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SPECIAL ISSUE

# Innovation in post-harvest biocontrol: novel strategies against *Botrytis cinerea* for grape preservation

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## ABSTRACT

Driven by the growing demand for sustainable agriculture, biocontrol is emerging as a key alternative to chemical fungicides in crop protection. This approach relies on living microorganisms, their metabolites, or other natural compounds to combat plant diseases and preserve food quality. In vitiviniculture, *Botrytis cinerea* poses a major challenge, particularly during grape storage, as it compromises fruit integrity. Although chemical fungicides are commonly used, their application is restricted in pre-harvest stages to avoid residues in the final product, prompting interest in natural solutions such as biocontrol. However, the number of microorganisms registered as active substances in the European Union remains limited, underscoring the need for improved selection and application strategies.

This study aims to evaluate the performance of novel microbial isolates—specifically grapevine wood endophytes—as bioprotective agents, comparing them with commercial strains of *Metschnikowia pulcherrima* and *Aureobasidium pullulans*. The focus is on post-harvest application, particularly the storage phase, which is critical for maintaining grape quality. Selected bacterial isolates, primarily Actinobacteria, were tested on grape bunches previously infected with *B. cinerea* under controlled laboratory conditions. These trials assessed the microorganisms' ability to colonise grape surfaces and remain viable during storage—key traits for effective biocontrol. Monitoring of disease incidence and severity revealed a significant reduction in *B. cinerea* growth on treated grapes.

Further trials were conducted under industrial conditions in withering warehouses used for passito wine production. Experiments at two wineries tested the efficacy of the isolates, both individually and in microbial consortia, with and without adhesives to enhance treatment adherence. Results were particularly promising in environments with high *Botrytis* pressure, demonstrating biocontrol efficacy comparable to commercial agents.

To our knowledge, this is the first study to investigate the use of novel endophytic microorganisms for biocontrol during the critical post-harvest grape drying phase, offering a promising alternative to chemical treatments.

**KEYWORDS:** bioprotection, grape withering, *Metschnikowia pulcherrima*, *Aureobasidium pullulans*, wood endophytes, winemaking

## INTRODUCTION

*Vitis vinifera* L. cv. Corvina and Rondinella are the principal red grape varieties cultivated in the Verona region of northern Italy, and Amarone wine, produced from withered grapes, stands as the most significant dry red wine of the area (Fedrizzi *et al.*, 2011). Despite its substantial economic importance, particularly in the last three decades, research focused on the unique characteristics of this wine remains limited, in particular regarding the withering process of these grape varieties, which plays a crucial role in imparting the distinctiveness and aromatic complexity to the wine.

Grape withering involves air-drying harvested bunches in large, well-ventilated warehouses, where they can lose up to 40 % of their initial mass over a period of 60 to 90 days, depending on the relatively low temperatures typical of late autumn and early winter (Binati *et al.*, 2023; Paronetto & Dellaglio, 2011). This gradual dehydration results in a concentration of sugars, alongside other compounds that influence the aromatic profile of the wine. During this process, the microbial composition of the grapes undergoes significant changes (Barbato *et al.*, 2025; Salvetti *et al.*, 2016; Stefanini *et al.*, 2017) and aromatic molecules critical to the wine's bouquet and phenolic compounds evolve (Consonni *et al.*, 2011; Tosi *et al.*, 2012; Consonni *et al.*, 2011; Fedrizzi *et al.*, 2011; Paronetto & Dellaglio, 2011; Tomasi *et al.*, 2021).

To this day, a significant portion of Amarone wine production still relies on the traditional withering process, where sometimes mould infection is challenging to control as humidity and ventilation are not controlled. These conditions often result in a higher impact of noble rot on the wine's aroma compared to grapes dehydrated in controlled chambers (Fedrizzi *et al.*, 2011; Tosi *et al.*, 2012). While the formation of noble rot by *Botrytis cinerea* can positively influence the organoleptic profile of the wine, uncontrolled environmental conditions in the drying rooms, particularly high relative humidity, encourage the development of grey rot and other mould pathogens, which can negatively affect the wine. The incidence and severity of these problems, especially during the post-harvest and withering phases, can significantly compromise grape quality and, as a result, diminish the economic value of the final product (Salvetti *et al.*, 2016; Stefanini *et al.*, 2017).

During grape ripening, when adverse climatic conditions occur before harvest, chemical pesticides are traditionally employed to reduce the incidence of infections by the saprophytic growth of filamentous fungi (Binati *et al.*, 2023). However, their indiscriminate use has led to increased pollution of agricultural ecosystems, with consequent risks also for human health. Many pesticides are limited pre-harvest to prevent contamination with residues in the final product. Additionally, concerns about pesticide residues have grown not only for human health but also can negatively affect yeast activity during alcoholic fermentation. Furthermore, the escalating costs of developing new chemicals due to the rise of fungicide-resistant strains have driven the search for alternative, less harmful solutions, such as the use of microorganisms for biocontrol (Galli *et al.*, 2024; Sellitto *et al.*, 2021).

In recent years, significant progress has been made in using microbial antagonists to control post-harvest diseases. For instance, the application of biocontrol agents to manage *Botrytis* bunch rot offers a viable alternative or can be used in conjunction with sulfur dioxide ($SO_2$), thereby contributing to a reduction in its content in grapes and minimising potential allergic reactions to sulfites in wine consumers (Galli *et al.*, 2021). Indeed, although $SO_2$ has become indispensable in the winemaking process, growing health concerns, particularly among sensitive individuals, have sparked efforts to reduce its use in food and beverages (Binati *et al.*, 2023; Nardi, 2020).

Among yeast species with antimicrobial properties, *Metschnikowia pulcherrima* stands out as one of the most extensively used for agri-food applications, owing to its broad biotechnological potential (Binati *et al.*, 2023; Sipiczki, 2022). Additionally, *Aureobasidium pullulans* has been commercialised for the management of *Botrytis* grey mould, particularly in relation to post-harvest rots of table grapes. *A. pullulans* has evolved exceptional tolerance to a wide range of ecological conditions, and its widespread occurrence is attributed to its high tolerance for environmental stresses and its strong antagonistic activity against bacteria and fungi (Bozoudi & Tsaltas, 2018; De Curtis *et al.*, 2012). It is frequently isolated from both diseased and healthy vine tissues, and it is one of the predominant yeast species found on the surface of grape berries at all stages of maturity (Barata *et al.*, 2012). The biocontrol mechanisms of *Metschnikowia pulcherrima* and *Aureobasidium pullulans* include various modes of action, such as competition for nutrients and the production of volatile organic compounds (VOCs) (Di Francesco *et al.*, 2015; Yalage Don *et al.*, 2021). Specifically, *M. pulcherrima* competes for iron, which has been shown to play a significant role in biocontrol interactions (Saravanakumar *et al.*, 2008), while *A. pullulans* primarily competes for nutrients and produces enzymes like glucanase, chitinase, protease, and extracellular proteases, further enhancing their antagonistic activity (Bencheqroun *et al.*, 2007; Castoria *et al.*, 2001; Ma *et al.*, 2012).

While different commercial products are currently available in the market, new candidates for controlling *Botrytis* bunch rot in vineyards are continually being explored (Galli *et al.*, 2021). However, the number of microorganisms registered as active substances within the European Union remains limited (Sellitto *et al.*, 2021), highlighting the need for improved selection and application processes (EU Pesticides Database—Active substances, s.d.).

Although initial aspirations to rely on a single biological control agent (BCA) and its ability to self-disperse throughout a crop canopy have proven to be overly optimistic in many

cases, significant progress has been made in understanding the biological modes of action of these agents. These advancements are paving the way for more effective and targeted applications of microbial biocontrol in agriculture. Additionally, they open up opportunities to study these biocontrol agents in their native environments, enhancing our understanding of microbiota and enabling further exploration of their potential.

This study is positioned within the broader context of exploring alternative biocontrol strategies for post-harvest fruit protection, focusing on the efficacy of new isolates derived from vinewood endophytes, specifically *Achromobacter xylosoxidans* (19VE21-3) and *Mycolicibacterium arabiense* (ACT34) (Bertazzoli *et al.*, 2024), as bioprotective microorganisms against *Botrytis cinerea* in the grape withering context. In addition to being recognised as a plant growth-promoting bacterium and a biological control agent of various plant pathogens in other crops (Forchetti *et al.*, 2010), *A. xylosoxidans* has been identified as a member of the grape cane endosphere microbial community that negatively correlates with *Xylella fastidiosa*, suggesting a potential role in the disease escape phenotype (Deyett *et al.*, 2017). Conversely, *M. arabiense* has not previously been associated with vineyards or grapes.

To our knowledge, no prior study has investigated biocontrol using novel endophytic microorganisms during the critical post-harvest phases of grape drying. The study emphasises the post-harvest application phase, particularly the storage period, crucial in preserving fruit quality. The selected endophytes were compared to *Metschnikowia pulcherrima* and *Aureobasidium pullulans,* two established commercial biocontrol agents.

# MATERIALS AND METHODS

## 1. Culture conditions

The tested bacteria were identified as *Achromobacter xylosoxidans* (19VE21-3) and *Mycolicibacterium arabiense* (ACT34) (ViMED – Biomebank, s.d.) as previously described (Bertazzoli *et al.*, 2024). Stock cultures of the endophytic bacteria were maintained in 20 % glycerol and stored at −80 °C for long-term storage. Liquid cultures were grown on MYA medium composed of yeast extract (Oxoid) 20 g/L, malt extract (Oxoid) 2 g/L, dextrose (Titolchimica) 20 g/L, and vitamin B12 (Sigma-Aldrich) 0.01 g/L. The number of cells grown in liquid culture was quantified by centrifuging the suspension at 4,500 rpm for 5 minutes. The resulting pellet was then resuspended in physiological solution, and cell counts were performed using a counting chamber. The required cell concentration for the experiment, both at lab scale and in the withering room, was $10^8$ CFU/mL.

*Metschnikowia fructicola* (nowadays reclassified as *pulcherrima*) GAÏA™ (Perdomini-IOC S.p.A., San Martino B.A. (VR), Italy), a commercial product already characterised for its biocontrol activity in post-harvest conditions as grapes and musts (Binati *et al.*, 2023; Sellitto *et al.*, 2021; Torriani & Tornielli, 2019), was used in this study as a positive control for both laboratory trials and the withering room experiments. Yeast cells were rehydrated following the supplier's instructions, then diluted in sterile water to a final concentration of $10^7$ cells/mL, before being used in the experiments.

In addition, another commercial product containing two strains of *Aureobasidium pullulans* (DSM 14940 and DSM 14941) was included in the tests for comparison, namely Botector® New (Manica S.p.A., Rovereto (TN), Italy). This product, a commercial product already characterised for its biocontrol activity in pre-harvest conditions, including grape ripening (He *et al.*, 2024; Sellitto *et al.*, 2021), was used as a bioactive positive control for laboratory trials following reconstitution according to the supplier's instructions (in 0.1 % sterile water). The final concentration of the yeast cells was adjusted to $10^7$ cells/mL, like the other yeast strain.

The phytopathogen *Botrytis cinerea* was maintained in internal collection at the CREA-VE laboratory in Conegliano, cultured on PDA (Potato Dextrose Agar, Oxoid). The spores of *B. cinerea* were collected by scraping the surface of 15-day-old colonies from Petri dishes using a sterile tip and diluted in a sterile 0.1 % Triton X-100 solution to a final concentration of $10^5$ spores/mL.

## 2. Set-up of lab-scale post-harvest experiment

The post-harvest experiment was conducted using table grape berries from the Victoria cultivar obtained from the commercial market. For each treatment, 20 grape berries were placed in plastic trays previously sterilised with UV light. The berries were sprayed with microorganism solutions, prepared as described earlier ("1. Culture conditions"). For microorganism inoculation, 2 mL of the culture was sprayed onto each tray, ensuring that the entire grape bunch was evenly coated (T0). The experimental setup consisted of 12 treatments (as detailed in Table 1), and each treatment was replicated three times. Three trays containing untreated grape berries served as the control group.

After 24 hours (T1), the trials that underwent artificial fungal inoculations were treated by spraying 1 mL of $10^5$ spores/mL of *Botrytis cinerea*, prepared as previously described. All the trays were kept at 25 °C. After 10-day post-inoculation with *B. cinerea* (T10), all grape berries were carefully examined for damage, and the percentage of damaged berries was used to calculate the incidence rate, which allowed for the evaluation of disease progression and treatment efficacy across different experimental groups.

Two different grape batches were used for the treatments that were grouped in trial groups "A" and "B" (Table 1). As a consequence, the non-inoculated *Botrytis* pressure was not comparable among them. Indeed, in the section "Results and discussion", findings of trials A and B have been analysed separately.

**TABLE 1.** Treatments applied to *Vitis vinifera* cv. Victoria grape berries in trials A and B, including microbial applications at time 0 (T0) and *Botrytis cinerea* inoculation at time 1 (T1), 24 hours later.

| Treatment number | Grape batch/trial | Treatments code | T0 | T1 | T10 |
|---|---|---|---|---|---|
| 1 | A | CTR – NI | x | x | Count of infected berries |
| 2 | A | CTR – I | x | *Botrytis cinerea* | |
| 3 | A | ACT34 – NI | *Mycolicibacterium arabiense* ACT34 | x | |
| 4 | A | ACT34 – I | *Mycolicibacterium arabiense* ACT34 | *Botrytis cinerea* | |
| 5 | A | 19VE21-3 – NI | *Achromobacter xylosoxidans* 19VE21-3 | x | |
| 6 | A | 19VE21-3 – I | *Achromobacter xylosoxidans* 19VE21-3 | *Botrytis cinerea* | |
| 7 | A | 50:50 – NI | ACT34:19VE21-3 | x | |
| 8 | A | 50:50 – I | ACT34:19VE21-3 | *Botrytis cinerea* | |
| 9 | B | Gaia – NI | GAÏA™ | x | |
| 10 | B | Gaia – I | GAÏA™ | *Botrytis cinerea* | |
| 11 | B | Botector – NI | Botector® New | x | |
| 12 | B | Botector – I | Botector® New | *Botrytis cinerea* | |

CTR – NI = control non-inoculated; CTR – I = control inoculated.

## 3. Set up of an industrial-scale post-harvest experiment

Grapevine bunches of *V. vinifera* L. cv. Corvina was collected from two different wineries in the Valpolicella area of Italy during the 2021 and 2022 harvest seasons. We have collaborated with the Benedetti Winery, located in the Sant Ambrogio di Valpolicella area (45.51687, 10.853902), within the Valpolicella Classica zone as defined by the Consorzio Tutela Vini Valpolicella (Consorzio Tutela Vini Valpolicella, s.d.). The estate spans approximately 14 hectares and produces around 80,000 bottles per year, primarily Amarone della Valpolicella DOCG, Valpolicella Ripasso, and Recioto. The second site was San Cassiano Farm in Mezzane di Sotto (45.481395, 11.144379), which belongs to the eastern to the Valpolicella DOC area according to the same classification. San Cassiano manages about 14 hectares of vineyards and produces a range of Valpolicella wines, including Amarone and Ripasso, alongside olive oil. Both wineries manage the post-harvest withering process at their own premises, in well-ventilated *"fruttaio"* rooms, hereby described.

### 3.1. Withering room environmental conditions

To maintain their health, the grape bunches were placed on racks inside a traditional withering room to undergo dehydration under natural conditions. The airflow for the withering process was controlled by using fans for air circulation.

Grape was monitored during the withering process, and bunches were randomly sampled and analysed at three stages of water loss (WL): (i) 10 % water loss, (ii) 30 % WL, and (iii) 50 % WL, corresponding to 10, 45, and 90 days of drying, respectively. Grape bunches were then placed in sterile plastic bags and transferred to the laboratory for further analysis, as described in the section below. The indicators monitored included dehydration kinetics, determined by measuring weight loss, fungal infection levels, assessed by counting infected berries, and microbial count.

### 3.2. Experimental design, microorganisms employed, and analytical plan

The microorganisms tested included *Metschnikowia pulcherrima*, *Achromobacter xylosoxidans* (19VE21-3), and *Mycolicibacterium arabiense* (ACT34), along with a microbial consortium made up of 50 % ACT34 and 50 % 19VE21-3 and a negative control consisting only of water. A dose of 2.5 mL of cell suspension per 100 g of grape berries was applied by spraying at the beginning of the withering process. Microorganism suspensions were applied using a manual trigger sprayer (HDPE bottle 1 L capacity) equipped with an adjustable conical nozzle. The spray was applied at a distance of 50 cm from the grape surface, ensuring complete coverage by moving the nozzle in a uniform pattern for 10 seconds per tray. Each microbial suspension was prepared according to the specific culture conditions described in "1. Culture conditions".

A total of three batches (trays containing approximately 10 kg of fresh grapes) were treated for each microorganism and randomly placed in a pallet containing all treatments, which allowed a biological triplicate.

A total of 10 different treatments were tested, as shown in Table 2, among them five treatments containing microorganisms along with pinolene, an adhesive agent.

**TABLE 2.** Treatments applied to *Vitis vinifera* cv. Victoria grape berries, including microbial applications at time 0 (T0) and evaluations at multiple time points (T15, T45, T90) for weight loss, infection rate and microbial persistence.

| Treatment number | ID sample | T0 treatment | T15 | T45 | T90 |
|---|---|---|---|---|---|
| 1 | Negative control | x | Weight loss (%)<br>Count of infected berries<br>Microbial count<br>Grape chemical analyses | | |
| 2 | ACT34 | *Mycolicibacterium arabiense* ACT34 | | | |
| 3 | 19VE21-3 | *Achromobacter xylosoxidans* 19VE21-3 | | | |
| 4 | 50:50 | 50 % 19VE21-3<br>50 % ACT34 | | | |
| 5 | Gaia | *Metschnikowia pulcherrima* GAÏA™ | | | |
| 6 | Negative control + pinolene | Pinolene | | | |
| 7 | ACT34 + pinolene | *Mycolicibacterium arabiense* ACT34 + pinolene | | | |
| 8 | 19VE21-3 + pinolene | *Achromobacter xylosoxidans* 19VE21-3 + pinolene | | | |
| 9 | 50:50 + pinolene | 50 % 19VE21-3<br>50 % ACT34 + pinolene | | | |
| 10 | Gaia + pinolene | *Metschnikowia pulcherrima* GAÏA™ + pinolene | | | |

### 3.3. Kinetics of withering

The kinetics of the grape withering process were evaluated by monitoring post-harvest weight loss. The results are expressed as the percentage (%) of weight loss during the withering process. Specifically, data were collected by weighing three trays for each treatment, each containing approximately 10 kg of grapes. The trays were selected from representative positions within the drying chambers to ensure a comprehensive assessment. The trays were positioned evenly relative to the fans to facilitate uniform drying conditions. To standardise the drying process, the trays were randomly repositioned during the experiment.

The climatic conditions in the environment were measured. Specifically, temperature and humidity were measured using air sensors (Lascar Electronics EL–USB–2, Bergamo (BG), Italy) located in the environment of the study: two sensors per drying chamber, in contact with the withering grapes. The daily values recorded were the average of the measurements collected every 15 minutes. The outdoor climatic conditions were measured by the weather station belonging to ARPAV-Regional Agency for Environmental Monitoring (San Pietro in Cariano, s.d.) located in the San Pietro in Cariano area.

## 4. Microbial analysis and enumeration methods

For microbial enumeration, approximately 50 g of grapes were collected and placed in sterile bags. These berries were then placed in a sterile flask containing 100 mL of sterile physiological saline solution (9 g/L NaCl) to aid in the detachment of surface-associated microorganisms. The washing step was conducted at 20 °C for 2 hours under gentle agitation (100 rpm).

Next, 10 mL of the resulting solution was transferred to a sterile tube for serial decimal dilutions on specific, semi-selective or selective media.

For yeast enumeration, WL nutrient agar (Wallerstein Laboratory, Sigma), supplemented with 10 mg/L of chloramphenicol (Sigma-C0378), was used. For *Metschnikowia pulcherrima* enumeration, only yeast colonies compatible with morphology were enumerated throughout time, including at T0.

Bacterial growth was assessed using a MYA medium, supplemented with 10 mL/L of a 0.1 % cycloheximide (Sigma-1810) solution. Total microbial counts were recorded and compared between control and inoculated samples throughout time, including at T0.

To evaluate the presence of *Botrytis cinerea* and assess the bioprotective capacity of the tested microorganisms, BSM (Botrytis Selective Medium) was employed (Edwards & Seddon, 2001). BSM plates were incubated at 25 °C, while all other plates were incubated at 30 °C for optimal growth.

Colony and cell morphology were visually differentiated on MYA and WL media for bacteria and yeasts, respectively, and then counted (Tosi *et al.*, 2012). For BSM, morphologically similar saprophytes were not included in the enumeration because *B. cinerea* colonies were easily distinguishable by a dark brown halo clearly visible against the pink background of the medium, as described by Edwards and Seddon (2001).

## 5. Grape chemical analyses

Grape berries were analysed for soluble sugars (glucose and fructose) and for the quantification of organic acids (gluconic acid, glycerol, and acetic acid) expressed in g/L, using commercial enzymatic kits (Steroglass, Perugia, Italy).

For each sample, three subsamples of 250 g of berries were pressed, and the resulting product was filtered through Whatman filter paper. From the filtered samples, 250 µL were diluted 1:50 with distilled water for enzymatic assays. Enzymatic analyses were performed using the Hyperlab wine analyzer (Steroglass, Perugia, Italy) with dedicated kits thereof (SQPE078691 for glycerol – UV; SQPE076314 for gluconic acid; SQPE068205 for acetic acid; SQPE068207 for glucose-fructose). The pH was measured using a Hach Titralab AT1222.98 equipped with a PHC735 Hach pH electrode (Hach Lange GmbH, Willstätterstraße, Düsseldorf, Germany).

### 6. Assessment of biocontrol activity against *Botrytis cinerea* on grapes: disease incidence and McKinney index

For each experimental trial, the presence of grey mould caused by *Botrytis cinerea* on grape clusters was visually assessed in table grapes at the lab scale and in the withering room.

For lab-scale trials, all berries of the grapes used were analysed. For withering room trials, for each 10 kg batch, three bunches were randomly selected and transported to the laboratory. From these bunches, 60 berries were observed for severity and McKinney index calculation, while the remaining berries were used for microbial counts and then discarded. This sampling procedure was repeated at each time point.

Disease incidence was calculated using the following formula (1):

$$\text{Disease incidence (\%)} = (\text{Number of decayed grapes}/\text{Total number of grapes}) \times 100$$

In the case of the field experiment, grape berries were monitored at each checkpoint, as described earlier ("3.2. Experimental design, microorganisms employed, and analytical plan"), to assess the damage caused by *B. cinerea* infection. Disease severity was recorded using a modified 0-to-4 scale based on the criteria proposed by Carlucci *et al.* (2024). The severity scale was as follows:

- 0 = no symptomatic berries observed
- 1 = 1 %–25 % of berries affected
- 2 = 26 %–50 % of berries affected
- 3 = 51 %–75 % of berries affected
- 4 = 76 %–100 % of berries affected.

The infection index, or McKinney index, integrates both disease incidence and severity. It represents the weighted mean of the disease as a percentage of the maximum possible level. The McKinney index was calculated using the following equation (1):

$$I = d \times f/N \times D \times 100 \qquad \text{Eq. (1)}$$

where:

d = the severity category of rot intensity on the grapes

f = the frequency of that severity category

N = the total number of grapes examined (both healthy and infected)

D = the highest disease severity category recorded.

This method allows for a comprehensive evaluation of both the spread and intensity of the disease across the grape samples (Agarbati *et al.*, 2022).

### 7. Statistical analysis

Biocontrol assays were analysed at two experimental scales. For laboratory-scale trials, data were evaluated using one-way ANOVA followed by Tukey's multiple comparison test (GraphPad Prism software), based on three independent biological replicates per treatment. For withering trials, incidence data were analysed using two-way ANOVA, followed by Dunnett's post-hoc test to compare each treatment against the control at each time point. Prior to ANOVA, data were tested for normality and homogeneity of variances. Statistical significance was set at $p \leq 0.05$, and exact *p*-values are reported in the supplementary material (Tables S11 and S12). All analyses were performed on at least three biological replicates per treatment and repeated across two consecutive vintages.

## RESULTS AND DISCUSSION

### 1. Antagonistic potential of selected microorganisms against *Botrytis cinerea* on grape: A lab-scale analysis

To assess the effectiveness of putative biocontrol microorganisms and their ability to colonise the grape surface, laboratory conditions experiments were conducted on grape bunches. Selected microorganism strains were tested for their ability to inhibit *Botrytis cinerea* on grapes, based on disease incidence assessed 10 days after inoculation with a spore suspension ($10^5$ spores/mL). Incidence was expressed as the percentage of infected berries over the total number in treated bunches. Results are shown in Figure 1.

In all bunches not inoculated with the pathogen *B. cinerea* ("NI"), infection development was observed, albeit limited, with incidences lower than 20–30 %, and no significant impact was observed by any of the putative biocontrol microorganisms applied (Figures 1A and 1B). These results highlight how, in the context of a low-rate infection, already present before inoculation, the effect of a potential BCA (either endophytic bacteria or commercial yeasts) was not detectable, as indicated by the lack of significant differences in disease incidence with the CTR – NI.

Concerning samples inoculated with the pathogen *Botrytis* ("I"), *Achromobacter xylosoxidans,* among the endophytic microorganisms, showed a marked reduction in infection incidence, significantly decreased compared to the control inoculated only with *B. cinerea* (Figure 1A). This suggests a strong antagonistic capacity, likely mediated by competition for resources or the production of antimicrobial compounds, as previously proposed for related strains (Hayat *et al.*, 2010). In contrast, *Mycolicibacterium arabiense* exhibited no biocontrol activity under the tested conditions, with incidence values comparable to those of the control (Figure 1A). Compant *et al.* (2005) previously pointed out that certain bacterial strains may fail to express beneficial traits in suboptimal conditions, despite showing potential in other systems.

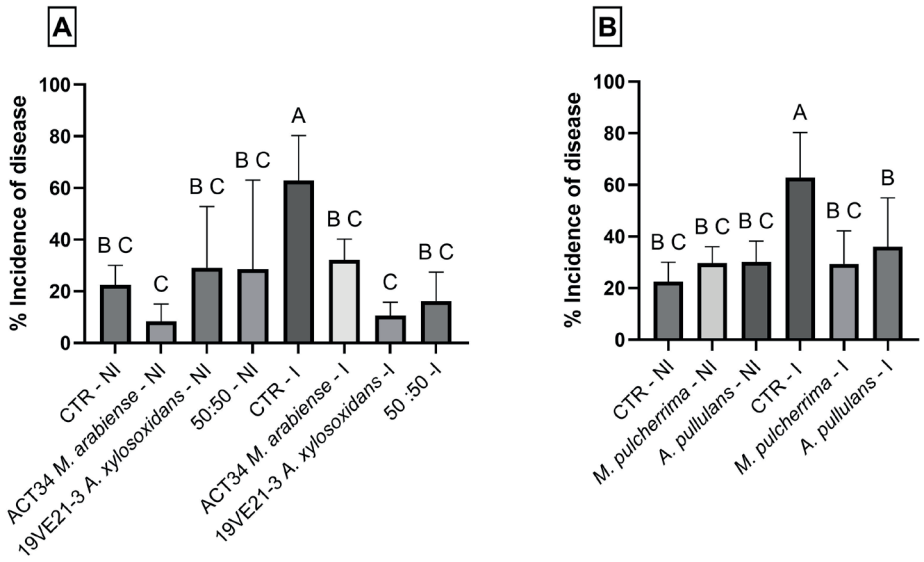



**FIGURE 1.** Incidence of *Botrytis cinerea* disease on grapes in lab-scale experiments.

Incidence of *Botrytis cinerea* disease: (A) treatments with endophytic bacteria (grape batch A), (B) treatments with yeast and yeast-like fungi (grape batch B). Vertical bars represent standard deviation. Different letters indicate statistically significant groups ($p < 0.05$). NI = non-inoculated; I = inoculated. Exact *p*-values for statistical tests are provided in the supplementary materials (Table S11).

Notably, the microbial consortium ACT34 + 19VE21-3 also showed a strong inhibitory effect, indicating that the presence of *A. xylosoxidans*, even at lower concentrations, was likely the primary driver of the observed antagonism. This aligns with findings by Niu *et al.* (2020), who showed that, in multi-strain microbial consortia, overall biocontrol efficacy is often dictated by the most active component.

In the context of *Botrytis* inoculation, both the commercial yeast-based products demonstrated a significant reduction in *B. cinerea* infection, supporting their effectiveness as biocontrol agents and the suitability of the selected protocol for visualising the effectiveness of biocontrol agents (Figure 1B).

## 2. Antagonistic potential of selected microorganisms against *Botrytis cinerea* on grape in industrial conditions

Following laboratory trials, experiments were carried out in real-scale withering rooms at two wineries in the Valpolicella region over a 90-day period to confirm the laboratory findings on an industrial scale.

As described in Table 2 (Methods, par. "3.2. Experimental design, microorganisms employed, and analytical plan"), batches of grapes were treated with: *Mycolicibacterium arabiense* ACT34, *Achromobacter xylosoxidans* 19VE21-3, a consortium 50 %:50 % of ACT34 + 19VE21-3 and a positive control *Metschnikowia pulcherrima* GAÏA™. The experiment was performed in triplicate (biological replicates consisting of three different trays (batches) for each microorganism) and was entirely repeated in the presence and absence of pinolene, chosen as a potential adhesivant for improving microorganisms' persistence and performance.

### 2.1. Kinetics of withering and quantification of grape sugars

The progress of grape withering was monitored by measuring the percentage weight loss of Corvina grape bunches relative to their initial weight. In Winery A, approximately 50 % weight loss was reached, while in Winery B, it was around 40 % after 90 days, consistent with the natural dehydration dynamics reported by Tomasi *et al.* (2021) (Figures 2A and 2B).

Temperature and relative humidity (RH) were also measured throughout withering to correlate them with weight loss and overall withering room conditions, as previously described (Tomasi *et al.*, 2021). In Winery B, grape dehydration progressed more rapidly during the first month due to higher temperatures (Figures 2B and 3A), while in Winery A, a slight acceleration in weight loss was observed in late December as temperatures rose again (Figures 2A and 3A). Relative humidity (RH) values ranged between 60 % and 90 % (Figure 3B), significantly influencing the dehydration dynamics. As previously highlighted by Lorenzini *et al.* (2013), a higher dehydration rate can be advantageous, as it allows the vinification process to begin earlier while minimising the risk of grape contamination during the drying phase.

Despite these environmental fluctuations (Figures 3A and 3B), no significant differences were observed in the dehydration curves among the various treatments with different microorganisms within each winery. Moreover, no significant differences were observed in terms of weight loss between samples treated and untreated with pinolene in either winery, suggesting that under the tested conditions, pinolene did not influence the dehydration rate of grape bunches. These findings partially contrast with those of Fahey and Rogiers (2019), who reported that the application of di-1-*p*-menthene reduced water loss by limiting bunch transpiration, although this was observed in different conditions: during the ripening season and for short periods (up to 36 hours).

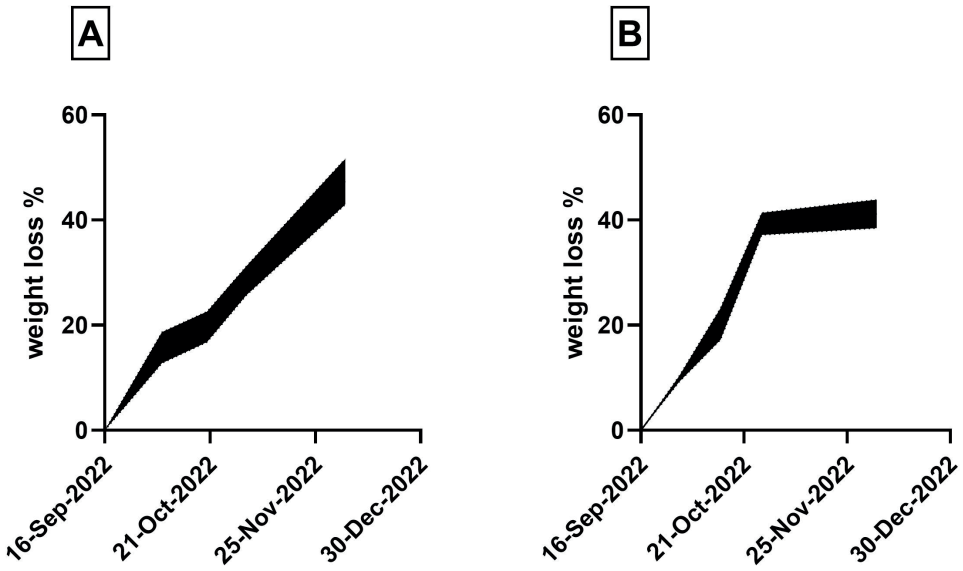



**FIGURE 2.** Withering-grape weight loss, expressed as % compared to fresh grapes.

Kinetics of withering: (A) Winery A, (B) Winery B. All thesis treatments were grouped within each winery.

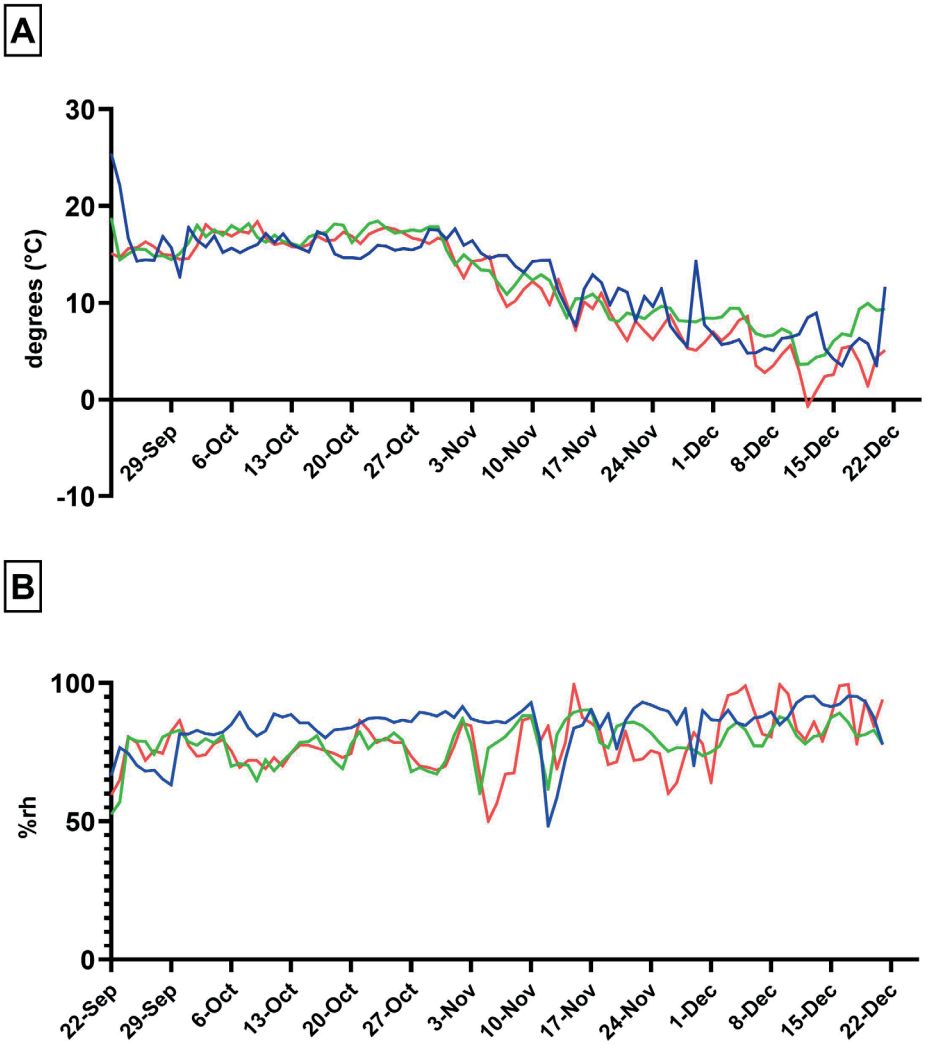



**FIGURE 3.** Temperature and relative humidity measured in the grape trays throughout the withering period.

Temperature trends in Winery A and Winery B compared to the average daily temperature (A). Relative humidity trends in Winery A and Winery B compared to average daily humidity (B). Winery A (green line), Winery B (blue line), and average daily (red line) recorded by the meteorological station of San Pietro in Cariano.

Along with the withering weight loss, key oenological parameters of the bunches were analysed and are provided in the supplementary material (Tables S1–S8). In agreement with the dehydration of the berries during the 90-day period, sugar concentration increased. However, no significant differences were observed between different samples, including pinolene-treated and untreated ones, suggesting that pinolene had no effect on sugar accumulation under post-harvest conditions. On this topic, other studies have shown that pinolene can reduce sugar accumulation, but again, when applied during the ripening phase in the vineyard (Brillante *et al.*, 2016; Gatti *et al.*, 2016). Conversely, acetic acid levels remained stable throughout the entire trial period, showing no difference between treatments (0.05 and 0.2 mg/L). Gluconic acid levels increased during the 90-day period, particularly in the control not inoculated with microorganisms, where levels rose from 0.2 to 1.9 g/L; although the trend was overall observed, significant differences were found only in Winery A (in the presence of pinolene), as reported in supplementary data (Figure S1). This trend is consistent with Ribéreau-Gayon *et al.* (2006), who identified gluconic acid accumulation as a typical consequence of *Botrytis cinerea* infection in grape tissues. The accumulation in the untreated control suggests a higher degree of fungal colonisation, likely due to the absence of antagonistic microbial competition or inhibition. Glycerol concentrations also increased in all samples, from approximately 0.09 to 1.0 g/L, with no significant differences between treatments. This is consistent with existing literature, as glycerol is known to accumulate both due to berry dehydration-induced metabolic stress and microbial activity (Modesti *et al.*, 2024). Together with gluconic acid, the increase of glycerol is another important indicator of the development of *B cinerea* in grapes.

## 2.2. Persistence and performance of microorganisms tested on grape at an industrial scale

The persistence of the tested strains, described in Table 2, was monitored in the withering room through plate counts on specific media. Yeast and bacterial populations were followed as described in methods: the experiment was performed in biological triplicate, and each replicate was plated in three technical repetitions (Petri dishes per dilution), which allows for the assumption that differences between inoculated and non-inoculated (control) samples are due to the presence of the inoculated microorganism (Agarbati *et al.*, 2022). Throughout the withering period, *Metschnikowia pulcherrima* samples maintained higher yeast concentrations, if compared to the performances of the bacteria *Mycolicibacterium arabiense* ACT34 and *Achromobacter xylosoxidans* 19VE21-3, as shown in Figure 4. This trend was consistent across both wineries and years (Figure 4); the yeast population in the *M. pulcherrima*-treated batch was higher than that in the control, and viable yeast counts remained stable throughout the trial, indicating that the withering environment is a suitable environment for its colonisation and persistence.

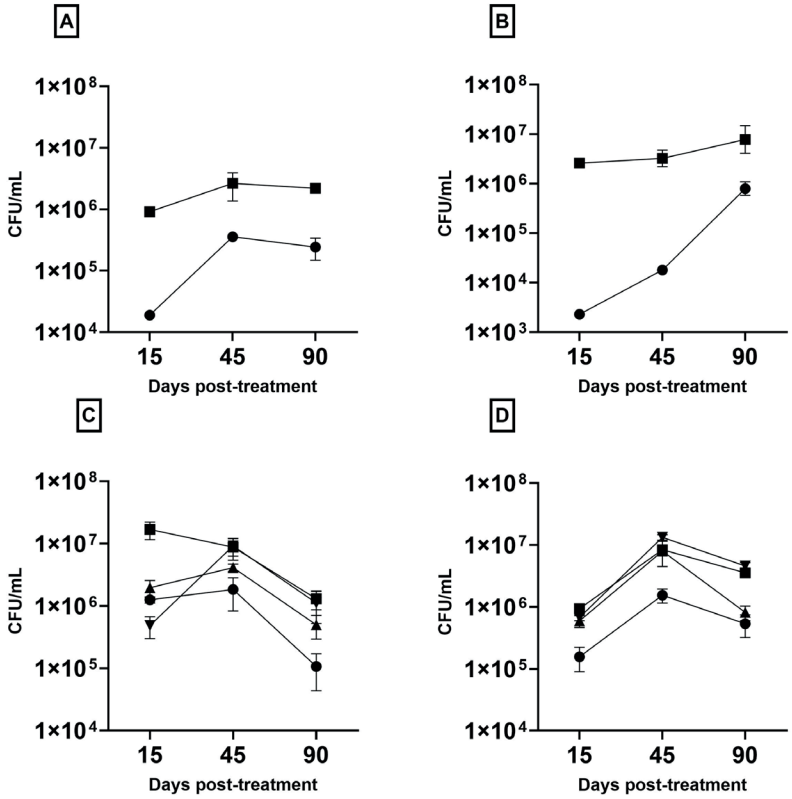



**FIGURE 4.** Yeast populations throughout withering in different grape batches.

Yeast counts (CFU) on WL medium of samples from: (A) 2021 vintage, Winery A, (B) 2021 vintage, Winery B, (C) 2022 vintage, Winery A, (D) 2022 vintage, Winery B. Rounds: control (untreated); squares: grapes inoculated with *M. pulcherrima*; triangles: pinolene control (water and pinolene); reverse triangles: grapes treated with pinolene and inoculated with *M. pulcherrima*.

This aligns with previous studies highlighting the robust performance and faster growth of *M. pulcherrima* in winemaking environments, particularly under stress conditions (Puyo *et al.*, 2023; Tatay-Núñez *et al.*, 2024).

In contrast, the batches treated with the two bacterial strains exhibited a decline in cell density from inoculation to 90 days post-treatment (Figure 5). After 90 days, the concentrations of treated samples, in terms of CFU/mL, were comparable to the untreated controls and, in some cases, even one magnitude order lower, as observed with *M. arabiense* ACT34 (Figures 5A, 5D and 5F). Specifically, *M. arabiense* ACT34 showed a consistent trend over both years, with a decrease in persistence after 90 days compared to the initial inoculum, even with the addition of pinolene (Figures 5D and 5F). The combination of the two tested strains, *M. arabiense* ACT34 and *A. xylosoxidans* 19VE21-3, showed better performance in terms of bacterial persistence over time, supported by data from a single year (Figure 5D). These findings are consistent with the literature suggesting that microbial consortia, when compatible, can enhance the shelf life and stability of individual strains by improving nutrient availability, stress tolerance, and resilience to environmental fluctuations such as temperature and humidity (Negi *et al.*, 2024; Santoyo *et al.*, 2021).

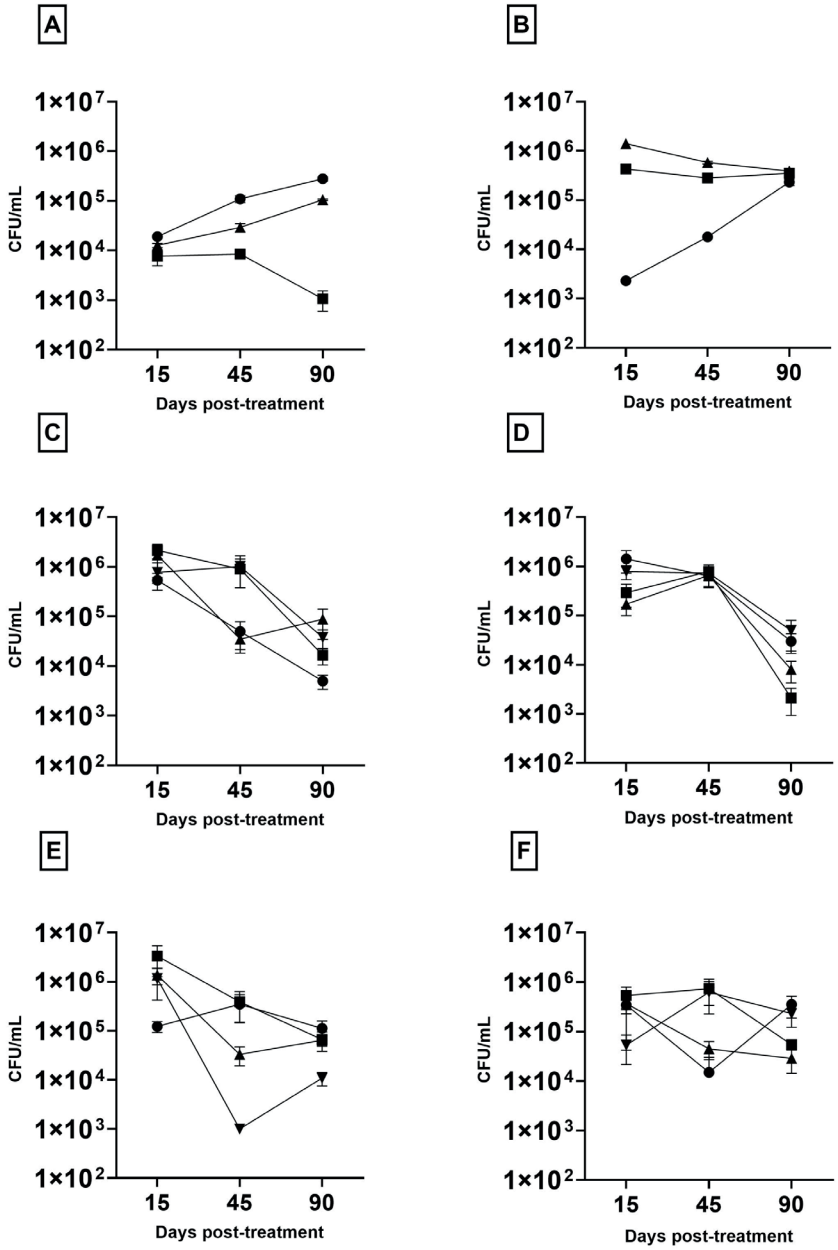



**FIGURE 5.** Bacterial populations throughout withering in different grape batches.

Bacterial counts (CFU) on MYA medium of samples from: (A) 2021 vintage, Winery A, (B) 2021 vintage, Winery B, (C and D) 2022 vintage, Winery A, (E and F) 2022 vintage, Winery B, (D and F) grapes treated with pinolene. Rounds: control (untreated); squares: grapes inoculated with *Mycolicibacterium arabiense* ACT34; triangles: grapes inoculated with *Achromobacter xylosoxidans* 19VE21-3; reverse triangles: grapes inoculated with microbial consortia of the two endophytic bacteria.

At 15 days post-inoculation, differences in growth between treated and untreated samples were observed, suggesting that the inoculum was viable and initially proliferated. However, this growth difference diminished over the subsequent 90 days. This indicates that during the withering period, *M. arabiense* and *A. xylosoxidans* populations were probably influenced by environmental conditions, remaining stable at 15–20 °C but declining below 10 °C. This is consistent with the mesophilic nature of both tested bacterial strains, which grow between 10 °C and 37 °C, with an optimum around 31 °C, and are unable to grow below 10 °C. According to Nedwell (1999), low temperatures reduce microbial substrate affinity, thereby limiting metabolic activity and growth. Consequently, a 10 °C drop in ambient temperature can significantly impair the persistence of inoculated strains under suboptimal environmental conditions.

*M. pulcherrima* instead seems to exhibit greater resistance to lower temperatures, which showed a stable population with the progression of low temperatures below 10 °C. This behaviour is consistent with recent findings demonstrating the species' ability to remain metabolically active and exert antagonistic effects at suboptimal temperatures (Larini *et al.*, 2025; Yuan *et al.*, 2024).

It is worth remarking, nevertheless, that bacterial counts are lower than yeast ones, even in the control samples, which is consistent with previous observations on grape berries that usually show lower bacterial than fungal populations (Barata *et al.*, 2012).

The addition of pinolene did not show to improvement in microorganism adhesion to the grape surface, as no growth differences were observed between microorganisms with and without pinolene, whether for bacteria or yeast (Figures 4C, 4D and 5C–5F). This is consistent with some other studies, which have shown that pinolene may reduce photosynthetic activity and sugar accumulation, without improving microbial colonisation (Brillante *et al.*, 2016).

### 2.3. Effect of potential BCAs on grape damage caused by *Botrytis cinerea*: impact on disease incidence and severity

To assess the effectiveness of the potential BCAs, disease incidence and culture-based quantification of *Botrytis cinerea* were evaluated during withering trials.

After treatment in the withering room, the damage caused by naturally occurring *B. cinerea* on grape berries and the effectiveness of the selected microorganisms were assessed based on disease incidence and the McKinney index, as previously described (Galli *et al.*, 2021; Marsico *et al.*, 2021).

During the first, explorative trials in 2021, *M. arabiense* ACT34 and *A. xylosoxidans* 19VE21-3 effectively reduced grey mould during storage, exhibiting lower incidence rates compared to the untreated controls across both vineyards and years (Figure 6).

In one instance, they also demonstrated greater efficacy than the positive control *M. pulcherrima* (Figure 6A). Specifically, in 2021 in Winery A, *M. arabiense* ACT34 and *A. xylosoxidans* 19VE21-3 reduced disease incidence by 80 % and 74 %, respectively, relative to the untreated control, outperforming *M. pulcherrima*, which achieved a 40 % reduction. In only one case, as shown in Figure 6B, the positive control *M. pulcherrima* did not reduce the incidence of infection. This outcome may be attributed to tray positioning, which potentially favoured higher levels of spontaneous infection in this specific experimental setup.

As for 2022 trials, the incidence assessment protocol was improved to get statistically significant data (in particular, incidence was calculated separately on each plot instead of on a pooled sample from the three plots constituting biological replicates). A notably lower baseline incidence of *B. cinerea* was recorded in the untreated negative control of Winery A (24 %; Figure 6C) compared to Winery B, where incidence reached approximately 50 % (Figure 6D). Nevertheless, a consistent trend was observed across both sites: all tested microorganisms, including the positive control *M. pulcherrima*, contributed to a reduction in disease incidence. In Winery A, the average reduction achieved by the potential BCAs and *M. pulcherrima* was approximately 40 %. In contrast, in Winery B, a significantly greater reduction in disease incidence was observed. Specifically, the consortium of *M. arabiense* ACT34 and *A. xylosoxidans* 19VE21-3 reduced incidence by 67 % during the 2022 vintage (Figure 6D). This combined application proved to be more effective than the individual strains, each of which achieved a 46 % reduction.

Similar trends were observed for disease severity, as measured by the McKinney index (Figure 7).

In particular, in Winery B in 2022 (Figure 7D), the consortium (*M. arabiense* ACT34 and *A. xylosoxidans* 19VE21-3) achieved a McKinney index of 6.19 %, further confirming its potential in reducing grey mould severity. To our knowledge, this is the first work demonstrating the efficacy of *M. arabiense* and *A. xylosoxidans* as biocontrol agents against *B. cinerea* on grape berries during withering. Recent studies have revealed how a strain of *A. xylosoxidans* has potential as a plant-associated bacterium with beneficial traits (Ansari *et al.*, 2025). In contrast, *M. arabiense* is a rarely described environmental bacterium with no prior reports of application in agriculture. Its strong performance in reducing grey mould incidence and severity, alone and in combination with *A. xylosoxidans*, suggests a synergistic interaction that merits further investigation.

### 2.4. Quantification of *Botrytis cinerea* on selective medium

Finally, evidence of infection by *Botrytis cinerea* was further confirmed through mould counts performed on a selective medium specific to *B. cinerea*. In almost all cases, the colony-forming units (CFUs) were higher for the untreated negative control compared to the batches treated with potential BCAs (Figure 8). In the 2022 vintage from Winery A, *B. cinerea* exhibited very low levels of viable cells, with the untreated negative control showing concentrations of $10^2$ CFU/mL or lower, which was significantly lower than the concentrations observed in other trials (around $10^4$ CFU/mL, 90 days after

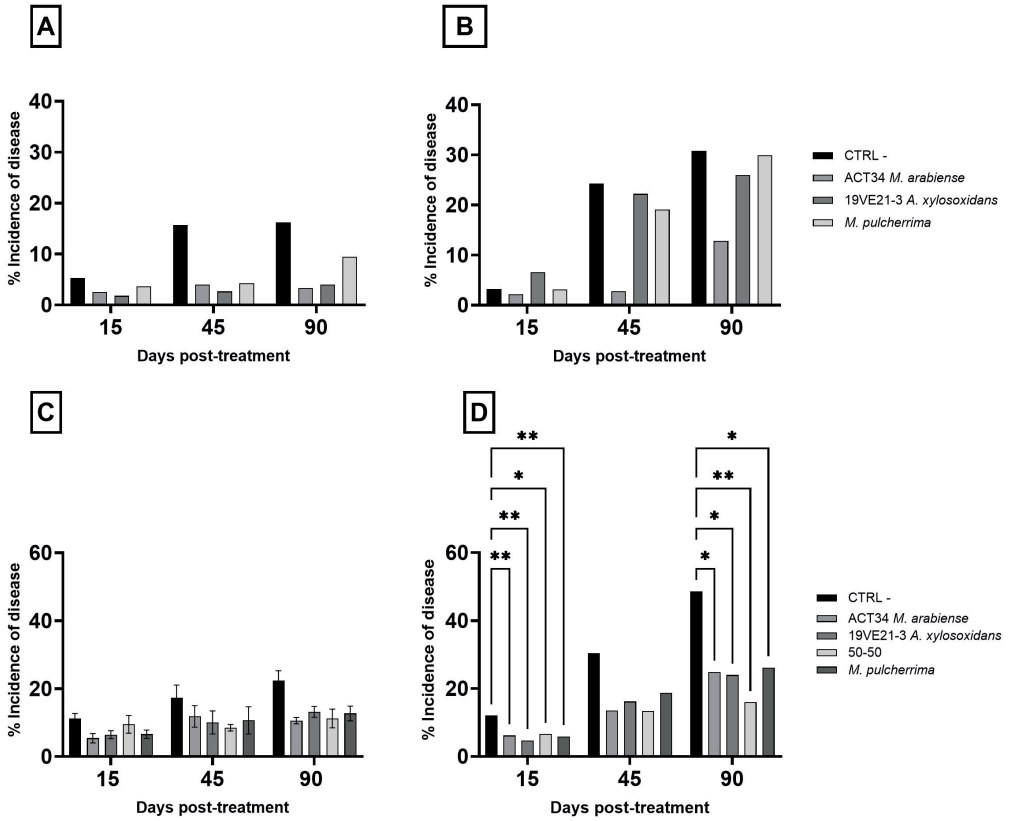



**FIGURE 6.** Incidence of *Botrytis cinerea* disease on grapes throughout the withering period.

Percentage of infected grapes from two wineries across two vintages. (A) 2021 vintage, Winery A, (B) 2021 vintage, Winery B, (C) 2022 vintage, Winery A, (D) 2022 vintage, Winery B. Vertical bars represent standard deviation. *, ** indicate statistically significant differences ($p \leq 0.01$ and $p \leq 0.001$, respectively) between grape inoculated with microorganisms and untreated controls (CTRL-). Exact *p*-values for statistical tests are provided in the supplementary materials (Table S12).

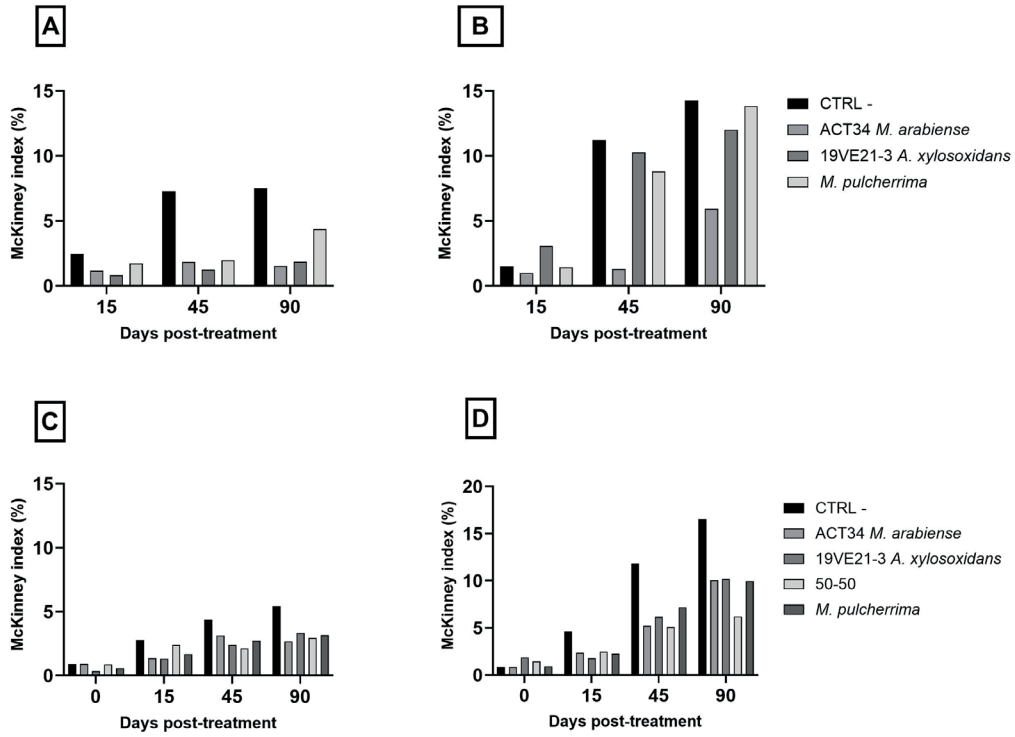



**FIGURE 7.** Severity of *Botrytis cinerea* disease on grapes throughout the withering period.

McKinney index (%) from two wineries across two vintages: (A) 2021 vintage, Winery A, (B) 2021 vintage, Winery B, (C) 2022 vintage, Winery A, (D) 2022 vintage, Winery B.

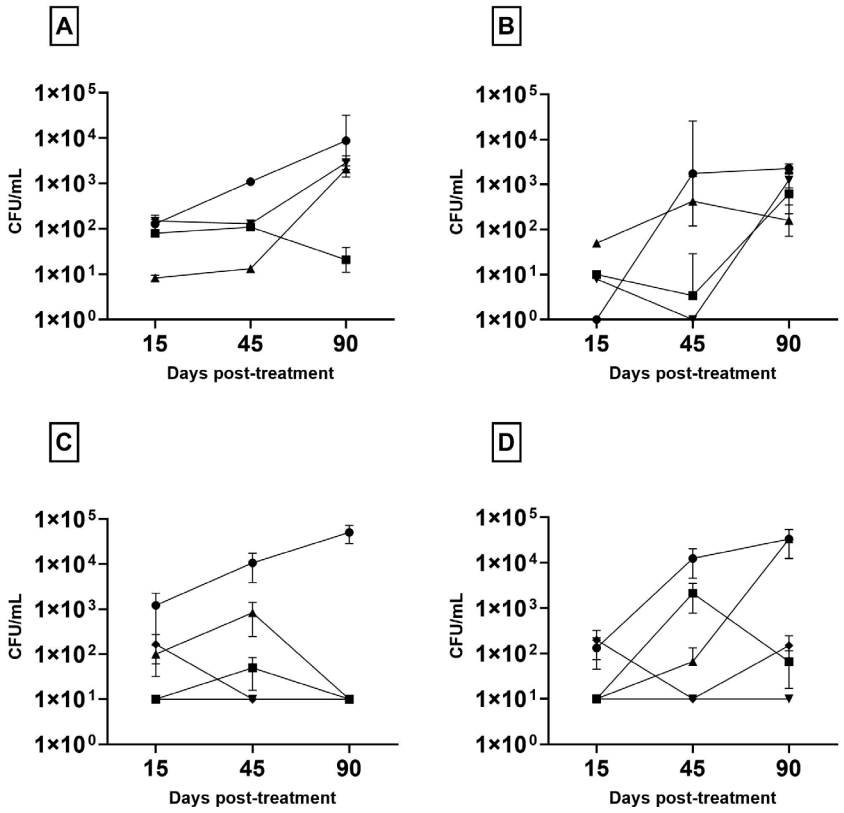



**FIGURE 8.** Fungal populations throughout withering in different grape batches.

*Botrytis cinerea* counts (CFU) on BSM selective medium of samples from: (A) 2021 vintage, Winery A, (B) 2021 vintage, Winery B, (C) 2022 vintage, Winery B, and treated with pinolene, (D) 2022 vintage, Winery B, not treated with pinolene. Rounds: control (untreated); squares: grapes inoculated with *Mycolicibacterium arabiense* ACT34; triangles: grapes inoculated with *Achromobacter xylosoxidans* 19VE21-3; reverse triangles: grapes inoculated with *M. pulcherrima*; diamonds: grapes inoculated with microbial consortia of the two endophytic bacteria.

treatment, as shown in Figure 8). This is consistent with the quite low values of incidence and severity observed in 2022 for grey-mould disease in Winery A. As a consequence, results from Winery A in 2022 did not show any significant impact of potential BCAs, and are therefore only reported in supplementary materials (Tables S9 and S10).

Overall, the reference strain of *M. pulcherrima* demonstrated the most consistent and effective performance. Notably, this yeast successfully maintained low *Botrytis* counts for 45 days in 2021 and remained effective for up to 90 days in 2022 (in Winery B).

Regarding the bacterial strains, each exhibited a distinct pattern in reducing *B. cinerea* counts compared to the control. *M. arabiense* ACT34 slightly outperformed *A. xylosoxidans* 19VE21-3, especially at the 90-day mark. Most significantly, the bacterial consortium (where present, Figures 8C and 8D) surpassed the effectiveness of both individual strains. Again, results are consistent with data about the incidence and severity of the grey-mould disease, described in paragraph "2.3. Effect of potential BCAs on grape damage caused by *Botrytis cinerea*: impact on disease incidence and severity".

In the context of the 2022 industrial trial (Winery B), the addition of pinolene appeared to partially enhance the antagonistic activity of the selected microorganisms (in the case of grapevine endophytes) and further inhibit the mycelial growth of *B. cinerea*, as shown by comparisons of samples from the same vineyard and year, with and without pinolene (Figures 8C and 8D). This was observed even though the pinolene itself did not show antifungal activity, as visible by comparing the control batches from Figures 8C and 8D, nor any other significant impact on grape chemical properties (supplementary data, Tables S1–S8). In the absence of microorganisms and pinolene, mycelial levels were generally higher, except in treatments with *M. pulcherrima*, whose efficacy remained consistent regardless of pinolene application. Since the addition of pinolene did not improve microorganism persistence for any of the tested potential BCAs, as described in paragraph 2.2. ("Persistence and performance of microorganisms tested on grape at an industrial scale"), the mechanism behind such a finding remains to be confirmed and clarified, possibly dealing with the tested bacteria's effectiveness rather than with their viability. Indeed, the confounding factor of microorganisms' preparation before spraying (lab-scale for endophytes, industrial-scale for *M. pulcherrima*, as described in methods) may contribute to explaining why the latter did not benefit from pinolene presence in terms of effectiveness (Figures 8C and 8D).

## CONCLUSION

Although a substantial body of literature now exists on potential biocontrol agents (BCAs) in the vitivinicultural sector, few studies have explored their use during the post-harvest phase, which remains highly vulnerable to microbial pathogens and spoilage. Furthermore, many of these studies do not assess the bioprotective efficacy of new BCAs under real industry-scale conditions, nor do they include comparisons with commercially available microbial-based products as benchmarks for pathogen control.

In this context, the present study contributes to the broader investigation of alternative biocontrol strategies for post-harvest fruit protection, focusing on the efficacy of novel isolates derived from vinewood endophytes—namely *Achromobacter xylosoxidans* (19VE21-3) and *Mycolicibacterium arabiense* (ACT34)—as bioprotective agents against *Botrytis cinerea*. These isolates were compared with two well-established commercial biocontrol agents, selected strains of *Metschnikowia pulcherrima* and *Aureobasidium pullulans*, and tested in industrial withering rooms representing grape post-harvest drying over a 90-day period. Moreover, several parameters were followed to gain robustness in the results: estimation of the persistence of the inoculated microorganisms, evaluation of *Botrytis* injury on the grapes through specific validated indices, and further validation through plate enumeration of the fungus.

Overall, the outcomes of this research are twofold: first, the effectiveness of newly selected microorganisms as potential BCAs was evaluated, giving promising results in particular on their mixed application as a microbial consortium; second, the performance of reference commercial BCAs was further validated in new application contexts, thus widening their potential application as biobased solutions. It is worth noting that the current research evidenced all the aforementioned biocontrol effects are significant under conditions of high pathogen pressure (as those observed in Winery B), whereas they are less evident in low-disease scenarios (Winery A). To the best of our knowledge, this is the first study to explore the use of novel endophytic microorganisms for biocontrol during the critical post-harvest grape drying phase.

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