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 (e.g., 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 Tordaspora 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 all 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 (Ramirez 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
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 KNO₃) 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; Agarbarati 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 spiroxamnia, copper-oxidechyl 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), rootox™ (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, spiroxamnia, 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-Bl+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.

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<th>Approach</th>
<th>Methodology</th>
<th>Advantages</th>
<th>Shortcomings</th>
<th>References</th>
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<tr>
<td>Agar surface dual culture assay</td>
<td>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.</td>
<td>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.</td>
<td>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.</td>
<td>Ayogu et al. (2023)</td>
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<td>Dual culture assay on liquid media</td>
<td>Coinoculation of fungal spores or mycelia into liquid media in test tubes containing the yeast; microscopic examination of preparation for antagonism features after incubation.</td>
<td>Ease of implementation.</td>
<td>Supports the evaluation of antifungal activity of only one yeast at a time.</td>
<td>Serna-Díaz et al. (2020)</td>
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<td>Agar plug diffusion assay</td>
<td>Transferring an agar plug (about 0.5 cm²) of filamentous fungi onto the middle of another agar plate previously inoculated with antagonistic yeast; measuring mycelia growth after incubation.</td>
<td>Gives idea of inhibitory effect of antagonists over hyphae proliferation; offers opportunity for testing processed antagonist products (heat denatured yeasts cells, cell-free supernatants).</td>
<td>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.</td>
<td>Zepeda-Giraud et al. (2016)</td>
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<td>Agar disk diffusion assay</td>
<td>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.</td>
<td>Simple, low cost; offers opportunity to test greater number of yeasts against one mould; ease of results interpretation.</td>
<td>The antagonistic activity of yeast may be affected by the permeability (quality) of the paper used in the assay.</td>
<td>Liu et al. (2017)</td>
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<td>Mouth-to-mouth dual assay</td>
<td>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.</td>
<td>Identification of yeasts producing VOCs with antagonistic action.</td>
<td>Less reliable than the in vivo assay; does not provide information about antagonistic mechanisms involving production of non-VOCs.</td>
<td>Parafati et al. (2015)</td>
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<td>In vivo dual assay</td>
<td>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.</td>
<td>Controlled in planta approach; relatively cheap, simple and quick characterisation of antagonistic potential in vivo.</td>
<td>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.</td>
<td>Parafati et al. (2015); Maluleke et al. (2022); Pollard-Flamand et al. (2022); Ayogu et al. (2023)</td>
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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...
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 (Agarbatì 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

**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 10⁸ CFU/mL towards fungi pathogens inoculated at 10⁷ 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).
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, Colletotrichum 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 completely effective, 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>
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<th>Characterisation</th>
<th>Causal agents</th>
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<tbody>
<tr>
<td>Bunch rot/Grey rot</td>
<td>Brown rot on bunches which can also affect other plant parts.</td>
<td>Botrytis cinerea</td>
<td>Copper (II) sulphate</td>
<td>Nally et al. (2012);</td>
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<td>Di Canito et al. (2021)</td>
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<td>Anthracnose/Ripe</td>
<td>Circular, reddish-brown spots, which enlargen to cover the whole fruit. Leaves</td>
<td>Colletotrichum acutatum, C.</td>
<td>Fludioxonil, folpet, captan</td>
<td>Ishii et al. (2022)</td>
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<td>rot</td>
<td>branches become covered with brown spots and wither.</td>
<td>nymphaeae, C. gloeosporioides,</td>
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<td>C. musae, C. fructicola, C.</td>
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<td>aestigma, C. pseudoacutatum</td>
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<td>Aspergillus rot</td>
<td>Grape clusters lighten and acquire a dark colour. Berries turn into a bluish-brown mass</td>
<td>Aspergillus carbonarius, A.</td>
<td>Copper (II) sulphate, fludioxonil, cyprodynil, captan</td>
<td>Di Canito et al. (2021)</td>
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<td></td>
<td>following shrinkage and cracking.</td>
<td>niger, A. Alliaceus, A.</td>
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<td>ochraceus</td>
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<td>Fruit rot</td>
<td>Light-brown soft, watery rot followed by the appearance of blue-green conidia on the fruit surface at advanced stages of decay.</td>
<td>Penicillium expansum, P.</td>
<td>Thiabendazole, imazalil, carbendazim</td>
<td>Di Canito et al. (2021)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>glabrum, P. purpurescens, P.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>decumbens, P. chrysogenum, P.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>crustosum, P. aurantiogriseum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Powdery mildew</td>
<td>Mycelial growth and sporulation occur on the surface of leaves and stems, resulting in a white fuzzy mildew appearance.</td>
<td>Erysiphe necator</td>
<td>Copper (II) sulphate, azoxystrobin, trifloxystrobin</td>
<td>Lukšić et al. (2022)</td>
</tr>
<tr>
<td>Downy mildew</td>
<td>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.</td>
<td>Plasmopara viticola</td>
<td>Copper oxychloride, copper hydroxide, captan</td>
<td>Koledenkova et al. (2022)</td>
</tr>
<tr>
<td>Black spot</td>
<td>A leaf spot that is characterised by small, water-soaked lesions.</td>
<td>Alternaria alternata, A.</td>
<td>Copper (II) sulphate, fludioxonil</td>
<td>Ji et al. (2023)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>brassicicola</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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, Starmerella thermotolerans, Lodderomyces elongisporus, Zygosaccharomyces 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 cephalosporiunum 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 BScl62, 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 BSc198, 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 (1×10⁶ 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 10⁶ CFU/mL, several other yeast species (S. cerevisiae, M. pulcherrima, P. guilliermondii, C sake, C. famata, C. parapsilosis, C. albidas, D. hansenii, H. vinace, P. anomala, P. membranifaciens, S. bayanus, S. cerevisiae, S. chevalieri, S. kuyveri, 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 thermod toleranters, Lodderomyces elongisporus, Z. meyerae, C. oleophila and H. urvarum 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. asperflouides (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 10⁶ 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 Zygosaccharomyces meyerae L29 from vineyards have also been shown to significantly reduce the incidence of P. glabrumin grapes (Cabañas et al., 2020). The 70% incidence reduction by Zygosaccharomyces 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 10⁶ 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 10⁶ CFU/mL) has been shown to protect grapefruits from decay caused by the green mould P. digitatum, likely due to
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 10^6 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.
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 3. Biocontrol yeasts that have been successfully field-tested for plant protection.

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Action mechanism</th>
<th>Target pathogen</th>
<th>Crop</th>
<th>Localisation</th>
<th>Vintage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cryptococcus laurentii</em> LS-28</td>
<td>Competition for nutrients and space, secretion of hydrolytic enzymes (β-1,3-glucanase), antibiotic</td>
<td><em>B. cinerea, P. niger, R. stolonifer, Geotrichum citri-auroanti</em></td>
<td>Grape, citrus, apple, pear, strawberry, kiwi, peach, plum, olive</td>
<td>NA</td>
<td>NA</td>
<td>Liu et al. (2013)</td>
</tr>
<tr>
<td><em>Aureobasidium pullulans</em> AP159-18</td>
<td>Competition for nutrients and space</td>
<td><em>B. cinerea</em></td>
<td>Grape</td>
<td>Siena, Italy</td>
<td>2015 - 2018</td>
<td>Galli et al. (2021)</td>
</tr>
<tr>
<td><em>A. pullulans</em> Y-1</td>
<td>Competition for space</td>
<td><em>A. carbonarius</em></td>
<td>Grape</td>
<td>Corinthis County and Rhodes Island, Greece</td>
<td>2004 - 2006</td>
<td>Dimakopoulou et al. (2008)</td>
</tr>
<tr>
<td><em>A. pullulans</em> S33 and 547</td>
<td>Competition for nutrients</td>
<td><em>B. cinerea</em></td>
<td>Table grape, sweet cherry</td>
<td>Southern Italy</td>
<td>1998 - 2001</td>
<td>Schena et al. (2003)</td>
</tr>
<tr>
<td><em>A. pullulans</em> SCSU18, SCSU1H15 and STR10</td>
<td>Competition for nutrients</td>
<td><em>Greeneria uvicola</em></td>
<td>Grape berry, leaf</td>
<td>Tambarumba, Australia</td>
<td>2011 - 2014</td>
<td>Rathnayake et al. (2018)</td>
</tr>
<tr>
<td><em>Rhodotorula glutinis</em> LS-11</td>
<td>Competition for nutrients, secretion of antifungal toxins</td>
<td><em>P. expansum</em></td>
<td>Grape, apple</td>
<td>Campobasso, Italy</td>
<td>NA</td>
<td>Lima et al. (1998)</td>
</tr>
<tr>
<td><em>Debaryomyces Hansenii</em> 18858</td>
<td>Competition for nutrients</td>
<td><em>digitatium</em></td>
<td>Grapefruit</td>
<td>Dagan, Israel</td>
<td>NA</td>
<td>Drobay et al. (1989)</td>
</tr>
<tr>
<td>Unassigned yeast taxa (ANYI-2, SATYI-1 and NYI-1)</td>
<td>Induction of phytoparasitism</td>
<td><em>E. necator</em></td>
<td>Grape</td>
<td>Maharashtra, India</td>
<td>NA</td>
<td>Raghuvanshi et al. (2017)</td>
</tr>
<tr>
<td><em>Aureobasidium pullulans</em> DSM 14940 and DSM 14941</td>
<td>NA</td>
<td><em>Plasmopara viticola</em></td>
<td>Grape</td>
<td>NA</td>
<td>NA</td>
<td>Harm et al. (2011)</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em> T39144</td>
<td>NA</td>
<td><em>P. viticola</em></td>
<td>Grape</td>
<td>San Michele all’Adige, Italy</td>
<td>NA</td>
<td>Vecchione et al. (2007)</td>
</tr>
</tbody>
</table>

NA: Not available.
TABLE 4. Biocontrol yeasts used in commercial formulations.

<table>
<thead>
<tr>
<th>Product</th>
<th>Yeast species</th>
<th>Target pathogens</th>
<th>Crops</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blossom Protect</td>
<td>Aureobasidium pullulans DSM 14940 and DSM 14941</td>
<td>E. amylovora, B. cinerea, C. gloeosporioides</td>
<td>Apple</td>
<td>Kołodziejska et al. (2021)</td>
</tr>
<tr>
<td>Botector</td>
<td>Aureobasidium pullulans DSM 14940 and DSM 14941</td>
<td>B. cinerea</td>
<td>Apple, pear, grape, strawberry</td>
<td>Schilder (2013)</td>
</tr>
<tr>
<td>BoniProtect</td>
<td>Aureobasidium pullulans</td>
<td>Pezicula sp., Nectria sp., B. cinerea, M. frutigena, P. expansum</td>
<td>Apple, pear</td>
<td>Lima et al. (2005)</td>
</tr>
<tr>
<td>Noli</td>
<td>Metschnikowia fruticola NRRL Y-27328 KMI1110 WDG</td>
<td>B. cinerea, Manilinia spp.</td>
<td>Table and wine grape, strawberry</td>
<td>Dukare et al. (2018)</td>
</tr>
<tr>
<td>Romeo</td>
<td>Saccharomyces cerevisiae cell walls</td>
<td>P. digitatum, Rhizopus stolonifer, Fusarium sp., B. cinerea, Sclerotinia sclerotium</td>
<td>Grape, tomato, strawberry, lettuce and cucumber</td>
<td>Dukare et al. (2018)</td>
</tr>
<tr>
<td>Aspire</td>
<td>Candida oleophila I-182</td>
<td>P. digitatum, Rhizopus stolonifer, Fusarium sp., B. cinerea, Sclerotinia sclerotium</td>
<td>Pome fruit, citrus, banana</td>
<td>Droby et al. (1998)</td>
</tr>
<tr>
<td>Nexy</td>
<td>Candida oleophila strain O</td>
<td>B. cinerea, Penicillium sp., Colletotrichum sp.</td>
<td>Pome, banana, citrus, sweet potato, peach, pepper, carrot</td>
<td>Droby et al. (2002)</td>
</tr>
<tr>
<td>CandifruitTM</td>
<td>Candida sake CPA-1</td>
<td>P. expansum, B. cinerea, Rhizopus nigricans</td>
<td>Apple</td>
<td>Teixidó et al. (2011)</td>
</tr>
<tr>
<td>Yieldplus</td>
<td>Cryptococcus albidos</td>
<td>Botrytis sp., Penicillium sp., Mucor sp.</td>
<td>Pome fruit</td>
<td>Teixidó et al. (2011)</td>
</tr>
<tr>
<td>Shemer</td>
<td>Metschnikowia fruticola</td>
<td>Botrytis sp., Penicillium sp., Rhizopus sp., Aspergillus sp.</td>
<td>Grape, apricot, citrus, peach, pepper, strawberry, sweet potato</td>
<td>Droby et al. (2002)</td>
</tr>
</tbody>
</table>

Field experiments on the biocontrol of powdery mildew have demonstrated that formulations based on *Pseudazyma 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 phyloosphere 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. *kurstaki* 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 typicality 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.

ACKNOWLEDGEMENTS

This work was supported by the “Contrato-Programa” UIDB/04050/2020 (DOI 10.54499/UIDB/04050/2020) and by DOI 10.54499/DL57/2016/CP1377/CT0027 to VM, funded by Portuguese national funds through the FCT I.P. The work was also supported by FCT, CCDR-N (Norte Regional Coordination and Development Commission) and European Funds (FEDER/POCU/COMPETE2020) through the projects GrapeMicrobiota (PTDC/BAA-AGR/2691/2020) and AgriFood XXI (NORTE-01-0145-FEDER-000041). This work benefited from the networking activities of CoLAB VINES & WINES.

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