 20D). In the CTY (Figure 20G, H and I) and CT method (Figure 20M, N and O), the three approaches showed strong relationships, with correlation coefficient values of 0.98 and 0.99. Nonetheless, in the CY method, the strongest relationships presented were between IER and IRG (Figure 20K) and IAC and IRG (Figure 20L) approaches, with a correlation coefficient values of 0.84 and 0.82, respectively. It suggests that the IRG can be a good approach to apply to the method data analyse

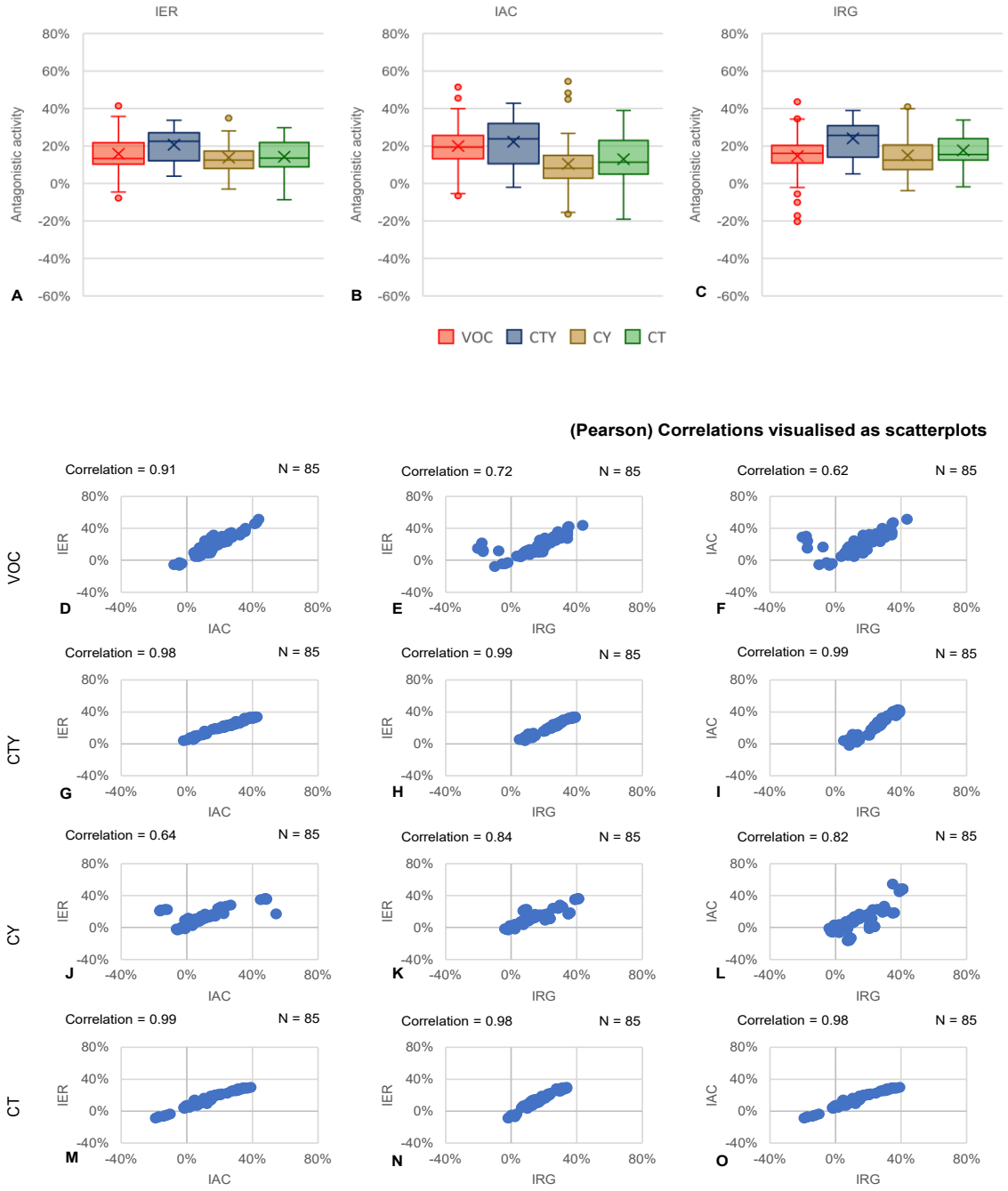

Figure 20 - Antagonistic activity overall according to the method VOC, CTY, CY, and CT by the three approaches application against TLS001; Correlation coefficient for each approach used in the four methods (VOC, CTY, CY and CT) against TLS001.

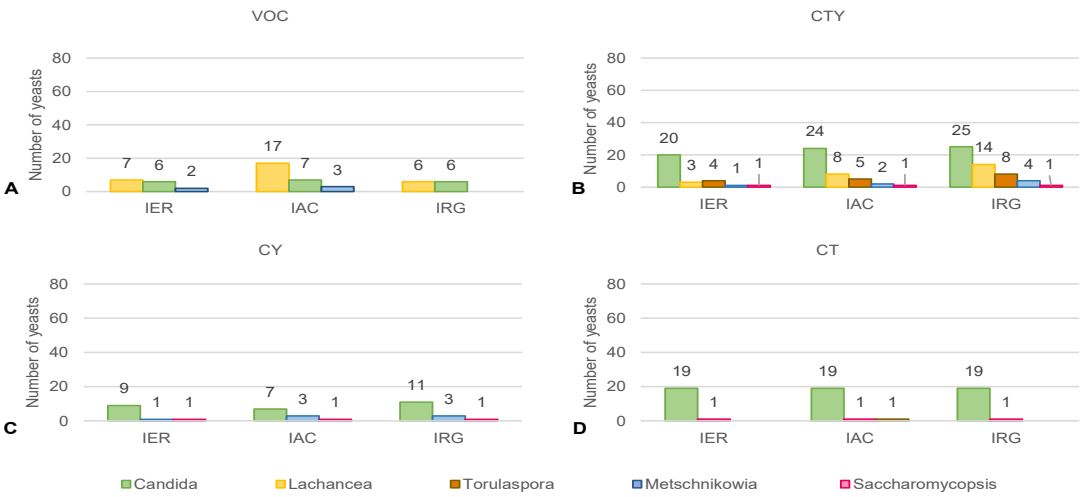

Figure 21 - Number of yeasts with equal or more than 25% of antagonistic activity against TSL001.

3.4. Does antagonistic activity against *Botrytis* rely on the yeast genus?

The results distribution obtained of yeasts of the five genera against FSC040 growth with the three approaches namely IER, IAC, and IRG were compared using boxplots (Figure 22). It was used the median value as a central trend measure. *Saccharomycopsis* yeast was not included in the genera comparison.

3.4.1. Production of volatile organic compounds

In the three approaches, the ***Candida* boxplot** displayed the data set widely dispersed which suggests a wide range of responses against FSC040 through VOC production. Considering the dispersion of antagonist activity data, in the IER (Figure 22A) ranged from -10 to 70%, in the IAC from -14% to 65% whereas in the IRG ranged from -5% to 68%. In IER and IAC approaches (Figure 22A and B), the ***Candida* boxplot** data set was slightly left-skewed, and the median values were 23% and 37%, respectively. It suggests that according to the IAC approach, half of *Lachancea* yeasts presented more than 37% antagonism activity. In the IRG approach (Figure 22C), the data set was right-skewed and presented the median value of 20%. ***Candida* boxplot** data presented the highest values of antagonism of the assay.

The followed genus with the most antagonistic activity yeasts was *Lachancea*, however it was lesser when compared with *Candida*. Considering the dispersion of antagonist activity data, in the IER (Figure 22A) ranged from -44 to 30%, in the IAC from -8% to 33% whereas in the IRG ranged from -8% to 30%. In IAC and IRG approaches the maximum antagonistic activity was defined by the two outliers of 38% and 55%, 42% and 57% respectively. In IER and IAC approaches (Figure 22A and B), the ***Lachancea* boxplot** data set was slightly left-skewed, and the median values were -4% and 12%, respectively. In the IRG approach (Figure 22C), the data set was right-skewed and presented the median value of 3%.

The *Metschnikowia* followed by the *Torulaspora* genus presented the yeasts with less antagonistic activity. Its antagonist activity data dispersion in the IER (Figure 22A), ranged from -17 to 25%, in the IAC from -0% to 26% whereas in the IRG ranged from -8% to 36%. The maximum antagonistic value in the IAC approach was defined by the one outlier with 33% of antagonist activity. In IER (Figure 22A), the ***Metschnikowia* boxplot** data set was left-skewed whereas in the IRG approach (Figure 22C) presented a right-skewed data, with the median values of 4% and 2%, respectively. In the IAC approach (Figure 22B), the data set symmetrical, presenting the median value of 12%.

At the three approaches, by ***Torulaspora* boxplot** data was showed that the yeasts of the genus did not present an antagonist efficacy in control the FSC040 growth through VOC producing. Indeed, in the three approaches, the antagonist activity was below 25%, being IAC the approach where they had a maximum of 22%. In the other two approaches, their action was negative or null.

3.4.2. Yeast and target inoculated simultaneously (CTY)

***Candida* boxplot** data presented the highest values of antagonism of the assay. Considering the dispersion of antagonist activity data, in the IER (Figure 22D) ranged from 16 to 35% with the median value sited at 27%. In the IAC approach (Figure E), the antagonist response ranged from -1% to 36% and with a median value of 23%. In both approaches, it displayed a left-skewed data which suggested that half of *Candida* yeasts presented more than 23% antagonism activity.

Followed *Candida*, ***Lachancea*** was the genus with the most yeasts with antagonistic activity. Considering the dispersion of antagonist activity data, in the IER (Figure 22D) ranged from 12 to 30% while in the IAC approach (Figure 22E) ranged from -1 to 27%. In both approaches, it displayed a left-skewed data with relatively right long tail and median values of 25% (Figure 22D) and 22% (Figure 22E).

In another hand, ***Metschnikowia*** followed *Torulaspora* were the genera with fewer yeasts with antagonistic activity, in which the maximum antagonistic activity was below 24%. The ***Metschnikowia*** and ***Torulaspora* boxplot**, both were comparatively short which suggests a less variable response in FSC040 growth inhibition. Considering the dispersion of *Metschnikowia* antagonist activity data, in the IER (Figure 22D) ranged from 13 to 23%, whereas in the IAC approach, ranged from -8 to 15% with the median values of 0% and 18%, respectively. The *Torulaspora* antagonist activity data dispersion in the IER (Figure 22A), ranged from 17 to 24%, in the IAC from 0% to 15%, with the median values of 19% and 5%, respectively.

Considering the IRG approach (Figure 22F), the antagonist activity values were quite similar between genera. Overall, the antagonistic activity varied from 15% to 35%. Considering the ***Candida* boxplot**, its antagonism response against FSC040 growth ranged from 25% to 34%, with the median value sited at 28%. This value was also observed for *Metschnikowia* and *Torulaspora* boxplot. Latter presented an outlier with 24% of antagonism activity. In the IRG approach, *Lachancea* was the genus with more data dispersion, ranging from 16% to 28% and with the median value of 26%.

3.4.3. Yeast inoculated before target (CY)

In the three approaches, the ***Candida boxplot*** displayed the data set widely dispersed which suggests a wide range of responses against FSC040 growth. Considering the dispersion of antagonist activity data, in the IER (Figure 22G) ranged from 17% to 43%, in the IAC from -15% to 23% whereas in the IRG ranged from 23% to 39%. In IER and IRG approaches, the ***Candida boxplot*** data set was right-skewed, and the median values were 24% and 28%, respectively. In the IAC approach, it presented a left-skewed data and with a median value of 10%.

Torulaspora was the second genus with the most antagonistic yeasts. Considering the dispersion of antagonist activity data, in the IER (Figure 22G) ranged from 19 % to 24%, in the IAC from 12% to 24% whereas in the IRG ranged from 19% to 35%. In IER and IRG approaches, ***Torulaspora boxplot*** data set was slightly left-skewed, and the median values were 23% and 26%, respectively. In the IER approach, it also presented a positive outlier of 32%. In the IAC approach, it presented a right-skewed data and with a median value of 13%.

Lachancea and *Metschnikowia* data was quite similar, however the first genus presented a higher level of data set agreement. The ***Lachancea* boxplot** (Figure 22G) data set presented an antagonistic activity ranging from 15% to 23% whereas ***Metschnikowia* boxplot** data, ranged from 9 to 23%. In the IAC approach, they displayed an antagonism activity ranging from 0 to 12% and -4 to 14%, whereas in the IRG approach ranged from 21% to 28% and 14% to 33%, respectively. ***Lachancea* boxplot** median values were 18% (Figure 22G), 5% (Figure 22H) and 24% (Figure 22I). Regarding ***Metschnikowia* boxplot** median values, were 6% (Figure 22G), 4% (Figure 22H) and 25% (Figure 22I).

3.4.4. Yeast inoculated after target (CT)

Regarding the IAC approach, the ***Candida* boxplot** presented the highest antagonistic activity values. Considering the dispersion of antagonism data, in the IER approach (Figure 22J), ***Candida* boxplot** presented an antagonism response against FSC040 ranging between 11% to 28% with two outliers of -4% and 4%. In the IAC approach (Figure 22H), it displayed a range from -2% to 26% presenting one outlier of -16%. ***Candida* boxplot** data set was left-skewed, and the median values were 22% and 19%, respectively.

Lachancea and *Metschnikowia* data was quite similar. The ***Metschnikowia* boxplot** (Figure 22K) data set presented an antagonistic activity ranging from 3% to 23% whereas ***Lachancea* boxplot** data, ranged from 7 to 25%. Similarly, to *Metschnikowia* data, it displayed a right-skewed data with a median value of 12%. In the IER approach, the ***Lachancea* boxplot** presents an antagonism response ranging between 19% to 32% whereas the ***Metschnikowia* boxplot** ranged from 15% to 32%, with the median values of 22% and 23%, respectively.

In the three approaches, the ***Torulaspora* boxplot** displayed the data set widely dispersed which suggests a wide range of responses against FSC040 growth. Considering the dispersion of antagonist activity data, in the IER (Figure 22J) ranged from -4 to 19%, in the IAC from -14% to 12% whereas in the IRG ranged from -1% to 28%. In the three approaches, the ***Torulaspora* boxplot** data set was left-skewed, and the median values were 14%, 4%, and 16%, respectively. ***Torulaspora* boxplot** data presented the lowest values of antagonism of the assay.

Considering the IRG approach (Figure 22L), the antagonist activity values were quite similar between genera. With exception of *Torulaspora*, the genera boxplot presented a median value of 28%. Considering the ***Candida* boxplot**, its antagonism response against FSC040 growth ranged from 25% to 34%.

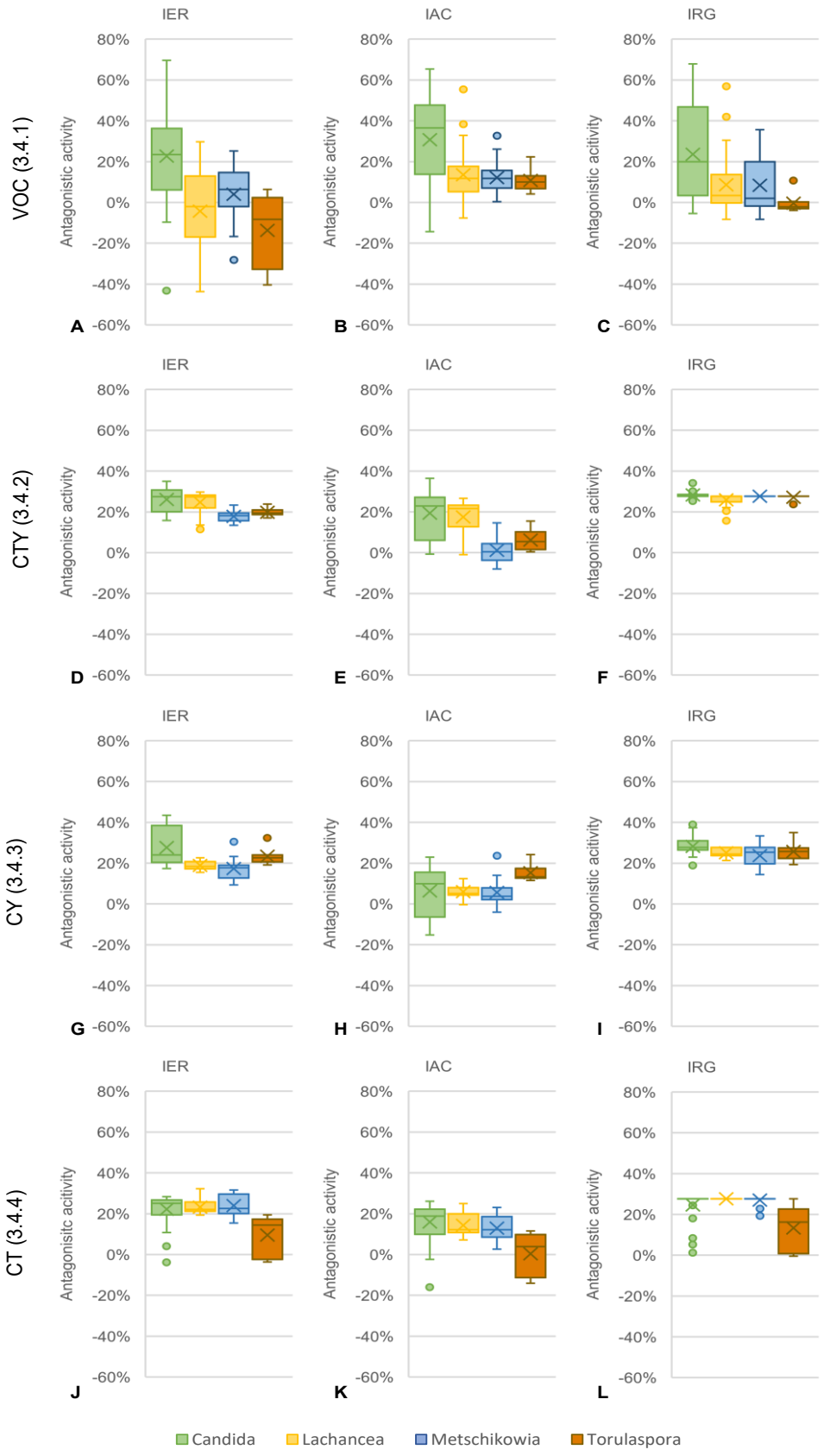

Figure 22 - Antagonistic activity data dispersion of yeast genera in the different methods VOC, CTY, CY, and CT by the three approaches application against FSC040 target.

3.4.5. Global analysis

Production of volatile organic compounds

Analysing the **IER overall boxplot** (Figure 23A) was showed a wide variability of the genera antagonism data which were bounded between -44% to 52%. Also, the data set was slightly left-skewed, with the median value of antagonism sited at 5%. The best antagonism result was defined by the outlier, with 70% of antagonistic activity. The data dispersion was also evident in **IAC** and **IRG Overall boxplot** (Figure 23B and C) in which were bounded between 7% to 28% and 0% to 23%, respectively. Both data set distribution was right-skewed. **Overall boxplot IAC** (Figure 23B) presented the median value of 13% and the best antagonism results were defined by the two positive outliers: 60 and 65% of antagonism activity. The **IRG approach Overall boxplot** (Figure 23C) had the median value of antagonism sited at 4%, the best antagonism result was defined by the one outlier, 68%. In a general, the IRG approach presented the highest values of antagonism. In comparison with the other assays, the VOC boxplot is the tallest, presenting the most dispersed data in the three approaches.

In order to select the yeasts with the best antagonism activity, a threshold was applied. In the three approaches data set (Figure 24A), the yeasts with equal or more than 25% of antagonism activity were selected. The set of number of yeasts selected varied according to the approach applied. Considering all the approaches, 30 yeasts were selected, namely 19 *Candida*, 6 *Lachancea*, 4 *Metschnikowia,* and 1 *Saccharomycopsis* yeasts. Regarding the threshold, the *Torulaspora* yeasts were not selected. In **IAC analysis** a total of 25 yeasts was selected, including 17 *Candida*, 5 *Lachancea*, 2 *Metschnikowia,* and 1 *Saccharomycopsis* yeasts. It was the approach where more yeasts were selected. In the **IER approach**, 16 yeasts were selected, namely 13 *Candida*, 2 *Lachancea*, and 1 *Metschnikowia* yeasts. In the **IRG approach**, 12 yeasts were included in the selection namely 4 *Candida* yeasts and 4 *Lachancea* yeasts. In this subset were selected 5 more yeasts were selected than in the previous approaches, namely 2 *Candida*, 1 *Lachancea*, and 2 *Metschnikowia* yeasts. Thus, it was the approach with lesser yeasts selected.

Concerning the potential antagonism of yeast collection, presented an antagonist potential value of 35%. To each approach, the ratio of potential antagonism yeasts within each genus was analysed. In the **IER approach**, half of the genus *Candida* yeasts were considered as potential antagonists, followed by 7% of the *Lachancea* and, 5% of *Metschnikowia* yeasts. According to the **IAC approach**, about 65% of the genus *Candida* yeasts were inserted as potential antagonists followed by 17% of the genus *Lachancea* yeasts and 10% of *Metchknowia* yeasts. This trend also was observed in the **IRG approach**, 46% of *Candida*, 19% of *Metchknowia,* and 14% of *Lachancea* genus yeasts were considered as potential yeasts to control FSC040 growth. Overall, *Candida* yeasts were represented in the three approaches as the major genus assembling potential antagonist yeasts.

Yeast and target inoculated simultaneously (CTY)

The antagonism activity results were different in the three approaches. The **IER Overall boxplot** data set (Figure 23A) displayed a variability of antagonism data ranging from 12% to 35% with a median

value of 23%. Regarding **IAC Overall boxplot** (Figure 23B), it presented the higher data dispersion, displaying long tails and left-skewed data, with an antagonistic activity data ranging from -8% to 36%. It also displayed the lowest median value,15%. Regarding the IRG approach (Figure 23C), it was that presented the less data dispersion, with an antagonistic activity ranging from 16% to 34% and a median value of 28%.

In order to select the yeasts with the best antagonism activity, a threshold was applied. In the three approaches data set (Figure 24B), the yeasts with equal or more than 25% of antagonism activity were selected. Considering the three approaches, 77 yeasts were selected (Figure 24B), namely 26 *Candida*, 22 *Lachancea*, 21 *Metschnikowia,* 7 *Torulaspora,* and 1 *Saccharomycopsis* yeast. This selection was the same to **IRG approach**. In **IAC analysis** a total of 11 yeasts were selected, including 9 *Candida*, 1 *Lachancea,* and 1 *Saccharomycopsis* yeasts. In the **IER approach**, 32 yeasts were selected, namely 9 *Candida*, 22 *Lachancea,* and 1 *Saccharomycopsis* yeasts.

Concerning yeast collection, presented an antagonist potential value of 88%. The *Saccharomycopsis* yeast was included in the three approaches. In the **IER approach**, 73% of *Candida* and 76% of *Lachancea* yeasts were considered. According to the **IAC approach**, 35% of *Candida* and 3% of *Lachancea* yeasts were inserted as potential antagonists. Concerning the **IRG approach**, where more yeasts were selected, including 76% of *Lachancea* and 88% of *Torulaspora* yeasts. Also, all *Metschnikowia* and *Candida* yeasts were considered as potential yeasts to suppress FSC040 growth.

Yeast inoculated before target (CY)

The three **Overall boxplot data** set presented a right-skewed data. IER approach (Figure 23A) displayed a variability of antagonism data ranging from 9% to 30% with five positive outliers of 32%, 34%, 38%, 40%, and 43%. The IRG approach (Figure 23C) displayed a data dispersion ranging from 17% to 35% and three negative outliers of -11%, -13%, and -15%. Also presented a left-skewed data. In AUC (Figure 23B) ranged from -7% to 24% antagonistic activity with two positive outliers of 37% and 39%.

In order to select the yeasts with the best antagonism activity, a threshold was applied. In the three approaches data set (Figure 24C), the yeasts with equal or more than 25% of antagonism activity were selected. Considering the three approaches 49 yeasts were selected (Figure 24C), namely 21 *Candida*, 12 *Lachancea,* 11 *Metschnikowia,* 5 *Torulaspora,* and 1 *Saccharomycopsis* yeasts. The same yeasts were selected by **IRG analysis**. In the **IER approach**, 15 yeasts were selected, namely 12 *Candida*, 2 *Metschnikowia,* and 1 *Torulaspora* yeasts. According to the threshold applied, no yeasts were selected in the **IAC approach**.

The yeast collection presented an antagonist potential value of 58%. In the **IER** approach, 46% of *Candida* yeasts were considered followed by 10% of *Metschnikowia* and 13% of *Torulaspora* yeasts. Concerning the **IRG**, was the approach where more yeasts were selected. In this approach were

included 81% of the *Candida,* 52% of *Metschnikowia*, 63% of *Torulaspora,* and 41% of *Lachancea* yeasts.

Yeast inoculated after target (CT)

The antagonism activity results were different in the three approaches. The **IER Overall boxplot** data set (Figure A) displayed a variability of antagonism data ranging from 13% to 32% with four outliers with values above 11%. It also presented a median value of 22%. In **IAC Overall boxplot** (Figure 23B) was presented a wide data dispersion with long tails and left-skewed data bounded from -6% to 26%, with two outliers of -16% and -14%. Both approaches presented right-skewed data. Considering the IRG approach (Figure 23C), the antagonist activity values were quite similar. The antagonistic activity varied from 15% to 35% and presented a median value of 28%.

In a total of the three approaches, 71 yeasts were selected (Figure 24D), namely 29 *Lachancea*, 21 *Candida*, 19 *Metschnikowia,* 1 *Torulaspora,* and 1 *Saccharomycopsis* yeasts. The same yeasts were selected by **IRG analysis**. In **IAC analysis** a total of 4 yeasts was selected, including 3 *Candida*, and 1 *Lachancea* yeasts. In the **IER approach**, 35 yeasts were selected, namely 17 *Candida*, 9 *Metschnikowia,* 8 *Lachancea,* and 1 *Saccharomycopsis* yeasts.

The yeast collection displayed an antagonist potential value of 84%. In the **IER approach**, 65% of *Candida* followed by 43% of *Metschnikowia* and 28% of *Lachancea* yeasts were considered. According to the **IAC approach**, 12% of *Candida*, and 3% of *Lachancea* yeasts were inserted as potential antagonists. Concerning the **IRG**, was the approach where more yeasts were selected. In this approach, 90% of *Metschnikowia* followed by 81% of *Candida,* and 13% of *Torulaspora* yeasts were considered as potential yeasts to suppress FSC040 growth. Also, all *Lachancea* yeasts were considered as potential antagonists.

The relations between the three approaches applied in the four methods are shown in Figure 23. In the CTY method, a strong relationship between IER and IAC was presented, with a correlation coefficient value of 1.00 (Figure 20G). Also, in the CT method, the same relationship between IER and IAC was presented with a correlation coefficient value of 1.00 (Figure 20M). It suggests that the IER can be a good approach to apply to the method data analyse. In the CY method, the strongest relationships presented were between IER and IRG (Figure 20K) approaches, with a correlation coefficient values of 0.79. Also, in the VOC method, the three approaches showed good relationships, with correlation coefficient values of 0.71 (Figure 20D), 0.68 (Figure E) and 0.73 (Figure F) which suggests IRG can be a good approach to apply in these method data analyse

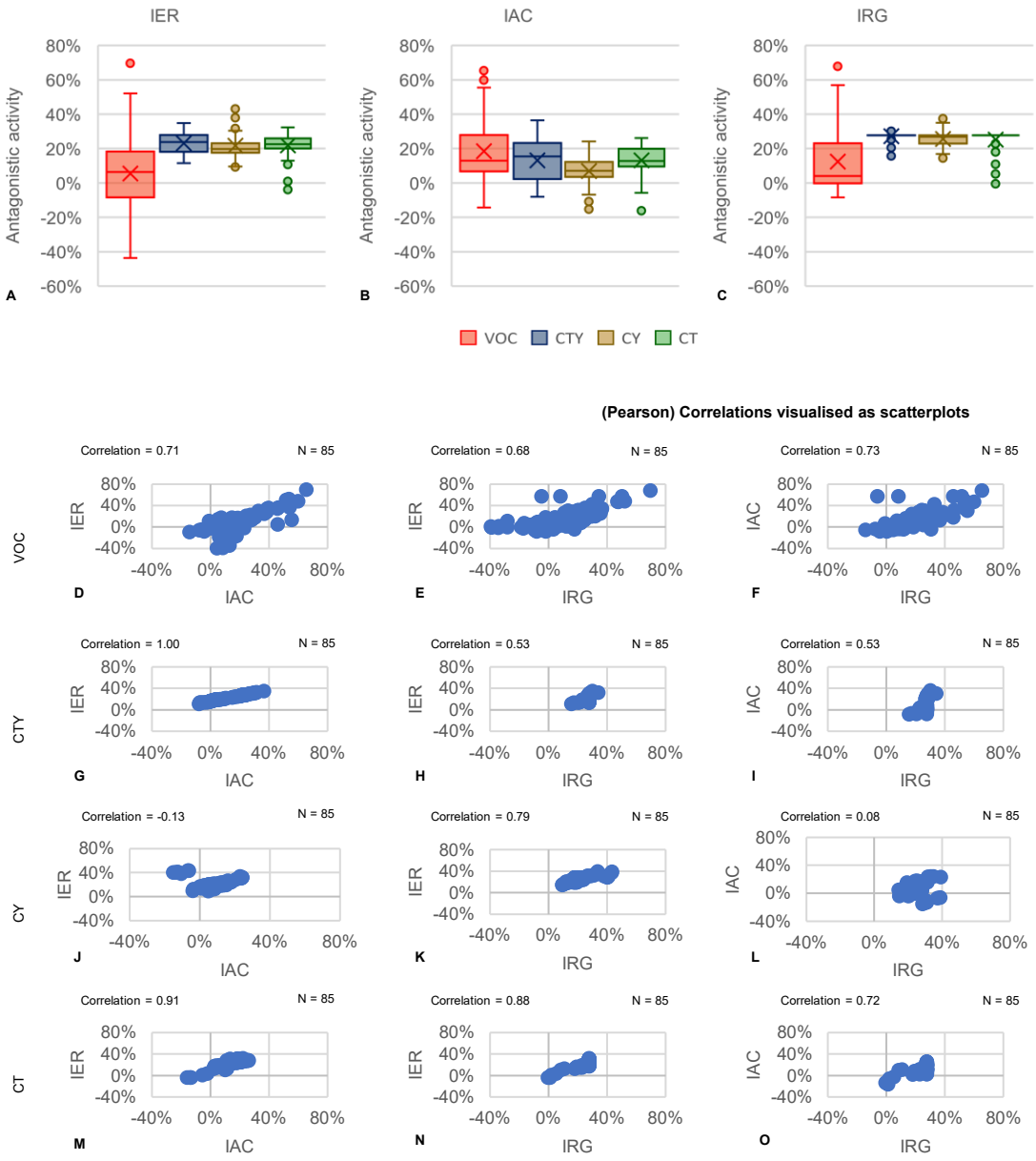

Figure 23 - Antagonistic activity overall according to the method VOC, CTY, CY, and CT by the three approaches application against FSC040; Correlation coefficient for each approach used in the four methods (VOC, CTY, CY and CT) against TLS001.

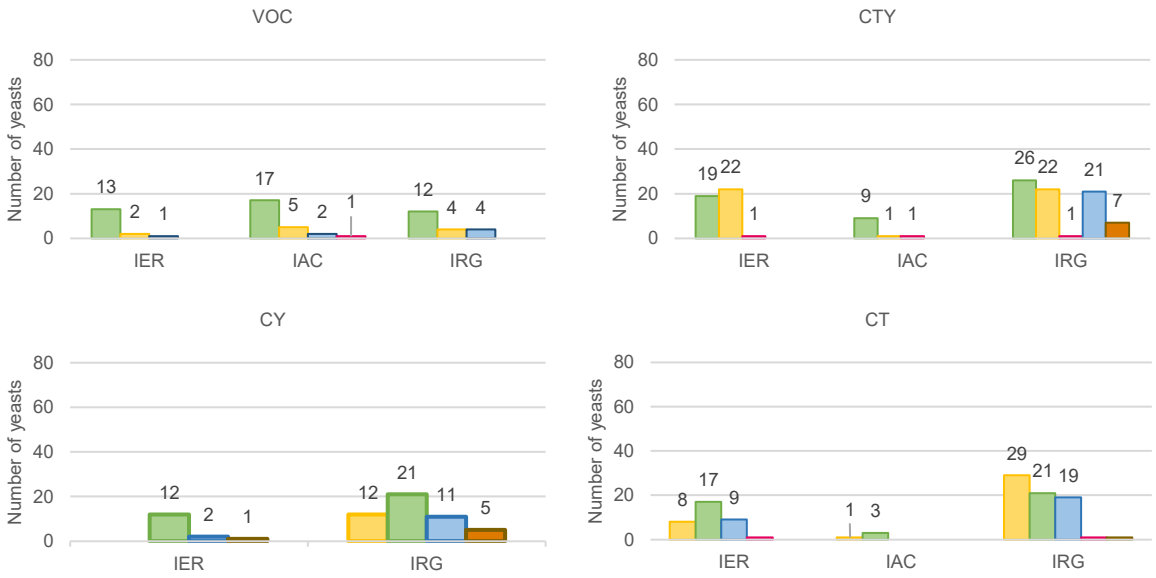

Figure 24 – Number of yeasts with equal or more than 25% of antagonism against *Botrytis* in VOC, CTY, CY, and CT according to the three approaches (IER, IAC, and IRG)

3.5. Selection of the best biocontrol yeast strains

The 10 best-ranked yeasts in all the methods of dual assays for *Aspergillus* and *Botrytis*, were selected. Firstly, the 10 best-ranked yeasts in the four methods (CTY, CT, CY, and VOC) analysed by the three approaches (IER, IAC, and IRG) were considered (Figure 25 and 26).

Aspergillus (Figure 25)

According to the CTY method, it was not selected any yeast using only the IER approach. When the IRG approach was applied exclusively, one yeast, Y72, was selected. With a combined approach, the Y641 was selected. In a combination of the IER and IAC approach, the Y678 was selected. The Y498 was selected by the IAC approach. Applying the three approaches, 8 yeasts were selected which means that 66.7% of the 10-best ranked yeasts were included. The selection included the yeasts Y86, Y182, Y298, Y391, Y395, Y490, Y611 and Y741.

In the CY method, when applied only the IER approach the Y365 was selected, while with the IRG approach two yeasts were selected namely Y366 and Y375. Regarding the IAC approach, when combined with the IER, two yeasts were selected namely Y86 and Y611. While, when combined with the IRG approach, the Y336 was selected. Applying the three approaches, seven common yeasts were selected including Y226, Y287, Y369, Y391, Y498, Y538, and Y678.

Concerning the CT method, the Y365 was selected by the IER approach, while Y4 was selected by the IAC approach. A combined approach selected the Y538. However, when the IRG approach was

applied exclusively, two yeasts were selected including Y72 and Y298. Combining the three approaches 8 yeasts were chosen, including Y182, Y391, Y395, Y490, Y523, Y641, Y678, and Y741.

In the VOC, when the IRG approach was applied exclusively selected the Y90 while, the IAC chosen the Y72. A combined approach selected one common yeast, the Y206. Applying the IER and IEG approaches, two yeasts were selected, including Y641 and Y298. Regarding IER and IAC approaches, selected the Y632 and Y675. Applying the three approaches, six yeasts were selected which means that 46.2 % of the 10-best ranked yeasts were included. The selection included the yeasts Y237, Y303, Y371, Y390, Y538, and Y594.

It was also integrated the data of the four methods (CTY, CY, CT, and VOC) analysed with the three approaches. According to IER, one yeast common to the four methods was selected, namely Y538. Considering the IAC and IRG approaches were not selected any yeast.

Botrytis (Figure 26)

According to the CTY method, it was not selected any yeast using only the IER or IAC approach. However, when both approaches were applied, two yeasts were selected, namely the Y237 and Y490. When the IRG approach was applied exclusively, two yeasts were selected including Y214 and Y4. Applying the three approaches, 8 yeasts were selected which means that 66.7% of the 10-best ranked yeasts were included. The selection included the yeasts, Y72, Y86, Y298, Y365, Y498, Y611, Y641, and Y741.

In the CY method, when applied only the IRG approach the Y365 was selected, while with the IER approach three yeasts were selected namely Y678, Y391, Y369. When both approaches were applied, four common yeasts were selected including Y226, Y287, Y498, and Y538. Regarding the IAC approach, five yeasts were selected namely Y92, Y232, Y503, Y641, and Y741. When combined with the IRG approach, two yeasts were elected namely Y206 and Y736. Applying the three approaches, three common yeasts were selected which include Y72, Y293, and Y468.

The CT was the method in which fewer common yeasts by the three approaches were selected. It selected two yeasts, the Y72 and Y214. When applied only the IER approach seven yeast were selected, namely Y94, Y134, Y167, Y171, Y172, Y173, and Y675. The combination of IER and IAC approach selected only one yeast, the Y632. However, when the IAC approach was applied exclusively, four yeasts were selected including Y86, Y287, Y375, and Y653. A combined approach applied by IAC and IRG chosen three yeasts, including Y4, Y226, and Y369. When applied only the IRG approach, five yeasts were selected namely Y182, Y395, Y490, Y498, and Y523.

In the VOC, when the IER approach was applied exclusively selected the Y490 and Y498 while, the IAC chosen the Y483 and Y369. A combined approach selected two common yeasts, Y72 and Y523. In the IRG approach, four yeasts were selected, including Y25, Y204, Y322, and Y735. Applying the three approaches, 6 yeasts were selected which means that 37.5% of the 10-best ranked yeasts were included. The selection included the yeasts Y4, Y391, Y538, Y641, Y678 and Y741

It was also integrated the data of the four methods (CTY, CY, CT, and VOC) analysed with the three approaches. According to IER and IAC approach, one yeast common to the four methods was selected, namely Y72. Considering the IAC approach was not selected any yeast.

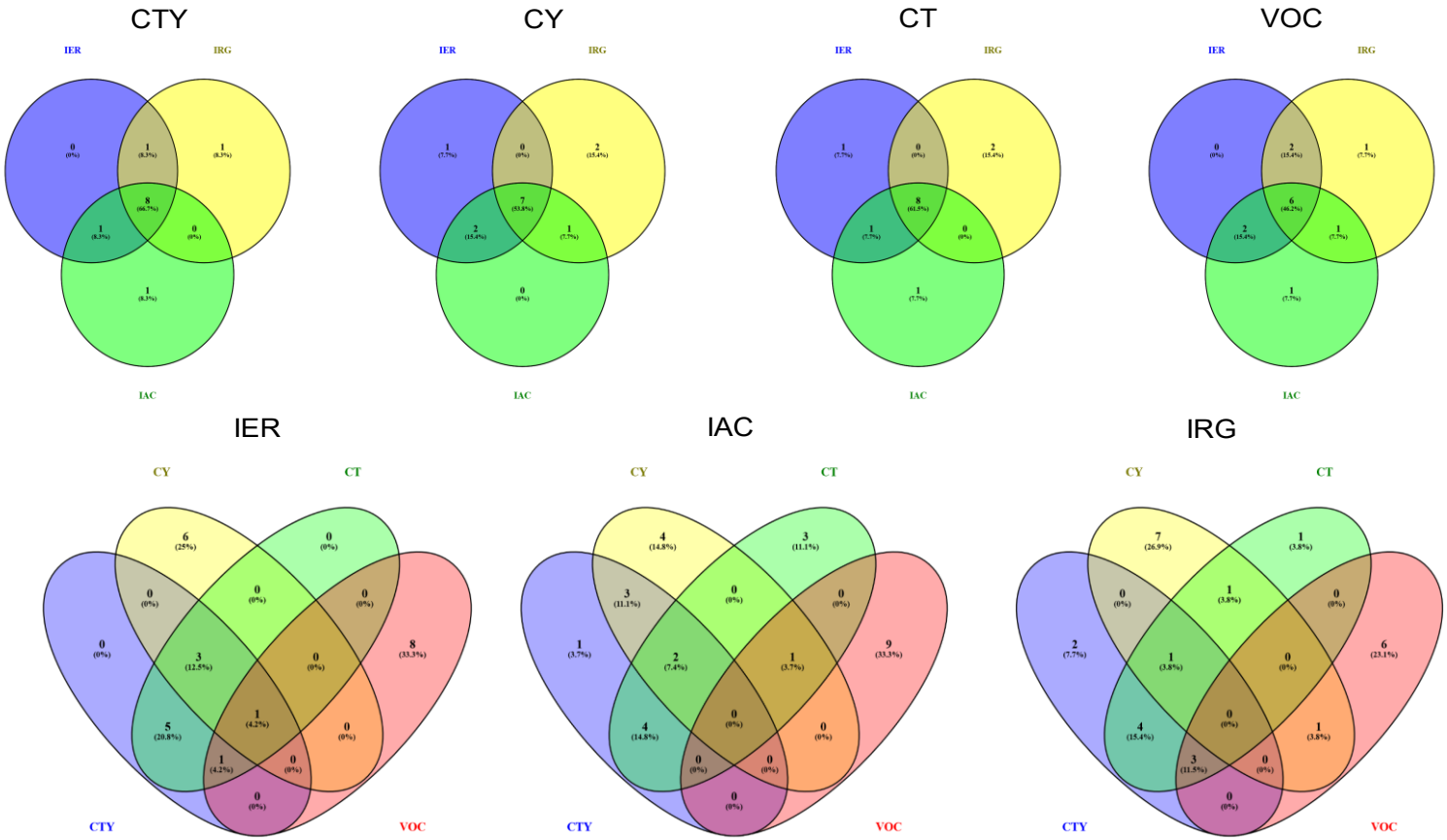

Figure 25 - The 10 best-ranked yeasts against *Aspergillus* sp. based in the four methods (CTY, CT, CY and VOC) and analysed by the three approaches (IER, IAC and IRG).

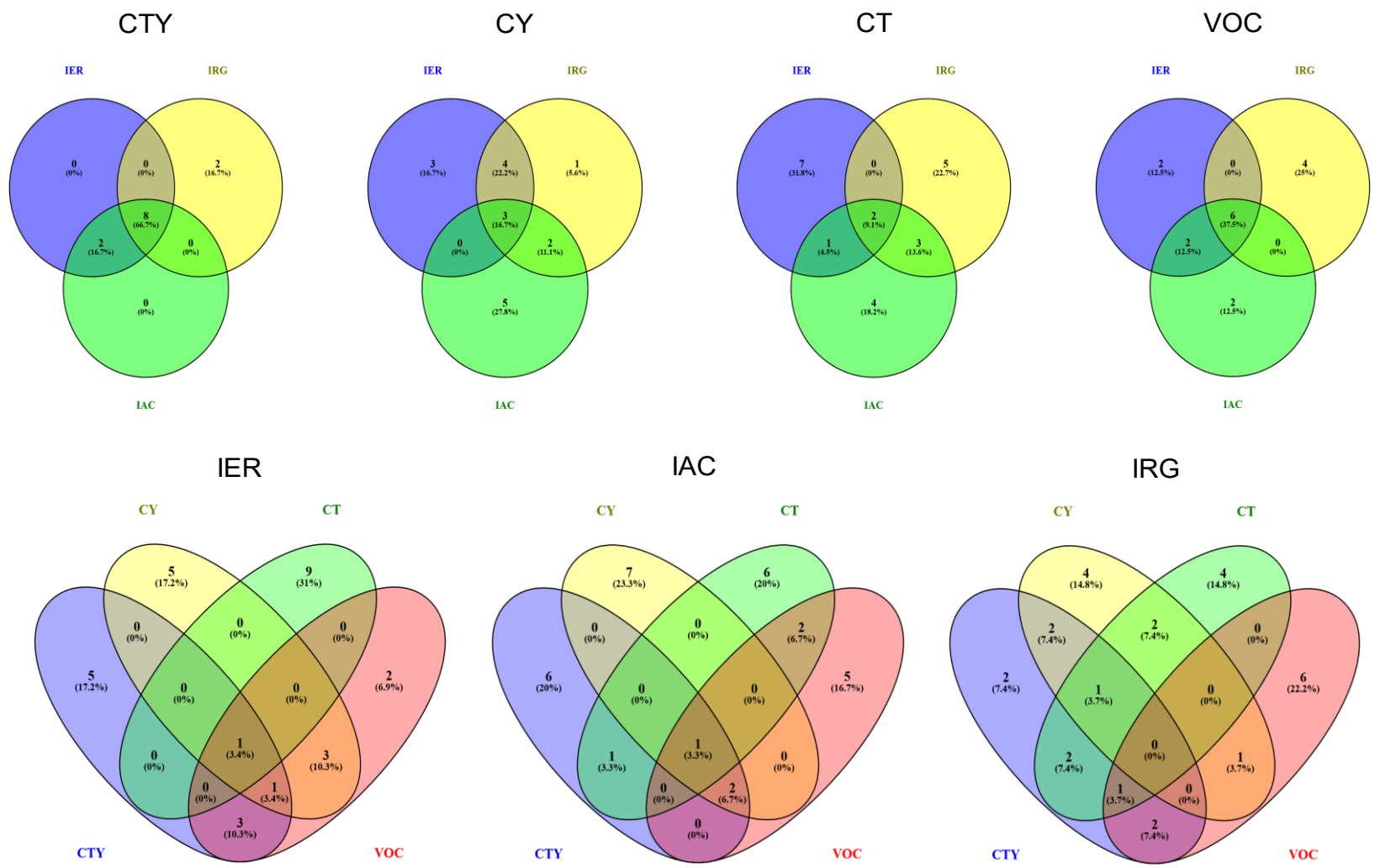

Figure 26 - The 10 best-ranked yeasts against *Botrytis* sp. based in the four methods (CTY, CT, CY and VOC) and analysed by the three approaches (IER, IAC and IRG).

Based on the selection of the best yeasts at the four methods by the three approaches, the main potentially antagonistic yeasts were considered (Table 1). Additionally, in order to select the best biocontrol yeasts results of spore gemination assay (IGSL and IGSA) (Supplementary Table II) and killer phenotype assay (KP) were integrated.

Table 1 - The best yeasts select by the four methods (VOC, CT, CTY, CY) using the three approaches (IER, IAC, and IRG). Integration of the best yeasts in spore germination assay realized in liquid medium (IGSL) and in agar medium (IGSA). Yeasts with killer phenotype (KP) were also considered. The black-coloured cell means that the yeast was selected as an antagonist in that assay. The green-coloured cell means the best-ranked antagonistic yeasts.

	Aspergillus				*Botrytis*						*Aspergillus*	
	VOC	CT	CY	CTY	VOC	CT	CY	CTY		KP	IGSL	IGSA
Y72												
Y86												
Y298												
Y365												
Y498												
Y611												
Y641												
Y741												
Y293												
Y468												
Y214												
Y4												
Y391												
Y538												
Y678												
Y182												
Y395												
Y490												
Y226												
Y287												
Y369												
Y523												
Y237												
Y303												
Y371												
Y390												
Y594												

It was selected a total of 27 yeasts. In the different methods, Y678 was the yeast more included (bold). However, there was not a yeast that was selected by all the methods. In fact, some yeasts displayed more antagonism activity with an assay than in others. Also, it was possible to verify that the same yeast can have different antagonistic activity against different fungal targets using the same assay.

Regarding the VOC method, there was one antagonistic yeast commonly selected for *Aspergillus* and *Botrytis* target, which means a relevant result, especially for industrial production. The efficacy against both fungi permits a singleness biocontrol agent application. Additionally, is also promising for the postharvest approach. The application of VOCs during postharvest transit is an eco-friendly, cost-effective, and do not represent a health concern. The VOCs producing do not contact with fruit surface, they can be used as bags containing lyophilized culture that is reactivated by hydration. Also, VOCs are produced in low amounts, are biodegradable so they do not leave residues on the fruit surface (Ippolito

and Nigro, 2000; Tilocca *et al.*, 2019). Also, yeasts are part of grape microbiota, taking the advantage of their by-products do not interfere with the equilibrium of the natural environment (Bleeve *et al.*, 2005). In the literature, is widely reported the efficacy of yeasts volatiles produced in controlling or suppressing the fungal pathogen control. Hua *et al.*(2011) reported a *Candida intermedia* strain C410 which suppressed the growth of *Botrytis cinerea in vitro* and *Botrytis* fruit rot in strawberry through the production of volatiles. Also, Tilocca *et al.* (2019) reported the antagonistic activity of *Candida intermedia* against *A. carbonarius*. The yeast was efficient in inhibit the radial growth, sporulation, and the production of ochratoxin A by the 2-phenylethanol production. Farbo *et al.* (2018) reported a *Lachancea thermotolerans* strain which was effective in reducing mycelial growth, sporulation, and *in vitro* OTA production by *A. carbonarius* and *A. ochraceus* through VOCs release.

In biocontrol, there are two application methods: pre-harvest and postharvest application. Pre-harvest application is a preventive measure that includes the application of the biological control agent in the field until the harvest. The biocontrol agent can colonize the fruit before the colonization by the pathogenic fungi. The postharvest approach is a curative measure, in which through the biofumigation or antagonistic application, the fungal growth is controlled, therefore the fruit spoilage is reduced. (Sharma *et al.*, 2009; Pliego *et al.*, 2011). Regarding this, the CY method was designed to screen antagonistic yeasts to be applied at pre-harvest while CT and CY were designed to screen yeasts potentially antagonistic for the postharvest application. In the CY method, 7 yeasts antagonistic yeasts for *Aspergillus* and only three for *Botrytis* were selected. Also, none of the selected yeasts showed antagonistic activity for both fungi. Similarly, in the CT method, more antagonistic yeasts for *Aspergillus* than for *Botrytis* were selected and none of the selected yeasts showed antagonistic activity for both fungi. Select individual strains antagonists against a broad spectrum of pathogens is difficult, in order to enhance their efficacy, antagonistic yeasts mixtures can be applied. Antagonistic yeasts gather several mechanisms of action thus when combined in a mixture, the antagonistic action will not rely on an activity of one strain but the result of the action of the antagonistic mixture (Sharma *et al.*, 2009).

CTY was the method with the most yeasts selected (both for *Aspergillus* and *Botrytis*), and four selected yeasts inhibited the mycelial growth of both fungi. This antagonistic activity could be underlying on the production and release of antimicrobial enzymes, metabolites, and toxins as well as a strategy of competition for space and nutrients. Indeed, the latter strategy seems to be the main mode of action of antagonists to control the growth of the fungal pathogens (Sharma *et al.*, 2009, Freimoser *et al.*, 2019). It also seems to be the principal mode of action of *Candida sake* strain, which was effective in controlling the *Penicillium* (Usall *et al.*, 2001; Morales *et al.*, 2008), *Botrytis* (Wilson *et al.*, 1993), and *Rhizopus* rot of apple (Viñas *et al.*, 1998). Droby *et al.* (1999) also showed that *Candida sake* was effective in inhibiting the *Penicillium digitatum* growth on grapefruit. Nally *et al.*(2012; 2015) reported the efficacy of the yeast strain to control the *Aspergillus terreus, Aspergillus carbonarius,* and *Penicillium commune* growth on grape. In the same study, it was described the efficacy of *Torulaspora delbrueckii* to control *Penicillium commune, Aspergillus caelatus, Aspergillus terreus* and, *Fusarium oxysporum* growth on grape.

Inhibition of spore germination assays were only tested with the *Aspergillus* strain and were recorded as qualitative results. To select the best antagonistic yeasts against *Aspergillus* the results obtained

were integrated into the other four methods. The assay in a liquid medium assembled the greatest number of yeasts selected, 11 antagonistic yeasts. In the agar medium, only four yeasts were selected. This result differs from that obtained by Swadling *et al.*(1996) in which from the 108 isolates selected by fungal mycelial growth inhibition assay, only 27 showed antagonism by inhibition of spore germination.

Spore germination inhibition of the *Aspergillus* strain could be due to competition for nutrients since it required the presence of nutrients for germination to occur (Hayer *et al.*, 2014). Yeasts were inoculated before the fungal target, which gave the advantage of consumption of nutrients more rapidly with the target, consequently inhibiting their spores germination.

It is documented that the efficacy of the biocontrol agents *in vitro* is not guaranteed *in vivo* or the field (Kasfi *et al.*, 2018; Besset-Manzoni *et al.*, 2019) and for that reason, the design of the screening step is crucial. Concerning this, in this study was not designed a stepwise screening of the tested yeasts, but they were considered in all the screening assays. Also, the results obtained evaluated by three approaches permitted more confidence in selection analyse. Lemos Junior *et al.* (2016) used *in vitro* followed by *in vivo* assays (wounded grape berries) and reported a positive correlation of the efficacy of *S.bacillaris* against *Botrytis cinerea*, in both methods. It suggests that results obtained by *in vitro* screening are indicative of the antagonism mechanisms. The *in vitro* screening permitted a quick, cost-effective, and a massive screening of the 85 yeast strains. From the 27 best- ranked yeasts, a subset of 6 yeasts were selected according to antagonism action displayed, namely Y72, Y237, Y287, Y298, Y395 and Y538. Regarding this subset, will be applied further *in vivo* screenings aiming a selection of an efficient biocontrol agent.

4. Conclusion and Future Perspectives

It was characterized 6 *Aspergillus* sp. isolates and 7 *Botrytis* isolates using the PCR-fingerprinting method. It was a rapid and highly reproducible method. It permitted verify an intraspecific variation for *Aspergillus* isolates. However, a higher number of isolates is needed to infer about the population and its structure. Characterization of the pathogens is important to biocontrol approach since the incidence of same fungi, namely *Botrytis* and *Aspergillus* are correlated with climatic conditions as well with the different practices used in grape cultivation and the specific microclimate of the vineyard. Regarding this MSP-PCR fingerprinting should be addressed with further analysis including with RFLPs analysis.

To evaluate the antagonistic potential of yeast collection the killer phenotype assay was applied, revealing that about 11% of collection strains can have biocontrol potential. The killer phenotype was not widely distributed amongst the yeasts tested, just only in *Candida* and *Metschnikowia* genus however, the sensitive phenotype was not screened in this assay. Thus, in the future the sensitive phenotype should be screened.

Regarding the selection of the best antagonistic yeasts, the application of the three approaches permitted more confidence in the yeasts selected to each method. This integration approach allowed to verify what the better yeasts against *Botrytis* and *Aspergillus*. Also, were verified that antagonistic yeasts relied on the method applied.

On balance, as a result of this study a subset of 6 yeasts was selected. These potential antagonists expressing the ability to inhibit the growth of pathogens are chosen to the next screen where they are carried throughout complex, costly, and time-consuming assays. These assays integrate a higher number of factors besides the interaction between antagonist and pathogen and mimic the field situation. This screening may be performed *in vivo*, using the host under controlled environmental conditions, but further goes through field trials in order to be a commercial biocontrol product.

5. References

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APPENDIX A - Supplementary table

Supplementary Table 1 - Commercially available antagonistic microbe-based biocontrol products for control of spoilage diseases in harvested fruits, based on Teixidó *et al.*, 2010; Wisniewski *et al.*, 2016; Carmona-Hernandez *et al.*, 2019; Dukare *et al.*, 2019

Biocontrol Product	Active ingredient	Producing firm/ country	Commodity	Target pathogen	Currently in use	Reference
Biocontrol products bases on bacterial antagonists:						
Biosave® 10 LP	*Pseudomonas syringae* strain ESC-10	Jet Harvest Solutions, USA	Pome, Strawberry, Cherry, Potato, Citrus	*Botrytis cinerea, Fusarium sambucinum, Geotrichum candidum, Mucor piriformis, Penicillium expansum, Penicillium digitatum, Penicillium italicum*	Yes	Janisiewicz *et al.*, 1992
Biosave® 11 LP	*Pseudomonas syringae* strain ESC-11	Jet Harvest Solutions, USA	Pome, Potato, Sweetpotatoes	*Botrytis cinerea, Fusarium sambucinum, Mucor piriformis, Penicillium expansum, Rhizopus stolonifer*		
Amylo-X®	*Bacillus amyloliquefaciens* subsp. *plantarum* strain D747	Intrachem Bio Italia	Pome fruit, Table grapes, Strawberry	*Botrytis cinerea Pseudomonas syringae Monilia laxa, Monilia fructigena Podosphaera aphanis Slerotinia slerotiorum Trichoderma aggressivum*	Yes	Borriss *et al.*, 2015
Avogreen®	*Bacillus subtilis*	Pretoria University, South Africa	Avocado	*Cercospora sp., Colletotrichum sp.*	No	Van Eeden *et al.*, 2004
Pantovital	*Pantoea agglomerans*	IRTA/ Sipcam-Inagra, Spain	Citrus, Pome	*Penicillium expansum, Penicillium digitatium, Penicilium italicum,*		Viñas *et al.*, 2001

				Botrytis cinera, *Rhizopus stolonifer,* *Monilinia* sp.		
Biocontrol products bases on yeasts antagonists:						
Candifruit®	*Candida sake* strain CPA-1	IRTA/ Sipcam Inagra, Spain	Pome	*Penicillium expansum ,* *Botrytis cinerea,* *Rhizopus stolonifer*	No	Carbó *et al.*, 2017
Aspire®	*Candida oleophila*	Ecogen, USA	Pome, Citrus, Stone fruit, Strawberry	*Botrytis* sp., *Penicillium* sp., *Monilinia*		Droby *et al.*, 1998
Yield plus®	*Cryptococcus albidus*	Lallem, South Africa	Pome, Citrus	*Botrytis* sp., *Penicillium* sp., *Mucor* sp.,		Wachowska U., 2009
Nexy®	*Candida oleophila* strain O	Lesaffre, Belgium	Pome	*Botrytis cinerea,* *Penicillium expansum*	Yes	Jijakli *et al.*, 1999
Boni Protectt®	*Aureobasidium pullulans* strains DSM14941 and DSM14940	Bioferm, Austria	**Apple, pear, quince and medlar**	*Botrytis cinerea,* *Monilinia fructigena,* *Penicillium expansum,* *Pezicula malicortici*		Weiss *et al.*, 2006
Shemer®	*Metschnikowia fructicola* strain NRRL Y-27328	Bayer/Koppert, TheNetherlands	Table grape, Pome, strawberry, Stone fruit, Sweet potato	*Botrytis cinerea,* *Penicillium expansum,* *Penicilium digitatum,* *Penicilium italicum,* *Rhizopus stolonifer,* *Aspergillus niger*		Kurtzman *et al.*, 2001

APPENDIX B - Supplementary table

Supplementary Table 2 - **Dual culture assay for inhibition of spore germination on liquid medium** - growth inhibition of TLS001 target was evaluated with a 4-values scale (0 = no growth; 1 = growth without sporulation; 2 = growth less than the control; 3 = growth equal to the control).

	10^5 spores/mL	10^4 spores/mL	10^3 spores/mL	10^2 spores/mL
Y25	0	0	0	0
Y29	3	1	2	0
Y121	3	3	1	0
Y122	3	3	3	0
Y139	3	0	0	0
Y177	3	3	2	2
Y188	3	3	3	1
Y200	1	1	0	0
Y204	3	3	3	3
Y218	3	2	0	0
Y220	3	3	3	0
Y237	0	0	0	0
Y254	1	1	3	3
Y303	0	0	0	0
Y304	3	3	3	3
Y322	3	3	3	3
Y351	0	0	0	0
Y371	0	0	0	0
Y390	0	0	0	0
Y452	3	3	3	1
Y460	3	3	3	0
Y470	1	3	0	1
Y483	0	0	0	0
Y484	1	3	3	3
Y540	1	3	0	0

Y594	0	0	0	0
Y632	2	2	2	2
Y653	2	1	1	1
Y675	2	2	1	0
Y94	0	0	0	0
Y134	0	0	0	0
Y167	0	0	0	0
Y171	0	0	0	0
Y172	0	0	0	0
Y173	0	0	0	0
Y242	0	0	0	0
Y250	0	0	0	0
Y274	0	0	0	0
Y306	0	0	0	0
Y315	0	0	0	0
Y316	0	0	0	0
Y322	0	0	0	0
Y336	0	0	0	0
Y366	0	0	0	0
Y375	0	0	0	0
Y415	0	0	0	0
Y435	0	0	0	0
Y448	0	0	0	0
Y468	0	0	0	0
Y736	0	0	0	0
Y4	2	2	2	2
Y26	3	3	3	3
Y72	2	2	2	2
Y86	2	2	2	1
Y137	0	0	0	0

Y182	1	0	0	0
Y206	0	0	0	0
Y214	0	1	0	1
Y226	0	0	0	0
Y229	3	3	3	3
Y287	0	0	0	0
Y298	0	0	0	0
Y365	2	1	1	0
Y369	3	3	3	3
Y391	0	1	0	0
Y395	0	0	0	0
Y429	3	3	3	3
Y490	2	2	2	2
Y498	3	3	3	3
Y503	0	0	0	0
Y523	3	3	3	3
Y538	3	3	2	2
Y641	2	1	1	0
Y678	0	0	0	0
Y735	0	0	0	0
Y741	0	0	0	0
Y92	0	0	0	0
Y109	0	2	2	2
Y232	0	0	0	0
Y289	0	0	0	0
Y293	3	2	0	0
Y341	0	0	0	0
Y691	2	2	1	0
Y706	2	1	0	0
Y611	2	1	1	0