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Study of the efficacy of *Aureobasidium* strains belonging to three different species: *A. pullulans, A. subglaciale,* and *A. melanogenum* against *Botrytis cinerea* of tomato

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Abstract

The difference in antagonistic activity against the causal agent of grey mould (*Botrytis cinerea*) of tomato between *Aureobasidium* strains belonging to three different species, namely *A. pullulans, A. melanogenum,* and *A. subglaciale,* was evaluated by *in vitro* and *in vivo* assays. In the yeast-pathogen direct interaction experiment, all the strains significantly reduced *B. cinerea* growth, with *A. melanogenum* the least efficient species (17.8% of reduction) compared to *A. pullulans* and *subglaciale* (22% and 27.8%). The non-volatile metabolites produced by all three species reduced mycelial growth between 95–100%. These metabolites were characterized by FT-IR spectroscopy as polysaccharides, lytic enzymes, siderophores, and antibiotics. The inhibitory effect of *Aurebasidium* strains on pathogenic enzymes such as xylanase, polygalacturonase, and pectinase was measured showing *A. pullulans* strains as capable of strong inhibition of xylanase, an enzyme

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directly related to the virulence of necrotrophic pathogens such as *B. cinerea.* Our data demonstrate that the different species of *Aureobasidium* isolated from a range of non-conventional environments exerted variable efficacy against *B. cinerea,* with *A. pullulans* as the most active species followed by *A. subglaciale,* and *A. melanogenum* as ineffective and not suitable for biocontrol applications.

Keywords

Yeasts, Non Volatile Metabolites, Enzymes, Bio Control

1 | Introduction

Aureobasidum pullulans (De Bary) G. Arnaud is a former species complex of black yeast-like fungi, particularly known for their biotechnological significance as producers of an extracellular polysaccharide known as ‘pullulan’, a promising biomaterial for the packaging of food (Rekha & Chandra, 2007; Singh *et al.,* 2008). Importantly, in the last years, *A. pullulans* has been considered as one of the most efficient microbial species to control fungal diseases of fruits and vegetables mainly during the post-harvest phase, used as a biocontrol agent (BCA) alone or associated with sustainable physical methods (Zhang *et al.,* 2010; Di Francesco & Mari, 2014; Di Francesco *et al.,* 2018a, 2020).

The multiloci DNA analysis distinguished four varieties of the species *A. pullulans* – var. *pullulans*, *melanogenum*, *subglaciale,* and *namibiae* (Zalar *et al.,* 2008), and later on distinguished into four well-defined species (Gostinčar *et al.,* 2014).

Aureobasidium pullulans is most frequently found in the phyllosphere and carposphere of various fruits and vegetables and is associated with the endophyte population of many plant species (Bozoudi & Tsaltsas, 2018). Moreover, its ecology is highly diverse and cosmopolitan, as it can be found in environments ranging from hypersaline water of salterns, glacial ice, polluted water, frozen and salt-preserved food, household surfaces and house dust, synthetic polymers, and aviation fuel

tanks (Gostinčar *et al.,* 2019). The species *A. melanogenum* is mainly found in oligotrophic and aqueous environments. Importantly, it grows at 37°C, when other species of the genus cannot, and is considered as an emerging human pathogen (Chan *et al.,* 2011). Conversely, *A. subglaciale* shows a psychrotolerant nature (it grows at 4°C) and occurs mainly in glacial habitats. Finally, *A. namibiae* was named based on a single isolate from Namib Desert marble, able to tolerate up to 10% of NaCl and grows between 10°C and 30°C (Zalar *et al.,* 2008; Gostincar *et al.,* 2014).

In the most recent studies, *A. pullulans* was shown to have substantial biocontrol efficacy for the control of postharvest pathogens such as *Monilinia* spp. of stone fruits (Di Francesco *et al.,* 2020), *Botrytis cinerea* of grape, strawberry, and kiwifruit (Parafati *et al.,* 2015, 2017; Di Francesco *et al.,* 2018b) and *Penicillium expansum* of apple (Mari *et al.,* 2012); this efficacy can be attributed to different mechanisms of action such as the production of antifungal compounds (volatile and non-volatile) and cell wall degrading enzymes (Parafati *et al.,* 2015; Zhang *et al.,* 2010), the competition for nutrients and space (Janisiewicz *et al.,* 2000, Di Francesco *et al.,* 2018b; Klein & Kupper, 2018), induction of resistance (Madhupani & Adikaram, 2017; Di Francesco *et al.,* 2017), and mycoparasitism (Klein & Kupper, 2018). In fact, the application of BCAs, such as *A. pullulans*, is considered a safe and environmentally friendly alternative (Zhang *et al.,* 2010) to synthetic fungicides, considering their massive use in crop protection with possible harmful effects on human health, and the emergence of pathogen resistance to single-site mode of action fungicides (Eckert & Ogawa, 1988).

No studies have been conducted to explore the efficacy of utilizing *Aureobasidium* spp. for the control of postharvest fungal pathogens. The objective of this study was to evaluate the principal differences of efficacy between the strains belonging to the three species: *A. pullulans, A. subglaciale,* and *A. melanogenum* by testing their ability to inhibit *B. cinerea,* one of the most important pre-harvest and post-harvest fungal pathogen on fresh-market tomatoes (Mari *et al.,* 1996).

This research was conducted through i) the evaluation of the pathogen mycelial growth inhibition in co-culturing; ii) the activity of non-volatile metabolites; iii) the analysis of the non-volatile compounds by FT-IR spectroscopy; iv) the evaluation of the inhibitory effect of *Aureobasidium* species on the fungal pathogenic enzymes; v) and the reduction in grey mould incidence on tomato fruit.

2 | Materials and methods

2.1 | *Aureobasidium pullulans* strains

The selected *Aureobasidium* spp. strains (Table 1) were obtained from the Culture Collection Ex Infrastructural Centre Mycosmo, (Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia), and the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). Strains were maintained on nutrient yeast dextrose agar (NYDA: 8 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose, and 15 g of technical agar, Oxoid, Basingstoke - UK) in 1 L of distilled water) and stored at 4 °C until use. Two days before the experiments, each antagonist was grown on NYDA at 25°C, and yeast cells were collected in sterile distilled water (SDW) containing 0.05% (v/v) Tween 80 (Sigma-Aldrich, St. Louis, MO, USA) then adjusted to a final concentration of 10^8 cells mL^{-1}.

2.2 | Pathogen

Botrytis cinerea (CRIOF-DipSA collection), derived from a diseased tomato, was grown on oatmeal agar (60 g of oatmeal, 10 g of sodium nitrate, 30 g of sucrose, 12 g of agar per 1 L of SDW, Oxoid, Basingstoke - UK) and incubated at 25°C for 10 days. Pathogen conidia were collected and suspended in SDW containing 0.05% (v/v) Tween 80 and the suspension was adjusted to 10^5 conidia mL^{-1}, with a haemocytometer.

2.3 | Tomato

"Datterini" tomatoes (*Solanum lycopersicum*, L.) obtained from an organic orchard located in Cesena (Italy) were used. Tomatoes were harvested at commercial maturity and immediately used. Fruits were wounded with a sterile nail (3 mm × 3 mm × 3 mm) on the middle line (one wound per fruit) and inoculated with yeast strains cell suspensions and pathogen conidial suspension.

2.4 | *In vitro* assays

In the co-culturing assay, *B. cinerea* was separately co-cultured with each yeast strain on potato dextrose agar (PDA: 39 g per 1 L of SDW, Oxoid, Basingstoke - UK) plates. In each plate, a mycelial plug (6 mm in diameter) derived from a 6-day-old colony of *B. cinerea* was inoculated 30 mm from the plate edge and *A. pullulans* cells, from a 48 h-old colony grown on NYDA, were streaked by a sterile loop 30 mm from the fungal plug (Di Francesco *et al.,* 2017). In control plates, only *B. cinerea* plugs were inoculated. Plates were incubated at 25°C in the dark for 5 days, and the radius of the fungal colony was measured on a line from the centre of the plug to the yeast swipe.
For the No-VOCs (Non-Volatile Organic Compounds) assay, cells suspension (100 µL; 10^8 cells mL^{-1}) of each *Aureobasidium* spp. strain obtained from 48 h-old colonies grown on NYDA, was spread on a sterile cellophane layer (Safta, PC, Italy) previously positioned on NYDA plate. In control plates, 100 µL of SDW were used.
After 48-h of incubation at 25°C, cellophane was removed, and a mycelial plug (6 mm diameter) of *B. cinerea* was inoculated in the centre of the plate. The plates were incubated at 25°C and the colony diameter was measured after 5 days from the inoculation.
In all experiments, five plates (replicates) were used for each combination and the controls. The experiments were conducted twice.

2.5 | Antagonists metabolites influence on *Botrytis cinerea* pathogenic enzymes

To verify the effect of secondary metabolites produced by *Aureobasidium* spp. strains on the pathogenic enzymes activity of *B. cinerea,* tissue culture plates (Costar, Corning Inc., Corning, NY) and culture plate inserts Millicell-CM (Millipore Corp., Bedford, MA) were used as reported by Janisiewicz *et al.,* (2000). The system allows for the interchange of metabolites without physical contact between antagonists cells and pathogen conidia. Aliquots (120 µL) of fungal suspension (10^5 conidia mL^{-1}) derived from 6 day-old colony or water (control) were dispensed in the wells of culture plates, while the same aliquots of *Aureobasidium* spp. cells suspensions (10^8 cells mL^{-1}) were dispensed inside the cylinder inserts. The plates with the cylinders were placed at 25°C on a rotary shaker at 50 rpm; after 6 h of incubation, cylinders were removed from the wells and fungal conidia aliquots were used for the enzymes assays. For each *Aureobasidium* spp. strain three wells of culture plate were used. The experiment was performed twice.

2.6 | Enzyme assays

Xylanase, polygalacturonase, and pectinase assays were performed in Petri dishes containing specific agar medium previously described in Di Francesco et al. (2018c). For the xylanase assay, the medium contained 0.5% beechwood xylan (Sigma-Aldrich) in a minimal medium consisting of $NaNO_3$ 0.3%; KH_2PO_4 0.1%; $MgSO_4$ 0.05%; yeast extract 0.1%; agar 1.2% (St Leger *et al.,* 1997).

Polygalacturonase activity was determined through the Eriksson & Pettersson culture medium (Eriksson & Pettersson, 1975) enriched with the sodium salt of polygalacturonic acid (Sigma-Aldrich) from citrus fruit (0.5%) and agarized with low calcium Oxoid No. 1 agar (2%, Oxoid, Basingstoke, UK). The medium was sterilized following the procedure of Ayers *et al.* (1966), then the pH was adjusted to 5 and 8, respectively, by adding NaOH 1 M. Polymethylgalacturonase activity was determined through the above-cited culture medium (Eriksson & Pettersson, 1975) enriched with Sigma citrus pectin (0.5%) and agarized with calcium-rich Oxoid No. 3 agar (2%).

The growth media was sterilized following the procedure of Durrands & Cooper (1988) and the pH was adjusted to 8 by adding HCl 1 M.

Forty microlitres of each treated pathogen suspension (10^5 conidia mL^{-1}) were placed in holes (three equidistant holes per dish) punched in the medium with a 5 mm-sterile cork borer. Control plates were inoculated with the same volumes of conidial suspension exposed to distilled water. After incubation at 20°C for 48 h, enzyme activities were determined with specific colorimetric methods visualizing a clear zone of substrate degradation around the holes. For xylanase, the clearing halo was visualized by staining with Congo red, then destaining with NaCl and measured as described above. Instead, the halo produced by pectic hydrolase activities was visualized by 1% cetyl methyl ammonium bromide (CTAB) dissolved in distilled water, heated at 30°C. The diameter of enzymes halos (mm) was measured with a ruler. For each treatment, five plates (replicates) were considered. Each assay was repeated twice.

2.7 | Spectroscopic analysis of yeasts secondary metabolites

Flasks containing 100 mL of NYDB medium (NYDA without agar) were inoculated with 100 µL of antagonists cell suspensions (10^8 cells mL^{-1}) and then incubated at 25°C in a rotary shaker (250 rpm) for 1, 24 and 48 h. After each incubation time, the liquid cultures were centrifuged at 5000 × ***g*** for 20 min at room temperature and the supernatant collected in sterile tubes (2 mL), stored at -80°C and lyophilized. The control consisted of a liquid medium (NYDB) depleted of yeast cells. The supernatant was analyzed by FT-IR spectroscopy to obtain a rapid and non-destructive characterization of their main molecular components. Infrared spectra were recorded with a Bruker ALPHA series FT-IR spectrophotometer (Bruker, Ettlingen, Germany) equipped with an apparatus for attenuated total reflectance (ATR-Diamond crystal). The spectra were collected from 4000 to 400 cm^{-1} and averaged over 100 scans (resolution = 4 cm^{-1}): four spectra were measured for each sample for each sampling time.

2.8 | *In vivo* assay

Tomatoes were surface disinfected by 1 min immersion in sodium hypochlorite solution (1%), rinsed with tap water, air-dried at room temperature and inoculated through artificial wounds by a sterile nail (3 mm × 3 mm × 3 mm) on the equator (one wound) with 15 μL of each antagonist suspension (10^8 cells mL^{-1}) (15 strains). After 1 h, 15 μL of *B. cinerea* suspension (10^5 conidia mL^{-1}) were inoculated by placement in the treated tomato wound. The control consisted of fruit inoculated with SDW. Tomatoes were kept at 20°C and 90% of relative humidity (RH). The disease incidence was detected after 6 days from the inoculation. Sixty tomatoes for each antagonist and control were used. The experiment was conducted three times.

2.9 | Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA). Statistical comparison of means was carried out by using Fisher's LSD test ($P < 0.05$). Data were reported as mean values ± standard deviation (SD) of two and three independent experiments. All analyses were performed with the software Statgraphic Plus Version 2.1 (Statistical Graphics Corp., USA 1996).

3 | Results

3.1 | *In vitro* assays

The effect of the three-tested *Aureobasidium* species on *B. cinerea* mycelial growth in the co-culturing condition and to the exposure of non-volatile metabolites produced by the strains was determined.

All the strains significantly reduced the pathogen colony growth. *Aureobasidium melanogenum* showed a lower efficacy compared to *A. pullulans* and *A. subglaciale* (17.8%, 22% and 27.8% respectively on average). Within each species, the strains AP3 (*A. pullulans*), AM9 (*A.

melanogenum), and AS15 (*A. subglaciale*) resulted the most active against *B. cinerea* compared to the other tested strains (Fig. 1).

Concerning the pathogen colony growth, the non-volatile metabolites of all the tested strains significantly inhibited *B. cinerea* mycelial growth ranging from 95% to 100% (data not shown).

3.2 | Antagonists metabolites influence on *Botrytis cinerea* pathogenic enzymes

The effect of secondary metabolites produced by *A. pullulans, A. melanogenum, A. subglaciale* strains was evaluated on the activity of virulence key factors of *B. cinerea*. Three lytic enzymes: xylanase, pectinase, and polygalacturonase, were considered. Among these, xylanase activity produced by *B. cinerea* was the prevalent as shown by control samples (320 mm^2 halo area) compared to pectinase and polygalacturonase (180 and 90 mm^2, respectively) (Fig. 2). Almost all the strains inhibited these enzymes. Conversely, the metabolites produced by the strain *A. melanogenum* AM10 and almost all the strains of *A. subglaciale* seemed to stimulate the fungal xylanase activity. An increase of 51.9% and 12.6% (on average, AS11, AS12, AS14 strains enzyme activity) was recorded, respectively for *A. melanogenum* AM10 strain and *A. sublglaciale* strains. Conversely, *A. pullulans* showed the highest percentage of reduction (54.8%) (on average, AP1 and AP3 strains) of the above-mentioned enzyme.

Pectinase activity was mainly inhibited by *A. pullulans* strains on average by 47.6%, followed by *A. subglaciale* (40.1%), and *A. melanogenum* (30.5%). The most efficient strains were AP5 and AS15. Polygalacturonase enzyme resulted in the lowest detectable activity; however, *A. pullulans* AP3 and AP5, *A. subglaciale* AS13, and AS15 strains were confirmed as the most active to inhibit the target enzyme (by 42.7%, 42.7% and 47.9%, 46.8%, respectively).

3.3 | Yeasts secondary metabolites characterization

The FT-IR spectroscopic measurements were carried out to determine the main non-volatile compounds produced by three randomly chosen yeast strains (AP5, AS15, and AM6), that previously showed a high inhibition of *B. cinerea* mycelial growth in the No-VOCs assays.

The spectra differences between the cultures of the various strains at 48 h and control are reported in Fig. 3: positive peaks are related to yeasts secondary metabolites produced during the 48 h of incubation, while negative peaks should be attributed to the consumption of the culture media. The peaks attributed to the nutrient medium were derived from the FT-IR spectrum of pure NYDB (data not shown), while the attribution of carbohydrates, enzymes, siderophores and antifungal compounds was based on previously reported protocols (Aghatabay *et al.,* 2014; Bae *et al.,* 2015; Cozar *et al.,* 2006; Dahiya *et al.,* 2016; Higgins *et al.,* 2006; Holmen *et al.,* 1997; Shingel, 2002; Tu, 1982; Um *et al.,* 2013; Yang *et al.,* 2006; Zhbankov *et al.,* 2000). Positive spectroscopic peaks were attributed to different classes of compounds already known to be produced by *Aureobasidium* strains (Freimoser *et al.,* 2019): complex carbohydrates (Bozoudi & Tsaltas, 2018), as pullulan and other glucans involved in the biofilm formation used as biocontrol mechanism by the yeasts; lytic enzymes (Bozoudi & Tsaltas, 2018), as amylases, cellulases, lipases, xylanases, proteases, laccase, chitinases and mannanases; antifungal compounds (Bozoudi & Tsaltas, 2018), as aureobasidins (derivatives of cyclic depsipeptides possessing, as typical amino acids, proline, leucine and phenylalanine) and liamocins (polyols containing mannitol headgroup acetylated with polyesters tails, Freimoser *et al.,* 2019); siderophores used as iron depriving agents (Bozoudi & Tsaltas, 2018).

The three spectra differences showed in Fig. 4 present a quite similar pattern of negative and positive peaks (in particular AP5 and AS15), while AM6 showed some differences in several spectral regions (highlighted in blue): between 3000 and 2800 cm^{-1} (C-H stretching vibrations), 1750–1500 cm^{-1} (carbonyl derivatives, as amides, and aromatic compounds vibrations), 1450–1350 cm^{-1} (C-H bending and carboxylate groups vibrations), 1200–900 cm^{-1} (C-O and C-H vibrations); 850-650 cm^{-1} (chain deformation and aromatic rings bending vibrations) and between 550 and 500 cm^{-1} (chain deformation). More in detail, the intense positive peaks observed in AM6 at 1720, 1614,

1170, 1075, 1025, 792 and 724 cm^{-1} can be all attributed to melanin-related vibrations (Pierce & Rast, 1995): melanin is a heterogeneous class of natural pigments produced by *A. melanogenum.* The other positive peaks present in both AP5 and AS15, but absent in AM6 are: 1630, 1589, 1520, 1396, 1088, 934, 664 and 535 cm^{-1}: the attribution of most of them can be ambiguous, because of the chemical similarities among the secondary metabolites produced by the different *Aureobasidium* strains. Enzymes, aureobasidins, and siderophores are all characterized by the presence of the amide group (HN-C=O), that can be attributed to 1630 (amide I), 1520 (amide II) and 664 (amide IV) cm^{-1} bands. Moreover, other common chemical groups to these three classes of metabolites are carboxylate ions (COO^-) and aromatic rings to which the 1396 and 1589 cm^{-1} bands can be attributed, respectively. Only the 535 cm^{-1} band can be uniquely attributed to hydroxymate siderophores (Higgins *et al.,* 2006; Holmen *et al.,* 1997; Yang *et al.,* 2006) bound to ferric ions. Again, another source of ambiguity concerns carbohydrates-related vibrations (2854, 1088, 934 and 822 cm^{-1}): biofilm produced by *Aureobasidium* is composed of pullulan and a mixture of α and β-glucans; most of the lytic enzymes (i.e. polygalacturonase) are heavily glycated proteins, while liamocins contain the mannitol moiety.

To sum up the previous findings, it can be stated that both AP5 and AS15 strains showed an increased concentration of complex carbohydrates, enzymes, siderophores and antifungal compounds that are not present in AM6 spectrum or are even negative (i.e. 2854, 1396, 934 and 822 cm^{-1} bands).

3.4 | *In vivo* assay

Antagonists strains were applied as a preventative treatment against grey mould on tomato *cv* "Datterini". After 6 days of incubation at 20°C, grey mould disease incidence was evaluated. AP5 and AS15 reduced *B. cinerea* incidence by 80.9% and 55.5% respectively, showing the best efficacy respect to all the other strains (Fig. 4). All the remaining strains inhibited rot of less than

50%. Interestingly, the strain AM10 increased disease incidence compared to the control, suggesting a stimulatory effect of this strain on the fungal pathogen.

4 |Discussion

Aureobasidium pullulans, now separated as different species, is a ubiquitous oligotroph that can be found in various environments such as hypersaline waters in salterns (Cimerman *et al.,* 2000), on rocks and monuments (Urzi *et al.,* 1999), in the atmosphere as airborne spores (Punnapayak *et al.,* 2003), in food and feeds (Sasahara & Izumori, 2005), in deserts (Jiang *et al.,* 2017), in freshwater, and even in glaciers (Gostinčar *et al.,* 2014). This broad ecology can be attributed to its great adaptability to various environmental conditions, poly-extremo tolerance to cope with different stresses and to their nutritional versatility (Gostinčar *et al.,* 2011) being armoured with a plethora of extracellular enzymes (Gostinčar *et al.,* 2014). All these characteristics contribute importantly to the biocontrol potential of this microorganism.

In the present work, the antagonistic differences between the strains of three different *Aureobasidium* species, namely *A. pullulans, A. melanogenum,* and *A. subglaciale*, against the grey mould of tomato were evaluated for the first time. The selection of *Aureobasidium* strains for this study was based on previous experiments, where yeast strains from heterogeneous and extreme environments were tested as potential biocontrol agents (Zajc *et al.,* 2018). On the other hand, strains of *A. melanogenum* were used as a highly related outgroup of strains that could not be used in biocontrol due to their potential pathogenicity (Zajc *et al.,* 2019).

The phylogenetic diversity of these species could be linked also to their different antagonistic behaviour and efficacy to control plant pathogens. The present study confirmed *A. pullulans* strains together with *A. subglaciale* as being the most active to control *B. cinerea* in *in vivo* assay by reducing the mould incidence by 40.3% and 42.8% (on average) on tomatoes as well as in *in vitro*

co-culturing assay by showing inhibition of 22% and 27.8% (on average), respectively; also, almost a total inhibition by No-VOCs production of the pathogen mycelium growth was displayed.

Gostinčar *et al.,* (2014) demonstrated that, due to the abundant production of extracellular enzymes and the high number of sugar transporters, *A. pullulan*s has ecological preference associated to plants compared to the other species. In fact, previous studies of post-harvest diseases control were all conducted by using strains of *A. pullulans* isolated from plant or fruit (Di Francesco *et al.,* 2018; Banani *et al.,* 2014; Parafati *et al.,* 2015; Schena *et al.,* 2003; Castoria *et al.,* 2001; Ippolito *et al.,* 2000). This was recently confirmed in a study of characterisation of various strains of antagonistic *A. pullulans* from leaves and fruits from different parts of the world (Zajc *et al.,* 2020).

With regards to *A. subglaciale*, its extreme tolerance to various environmental factors could confer it a major ability to compete with fungal pathogens in storage conditions. On the other hand, *A. subglaciale* has a much more restricted distribution and showed a great ability to resist to gamma irradiation, UV light, and heavy metal ions (Liu *et al.,* 2017). According to its physiology, *A. subglaciale* displayed two important characteristics: it is a typical epiphyte and it belongs to the group of psychrophilic species (Kachalkin, 2010). Nevertheless, some strains belonging to the two above-mentioned species can present different efficacy, probably because the efficacy may vary based on fungal pathogen and pathosystem (Whipps, 2001; Botha, 2011).

Aureobasidium melanogenum was previously recognized as an opportunistic human pathogen (Gostinčar *et al.,* 2014) and our results showed how it was the least active species against *B. cinerea*, confirming its inadequate use in biological control of post harvest fruit fungal diseases.

Aureobasidium pullulans is also well known for its biotechnological potential, among other things for the production of the polysaccharide pullulan (Cheng *et al.,* 2011) and its biocontrol use in agriculture (Sharma *et al.,* 2009). It also produces a large spectrum of extracellular enzymes (Molnarova *et al.,* 2013) and an antifungal peptide such as aureobasidin A (Takesako *et al.,*1991). Yeast-like cells, mycelia and melanin-pigmented chlamydospores of genus *Aureobasidium* are particularly known for their biotechnological significance since this organism is a producer of the

extracellular polysaccharide (EPS) pullulan (poly-α-1,6-maltotriose) (de Hoog, 1993, Gostinčar *et al*., 2014, Peterson *et al.,* 2013). The FT-IR characterization revealed that *A. pullulans* and *A. subglaciale* strains showed many similarities in the production of several classes of secondary metabolites compounds: in particular, peaks attributed to complex carbohydrates (such as pullulan or other α and β-glucans), degrading enzymes, cyclic depsipeptides (aureobasidins), siderophores and other antifungal compounds were detected. Pullulan and other polysaccharides may improve the biofilm formation and therefore adhesion, thus explaining the better performance of these two species in both *in vitro* and *in vivo* assays, together with a higher production of degrading enzymes (i.e amylases, proteases or lipases), aureobasidins (Bozoudi & Tsaltas, 2018; Freimoser *et al.,* 2019), and siderophores, confirmed by the diagnostic band at 530 cm^{-1} (Cozar *et al.,* 2006). On the contrary, the spectrum of AM6 was characterized by both the absence of many of the bands attributed to the previous classes of non-volatile metabolites and the presence of several positive peaks attributed to melanin pigments that could explain its worst performance in the inhibition of *B. cinerea*.

Nigam (2013) asserted that filamentous microorganisms can produce and secrete a wide array of active compounds including hydrolytic enzymes and enzyme inhibitors. Indeed, yeast-like fungus *A. pullulans* produces different polymers, lipids, volatile compounds, enzymes, and secondary metabolites, that confer it a marked antagonistic activity against bacteria and fungi (Freimoser *et al.,* 2019; Prasongsuk *et al.,* 2018; Price *et al.,* 2013, 2017; Takesako *et al.,* 1991; Zain *et al.,* 2009) by inhibiting their virulence. Our results showed that almost all the tested yeast strains inhibited the enzymes pathogenic activity on *B. cinerea*. The species *A. pullulans* displayed the highest xylanase and pectinase inhibitory activity, enzymes directly related to the pathogenicity/virulence of a microorganism (Douaiher *et al.,* 2007; Kikot *et al.,* 2009) and responsible for the fruit spoilage (Al-Hindi *et al.,* 2011), and one of the major Cell Wall Degrading Enzyme (CWDE) of fruit fungal pathogens, respectively.

Nevertheless, in some cases, xylanase enzyme was enhanced by individual strains mainly belonging to *A. melanogenum* and *A. subglaciale* acting like pathogenic enzyme elicitors. It can be assumed that, thus, the efficacy of BCAs strongly depend on their ecological competence (Khol *et al.* 2019). Our data demonstrated that different species of *Aureobasidium* exerted a different efficacy against *B. cinerea.* This was especially obvious for *A. melanogenum,* that showed the lowest antagonistic activity compared to *A. pullulans* and *A. subglaciale*.

Moreover, all the tested varieties were able to produce No-VOCs, essential for biocontrol activity of *B. cinerea*. More investigations on the study of other mechanisms of action should be performed to understand the exact contribute of *A. pullulans,* and also of *A. subglaciale* mainly for the control of fungal pathogens in cold storage conditions, to improve their efficacy, whereas *A. melanogenum* cannot be considered a candidate for biocontrol as it was previously linked to human infections.

Interestingly, our study uncovers the wide potential of biocontrol strains of *Aureobasidium* spp. isolated from a range of different environments not linked to plants, such as car reservoir, clouds, and glaciers of Arctica. This could change the strategy of the search for new, more potent biocontrol strains of already known antagonists.

Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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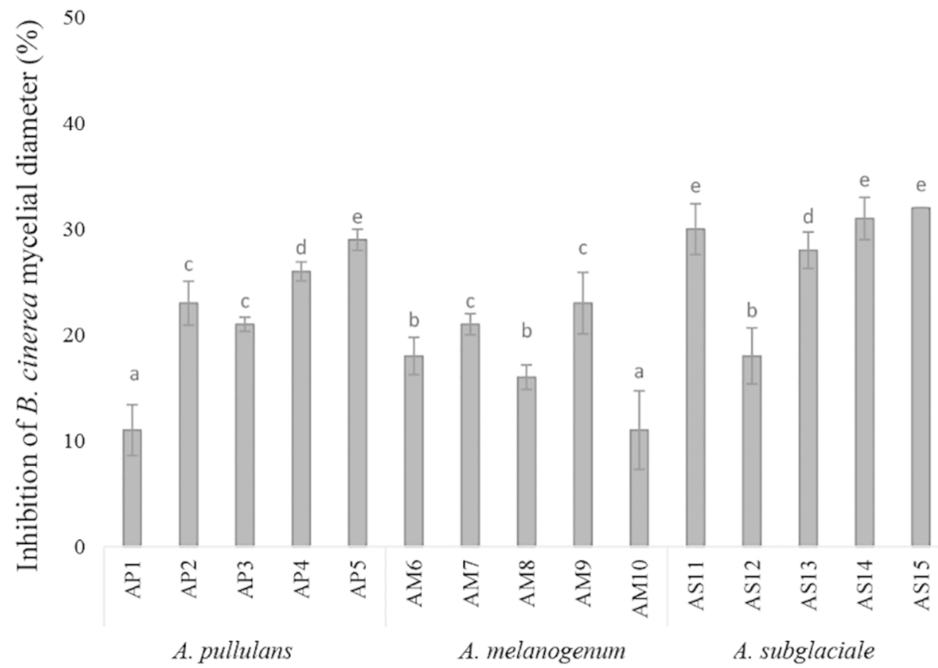
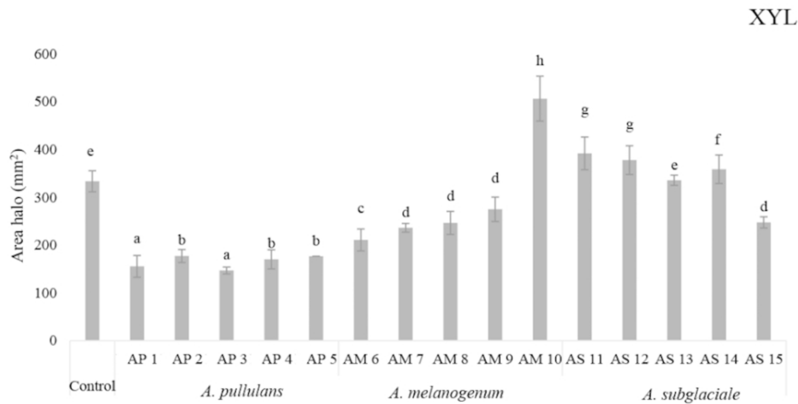
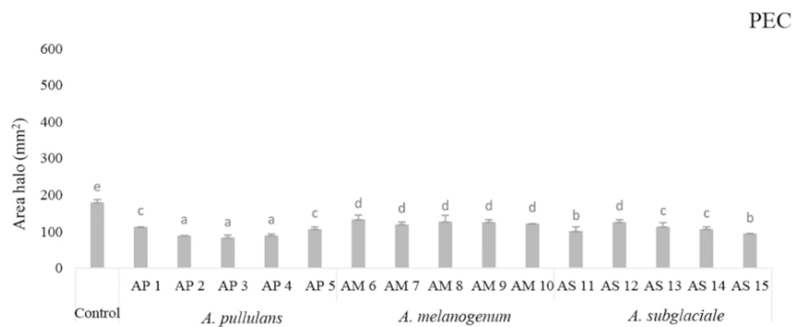
Table 1 List of strains used in this study.

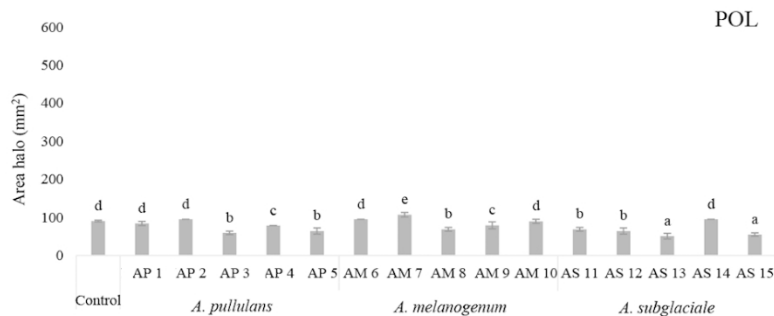
Species	Culture collection strain number	Present study number	Isolation habitat	Sampling site location
Aureobasidium pullulans	EXF-6519	AP1	felt of a metal roof tile	Slovenia (Mengeš)
	EXF-10507	AP2	marble block surface	Italy (Messina)
	EXF-10629	AP3	car petrol reservoir	Slovenia (Jezero)
	EXF-10650	AP4	acrylic painting	Slovenia (Solkan)
	EXF-10751	AP5	cloud sample	France
Aureobasidium melanogenum	EXF-3378 / CBS 110374	AM6	public fountain	Thailand
	EXF-3397	AM7	endoperitoneal fluid	Greece
	EXF-8016	AM8	bathroom, between faucet and sink	nd
	EXF-8429	AM9	tap water	Slovenia
	EXF-11028	AM10	aquarium water	Slovenia, Ljubljana
Aureobasidium subglaciale	EXF-2481 / CBS 123387	AS11	subglacial ice	Arctic; Svalbard, NyAlesund
	EXF-4632	AS12	leaves of Convallaria	Slovenia
	EXF-2425	AS13	subglacial ice	Arctic; Svalbard, NyAlesund
	EXF-2428	AS14	subglacial ice	Arctic; Svalbard, NyAlesund
	EXF-2450	AS15	glacial ice	Arctic; Svalbard, NyAlesund



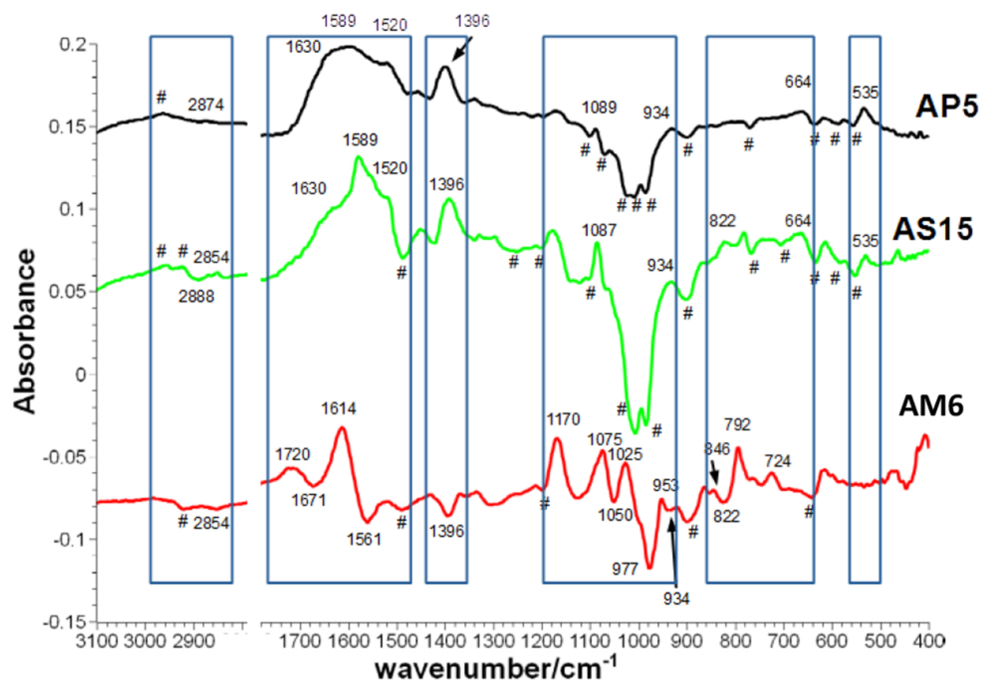
Percentage of inhibition of <I>Botrytis cinerea</I> mycelial growth co-cultured with <I>Aureobasidium pullulans</I>, <I>A. melanogenum</I> and <I>A. subglaciale</I> for 5 days at 25°C.



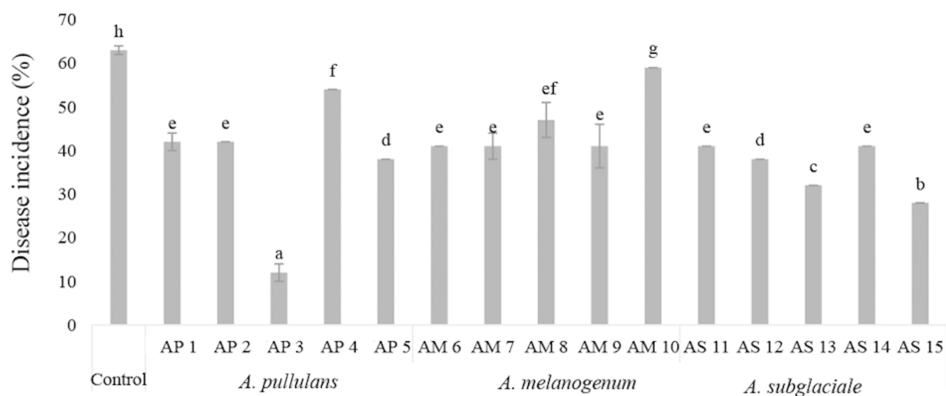




Xylanase (XYL), pectinase () of <I>Botrytis cinerea</I> conidia suspension (10^{5} conidia mL^{-1}</suo>) after exposure to secondary metabolites (No-VOCs) of <I>A. pullulans</I>, <I>A. melanogenum</I> and <I>A. subglaciale</I> strains (6 hours of incubation at 25°C) or to distilled water (control). Data are the means of two independent experiments ± standard deviation, each consisting of five replicates per treatment. Data were analysed using one-way ANOVA; Fisher's Least Significant Differences (LSDPEC), and polygalacturonase (POL) activity (measured as area of cleared halo in mm²) Test at <I>P</I> < 0.05 was used to separate differences among the means.



Difference FT-IR spectra of <I>A. pullulans</I> (NAP5), <I>A. melanogenum</I> (AM6) and <I>A. subglaciale</I> (AS15) strains after 48 h of incubation and the beginning (1 h). Positive peaks are related to the production of non-volatile compounds discussed in the text. Negative peaks are usually related to the consumption of the culture medium and are highlighted by the # symbol. The blue rectangles indicate the spectral regions in which AM6 spectrum differs the most from both NAP5 and AS15.



<I>In vivo</I> antagonistic effect by <I>Aureobasidium pullulans</I>, <I>A. melanogenum</I> and <I>A. subglaciale</I>
against <I>Botrytis cinerea</I> of tomato. ‘Datterini’ were artificially inoculated with antagonists’ cells mL.1) suspensions (15 .L, cells
and after 1 hour with conidial suspension (15 .L,
conidia mL.1) of <I>B. cinerea</I> and incubated for 6 days at 20°C and 90% RH. Tomato inoculated with water and <I>B. cinerea</I> suspension was considered as a control treatment.
Each value is the mean of three replicates (60 fruits) ± standard deviation. Different letters represent significant differences among the strains treatments according to Fisher’s Least Significant Difference (LSD) Test at <I>P</I> < 0.05.