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REVIEW ARTICLE

# Grape berry native yeast microbiota: advancing trends in the development of sustainable vineyard pathogen biocontrol strategies

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## ABSTRACT

Grape berry is an ecological niche for a myriad of microbes, whose interactions with one another modulate fruit health and can play a role in fermentation, imparting character and distinctiveness to wines. With the growing concerns about and awareness of the risks associated with the overuse of chemical pesticides in viticulture, microbial-based pest control is increasingly encouraged as a more sustainable and environmentally friendly strategy. The use of yeasts from grape berries is a promising alternative for the control of vineyard diseases and their increasing acceptance is rapidly changing our perception of fungicides. In this paper, we provide an overview of the latest methodologies for characterising the dynamics of grape berry yeasts in the context of grape disease management, and discuss the prospects for their effective use as biocontrol agents in viticulture. Most research has focused on the control of fruit rots produced by *Botrytis, Aspergillus, Colletotrichum* and *Penicillium* spp. using formulations that comprise single strains of specific yeast genera, including *Aureobasidium, Metschnikowia, Saccharomyces, Pichia, Candida* and *Rhodotorula*. However, the challenging disparity between successful biocontrol outcomes obtained *in vitro* and their low applicability in the field is a major limitation for the large-scale implementation of these strategies. Novel research approaches for maximising the stability and efficiency of yeast-derived bioactives are discussed in this review.

**KEYWORDS:** Biological control, pest management, grape-associated yeasts, phytopathogens, viticulture, *Vitis vinifera*

## INTRODUCTION

Grape berries are natural niches for a diverse microbial community of yeasts, bacteria and filamentous fungi. The composition of this microbial community depends on various factors, such as the ripening stage of the berry, pedoclimatic conditions, viticultural practices and other parameters that modulate fruit integrity (Barata *et al*., 2012; Pinto *et al*., 2015; Martins *et al.*, 2022). The coexistence of non-virulent microbes with disease-causing microbes creates a balance that ensures the healthiness of the fruit (Bettenfeld *et al*., 2022). Yeasts produce essential plant hormones (eg., indole-3-acetic acid), ammonium, siderophores and nutrients, and they provide protection against spoilage microorganisms (Mukherjee *et al*., 2020; Hernández-Fernández *et al*., 2021; Carvajal *et al*., 2023).

The grape berry is composed predominantly of the sugars glucose and fructose, and contains trace amounts of sucrose, amino acids (e.g., proline, arginine, threonine, and phenylalanine) and organic acids (e.g., oxalic, citric, tartaric, malic, succinic and acetic acids) (Adams, 2006; Teixeira *et al*., 2013). This environment has the ideal water activity and a pH (i.e., low) for microbial growth, particularly during ripening (Prakitchaiwattana *et al*., 2020). Thus, the abundance of numerous microorganisms is influenced by the composition of the berry at various stages of development and ripening (Barata *et al*., 2012).

In traditional viticulture, chemical pesticides, such as the copper-based Bordeaux mixture, azoles, and their derivatives are extensively used in disease management, including downy mildew caused by *Plasmopara viticola* (Yeon *et al*., 2022). However, the long-term use of pesticides can result in less effective outcomes, as some target pathogens can develop resistance against these compounds (La Torre *et al*., 2018). Furthermore, the pollution of the environment via heavy metal accumulation in the soil and water bodies can pose risks to human health and other non-target soil microorganisms (Alengebawy *et al*., 2021; Bakshi and Kumar, 2021). Consequently, the European Union Regulation 473/2002 has restricted the use of copper fungicides in organic agriculture (European Commission, 2002). Additionally, a proposal adopted in June 2022 legally binds the EU to reducing the use of harmful chemical pesticides by 50 % by 2030; this is in line with the EU Farm to Fork and Biodiversity strategies, aiming to accelerate our transition to a sustainable food system as a core part of the European Green Deal (European Commission, 2022). In this context, new EU rules facilitate the approval of microorganisms for use as active substances in plant protection products, aiming to provide farmers with better access to more sustainable solutions (European Commission, 2022). In this regard, the identification and characterisation of natural pathogen antagonists in grape berries have an important role in the development of biocontrol agents, which could be beneficial for the growth and health of crop plants.

Part of the grape berry yeast microbiota, the non-*Saccharomyces* yeasts, such as *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Lachancea thermotolerans*, alongside *Saccharomyces cerevisiae*, are vital for imparting distinctiveness and character to wines (Barata *et al*., 2012; Pinto and Gomes, 2016; Fernandes *et al*., 2021). Studies have also shown that not only do these yeasts promote wine quality, but they also provide protection against spoilage species (Yao *et al*., 2022), as discussed hereafter.

Previous reviews concerning the use of yeasts as biocontrol agents have mainly focused on the control of postharvest diseases caused by pathogens, including *Aspergillus*, *Penicillium* and *Botrytis*, and they explore the desirable characteristics of yeasts used for this purpose and their modes of action (Liu *et al*., 2013; Freimoser *et al*., 2019; Di Canito *et al.*, 2021). The present review complements previous works by focusing exclusively on native grape yeasts and extending knowledge obtained from the results of basic *in vitro* studies to the application and efficiency of the yeasts in the field, at both pre- and postharvest stages, and when used against different fungal pathogens, including the causal agents of grape mildews. An overview is given of the development of yeast microbiota during fruit ripening, as well as the effect of conventional fungicides on their diversity. Various culture-dependent and culture-independent high-throughput methods to isolate and characterise different yeast species and strains are described. Different methodologies for assessing antagonistic potential are explored, alongside up-to-date knowledge on the efficacy of the agents for biocontrol purposes. Finally, the applicability of the agents in viticulture in the light of achieving the EU goals for sustainable agriculture is discussed.

### 1. The dynamics of grape berry-associated yeasts: insights into environmental and genotypic influences

The origin of grape berry-associated microbes is so far not fully understood (Martins *et al*., 2013). The microbial community of the fruit surface is thought to originate from the vineyard environment, with each locality having its own embedded microbial signature underlying regional wine characteristics (Barata *et al*., 2012). While soil, grapevine bark, leaves and other nearby plants are natural reservoirs for the microbial community associated with the grape berry, microbial transmission is largely induced by humans, insects and weather events (e.g., wind and rain) during the different developmental stages (Ramírez *et al*., 2020). The composition of these microbes on the grape surface change with fruit developmental stage, climate and sanitary conditions, and a core microbiota comprising the yeast genera *Aureobasidium*, *Hanseniaspora* and *Metschnikowia* is ubiquitously present regardless of environmental factors and plant genotype. A catalogue of cultivable yeasts isolated from grape berries and wines from the Douro wine region is shown in Figure 1 (Martins *et al*., 2014a).

An extensive review by Barata *et al*. (2012) categorises berry yeasts according to three main genera, with oligotrophic species populating green berries when nutrient availability is low and skin firmness and integrity are high. Conversely, after veraison, the yeast microbiota

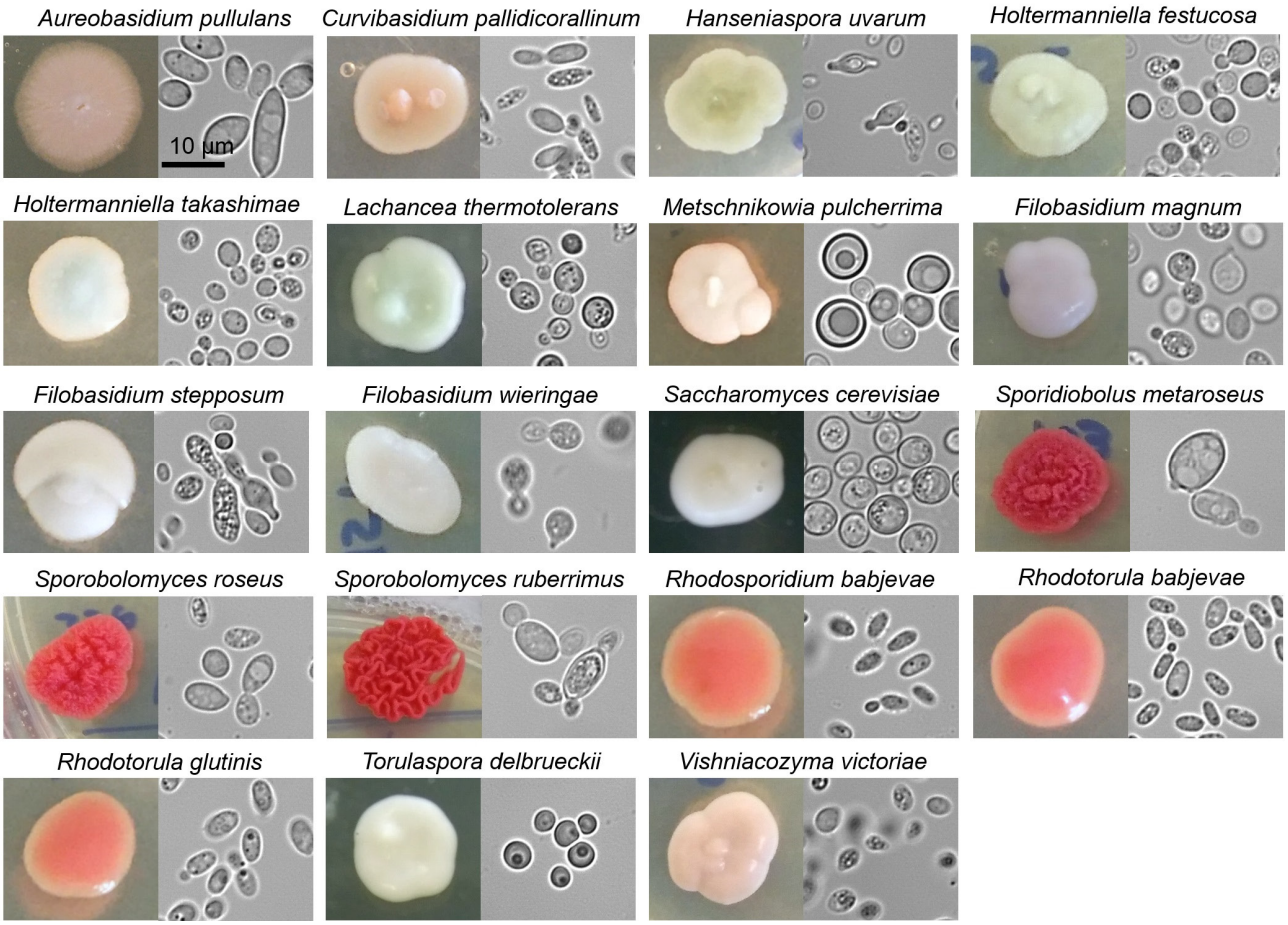



**FIGURE 1.** Catalogue of cultivable yeasts isolated from grape berries and wines of cv. Sousão, Touriga Nacional and Viosinho of Douro wine region in the vintage of 2021.

For each species, images show the colony morphology (left) and the cell architecture observed by DIC microscopy (right). Original photographs by Viviana Martins.

is dominated by basidiomycetous yeasts of the genera *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, and the (yeast-like) *Aureobasidium*, with an increase in oxidative or weakly fermentative ascomycetous populations of different genera, of which *Candida*, *Hanseniaspora*, *Metschnikowia* and *Pichia,* close to harvest time (Mane *et al*., 2017). This suggests that the yeast species found in grape berries at harvest are also the major taxa found at the onset of the alcoholic fermentation. During winemaking in particular, native wine grape yeasts and winery equipment-associated yeasts belonging to the genera *Kloeckera*, *Cryptococcus*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, and *Issatchenkia* grow, representing the majority of the yeast microbiota in the early stages of fermentation. These are succeeded by other non-*Saccharomyces* yeasts, including those of the genera *Hanseniaspora*, *Starmerella*, *Metschnikowia*, *Torulaspora* and *Lachancea* (Mane *et al*., 2017; Ciani and Comitini, 2019; Costantini *et al*., 2022), which are gradually replaced by more resilient *Saccharomyces* species that remain active until the end of the fermentation process, suggesting that the winery is a potential reservoir for this genus (Perrone *et al*., 2013). Although *S. cerevisiae* is rarely found in grapes and vineyards and can be more easily isolated from the surface of winery equipment, where it is considered a resident species (Le Jeune *et al*., 2006; Aponte and Blaiotta, 2016), recent studies suggest that it behaves like an endophytic organism intimately connected with the berry tissues, its proliferation depending on its adaptation to the grape skin ecosystem (Cordero-Bueso *et al*., 2022; Watanabe and Hashimoto, 2023; Martins *et al*., 2024). The permanent presence of *S. cerevisiae* in the must during alcoholic fermentation - which is closely linked to its higher tolerance of ethanol compared with other yeasts present in the wine environment - makes it indispensable in the production of quality wines (Goddard and Greig, 2015; Albergaria and Arneborg, 2016; González-Alonso *et al*., 2021). In this regard, the active participation of these non-*Saccharomyces* yeasts is being increasingly explored in mixed starter cultures for enhancing the complexity of the wine aroma bouquet (Varela *et al*., 2021).

Besides winemaking, grape yeasts have been explored for other biotechnological applications, including for the production of pigments, oils and fatty acids in the pharmaceutical and food industries (Varela *et al*., 2021), and

the production of other metabolites and enzymes that are now known to underlie their antagonistic potential, as discussed in the following sections.

## 2. An overview of the methodological approaches used to isolate grape yeasts and characterise microbial biodiversity

Culture-dependent approaches can be used to study the cultivable fraction of grape yeasts by growing and isolating them on selective and non-selective media. The macroscopic identification of a genus or species is mainly based on the observation of elevation, opacity, production of polysaccharide capsular material, pigmentation, pastiness, consistency of cream, smoothness, dryness and texture of the yeast colony (Pallmann *et al*., 2001). Microscopic identification is carried out via the observation of structures in culture, such as shape of cells, buds, pseudohyphae and mature hyphae. Other detailed structures, such as organelles, bud scar and fermentation capacity, can be visualised by transmission electron microscopy (Osumi, 2012). In addition, biochemical phenotypic assays are also used to screen for certain groups of the microbiome; for example, enzymatic tests (for example, urease, glycosidase, aminopeptidase and phosphatase), cycloheximide resistance, phenoloxidase production and esculin hydrolysis. In addition, it is possible to determine the potential of different isolates to ferment and assimilate in microtubes containing different colorimetric substrates (carbohydrates, organic acids, nitrogen and $KNO_3$) in the medium (Bascomb and Manafi, 1998; Hafeez and Aslanzadeh, 2018). Substrate utilisation or hydrolysis can be determined from increased turbidity, the generation of coloured products or the generation of fluorescent products after incubation. Biochemical tests can be used to identify different taxa and at different levels of performance, based on the type of biochemical substrates used (Pincus *et al*., 2007). Drumonde-Neves *et al*. (2016) cultivated yeasts on differential Wallerstein Nutrient Media as part of microbiota analyses carried out in vineyards in Europe (during the 2012 and 2013 to 2016 vintages) with the aim of identifying and characterising yeasts from grapes which can contribute to wine typicity when used in winemaking. By sequencing 26S rDNA D1/D2 domains, 5.8S rRNA gene and ITS region, the authors were able to identify *Saccharomyces cerevisiae* and non-*Saccharomyces* species: *Candida boidinii, Candida zemplinina, Hanseniaspora guilliermondii, Issatchenkia terricola, Zygosaccharomyces bailii, Hanseniaspora uvarum, Zygoascus hellenicus* and *Hanseniaspora opuntiae*. Thus, after a pre-selection of yeast isolates, their identity at species and strain level can be routinely confirmed by carrying out ITS/LSU barcoding, following the PCR amplification and Sanger sequencing of these regions (Martins *et al.*, 2022). At advanced level, microorganisms can also be identified using a character strain approach in which microbial strains are analysed in more detail, taking into account factors such as genomic variations, metabolic pathways and physiological traits specific to each strain (Ghazi *et al*., 2022). This approach enables a more accurate and granular classification of microorganisms, leading to a deeper understanding of microbial diversity. Such recognition and differentiation of microbial strains can provide insight into the functional capabilities and ecological roles of specific strains – information that is necessary for targeting applications and ensuring their efficiency in various scientific and industrial contexts (Van Rossum *et al*., 2020). The incorporation of molecular tools, such as ITS ribotyping and bioinformatic analysis, has enhanced the study of the cultivable yeast occurrence and diversity in grape berries.

Recently, studies are increasingly applying DNA-based community fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE), single-strand conformational polymorphisms (SSCP), terminal restriction fragment length polymorphisms (T-RFLP), and automated ribosomal intergenic spacer analysis (ARISA), which can be used for the identification and analysis of grape berry microbiota without cultivation (Laforgue *et al*., 2009; Morgan *et al*., 2017). DGGE coupled to PCR can be used for evaluating microbiota evolution during the ripening and fermentation stages, but this method is culture-dependent and is not highly sensitive (Laforgue *et al*., 2009). On the other hand, SSCP, T-RFLP and ARISA have been found to be useful tools for characterising the microbial diversity of wine (Martins *et al*., 2014a). However, these methods do not always provide a complete inventory of the microbial consortium living on grapes, as they sometimes do not reveal species of low abundance.

More recently, metagenomics involving high-throughput techniques is changing the paradigm in this line of research, laying the foundation for the characterisation of microbial diversity and composition, microbial functional analyses and potential biocontrol applications (Urich *et al*., 2008; Mackelprang *et al*., 2011; Zhou *et al*., 2022). These approaches are preferred to culture-dependent methods, because they are more accurate and less labour-intensive; they provide extensive microbial profiling of a sample, as both cultivable and non-cultivable taxa are detected. In metabarcoding approaches, microbial DNA is extracted from grape samples using commercial kits, and amplicon library paired-end sequencing can be carried out using primers for specific regions, such as ITS1; the generated raw sequence data can be deposited in public repository databases. The development of whole metagenome sequencing has further provided excellent conditions for detecting even species of the lowest abundance, making it one of the most preferred microbiota fingerprinting methods (Constantini *et al*., 2022).

Analysis of grape berry microbiota using culture-dependent and -independent methods (such as Next Generation Sequencing approaches) has made it possible to not only identify the yeast fingerprints of grape berries and other vine organs in many wine regions, but also to assess important aspects of plant-microbe interactions (such as the impact of organic and conventional fungicide treatments on grape berry yeast community) and the evolution of yeast biodiversity during spontaneous fermentations (Martins *et al*., 2014a; Agarbati *et al*., 2019). Although the majority of taxa in a sample can be detected using culture-independent methods,

some species, including highly fermentative yeasts, can only be detected using culture-dependent methods involving enrichment procedures; these also enable the utilisation of isolates in subsequent experiments, such as inoculated fermentations. Studies on grape microbiota in which a combination of culture-dependent and -independent methods have been used are scarce (Martins *et al*., 2014a; Agarbati *et al*., 2019); however, the use of both methods is essential for ensuring the detection of the highest possible number of taxa, and thus accurately reflecting yeast diversity in each sample.

## 3. Chemical pesticides and grape yeast microbiota: impacts of organic and conventional farming systems on population diversity

Over the years, it has been acknowledged that farming systems and vineyard soil management strategies can have an indelible impact on the grape microbial community. The low abundance of beneficial fungal diversity in grapes grown using common chemical fertilisers and pesticides is an indicator of toxicity of the chemical compounds to organisms (Wu *et al*., 2021). Increasing research shows that the population and composition of yeast communities resident on grape berries are modified as a result of antifungal treatments (Rogiers *et al*., 2005; Martins *et al*., 2014a; Yao *et al*., 2022). The foliar application of the conventional fungicides spiroxamina, copper-oxychloride and sulfur copper sulfate on cv. Montepulciano vines has been found to cause a reduction in yeast biodiversity, negatively affecting the fermenting yeasts, except for oxidative yeasts (such as *Aureobasidium pullulans*) at harvest time (Agarbati *et al*., 2019). In another study, the application of iprodione, pyrimethanil and fludioxonil plus cyprodinil on cv. Rebula vines had only a minor impact on the overall composition of grape berry microbial communities, but affected specific yeast species at harvest time (Čadež *et al*., 2010). Meanwhile, Setati *et al*. (2012) also reported higher yeast species diversity in the biodynamic vineyard and the lower diversity in the conventional vineyard where chemical fungicides like folpan® (N-(trichloromethylthio)-phthalimide), rootex™ (phosphorous acid), kumulus® (sulphur), dithane® (mancozeb), acrobat® (dimethomorph), talendo® (proquinazid), cungfu® (copper hydroxide) and topaz® (mono- and di-potassium salts of phosphorous acid) had been applied. In spite of their pest control benefits, the intensive application of copper- and sulphur-based fungicides in organic agriculture can negatively affect the epiphytic yeasts and yeast-like community on grape berry surfaces due to their wide fungicidal spectrum, causing sluggish or stuck fermentations (Martins *et al*., 2014a; Martins *et al*., 2014b). Meanwhile, other studies involving the application of synthetic-organic fertilisers and fungicides (e.g., cyprodinil, fludioxonil, metiram, penconazole and pyraclostrobin) used in conventional agriculture showed an increase in fungal diversity, as well as in the relative abundance of beneficial fungi on grape berry surfaces, thereby improving grape quality and storability (Čadež *et al*., 2010; Wu *et al*., 2021). However, many active components of conventional fungicides, including penconazole, spiroxamina, quinoxyfen, myclobutanil, among others, can have negative effects on the grape berry yeast biodiversity (Agarbati *et al*., 2019).

The preharvest field application of fungicides like potassium phosphate + sulphur + copper hydroxide for the control of vineyard diseases has also been shown to modulate the abundance of yeast microbiota on grapes and in musts (Grangeteau *et al*., 2016; Grangeteau *et al*., 2017); for instance, *H. uvarum* and *P. kudriavzevii* were predominant during spontaneous winery fermentations and *S. cerevisiae* was predominant during inoculated winery fermentations. The lower abundance of *A. pullulans* with potassium phosphonate (K-Pho) treatment against powdery mildew in conventional vineyards and its predominance in berries treated with potassium bicarbonate + copper hydroxide (K-Bic+C-h) and sulphur + metiram (S+Me) supports the above findings (Englezos *et al*., 2022), further suggesting that this yeast-like fungus could contribute to the effective control of nonbeneficial microbes. The impact of fungicides on yeast microbiota thus likely depends on other variables, since studies have shown either a reduction in biodiversity in both organic and conventional regimes or no significant effects of fungicide treatments.

## 4. The search for vine yeasts with antagonistic activity: methodologies for *in vitro* and *in vivo* screening

Biocontrol agents for plant disease management are receiving increasing attention, and thus the focus of research is now on indigenous yeast strains in vineyards that exhibit antagonistic activity against pathogenic filamentous fungi. At primary level, to assess the biocontrol properties of the yeasts, researchers rely on methodologies involving a wide range of *in vitro* assays (e.g., the dual culture assay, the mouth-to-mouth dual assay, the agar plug diffusion assay and the agar disk diffusion assay), as well as *in vivo* investigations conducted in controlled laboratory, greenhouse or field conditions (Table 1).

*In vitro* dual assays are of primary importance when screening for biocontrol activity, since they are easy to perform and broadly applicable to the identification of bacterial, yeast, fungal, oomycete and viral biocontrol agents. Dual culture assays can be used to detect the release of antifungal compounds by yeasts that are in the proximity of filamentous fungi, and to study antagonist-pathogen interactions. They have been extensively used for determining the antifungal activity of native grape yeasts (Cordero-Bueso *et al*., 2017; Ayogu *et al*., 2023).

The agar plug diffusion method can be used not only for determining the inhibitory effects of antagonists, but also for testing processed antagonist products, such as heat-denatured yeast cells and cell-free supernatants (Zepeda-Giraud *et al*., 2016). However, this method still needs to be refined and complemented with other *in vitro* or *in vivo* assays. In addition, a major limitation is that the concentration of the pathogen may not be known, as a cut out plug of an actively growing mycelia of fungi is mainly used for inoculation.

**TABLE 1.** Approaches for screening antagonistic activity of native vine yeasts.

| Approach | Methodology | Advantages | Shortcomings | References |
|---|---|---|---|---|
| Agar surface dual culture assay | Co-inoculation of suspensions of antagonist and pathogen spores on the same Petri dish containing appropriate solid growth media, 2-3 cm apart; measurement of the inhibition zone around pathogen following incubation. | Ease of execution and broad applicability; allows the simultaneous screening of several yeast species towards the same pathogen; provides information on the effect of antagonist on pathogen spore germination and growth. | Result less reliable than *in vivo* assays; inhibition zone diameter depends on the initial distance between the inoculation spots of the antagonist and the pathogen. | Ayogu *et al.* (2023) |
| Dual culture assay on liquid media | Coinoculation of fungal spores or mycelia into liquid media in test tubes containing the yeast; microscopic examination of preparation for antagonism features after incubation. | Ease of implementation. | Supports the evaluation of antifungal activity of only one yeast at a time. | Serna-Díaz *et al.* (2020) |
| Agar plug diffusion assay | Transferring an agar plug (about 0.5 $cm^2$) of filamentous fungi onto the middle of another agar plate previously inoculated with antagonistic yeast; measuring mycelia growth after incubation. | Gives idea of inhibitory effect of antagonists over hyphae proliferation; offers opportunity for testing processed antagonist products (heat denatured yeasts cells, cell-free supernatants). | No information provided on the effect of antagonist on spore germination; determination of antifungal activity of only one yeast per assay; agar plugs may have different pathogen loads. | Zepeda-Giraud *et al.* (2016) |
| Agar disk diffusion assay | Overlay of agar plates with the fungal suspension; placement of the yeast-laddened filter paper discs evenly on the agar surface; measurement of diameters of inhibition growth zones around the disks after incubation. | Simple, low cost; offers opportunity to test greater number of yeasts against one mould; ease of results interpretation. | The antagonistic activity of yeast may be affected by the permeability (quality) of the paper used in the assay. | Liu *et al.* (2017) |
| Mouth-to-mouth dual assay | Inoculation of pathogen suspension or agar plug in solid growth media and replacement of the Petri dish lid by an inverted open Petri dish containing yeast solid agar culture, allowing diffusion of yeast VOCs to the Petri dishes' headspace; measurement of mycelia growth of mould after incubation. | Identification of yeasts producing VOCs with antagonistic action. | Less reliable than the *in vivo* assay; does not provide information about antagonistic mechanisms involving production of non-VOCs. | Parafati *et al.* (2015) |
| *In vivo* dual assay | Inoculation of purposely wounded healthy berries, detached leaves, young vines, canes or root stocks with the yeast suspension, followed by a mould spore suspension at given concentrations; measuring antagonism by severity or disease incidence after incubation. | *Controlled in planta* approach; relatively cheap, simple and quick characterisation of antagonistic potential *in vivo*. | Antagonist activity is often lower than in in vitro approaches; does not yet contemplate the influence of environmental factors on antagonist efficiency; possible risk of affecting non-target microbial residents, especially in field setting. | Parafati *et al.* (2015); Maluleke *et al.* (2022); Pollard-Flamand *et al.* (2022); Ayogu *et al.* (2023) |

The agar disk diffusion method - also known as the NCCLS (National Committee for Clinical Laboratory Standards) or Kirby-Bauer method and which was primarily developed for routine antimicrobial susceptibility testing - has found use in some yeast antagonism assays (Balouiri *et al*., 2016). It is a simple, low-cost method that can be used to test high numbers of microorganisms and antimicrobial agents, and the results can be interpreted easily.

The production of volatile organic compounds (VOCs) (e.g., ethyl acetate, isoamyl alcohol and 2-phenethyl ethanol) in the presence of a pathogen is a very common mechanism of antagonistic activity of many yeasts. The mouth-to-mouth dual assay can be carried out to assess strains exhibiting this type of behaviour (Parafati *et al*. 2015; Zhao *et al*., 2023).

The *in vivo* dual assay has been widely reported for its effectiveness in evaluating the biocontrol potential of various yeast strains against serious phytopathogens (Parafati *et al*., 2015; Agarbati *et al*., 2022). It is often performed on intentionally wounded plant materials, including healthy berries. Other *in vivo* and *in planta* approaches involving

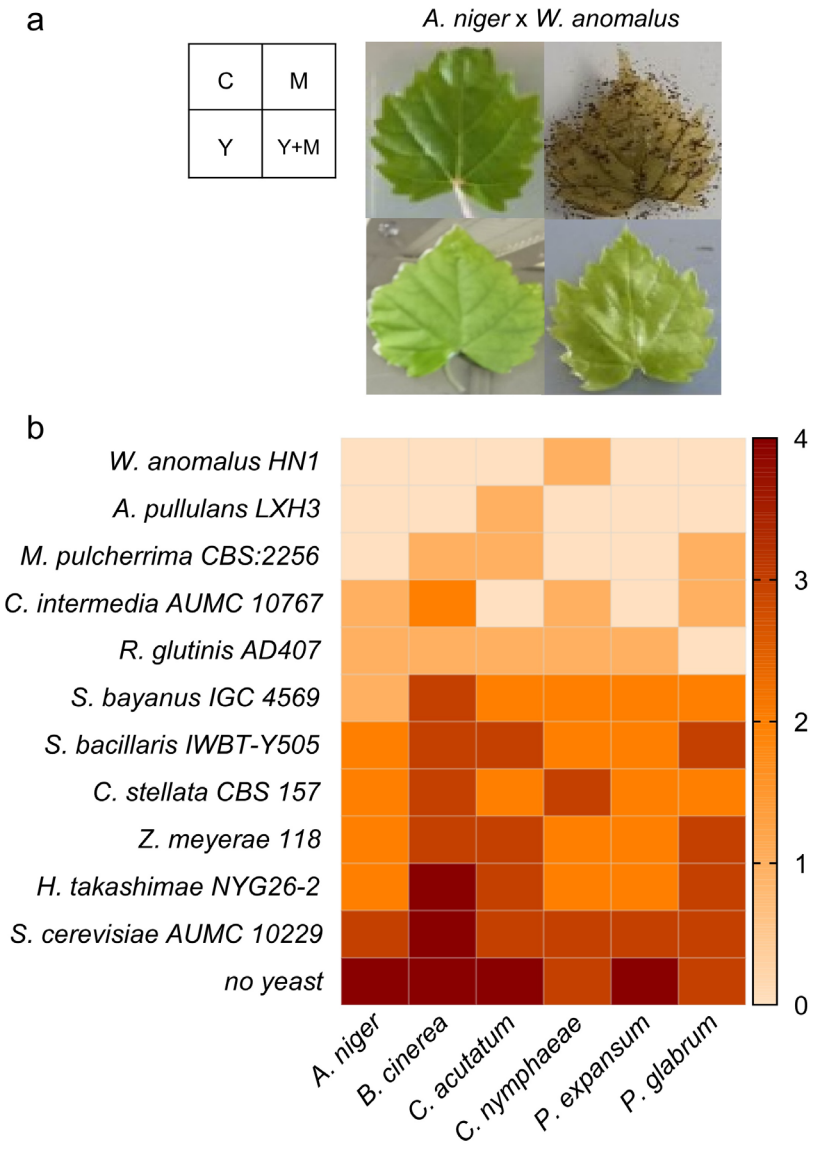



**Figure 2.** Incidence of disease development in young grapevine leaves after inoculation with native grape yeasts and moulds, after 7 days of incubation at 25 °C.

Inhibition of *A. niger* infection by *W. anomalus* (a), with images showing a non-inoculated control leaf (C), a leaf inoculated with only the mould (M), a leaf inoculated with only the yeast (Y) and a leaf inoculated with both yeast and mould (Y+M). Heatmap with antagonistic activity of yeasts inoculated at $10^8$ CFU/mL towards fungi pathogens inoculated at $10^7$ spores/mL (b); values were centred and scaled in the row direction to form virtual colours, as presented in the colour key, and indicate the disease incidence index, as follows: 0 = no visible symptoms (0-5 % leaf area infected); 1 = trace symptoms (6-25 % leaf area); 2 = slight lesions (26-50 % leaf area); 3 = pale/yellowing (51-75 % leaf area); 4 = extensive deformation/loss of integrity (> 75 % leaf area). Data was retrieved from Ayogu *et al.* (2023).

the use of sterile detached leaflets, young vines, canes and root stocks can also be applied to evaluate the biocontrol activity of various microbial species without going to the field (Agarbati *et al.*, 2022). In particular, non-destructive detached leaf and young vine experiments can be carried out to determine the interactions between plants and phytopathogens, as well as to quickly characterise the infectivity of pathogens and plant resistance to them. In a recent study, native yeasts of grape berry, *A. pullulans*, *W. anomalus*, *M. pulcherrima*, *C. intermedia*, and *R. glutinis,* were identified as potential biocontrol yeasts for the control of *A. niger*, *B. cinerea*, *C. acutatum*, *C. nymphaeae*, *P. expansum* and *P. glabrum* both *in vitro* and *in vivo* using detached leaves and *in vitro*-grown grapevine plantlets (Figure 2) (Ayogu *et al.*, 2023).

Translating *in vitro* results into *in vivo* efficacy is a multifaceted undertaking shaped by numerous factors, including the intricacies of *in vivo* environments, host immune responses, microbial adaptability, inoculum concentration, and more. Therefore, the validation of the biocontrol potential of

antagonistic yeasts requires a comprehensive evaluation employing diverse approaches, such as *in vitro* assays, *in vivo* studies, *in planta* investigations and field foliar spraying tests (Liu *et al*., 2013).

## 5. Towards the biocontrol of grapevine fungal pathogens: fundamental studies and action mechanisms

While pathogenic fungi, such as *Aspergillus niger*, *Botrytis cinerea*, *Colletothricum* spp. and *Penicillium* spp., represent the most studied grapevine spoilage moulds that cause pre- and postharvest losses worldwide (Di Canito *et al.,* 2021), the obligate biotrophs *Erysiphe necator* and *Plasmopara viticola* are also of grave concern, causing highly devastating mildews (Koledenkova *et al*., 2022). Compared to other fungi, the initial screening of these two biotrophs *in vivo* at laboratory scale is more challenging (performed mostly on plant tissues), as they are both difficult to grow on culture media (Colcol and Baudoin, 2016). To date, their complete eradication is still difficult to achieve, as the traditional practices of pruning, defoliation, vineyard cover management, hot water treatment, irradiation, as well as chemical treatments, will not always be completelyeffective, and the outcome will depend on the product, method and organism involved (Table 2)**.** Thus, the FAO estimates that plant diseases still cost the global economy $220 billion/ year, roughly estimated as 20 - 40 % annual productivity losses (FAO, 2019). Of the grapevine fungal diseases, grey mould caused by *B. cinerea* and downy mildew caused by *E. necator* represent the endemic diseases responsible for economic losses for grape growers and wine producers worldwide (Rienth *et al*., 2021).

With the aim of reducing by 50 % the use of harmful chemical pesticides by 2030, new EU rules encourage the use of microorganisms as biocontrol agents (European Commission, 2022). The results of a large number of studies support the use of native grape yeasts in the management of

**TABLE 2.** Grapevine diseases and traditional management options.

| Disease | Characterisation | Causal agents | Active ingredients approved in EU | References |
|---|---|---|---|---|
| Bunch rot/Grey rot | Brown rot on bunches which can also affect other plant parts. | *Botrytis cinerea* | Copper (II) sulphate | Nally *et al.* (2012); Di Canito *et al.* (2021) |
| Anthracnose/Ripe rot | Circular, reddish-brown spots, which enlargen to cover the whole fruit. Leaves and branches become covered with brown spots and wither. | *Colletotrichum acutatum, C. nymphaeae, C. gloeosporioides, C. musae, C. fructicola, C. aenigma, C. pseudoacutatum* | Fludioxonil, folpet, captan | Ishii *et al.* (2022) |
| *Aspergillus* rot | Grape clusters lighten and acquire a dark colour. Berries turn into a bluish-brown mass following shrinkage and cracking. | *Aspergillus carbonarius, A. niger, A. Alliaceus, A. ochraceus* | Copper (II) sulphate, fludioxonil, cyprodynil, captan | Di Canito *et al.* (2021) |
| Fruit rot | Light-brown soft, watery rot followed by the appearance of blue-green conidia on the fruit surface at advanced stages of decay. | *Penicllium expansum, P. glabrum. P. purpurescens, P. decumbens, P. chrysogenum, P. crustosum, P. aurantiogriseum* | Thiabendazole, imazalil, carbendazim | Di Canito *et al.* (2021) |
| Powdery mildew | Mycelial growth and sporulation occur on the surface of leaves and stems, resulting in a white fuzzy mildew appearance. | *Erysiphe necator* | Copper (II) sulphate, azoxystrobin, trifloxystrobin | Lukšić *et al.* (2022) |
| Downy mildew | Light green to yellow spots on upper leaf surface which eventually turn brown, berries turn a mottled dull green or reddish purple and readily fall from the cluster. | *Plasmopara viticola* | Copper oxychloride, copper hydroxide, captan | Koledenkova *et al.* (2022) |
| Black spot | A leaf spot that is characterised by small, water-soaked lesions. | *Alternaria alternata, A. brassicicola* | Copper (II) sulphate, fludioxinil | Ji *et al.* (2023) |

vineyard diseases, showing that various species and strains have differing efficacy and pathogen specificity. A recent review explored the distinct mechanisms involved in yeast-mediated biocontrol (Freimoser *et al*., 2019).

### 5.1. Alternaria

Killer toxins produced by isolates of winegrape-associated yeast strains have been identified as inhibitors of *Alternaria* spp. that cause spoilage in fruit crops. *In vitro* assays have demonstrated that native grape yeasts, such as *Metschnikowia pulcherrima* GP8, *Starmerella bacillaris* FE08.05 and *Hanseniaspora uvarum* GM19, reduce the radial growth of *A. alternata* (Prendes *et al*., 2021), while *Metschnikowia* sp. (LP122.2, LP123.2, LP125.2, LP128.2, LP131.2 and LP132.1) and *Starmerella bacillaris* (LP6.4.1, LP8.5.1, LP8.5.2) strains were effective in reducing *A. alternata* growth and the production of mycotoxin, such as alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA), at different temperatures (Prendes *et al*., 2021).

### 5.2. Aspergillus

The autochthonous yeast-like fungi *Aureobasidium pullulans* LS-30 has been tested and found to be effective against *A. niger* incidence table grape; antagonism was mainly due to competition for nutrients and the secretion of lytic enzymes in the presence of the pathogen (Castoria *et al*., 2001). *A. pullulans* Y-1 was also found to be effective against grapevine sour rot infection due to *A. carbonarius* in an *in vivo* laboratory investigation (Dimakopoulou *et al*., 2008). *Wickerhamomyces anomalus*, *Hyphopichia burtonii*, *Lachancea thermotolerans*, *Lodderomyces elongisporus*, *Zygoascus meyerae*, and *Candida oleophila* have been described as effective antagonists against the Ochratoxin A producing mould (Maluleke *et al*., 2022). In addition, *C. guilliermondii* A42 and *Acremonium cephalosporium* B11 have shown strong antagonism against *A. niger* (Zahavi *et al*., 2000). Assays carried out on vineyard native yeasts in *in vivo* laboratory experiments have demonstrated high antagonistic activity in *Dekkera anomala* BDa184, *Saccharomyces chevalieri* BSch25, *S. cerevisiae* BSc119, *Torulaspora delbrueckii* BTd156 and *C. rugosa* BCr182 against *A. caelatus*, as well as complete inhibition of the growth of *A. terreus* by *Issatchenkia orientalis* BIo148, *S. cerevisiae* BSc62, *C. catenulata* BCc185, *C. sake* BCs54, and *T. delbrueckii* BTd161 (Nally *et al*., 2013). The antagonistic effect of native *Saccharomyces* and non-*Saccharomyces* species (*Dekkera*, *Torulaspora*, *Pichia*, *Kluyveromyces*, *Candida* and *Debaryomyces*) against *A. versicolor* has also been shown: *A. carbonarius* was specifically antagonised by *Candida sake* BCs198, thus demonstrating the ability of vineyard yeasts to control diseases caused by *Aspergillus* in grape berries at postharvest (Nally *et al*., 2013).

### 5.3. Botrytis

*A. pullulans* LS-30 ($1\times10^8$ cells/mL) has been shown to have biocontrol potential against bunch rot caused by *B. cinerea* in wounded table grapes, apparently occurring through the production of unidentified volatile organic compounds and not by the secretion of lytic enzymes (Castoria *et al*., 2001). At $10^6$ CFU/mL, several other yeast species (*S. cerevisiae*, *M. pulcherrima*, *P. guilliermondii*, *C sake*, *C. famata*, *C. parapsilosis*, *C. albidus*, *D. hansenii*, *H. vinae*, *P. anomala*, *P. membranifaciens*, *S. bayanus*, *S. cerevisiae*, *S. chevallieri*, *S. kluyveri*, *S. steineri*, *T. delbrueckii*, and *Sch. pombe)* have also been shown to antagonise the development of grey mould *in vitro* (Nally *et al*., 2012). In other trials, *P. kudriavzevii*, *A. pullulans*, *A. melanogenum*, *A. subglaciale*, *W. anomalus*, *Hyphopichia burtonii*, *Lachancea thermotolerans*, *Lodderomyces elongisporus*, *Z. meyerae*, *C. oleophila* and *H. uvarum* have been reported to reduce the growth of *B. cinerea* in postharvest table grapes as a result of their ability to secrete lytic enzymes and volatile organic compounds, including ethanol, 2-methyl-1-propanol, and 3-methyl-1-butanol, in the presence of the mould (Di Francesco *et al*., 2020; Maluleke *et al*., 2022).

### 5.4. Colletotrichum

Having caused a significant reduction in strawberry anthracnose and grey mould, beneficial grape-associated fungi of the Trichoderma genus, such as *Trichoderma harzianum* (T-39), *T. hamatum* (T-105), (T-DAOM 167057), *T. atroviride* (T-161), T. asperlloides (T-GJS O4-116) and *T. longibrachiatum* (T-166), have been identified as promising biocontrol agents (Pollard-Flamand *et al*., 2022). The killer strains of *S. cerevisiae* (GA8, LFA802 and L24) at $10^8$ CFU/mL have been found to be effective in the control of *C. gloeosporioides,* inhibiting its conidial germination *in vitro;* meanwhile, in *in vivo* tests, the antagonistic activity (highest in *S. cerevisiae* GA8) was reported to be the result of the production of antifungal compounds, hydrolytic enzymes β-1,3- glucanase and chitinase (Liu *et al*., 2017).

### 5.5. Penicillium

Strains of the indigenous antagonistic yeasts *Pichia kudriavzevii* L18 and *Zygoascus meyerae* L29 from vineyards have also been shown to significantly reduce the incidence of *P. glabrum* on grapes (Cabañas *et al*., 2020). The 70 % incidence reduction by *Zygoascus meyerae* L29 was reported to occur through different mechanisms: the production of lytic enzymes pectinase, chitinase and β-glucanase, the formation of biofilms, and competition for nutrients. Meanwhile, the reduction in disease incidence by *Pichia kudriavzevii* L18 resulted from the production of volatile organic compounds (VOCs). The effectiveness of *A. pullulans*, *Cryptococcus magnus*, *M. pulcherrima* and *Rhodotorula glutinis* in the control of blue mould caused by *P. expansum* has also been found to be promising (Rodriguez Assaf *et al*., 2020). In another laboratory study by the same authors, three strains of the native yeast *M. pulcherrima* (Mp22, Mp36 and Mp43) at $10^6$ cells/mL were found to inhibit the 4 most aggressive strains of *P. expansum* tested on seedless table grapes, indicating their ability to control fruit rot at postharvest (Rodriguez Assaf *et al*., 2020).

Using minimal synthetic media, *Debaryomyces hansenii* (at $10^9$ CFU/mL) has been shown to protect grapefruits from decay caused by the green mould *P. digitatum*, likely due to

competition for nutrients (Droby *et al*., 1989). Furthermore, the native vineyard yeast *Yarrowia lipolytica* showed high antagonistic activity against *P. rubens* (at $10^9$ cells/mL), both *in vitro* and *in vivo,* by significantly inhibiting the postharvest decay and Ochratoxin A production in grapes infected with this pathogen. The antagonism was likely due to the stimulation of the activities of the defense-related plant enzymes polyphenoloxidase (PPO), peroxidase (POD), catalase (CAT), phenylalanine ammonialyase (PAL), ascorbate peroxidase (APX) and β-1,3 glucanase (GLU) (Wang *et al*., 2019). The results of these trials reveal a consistency in the inhibition activity of wine grape-associated yeasts, lending support to their consideration in biocontrol programmes.

### *5.6. Erysiphe*

Microbial preparations of antagonistic yeasts that produce antibiotics and hydrolytic enzymes have been found to collapse the hyphae and conidia of *Erysiphe necator*, making them viable alternatives for the biocontrol of powdery mildew (Singh *et al*., 2017; Lombardo *et al*., 2023). Specifically, species of the genera *Pseudozyma,* occuring as saprophytic yeast-like fungi epiphytes on the leaf surface of various plant species, and of *Tilletiopsis* (*T. albescens*, *T. minor*, *T. pallescens* and *T. washingtonensis)* were shown to have biological control activity against powdery mildew diseases (Avis and Bélanger, 2002). Remarkably, the application of culture filtrates and biomass of *Saccharomyces cerevisiae* (NFCCI 4094), along with *Ampelomyces quisqualis* (NFCCI 222) and *Trichoderma harzianum* (NFCCI 4090), and subsequent inoculation with *E. necator* in greenhouse conditions resulted in the reduction of the incidence of powdery mildew by 72.03 %, 80.42 % and 86.41 % respectively after 30 days of foliar application (Singh *et al*., 2017); this indicates that antagonism against the mould is more pronounced when culture filtrates are applied prior to mould infection.

### *5.7. Plasmopara*

In laboratory assays using leaf discs, a *Aureobasidium pullulans*-based formulation was found to provide low protection against *P. viticola*, while no direct effect on zoospores was observed *in vitro* (Harm *et al*., 2011). Subsequent application of an inactivated suspension of apple-epiphytic yeast *A. pullulans* on grapevine callus culture and *in vitro* plantlets increased the gene expression and enzyme activity of PAL and STS, indicating the possibility of a resistance induction mechanism (Harm *et al*., 2011). In a similar assay using leaf discs, the fungi *Trichoderma harzianum* T39144 and a combination of *Clonostachis rosea* and *T. harzianum* were identified as promising candidates in the management of downy mildew disease through the prevention of sporangia germination and/or inhibition of sporangia development (Vecchione *et al*., 2007). The mechanisms underlying the biocontrol of these pathogens were mainly uncovered in field trials as discussed in the following section.

## 6. Yeasts as biocontrol agents in the vineyard: the challenging *in vitro* to field transition

Although an assortment of native yeasts is continuously being evaluated for biocontrol potential and plant growth enhancement, little information is available regarding confirmation via studies in the field of the various promising results obtained *in vitro;* this is partially due to the variable conditions that characterise disease incidence and vine response across regions (Droby *et al*., 2016). A summary of successful biocontrol studies in field conditions is provided in Table 3. The most efficient formulations were found to be those for the control of rots caused by *Botrytis* and *Aspergillus* spp., and new information has also been obtained regarding the mechanisms underlying the antagonistic activity of yeasts for the biocontrol of grapevine mildews in field trials.

In a Greek vineyard containing cv. Grenache Rouge and Agiorgitiko, the grape berry-isolated yeast *A. pullulans* Y-1 (at $10^7$ cells/mL) was field-tested for use against *A. carbonarius* (at $10^4$ spores/mL): the yeast efficiently reduced the infection symptoms in berries at harvest and ochratoxin A contamination in must, showing a similar pattern to the commercial fungicide fludioxonil + cyprodinil (Dimakopoulou *et al*., 2008). Another field trial with *A. pullulans* strains L47 and 547 on table grapes showed a significant reduction of incidence and severity of grey mould (Schena *et al*., 2003): treatment with strain L547 significantly reduced postharvest rots in sweet cherries (by 47 %) and table grapes (by 38 %), whereas the application of strain L47 alone or in combination with calcium chloride and sodium bicarbonate effectively controlled rot incidence caused by *B. cinerea* in wounded sweet cherries. A strain of the yeast-like fungi *A. pullulans* LS30 field-tested for its biocontrol activity against *Aspergillus* spp. on wine grapes in the vineyard was also demonstrated to significantly reduce the severity of grape rots and OTA contamination by 81.9 % (de Felice *et al*. 2008).

Other *in vivo* field trials have demonstrated the efficacy of *A. pullulans* Y-1 in the control of *A. carbonarius,* which causes sour rot in grapevines (Dimakopoulou *et al*., 2008). Remarkably, in a commercial organic vineyard in Catalonia, Spain, when applied without the addition of biodegradable coatings, *Candida sake* CPA-1 significantly reduced sour rot incidence and severity, while novel film-forming formulations and the liquid (potato-starch) formulations of *C. sake* CPA-1 controlled *B. cinerea* by reducing disease severity from 56 % (CS) to 84 % (Carbó *et al.*, 2019).

The seasonal application of the vineyard isolates *Candida guilliermondii* A42 and *Acremonium cephalosporium* B11 to table (Thompson Seedless and Superior Seedless) and wine (Sauvignon blanc) grapes in Israeli vineyards showed *C. guilliermondii* A42 to be effective in reducing decay due to moulds in both types of grapes in the given seasons (Zahavi *et al*., 2000). In this study, although *A. cephalosporium* B11 did not effectively reduce the sour rot of grape caused by *A. niger*, the findings indicate that both native yeasts can prevent disease progression in the vineyard and reduce postharvest decay.

**TABLE 3.** Biocontrol yeasts that have been successfully field-tested for plant protection.

| Yeast strain | Action mechanism | *Target pathogen* | Crop | Localisation | Vintage | References |
|---|---|---|---|---|---|---|
| *Candida sake* CPA-1 | Antifungal toxins | *B. cinerea*, *P. expansum* | Grape | Lleida, Spain | 2005 - 2006, 2010 - 2011 | Calvo-Garrido *et al.* (2013) |
| *Candida oleophila* 182 | Induction of systemic resistance | *P. digitatum* | Grapefruit | Dagan, Israel | NA | Droby *et al.* (2002) |
| *Cryptococcus laurentii* LS-28 | Competition for nutrients and space, secretion of hydrolytic enzymes (β-1,3- glucanase), antibiotic | *B. cinerea*, *P. expansum*, *A. niger*, *R. stolonifer*, *Geotrichum citri-aurantii* | Grape, citrus, apple, pear, strawberry, kiwi, peach, plum, olive | NA | NA | Liu *et al.* (2013) |
| *Metschnikowia fruticola* NRRL Y-27328, and CBS 8853 | Competition for nutrients and space | *B. cinerea*, *A. niger*, *Alternaria* spp. | Grape, strawberry | California, United States | 2000 - 2002 | Karabulut *et al.* (2003) |
| *Aureobasidium pullulans* AP159-18 | Competition for nutrients and space | *B. cinerea* | Grape | Siena, Italy | 2015 - 2018 | Galli *et al.* (2021) |
| *A. pullulans* Y-1 | Competition for space | *A. carbonarius* | Grape | Corinthos County and Rhodes Island, Greece | 2004 - 2006 | Dimakopoulou *et al.* (2008) |
| *A. pullulans* 533 and 547 | Competition for nutrients | *B. cinerea* | Table grape, sweet cherry | Southern Italy | 1998 - 2001 | Schena *et al.* (2003) |
| *A. pullulans* SCSUI8, SCSUH15 and STR10 | Competition for nutrients | *Greeneria uvicola* | Grape berry, leaf | Tumbarumba, Australia | 2011 - 2014 | Rathnayake *et al.* (2018) |
| *A. pullulans* LS30 | NA | *Aspergillus* carbonarius. | Grape | Abruzzo, Italy | 2006 - 2007 | de Felice *et al.* (2008) |
| *Candida guilliermondii* A42 | Competition for nutrients | *B. cinerea*, *A. niger*, *R. stolonifer* | Grape | Beijin, China | 1996 - 1998 | Qing *et al.* (2000) |
| *Rhodotorula glutitnis* LS-11 | Competition for nutrients and space, secretion of antifungal toxins | *P. expansum* | Grape, apple | Campobasso, Italy | NA | Lima *et al.* (1998) |
| *M. pulcherrima* NCAIM Y0177071, NCAIM Y0177072, NCAIM Y0177071 | Iron depletion | *A. clavatus*, *A. niger*, *B. cinerea*, *Mucor piriformis* | Grape | Tokaj, Hungary | 2002-2003 | Sipiczki (2006) |
| *Debaryomyces hansenii* 18858 | Competition for nutrients | *digitatum* | Grapefruit | Dagan, Israel | NA | Droby *et al.* (1989) |
| *Pseudozyma flocculosa* | Secretion of antifungal unsaturated fatty acids | *Erysiphe necator* | Grape | Patras, Greece | 1999 - 2001 | Konstantinidou-Doltsinis *et al.* (2007) |
| Unassigned yeast taxa (ANYI-2, SATYI-1 and NYI-1) | Induction of phytoparasitism | *E. necator* | Grape | Maharashtra, India | NA | Raghuwanshi *et al.* (2017) |
| *Aurereobasidium pullulans* DSM 14940 and DSM 14941 | NA | *Plasmopara viticola* | Grape | NA | NA | Harm *et al.* (2011) |
| *Trichoderma harzianum* T39144 | NA | *P. viticola* | Grape | San Michele all'Adige, Italy | NA | Vecchione *et al.* (2007) |

NA: Not available.

Other studies, like that of Calvo-Garrido *et al*. (2017), have shown that formulations of *C. sake* CPA-1 combined with Fungicover to reduce *Botrytis* incidence and severity by 35 - 85 %. The overall results show a synergy between antagonistic yeasts and most phytosanitary products commonly used in viticulture, with mixed formulations being the most effective for biocontrol (Calvo-Garrido *et al*., 2017). Furthermore, the efficiency of the same formulations has been found to be comparable to that of commercialised bio-based products, such as *BOTRY-Zen*® and *ARMOUR-Zen*®, when used against *Botrytis* infection in organic vineyards (Calvo-Garrido *et al*., 2013).

**TABLE 4.** Biocontrol yeasts used in commercial formulations.

| Product | Yeast species | Target pathogens | Crops | References |
|---|---|---|---|---|
| Blossom Protect | *Aureobasidium pullulans* DSM 14940 and DSM 14941 | *E. amylovora, B. cinerea, C. gloeosporioides* | Apple | Kołodziejska *et al.* (2021) |
| Botector | *Aureobasidium pullulans* DSM 14940 and DSM 14941 | *B. cinerea* | Apple, pear, grape, strawberry | Schilder (2013) |
| BoniProtect | *Aureobasidium pullulans* | *Pezicula sp., Nectria sp., B. cinerea, M. fructigena, P. expansum* | Apple, pear | Lima *et al.* (2005) |
| Noli | *Metschnikowia fruticola* NRRL Y-27328 KM1110 WDG | *B. cinerea, Monilinia spp.* | Table and wine grape, strawberry | Dukare *et al.* (2018) |
| Romeo | *Saccharomyces cerevisiae* cell walls | *B. cinerea, Erysiphales, Plasmopara* | Grape, tomato, strawberry, lettuce and cucumber | Dukare *et al.* (2018) |
| Aspire | *Candida oleophila* I-182 | *P. digitatum, Rhizopus stolonifer, Fusarium sp., B. cinerea, Sclerotinia sclerotium* | Pome fruit, citrus, banana | Droby *et al.* (1998) |
| Nexy | *Candida oleophila* strain O | *B. cinerea, Penicillium sp., Colletotrichum* sp. | Pome, banana, citrus, sweet potato, peach, pepper, carrot | Droby *et al.* (2002) |
| CandifruitTM | *Candida sake* CPA-1 | *P. expansum, B. cinerea, Rhizopus nigricans* | Apple, pear | Teixidó *et al.* (2011) |
| Yieldplus | Cryptococcus albidus | *Botrytis* sp., *Penicillium* sp., *Mucor* sp. | Pome fruit | Teixidó *et al.* (2011) |
| Shemer | Metschnikowia frutícola | *Botrytis* sp., *Penicillium* sp., *Rhizopus* sp., *Aspergillus* sp. | Grape, apricot, citrus, peach, pepper, strawberry, sweet potato | Droby *et al.* (2002) |

Field experiments on the biocontrol of powdery mildew have demonstrated that formulations based on *Pseudozyma flocculosa* (Sporodex® L) reduce disease incidence, thus representing promising biocontrol strategies (Konstantinidou-Doltsinis *et al*., 2007). The antagonistic action of *P. flocculosa* is linked to the secretion of extracellular fatty acids like flocculosin and cis-9-heptadecenoic acid, which disrupt the cytoplasmic membrane in a variety of fungi (Konstantinidou-Doltsinis *et al*., 2007). Under field conditions, other native grapevine leaf yeast isolates with no assigned taxa (ANYI-2, SATYI-1 and NYI-1) have been shown to reduce powdery mildew incidence by ≈80 % (Raghuwanshi *et al*., 2017): the yeast treatment caused a shift in the leaf microflora, reducing phyllosphere microbial biodiversity while increasing the abundance of yeast population, and was suggested to induce phytoparasitism via adherence of yeast spores to the fungal spore epidermis, leading to its shrinkage. Assays conducted in greenhouse conditions on potted vines have shown that a treatment based on *A. pullulans* (Aureo) provides some protection against *P. viticola;* after the treatments, the activity of the pathogenesis-related (PR) protein glucanase slightly increased, but not enough to consider this microorganism as a resistance inducer (Harm *et al*., 2011). However, the study highlights its potential to partly induce natural resistance metabolites that enhance the tolerance of grapevines to *P. viticola* through synergistic effects with conventional fungicides, thereby reducing their ecological burden, while not being sufficiently effective to entirely replace them. Similarly, under greenhouse conditions using potted vines of cv. Pinot gris and cv. Chardonnay, microbial formulations comprising *Trichoderma harzianum* T39144 (Trichodex) and a combination of *Clonostachis rosea* and *T. harzianum* demonstrated good ability to reduce the disease incidence of *P. viticola* (Vecchione *et al*., 2007). The performance of microbial formulations lasts for only a short time after inoculation and it is possible that the disease will not be completely controlled (Vecchione *et al*., 2007); therefore, for effective disease management, a strategy needs to be found for controlling the fungi at multiple site and stages of its life cycle.

As mentioned previously, the outcomes of vineyard foliar spraying of yeast formulations do not always align with those obtained in laboratory scale experiments. For the optimal performance of an antagonistic yeast, the cells must colonise the plant surface and consequently remain active during periods conducive to plant infection (Dimakopoulou *et al*., 2008); this requires the optimisation of several variables: the composition of the yeast formulations, the treatment timings and the operative methods for applying them in the vineyard.

### 7. First steps towards the commercialisation of yeast-based biocontrol formulations

The use of yeast isolates from wine grape microbiota for developing biofungicides is favored by their environmental friendliness, ease of manipulation and GRAS status (Freimoser *et al*., 2019; Gonzalez and Morales, 2022). The grape berry-associated yeasts play an essential role in wine fermentations and co-fermentations, as the metabolic interactions that occur after controlled inoculations using *Saccharomyces* and non-*Saccharomyces* yeasts can enhance the complexity of wine flavour, contributing to the production of innovative and distinct wines (Álvarez *et al*., 2023). These yeasts are considered safe, but their potential risks to consumers should not be excluded (Riesute *et al*., 2021). In terms of cost, the use of yeasts seems to be cheaper than using conventional pesticides. Apart from products such as Botector®, Noli®, Romeo® and Shemer®, relatively few bioproducts are commercially available for use with grapes (Table 4). Certain microbes that have been studied in the laboratory for biocontrol may not be accepted for commercialisation due to inconsistent results in the field (Droby *et al*., 2016).

Botector acts competitively and does not attack the metabolism of fungal pathogens, indicating that the product could be used to combat *Botrytis cinerea* in grape berries and other crops without the risk of resistance developing after several applications (Bio-Ferm, 2017). More research is needed to identify other microbial species, including fungi and bacteria, that could be marketed as novel agricultural bioproducts for other crops. It should be noted that some of the earlier commercialised biocontrol yeast formulations, such as Aspire and Yieldplus, were eventually withdrawn from distribution due to inconsistent efficacy, as well as low marketing success and profit (Spadaro and Droby, 2016). Most commercialised bioproducts are still subject to regulation by local authorities (Table 4). In this regard, 65 strains of microorganisms have to date been approved for plant protection in the European Union: 36 strains of fungi (e.g., *Trichoderma harzianum* strains ICC012, T25 and TV1, *T. afroharzianum* T-22, *Isaria fumosorosea* 97), yeasts (e.g., *Aureobasidium pullulans* DSM 14940 and DSM 14941, *Metschnikowia fructicola* NRRL Y-27328, *Saccharomyces cerevisiae* LAS02, *Candida oleophila* strain O) and oomycetes (e.g., *Pythium oligandrum* M1); 21 strains of bacteria (e.g., *Bacillus amyloliquefaciens* AH2, *Bacillus thuringiensis* subsp. k*urstaki* SA-12 ); and 7 viruses (e.g., *Cydia pomonella* Granulovirus (CpGV), *Adoxophyes orana* granulovirus). Of these agents, 32 strains have been recognised as fungicidal agents and can be used as such (see: ec.europa.eu, June 2020 data).

The shortcomings of using biocontrol agents are that they may not eradicate all the pests if they are not applied at the right time (i.e., before the pathogen's establishment in the host), and they might not provide a high level of protection when used on a large scale (Spadaro and Droby, 2016). In addition, there is need for skillful management of the process; the farmers need to have an understanding of the biology of both the target pests and their natural enemies before they can use the agents. However, despite these shortcomings, the use of biological control agents can majorly contribute to safer food production by eliminating the occurrence and accumulation of pesticides within the global food chain and food delivery system.

## CONCLUSION - PROSPECTS FOR THE USE OF GRAPE NATIVE YEASTS AS SUSTAINABLE BIOCONTROL AGENTS IN VITICULTURE

Biological control yeasts represent a family of fungi with high potential for use as plant protection tools in integrated pest management initiatives aiming to kill, disable or restrain target pests. Most commercial formulations containing yeasts have a very limited spectrum and biocontrol efficacy against most grapevine pathogens due to the myriad of variables conditioning disease incidence and plant response across regions. Additionally, their use involves the introduction of foreign genetic material into habitats, potentially impacting the genetic pool of the indigenous microflora and the diversity of the vine holobiont. Using autochthonous grape yeast isolates as biocontrol agents *in loco* is an emerging trend (Galli *et al*., 2021), and they could substantially increase the biocontrol efficacy on a local scale, as well as contribute to the preservation of the microbial terroir, and thus to the typicity of regional wines. The advantage of using native yeasts in biocontrol formulations is that because they are thriving in their natural habitat the period of adaptation to a new environment that is imposed to non-autochthonous yeasts is circumvented. The omics exploitation of the bioactive compounds underlying antagonistic activity will also open doors for optimising the production of more stable and efficient formulations with wide crop applicability. A combination of antagonistic yeasts with eco-friendly and compatible binding compounds, such as chitosan, alginate, edible starch, kaolin and calcium, have been shown to improve colonisation, enhance the adherence of cells to plant tissues and maintain activity for a longer duration (Gramisci *et al*., 2018). Thus, modelling the microencapsulation of yeast formulations using these compounds seems to be a promising approach for achieving optimal stability and efficiency of biocontrol products, whereby maximising the delivery of bioactives to target compartments could accelerate plant defense against biotic stress (Peil *et al*., 2020).

Recently, research has opened up new avenues for increasing the effectiveness of biocontrol agents in agriculture and environmental management, leading to the development and exploration of synthetic microbial communities. With the aim of replicating the structural and functional characteristics of a microbiome and improving the stability of microbial communities, this construct leverages on the synergistic interactions between diverse microbial species to combat pathogens and tackle other ecological challenges (Shayanthan *et al*., 2022). Interestingly, the co-cultivation of multiple taxa under precisely-defined conditions, allowing the power of synthetic biology and microbial ecology to be harnessed, could lead to the establishment of synergistic

interactions between their constituents in such a way as to outperform individual biocontrol agents; the produced microbes could thus be used as inoculum for different crops in different soils (Marín *et al*., 2021).

The increasing awareness of producers and consumers regarding the benefits of implementing green technologies is pivotal for the preservation of the environment and human health, and for achieving a more sustainable and circular viticulture in line with OIV guidelines for sustainable wine production (International Organisation of Vine and Wine, 2020). Streamlining the processes underlying the validation of new biocontrol products is essential for their rapid implementation and market acceptance, in order to reach the goals of EU 2030 Agenda for sustainable development.

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