horticulturae

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Exploring Wild and Local Fruits as Sources of Promising Biocontrol Agents against *Alternaria* spp. in Apples

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Abstract: Biological control agents (BCAs) are a promising option for managing postharvest diseases. Their environmentally friendly nature makes them valuable for sustainable and eco-friendly postharvest disease management. This study evaluated the antagonistic potential of epiphytic yeasts isolated from a local apple genotype known as "Niğde Elması" and a range of wild fruits: rosehip, hawthorn, and wild pear. There were 375 yeast isolates obtained and screened in vitro and in vivo. Initially selected were 32 isolates able to inhibit the growth of *Alternaria alternata* mycelia in the in vitro experiments and identified using molecular methods as candidate BCAs. These isolates were identified as *Aureobasidium pullulans* using sequence analysis of the internal transcribed spacer (*ITS*) and the translation elongation factor EF-1α gene (*EF1α*). Based on two rounds of in vivo screening, four isolates were subsequently selected for their capability to control the infection of apple fruits under high inoculum pressure. Finally, their volatile and non-volatile antimicrobial activity was tested against *Alternaria* spp. These findings showed how wild fruits and a local apple genotype could represent a promising source for new BCA isolation. However, further studies are needed to reveal the mechanisms of action of these putative BCAs for application during the postharvest processing and storage of apples.

Keywords: *Aureobasidium* spp.; antagonist; black rot; rosehip; hawthorn; wild pear

Citation: Tulukoğlu-Kunt, K.S.; Özden, M.; Di Francesco, A. Exploring Wild and Local Fruits as Sources of Promising Biocontrol Agents against *Alternaria* spp. in Apples. *Horticulturae* **2023**, *9*, 1156. https://doi.org/10.3390/horticulturae9101156

Academic Editor: Jiatao Xiè

Received: 24 September 2023
Revised: 19 October 2023
Accepted: 20 October 2023
Published: 22 October 2023

1. Introduction

A rapidly growing world population leads to the need for an adequate and secure food supply all over the world. However, one-third of globally produced fresh fruits and vegetables are annually lost or wasted after harvesting. One of the main factors causing postharvest loss is represented by fungal pathogens. It is estimated that 25–50% of total production is lost due to fungal diseases, depending on countries' development levels [1].

Apple (*Malus domestica* L.) is one of the most cultivated temperate zone fruits in the world, and its annual production is over 87 million metric tons [2]. Moreover, they can be stored for 6 to 12 months under cold storage conditions, which makes them sufficiently available at the market all year around [3]. However, apple fruits are susceptible to several fungal infections during long-term storage, causing fruit rots and decay and decreasing fruit quality and quantity. Among these diseases, black rot caused by *Alternaria* spp. is considered one of the major postharvest diseases of apples [4,5]. Although *A. alternata* is known as a relevant species, recent studies have reported also that *Alternaria tenuissima* and *Alternaria arborescens* caused black rot symptoms in stored fruits [6,7]. Additionally, *Alternaria* spp. can produce toxic secondary metabolites such as mycotoxins, which can accumulate in the edible parts of fruits and can cause adverse effects on health [8]. Therefore, it is important to develop and improve suitable and sustainable disease management strategies to preserve the quality of and prolong the storage life of apple fruits.

Biological control using antagonistic microorganisms is receiving increasing attention as an eco-friendly alternative to synthetic fungicides for postharvest disease management.

Microbial antagonists, such as bacteria, yeasts, and fungi, are an innovative way to control postharvest losses while protecting the environment and addressing consumer concerns [9,10]. Yeasts, among these microorganisms, possess a unique set of characteristics that make them promising biocontrol agents (BCAs), including their ability to colonize fruit surfaces rapidly, effectiveness at low concentrations, resistance to adverse conditions, minimal nutrient requirements, and non-toxic metabolite production [1,9]. The screening and selection procedure is the key step in discovering novel antagonistic yeasts, as well as elucidating their modes of action for further development of commercial products [10]. The fruit surface is one of the most studied habitats for the isolation of beneficial epiphytic yeasts to apply them against postharvest fungal diseases. We hypothesized that wild relatives of domesticated species and local genotypes might be a viable source of novel yeast species with higher antagonistic activity. For this purpose, we sampled fruits of the local apple genotype "Niğde Elması", which has been cultivated in Niğde, Turkey, for almost centuries [11], and wild species including rosehip, hawthorn, and wild pear. To our knowledge, the epiphytic yeast community of "Niğde Elması" and wild pear has never been explored before as a potential source of BCA candidates.

The main aim of this study was to explore the antagonistic potential of yeast communities isolated from local apple genotypes and wild fruits against black rot disease. For this aim, high-throughput screening was undertaken to select the candidate yeasts, and the biocontrol activity of the candidates was tested by using two rounds of in vivo experiments and in vitro assays to test the effectiveness of volatile and non-volatile metabolites and reveal the most promising isolates against *Alternaria* spp.

2. Materials and Methods

2.1. Pathogens

Isolates of *A. alternata*, *A. tenuissima*, and *A. arborescens* were isolated from decayed apples and molecularly identified. Pure cultures of mycelium were grown on sterile filter paper pieces (2 × 2 cm and maintained at −25 °C until use. Before each experiment was set up, the *Alternaria* spp. cultures were reactivated on potato dextrose agar (PDA, VWR, UK) at 22 ± 1 °C for 7 days in the dark.

2.2. Fruits

The apples (*Malus domestica* L.) of cv "Starking Delicious" were harvested at commercial maturity in an experimental orchard at the Faculty of Agricultural Science and Technologies of Niğde Ömer Halisdemir University (Turkey) (37°56′35.2″ N 34°37′56.1″ E) and kept in cold storage (0 ± 1 °C, 95% ± 5 relative humidity) until their use. Apples free from decay and injuries and uniform in size were selected. These fruits were superficially sterilized with 2% (*v*/*v*) sodium hypochlorite (NaOCl), rinsed with pure water, and left at room conditions for 2 h to dry. The fruits were artificially wounded using a sterile tip on the equator side before inoculation and treatment.

2.3. Sampling and Yeast Isolation

Wild fruits of rosehip (*Rosa canina*), hawthorn (*Crataegus orientalis*), and wild pear (*Pyrus elaeagnifolia*) from non-agricultural sites, and the local apple genotype "Niğde Elması" (*M. domestica* L.), cultivated under organic agriculture certification in Niğde (Turkey), were collected for yeast isolation. The sampling sites were 29 km apart, with 37°56′36.0″ N 35°03′53.1″ E and 37°50′14.0″ N 34°58′39.7″ E latitude and longitude, respectively (Figure 1). Three trees were randomly chosen for each fruit species at each site. Fruit with no visible infections and disorders was aseptically collected from tree crowns in triplicate, placed in polyethylene plastic bags, immediately transported to a laboratory with a cold box, and then processed within 24 h. There were one to five fruits in each replicate depending on the type and size of the fruits. The sampling was carried out during the first week of October 2021 at optimal harvesting time.

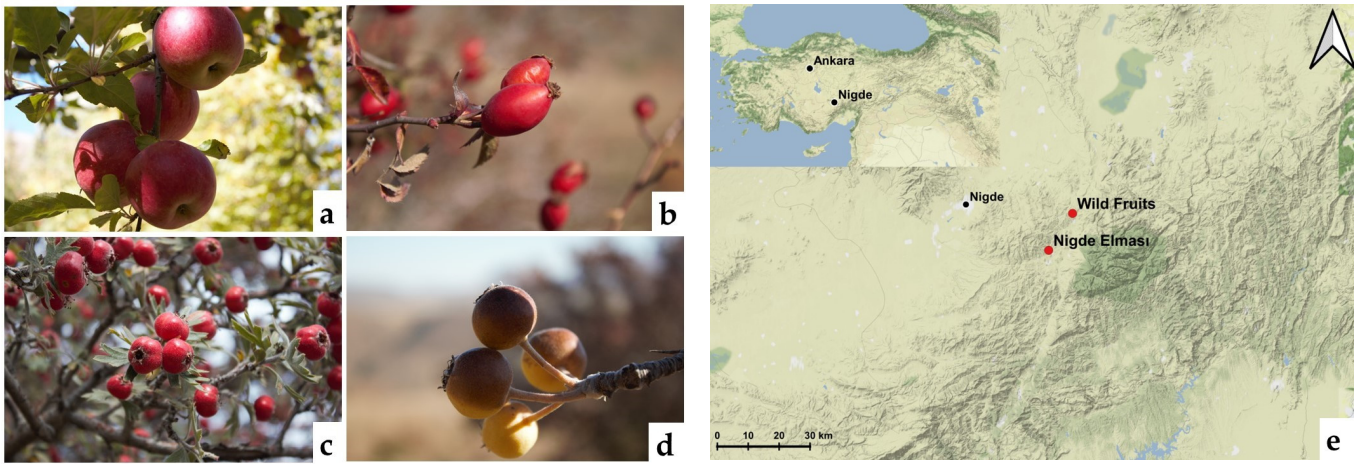


Figure 1. Epiphytic yeast source and the sampling sites. (**a**) Local apple genotype "Niğde Elması" (*M. domestica* L.); (**b**) rosehip (*R. canina*); (**c**) hawthorn (*C. orientalis*); (**d**) wild pear (*P. elaeagnifolia*), and (**e**) geographical location of the sampling sites.

Epiphytic yeast isolation was performed using the previously described method with slight modifications [12]. Wild fruits and apples from each sample were placed in a sterile 250 mL and 500 mL beaker, and then 50 mL and 100 mL sterile phosphate buffer (0.05 M, pH 6.8) was added to cover the fruit surface, respectively. The beakers were placed on a rotary shaker at 140 rpm for 5 min. The first washing solution was discarded and the fresh buffer solution was added, and then the shaking step was repeated for 1 h. Ten-fold serial dilutions were prepared from the washing solution and 100 µL of serial dilutions were spread on plates including nutrient yeast dextrose agar medium (NYDA; 8 g L^{-1} nutrient agar, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose (VWR, UK), 25 g L^{-1} agar (Merck, Germany)) and the plates were incubated at 25 ± 1 °C for 2 to 4 days. Single colonies with different phenotypic characteristics were randomly chosen from each plate and purified using triple streaking on NYDA plates. The pure cultures were preserved in 30% glycerol solution at −80 °C, and the yeast cultures were reactivated in the NYDA at 25 ± 1 °C for 48 h before use.

2.4. Primary Screening for Antagonistic Yeast

High-throughput primary screening of 375 yeast strains was tested against *A. alternata* using an in vitro dual-culture assay on PDA plates. Briefly, agar plugs (Ø, 6 mm) of the pathogen were obtained from the margin of the 7 day old culture and inoculated into the PDA plates, 2 cm away from the edge of the plates (Ø, 90 mm). On the other hand, a loopful of yeast cells from the 48 h culture was streaked vertically 2 cm away from the opposite side of the plate, and the plates inoculated only with the pathogens were considered a control. The assay was conducted in triplicate. The plates were incubated at 25 ± 1 °C until the control reached the edge of the plate. By the end of incubation, the mycelial diameter was measured using a ruler, and the following formula was used to calculate the mycelial inhibition rate.

$$\text{Mycelial inhibition (MI, \%)} = ((C - T)/T) \times 100 \quad (1)$$

where C is the diameter of the mycelial growth in the control plates, and T is the diameter of the mycelial growth in the dual-culture assay. The yeast strains with an MI of 40% or higher were selected and referred to as candidate yeast isolates. The candidate isolates were further subjected to two-stage biocontrol screening.

2.5. Two-Step In Vivo Biocontrol Screening

Two-step in vivo biocontrol screening experiments were conducted to reveal the most promising antagonist yeast strains against *A. alternata* among the 32 candidate strains. For this purpose, the crude cell suspension of the candidate strains was initially tested

by creating artificial wounds on the apples, and then the strains were selected based on the in vivo test results. Finally, the selected strains were tested under high inoculum pressure [13].

The conidia of *A. alternata* were collected by adding 5 mL of sterile water with 0.05% (*v*/*v*) Tween-20 (Sigma-Aldrich, Darmstadt, Germany) to the 7 day old culture plates, and the conidial suspension was filtered through a two-layer sterile gauze. The suspension was adjusted to different inoculum concentrations (conidia/mL) using a hemocytometer. Similarly, the yeast cells were collected from a 48 h yeast culture, and the cell concentration was adjusted as reported above.

For the first step of the in vivo biocontrol screening, one transfer loop of cells of each of the 32 candidate strains was suspended in 5 mL of sterile water with 0.05% Tween-20 and homogenized. While 20 µL of the cell suspension was applied into each wound in the apples, the control fruits were treated with the water Tween-20 solution, and air-dried for 2 h. After drying, the wounds were inoculated with 20 µL of a conidial suspension of the pathogen at 10^5 conidia/mL. The fruits were incubated at 25 ± 1 °C for 7 days, and the lesion diameter was measured along two perpendicular axes [12]. Five fruits for each yeast strain and a control were used. The disease lesion inhibition was calculated by using the following formula:

$$\text{Lesion inhibition (LI, \%)} = 100 - ((T/C) \times 100) \quad (2)$$

where C is the lesion diameter in the control fruit and T is the lesion diameter in the fruits treated with the candidate yeast. The most effective yeast strains from each fruit (LR ≥ 50%) were selected and subjected to the second step of the in vivo biocontrol screening. After the first screening, the 13 most effective candidates were evaluated under high inoculum pressure. For this aim, each strain was tested in the artificial wounds by using three different cell concentrations (10^8, 10^7, 10^6 cells/mL) against three pathogen conidial concentrations (10^5, 10^4, 10^3 conidia/mL). Forty-five fruits for each strain were used and each combination was tested on five apple fruits. A similar procedure was followed as in the first step of the biocontrol screening and the lesion reduction (LR, %) was calculated. The experiments were conducted once. The most active strains after this step were referred to as promising strains and were tested for their volatile and non-volatile organic compound productions.

2.6. Volatile and Non-Volatile Organic Compounds (VOCs and nVOCs)

The promising antagonist strains, M56, M69, R44, and C57, were evaluated for their ability to secrete VOCs and nVOCs against the postharvest black rot pathogens *A. alternata*, *A. tenuissima*, and *A. arborescens*.

To assess the effectiveness of the VOCs produced by the antagonist against the mycelium growth of the pathogens, a double petri dish assay was performed [14]. The yeast cells were collected from an actively growing culture, adjusted to 10^8 cells/mL, spread on NYDA plates using a sterile glass hockey stick, and incubated at 25 ± 1 °C for 48 h. After incubation, agar plugs (Ø, 6 mm) from each pathogen were placed on the PDA plates, and then the lids were replaced with the yeast-cultured plates. The plate assembly was sealed with double-layer Parafilm (Sigma-Aldrich, Darmstadt, Germany). The control plates were inoculated only with pathogens.

For the nVOCs assay, the previously described methodologies were performed with slight modifications [15,16]. Briefly, 1 mL of the yeast cell suspensions (10^8 cells/mL) from the active cultures was pipetted into flasks containing 100 mL nutrient yeast dextrose broth (NYDB). The flasks were placed on a rotary shaker (140 rpm) at room temperature for 2 days, the cultures were centrifuged at 6000 rpm for 30 min, and then supernatants were passed through a sterile cellulose membrane filter (Ø, 0.22 µm, Millipore, Darmstadt, Germany) for removing the cells and collected culture filtrate which contained nVOCs. The filtrate was mixed with PDA at a 1:1 ratio and the culture filtrate media was immediately poured into plates at 45 °C. The plates were inoculated with the pathogen plugs (Ø, 6 mm).

The control was represented by the PDA plates inoculated only with pathogen without the culture filtrate.

The plates were incubated at 25 ± 1 °C for 5 days. The mycelium inhibition (MI, %) was calculated as previously described. Five plates represented the sample unit as replicates of each pathogen and antagonist interaction. The experiment was repeated once.

2.7. Molecular Identification

The candidate yeasts were inoculated in flasks containing NYDB and incubated on a rotary shaker (140 rpm) at room condition for 48 h. The cells were harvested using centrifugation (4000 rpm, 40 min), and the supernatant was discarded and rinsed with sterile pure water twice using centrifugation. The harvested cells were ground with liquid nitrogen, collected in 2 mL microcentrifuge tubes (Eppendorf AG, Hamburg, Germany), and then stored at −80 °C. The CTAB method was used for genomic DNA extraction [17].

Genotypic characterization of the yeasts was performed using the amplification of the internal transcribed spacer region (*ITS*) and translation elongation factor EF-1α gene (*EF1*) using polymerase chain reaction (PCR). The *ITS* region was amplified by using the primers ITS1 (5′-TCC GTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCC TCCGCTTATTATTGATATGC-3′) described by [18], and for the amplification of the elongation factor 1α gene, the EF1-728F (5′-CATCGA GAAGTTCGAAGG-3′) and EF1-986R (5′-TACTTG AAGGAACCTTTACC-3′) primers [19] were used. The reactions were conducted in a 25 µL reaction mix containing 5 µL 5 × PCR buffer for Taq polymerase (Promega, Madison, WI, USA), 2 µL 2.5 mM of each dNTP (Thermo Fisher, Waltham, MA, USA), 1.5U Taq polymerase (GoTaq G2 Flexi, Promega), 1.5 µL 25 mM MgCl2, 0.5 µL 20 mM of each primer, and 2 ng DNA as a template for the ITS and 20 ng DNA as a template for the EF-1α primers. The PCR amplifications were carried out on a MiniAmp Plus thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA) and followed the PCR conditions described by [20] with minor modifications. The PCR program for the ITS primer was an initial denaturation of 2 min at 94 °C, followed by 40 cycles of 94 °C, 40 s; 55 °C, 40 s; and 72 °C, 1 min, with a final elongation of 7 min at 72 °C. For the EF-1α primer pair, it was an initial denaturation of 8 min at 95 °C, followed by 34 cycles of 95 °C, 15 s; at a gradient of 55 °C, 20 s; and 72 °C, 1 min, with a final elongation of 5 min at 72 °C. Finally, the amplicons were purified using a commercial purification kit (The Wizard® SV Gel and PCR Clean-Up System, Promega) according to the manufacturer's instructions and sent to sequencing services at BMR Genomics (Padova, Italy).

2.8. Data Analysis

The experiments were set up based on a completely randomized design and the data were processed using SPSS Version 25.0 (SPSS Statistics, IBM Corp, Armonk, NY, USA, 2017). All the data obtained from the VOCs and nVOCs assays were subjected to a one-way analysis of variance (ANOVA), and multiple comparisons of the means were conducted using Tukey's test ($p < 0.05$). The DNA sequences were aligned using Unipro UGENE version 34 [21], and compared with the NCBI GenBank database sequences via the Basic Local Alignment Research Tool (BLAST). Finally, the phylogenetic trees were generated separately for each gene using the reference sequences available in GenBank using the Seaview 5.0.5 software based on the pairwise distance method using the BioNJ algorithm [22], and the sequence of *A. microstictum* was included as the outgroup (Table 1).

Table 1. Accession numbers of reference sequences for phylogenetic analysis.

Species	Isolates	GenBank Accession Number *	
		ITS	*EF1*
Aureobasidium pullulans	CBS 100524	FJ150905	FJ157900
A. pullulans	CBS 584.75	FJ150906	FJ57895
A. pullulans	CBS 100,280	FJ150910	FJ157906
A. melanogenum	CBS 110,373	FJ150887	FJ039810

Table 1. *Cont.*

Species	Isolates	GenBank Accession Number *	
		ITS	*EF1*
A. melanogenum	CBS 105.22	FJ150886	FJ157887
A. subglaciale	EXF-2481	FJ150895	FJ157911
A. subglaciale	EXF-2479	FJ150893	FJ157910
A. namibiae	CBS 147.97	FJ150875	na
A. microstictum	CBS 114.64	FJ150873	FJ157914

* Genes: *ITS* = internal transcribed spacer region, *EF1* = translation elongation factor EF-1α; na: sequence not available in GenBank.

3. Results

3.1. Primary Screening for Antagonistic Yeast

We conducted an extensive survey of epiphytic yeast communities on a local apple variety (Niğde Elması) and several wild fruit species, including rosehip, hawthorn, and wild pear, isolating 375 yeast strains. These isolates were then subjected to a primary screening to assess their antagonistic potential against the postharvest black rot pathogen *A. alternata* using an in vitro dual-culture assay (Figure 2). Among the yeast isolates, 32 demonstrated notable antagonistic activity, with mycelial inhibition (MI) rates equal to or exceeding 40.00%. The average MI rates varied between 40.00% and 49.25% among these isolates. Specifically, we selected 10 isolates from apples, 9 from rosehips, 7 from hawthorns, and 6 from wild pears, each showing MI rates ranging from 40.15% to 45.77%, 40.29% to 46.76%, 40.00% to 49.25%, and 40.63% to 46.77%, respectively (Figure 3). These yeast isolates have been designated as candidate yeast isolates for further screening steps and subjected to molecular characterization.

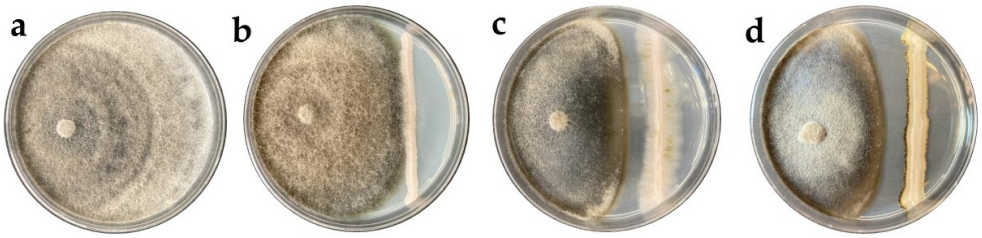


Figure 2. Dual-culture assay of yeast isolates for antagonistic activity against *A. alternata.* Control plate (**a**); ineffective yeast isolates (**b**); examples of candidate yeast isolates with antagonistic activity (**c**,**d**).

3.2. Molecular Identification of Candidate Yeast Isolates

To reveal the potential antagonist yeast community against postharvest black rot, we molecularly characterized 32 candidate yeast isolates by sequencing the target genes *ITS* and *EF-1α*. Notably, successful amplification of the *ITS* region was achieved for all, but the *EF-1α* gene could not be amplified for the isolate M31. Overall, the 32 candidate yeasts were then identified as *A. pullulans* according to BLAST analysis of the nucleotide sequences of the *ITS* gene with a $\geq$ 98% identity match, as detailed in Table 2. The phylogenetic analysis conducted with the target genes confirmed the identification and the *ITS* region showed no differences among the candidate isolates (Figure 4a). In contrast, the *EF-1α* gene revealed distinct clustering, indicating genetic diversity among the isolates (Figure 4b).

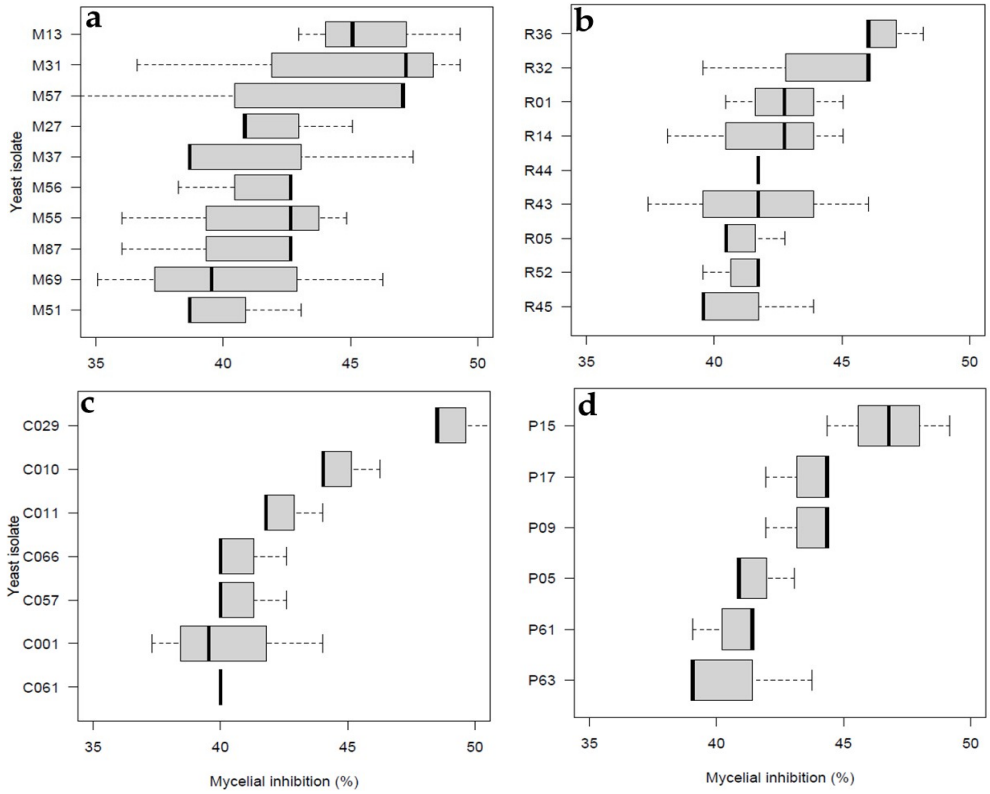


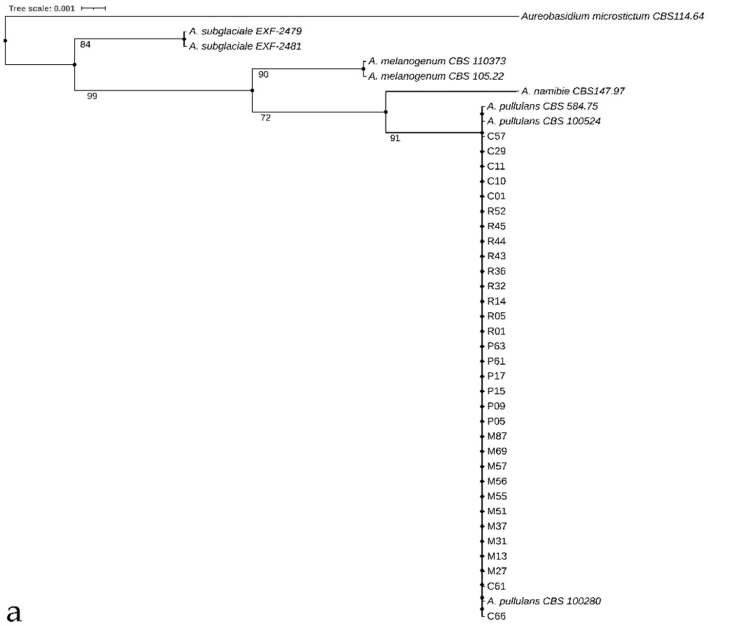
Figure 3. Primary screening of yeast isolates for antagonist activity against *A. alternata*. The candidate yeasts from (**a**) the local apple variety "Niğde Elması" (*M. domestica* L.); (**b**) rosehip (*R. canina*); (**c**) hawthorn (*C. orientalis*); (**d**) wild pear (*P. elaeagnifolia*).

Table 2. Identification of candidate antagonist yeasts based on the *ITS* region with the highest sequence identity found in BLAST analysis.

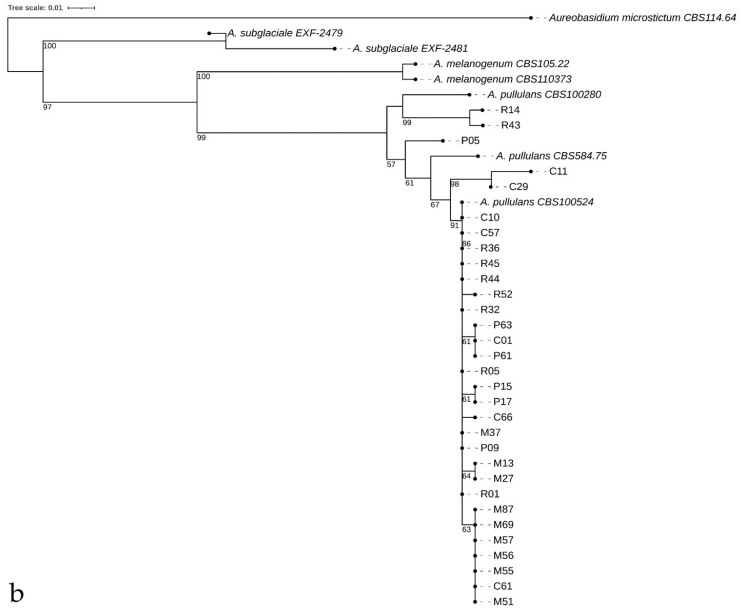
Isolate ID	Species Identification	Origin	Accession Number	Identity (%)
M13	*Aureobasidium pullulans*	Apple, fruit	JX462671	99.28
M27	*A. pullulans*	Apple, fruit	KT722604	99.45
M31	*A. pullulans*	Apple, fruit	MK460995	99.81
M37	*A. pullulans*	Apple, fruit	MK460995	100.00
M51	*A. pullulans*	Apple, fruit	MK460995	99.27
M55	*A. pullulans*	Apple, fruit	MK460996	100.00
M56	*A. pullulans*	Apple, fruit	HQ267772	99.63
M57	*A. pullulans*	Apple, fruit	KT722604	99.26
M69	*A. pullulans*	Apple, fruit	MK460995	99.27
M87	*A. pullulans*	Apple, fruit	MK460996	99.63
R01	*A. pullulans*	Rosehip, fruit	DQ640765	99.09
R05	*A. pullulans*	Rosehip, fruit	OR069592	99.08
R14	*A. pullulans*	Rosehip, fruit	DQ640765	99.08
R32	*A. pullulans*	Rosehip, fruit	KX444670	99.45
R36	*A. pullulans*	Rosehip, fruit	MK937951	99.63
R43	*A. pullulans*	Rosehip, fruit	MK460995	99.81
R44	*A. pullulans*	Rosehip, fruit	MK460996	98.92
R45	*A. pullulans*	Rosehip, fruit	MK460995	100.00
R52	*A. pullulans*	Rosehip, fruit	MN371866	99.45
C01	*A. pullulans*	Hawthorn, fruit	MK460995	99.81
C10	*A. pullulans*	Hawthorn, fruit	KT722604	99.82
C11	*A. pullulans*	Hawthorn, fruit	MT573468	99.25
C29	*A. pullulans*	Hawthorn, fruit	MT107050	99.81
C57	*A. pullulans*	Hawthorn, fruit	DQ640765	98.37

Table 2. *Cont.*

Isolate ID	Species Identification	Origin	Accession Number	Identity (%)
C61	*A. pullulans*	Hawthorn, fruit	MK460995	99.81
C66	*A. pullulans*	Hawthorn, fruit	OM237133	99.62
P05	*A. pullulans*	Wild pear, fruit	MT573468	99.44
P09	*A. pullulans*	Wild pear, fruit	HQ267772	99.81
P15	*A. pullulans*	Wild pear, fruit	MK937951	99.82
P17	*A. pullulans*	Wild pear, fruit	MT107050	99.81
P61	*A. pullulans*	Wild pear, fruit	MK460995	99.81
P63	*A. pullulans*	Wild pear, fruit	KT722604	99.45



a



b

Figure 4. Phylogenetic analysis of the isolates. Trees were constructed using BIONJ distance methods based on different regions: *ITS* (**a**) and *EF-1α* (**b**) genes. The numbers at nodes indicate the bootstrap values, indicated as a percentage of support from 1000 bootstrap replications, and the scale shows genetic distance.

3.3. Biocontrol Screening

To select the most promising isolates, a two-step in vivo screening assay was carried out. In the initial step, we tested the candidate yeast isolates by applying their crude cell suspensions, along with a conidial suspension of *A. alternata* (10^5 conidia/mL), to artificial wounds on the apples (Figure 5). Following a 7 day incubation period, we observed that several candidate isolates displayed a remarkable reduction in lesion size, some achieving a complete 100% reduction (Figure 6). Among these, the top-performing 13 isolates, each demonstrating a lesion reduction rate of 50% or higher, were chosen for further evaluation, as reported in Figure 6. Finally, these 13 isolates were tested under high inoculum pressure as the second step of in vivo screening. The second step confirmed the high efficacy of some of the best-performing isolates selected from the initial in vivo screening (Figure 7). Complete control was obtained on the fruit challenged with the lowest concentrations of the pathogen for the three promising antagonists, M56, M69, and R44. Furthermore, when applied to wounds at the highest concentration (10^8 cells/mL), three isolates M56, M69, and C57 consistently exhibited total control, irrespective of the pathogen concentration. As a result of this last challenge, we selected M56, M69, R44, and C57 as a promising biocontrol agent. These isolates showed significant efficacy in controlling the black rot pathogen under high inoculum pressure, reaffirming their potential for practical postharvest disease management.

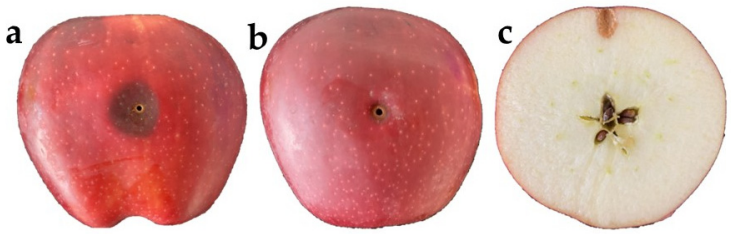


Figure 5. Apple artificial wounds were pre-treated with crude yeast cell suspension, inoculated with *A. alternata* (10^5 conidia/mL) after 2 h, and stored at 25 ± 1 °C for 7 days; control fruit without yeast treatment (**a**); apple with candidate yeast treatment (**b**); cross-section of apple with candidate yeast treatment (**c**).

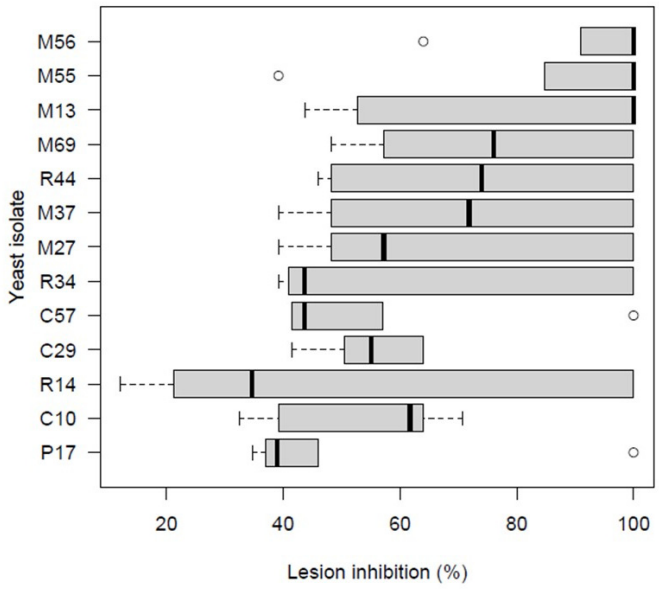


Figure 6. Primary in vivo screening of yeast isolates for biocontrol activity against *A. alternata*. A crude cell suspension was challenged with pathogen suspension (10^5 conidia/mL) in the wounded apples. Lesion inhibition (%) was determined after 7 days at 25 °C. Fruit inoculated with water was used as a control. ○ indicates outliers.

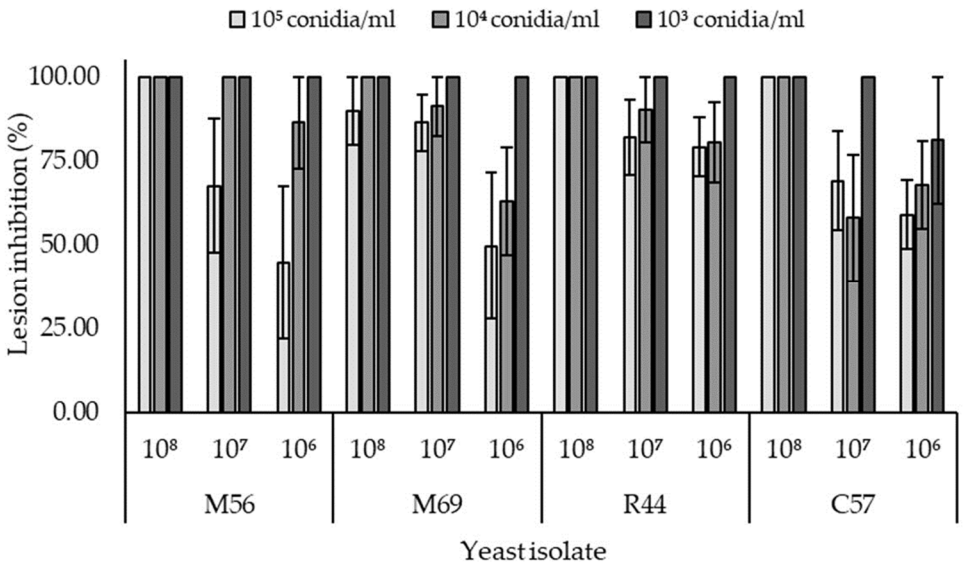


Figure 7. Secondary in vivo screening of yeast isolates for biocontrol activity against *A. alternata*. Yeast cell suspensions (10^8, 10^7, 10^6 cells/mL) were challenged with different pathogen suspensions (10^5, 10^4, 10^3 conidia/mL) in artificially wounded apples. Lesion inhibition (%) was determined after 7 days at 25 °C. Fruit inoculated with sterile water was used as a control. Each value is the mean of 5 five fruits ± standard error.

3.4. Volatile and Non-Volatile Organic Compounds (VOCs and nVOCs)

Although the antifungal effect of VOCs produced by four *A. pullulans* isolates (M56, M69, R44, and C57) on the mycelium growth of *A. alternata* was not significantly different, VOCs exhibited significant variability when tested against the mycelial growth of *A. tenuissima* and *A. arborescens* (Figures 8a and 9a). Specifically, the inhibition of *A. alternata* mycelium growth by the volatile metabolites of these isolates was as follows: M56 (22.39%), M69 (21.18%), R44 (15.77%), and C57 (21.85%). The M56 isolate metabolites inhibited the *A. tenuissima* mycelia more than the M69, R44, and C57 isolates (34.35%, 27.93%, 21.88%, and 20.29%, respectively). Similarly, the M56 isolate displayed the most pronounced inhibition of *A. arborescens* mycelium growth (20.87%). In contrast, R44 exhibited significantly lower activity in inhibiting *A. arborescens* mycelium growth compared to the other isolates, with values of 15.69% (M69), 3.95% (R44), and 14.31% (C57).

In the case of the nVOCs assay, the mycelium inhibition of *Alternaria* spp. pathogens using the culture filtrate of the promising yeast isolates is presented in Figures 8b and 9b. The data showed the inhibition activity of the isolates varied significantly for the tested pathogens. M69 recorded the highest growth inhibition against *A. alternata* (29.89%), followed by M56 (22.69%), C57 (16.05%), and R44 (15.77%). Similarly, the highest inhibition of mycelium was obtained by M69 for *A. tenuissima* (19.56%) and *A. arborescens* (13.91%). While C57 inhibited the growth of *A. tenuissima* by 14.39%, R44 and M56 reduced the growth by 12.48% and 11.68%, respectively. Lastly, regarding the inhibition of *A. arborescens* mycelial growth by the nVOCs, M56 exhibited an inhibition rate of 11.90%, followed by C57 (8.52%), and then R44 (4.28%).

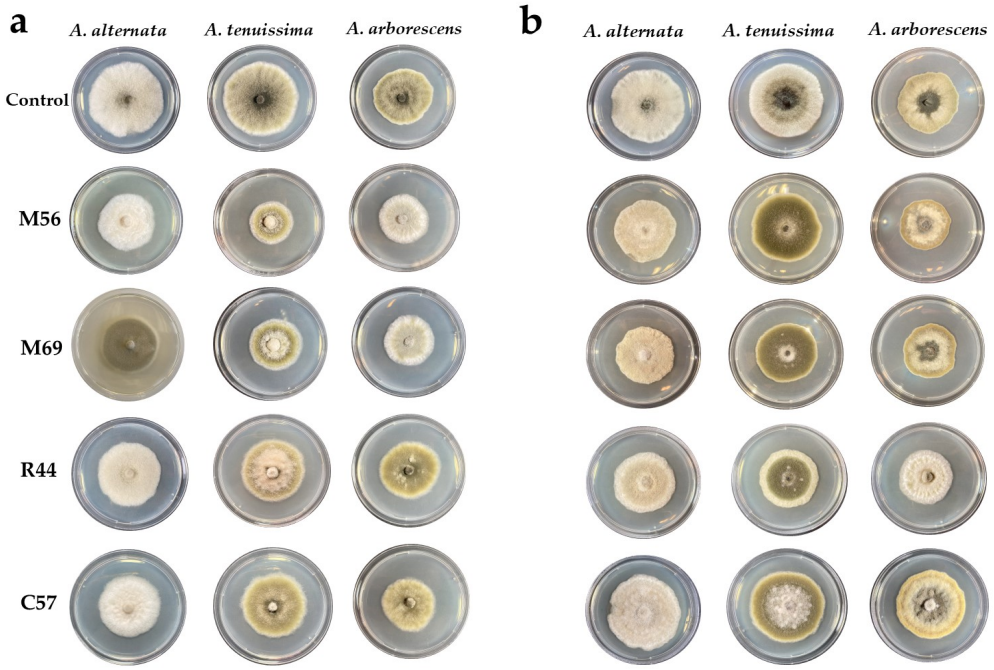


Figure 8. Effect of VOCs (**a**) and nVOCs (**b**) produced by promising antagonist isolates on mycelium growth of *Alternaria* spp.

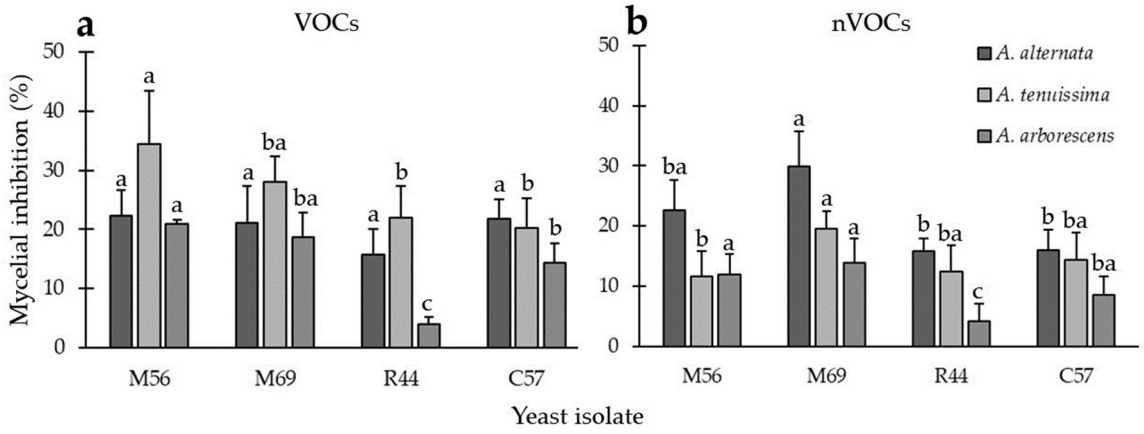


Figure 9. Effect of VOCs (**a**) and nVOCs (**b**) produced by promising antagonist isolates on *Alternaria* spp. Colony diameter (mm) was measured after seven days at 25 °C. Each value is the mean of five plates ± standard error. Different letters indicate significant differences according to Tukey's test ($p < 0.05$).

4. Discussion

Apples are a globally important fruit species not only because of their production rate but also because they can be stored for up to a year, ensuring a sufficient fresh fruit supply throughout the seasons. However, long storage makes the apple highly vulnerable to the postharvest diseases caused by fungal pathogens such as *Alternaria* spp., *Botrytis* spp., *Monilinia* spp., and *Penicillium* spp. [3,7,23], and these diseases are one of the main reasons for postharvest loss. The future of global food security depends on the development of sustainable and eco-friendly postharvest disease management strategies that reduce fruit decay. BCAs are considered promising alternatives for postharvest disease management [1]. Epiphytic populations found in natural environments serve as an important source of potential antagonists [24]. Yeasts or yeast-like microorganisms are abundant and are isolated frequently from different habitats [25]. In this study, the comprehensive sampling of epiphytic yeasts from the local apple genotype "Niğde Elması" (*M. domestica* L.), and

rosehip (*R. canina*), hawthorn (*C. orientalis*), and wild pear (*P. elaeagnifolia*) was carried out to discover native yeast species with potential antagonist activity. Screening is also a crucial step in finding promising biocontrol agents that fit into the applied conditions [26]. For this reason, we initially performed high-throughput in vitro selection and then the final selection was made based on in vivo screening of the candidate isolates using two rounds of experiments. The findings of this study represent an important step in the phylogenetic research of epiphytic yeast isolates collected from both the local apple genotype and wild fruits, since the potential of this biome has been relatively explored as a potential source of biocontrol agents for postharvest diseases. The candidate yeast isolates selected after the primary in vitro screening were molecularly characterized using the *ITS* and *EF1α* genes and further supported by the bootstrap values generated by BIONJ distance methods.

A. pullulans is the most frequently isolated yeast from the phyllosphere and can be found in different extreme habitats, from hypersaline waters [27] to polar zones [20], due to its remarkable phenotypic plasticity [28]. Recent taxonomic revisions have led to the redefinition of four previously considered varieties of *A. pullulans* into four separate species: *A. pullulans*, *A. melanogenum*, *A. subglaciale*, and *A. namibiae* [29]. Our candidate isolates with biocontrol potential belong to the *A. pullulans* species, which was previously classified as *A. pullulans var. pullulans*. *Metschnikowia* species have also been isolated from the rosehip and hawthorn and found as potential biocontrol agents [30]; however, all candidate BCA obtained from several wild fruits were identified as *A. pullulans* in the present study.

A. pullulans is a well-known and studied yeast species for its biocontrol potential against several postharvest diseases such as the gray mold caused by *B. cinerea* [31], brown rot caused by *Monilinia* spp. [32,33], and blue/green mold caused by *Penicillium* spp. [34]. In the present study, the promising isolates (M56, M69, R44, and C57) either completely prevented or reduced the infection of *A. alternata* in apple fruits when the different pathogen inoculation concentrations (10^5, 10^4, and 10^3 conidia/mL) were challenged with the higher antagonist concentration (10^8 cells/mL). Our findings align with previous studies that have reported similar results and highlighted the efficacy of biocontrol agents when applied at higher concentrations [13,35]. Such findings contribute to our understanding of the optimal conditions for biocontrol agent application. Furthermore, the application of isolates at the infection site showed a fungicidal effect, not a fungistatic one against *A. alternata*. This suggests a higher capacity of the isolates to compete for nutrients and space [10].

It is important to elucidate the mechanisms of action of BCAs for the development of commercial biocontrol products. For this purpose, we evaluated the antibiosis potential of the selected yeasts (*A. pullulans* M56, M69, R44, and C57) against *Alternaria* spp. of apples using in vitro assays (VOCs and nVOCs). *A. pullulans* is a well-studied BCA and the production of VOCs was reported as a potential mode of action for its antifungal activity on *Colletotrichum acutatum*, *Penicillium* spp. [14], *Monilinia* spp. [33], *B. cinerea* [33,36], and *A. alternata* [36]. A set of VOCs produced by *A. pullulans* was also identified in these studies, which are grouped into alcohols, ketones, and esters, and some of these reduced the growth of *A. alternata* by 47% [36]. Additionally, it is reported the growth and sporulation of *A. arborescens* were inhibited by the VOCs produced by *Torulaspora indica* [37]. In the present study, the VOCs produced by the isolate M56 showed the best inhibitory activity against *Alternaria* spp. among the tested isolates and the antifungal activity of the VOCs also depended on the pathogen species, more active against *A. alternata* than *A. tenuissima* and *A. arborescens*.

Secretion of nVOCs by antagonistic yeasts has been reported as a potential mechanism of action against several pathogens, including *Aspergillus flavus* [16], *Monilinia* spp. [32], and *B. cinerea* [38]. It is known that the cyclic peptides secreted by BCAs inhibit fungal pathogen growth by disturbing the fundamental components of the fungal cell walls [39]. Moreover, cyclic peptide production by antagonist yeasts has been recently identified as an nVOC against *B. cinerea* [38]. For this study, the isolate M69 showed the highest inhibitory activity against *Alternaria* spp. in terms of nVOCs secretion. Similarly to the VOCs activity, the antifungal activity of the nVOCs depended on the pathogen species. The highest nVOCs

activity among the isolates was observed against *A. alternata* and followed by *A. tenuissima* and *A. arborescens*. Furthermore, other studies state that the inhibitory activity of VOCs produced by antagonist yeasts was less than the activity of nVOCs on the mycelium growth of pathogens [16,31,38]. However, our results revealed similar mycelial growth inhibition of the pathogens by both the VOC and nVOC metabolites produced by the yeasts.

5. Conclusions

In this study, in vitro screening was conducted to reveal antagonistic strains against *A. alternata* in apples. All the isolates were identified as *A. pullulans*, a highly tolerant and active microorganism. Therefore, it is possible to hypothesize that the potential efficacy of the selected antagonists could be influenced also by isolating sources such as wild and local plants. However, future studies will be necessary to validate the selected BCAs' practical effectiveness in field and cold storage conditions, and further investigation on the possible physiological differences between species isolated from wild and common environments will be conducted.

Author Contributions: Conceptualization, M.Ö. and A.D.F.; methodology, K.S.T.-K., M.Ö. and A.D.F.; formal analysis, K.S.T.-K.; investigation, K.S.T.-K.; resources, M.Ö. and A.D.F.; writing—original draft preparation, K.S.T.-K.; writing—review and editing, A.D.F.; visualization, K.S.T.-K.; su-pervision, M.Ö. and A.D.F.; project administration, M.Ö. and K.S.T.-K.; funding acquisition, M.Ö. and K.S.T.-K. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been supported by the Scientific Research Projects Coordination Unit of Niğde Ömer Halisdemir University. Project number: TGT2020/20-BAGEP.

Data Availability Statement: The raw data are available at request.

Acknowledgments: We appreciate the expertise of Giusseppe Firrao for his assistance in the phylogenetic data analysis in this work; special thanks to Federico Bosetto for his help with the PCR analysis and also thanks to Rudy Cignola for his kind assistance. This research is a part of Ph.D. work.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hernandez-Montiel, L.G.; Droby, S.; Preciado-Rangel, P.; Rivas-García, T.; González-Estrada, R.R.; Gutiérrez-Martínez, P.; Ávila-Quezada, G.D. A sustainable alternative for postharvest disease management and phytopathogens biocontrol in fruit: Antagonistic Yeasts. *Plants* **2021**, *10*, 2641. [CrossRef]
2. Food and Agriculture Organization, FAOSTAT. Available online: https://www.fao.org/faostat/en/?#data/QCL (accessed on 1 September 2023).
3. Naets, M.; Bossuyt, L.; De Coninck, B.; Keulemans, W.; Geeraerd, A. Exploratory study on postharvest pathogens of 'Nicoter' apple in Flanders (Belgium). *Sci. Hortic.* **2020**, *260*, 108872. [CrossRef]
4. Jurick, W.M.; Kou, L.P.; Gaskins, V.L.; Luo, Y.G. First report of *Alternaria alternata* causing postharvest decay on apple fruit during cold storage in Pennsylvania. *Plant Dis.* **2014**, *98*, 690. [CrossRef]
5. Pavicich, M.A.; Cárdenas, P.; Pose, G.N.; Fernández Pinto, V.; Patriarca, A. From field to process: How storage selects toxigenic *Alternaria* spp. causing moldy core in Red Delicious apples. *Int. J. Food Microbiol.* **2020**, *322*, 108575. [CrossRef]
6. DeShields, J.B.; Kc, A.N. Morphological and molecular characterization of *Alternaria* spp. ısolated from European pears. *Plant Dis.* **2021**, *105*, 2531–2540. [CrossRef]
7. Elfar, K.; Zoffoli, J.P.; Latorre, B.A. Identification and characterization of *Alternaria* species associated with moldy core of apple in Chile. *Plant Dis.* **2018**, *102*, 2158–2169. [CrossRef] [PubMed]
8. Chen, A.; Mao, X.; Sun, Q.; Wei, Z.; Li, J.; You, Y.; Zhao, J.; Jiang, G.; Wu, Y.; Wang, L.; et al. *Alternaria* mycotoxins: An overview of toxicity, metabolism, and analysis in food. *J. Agric. Food Chem.* **2021**, *69*, 7817–7830. [CrossRef]
9. Wisniewski, M.; Droby, S. The postharvest microbiome: The other half of sustainability. *Biol. Control* **2019**, *137*, 104025. [CrossRef]
10. Teixidó, N.; Usall, J.; Torres, R. Insight into a successful development of biocontrol agents: Production, formulation, packaging, and shelf life as key aspects. *Horticulturae* **2022**, *8*, 305. [CrossRef]
11. Gencer, O.; Serçe, S. Determination of morphological, pomological and molecular variations among apples in Niğde, Turkey using iPBS primers. *Tarım Bilim. Derg.* **2021**, *28*, 296–306. [CrossRef]

12. Janisiewicz, W.J.; Kurtzman, C.P.; Buyer, J.S. Yeasts associated with nectarines and their potential for biological control of brown rot. *Yeast* **2010**, *27*, 389–398. [CrossRef]
13. Janisiewicz, W.J.; Jurick, W.M.; Peter, K.A.; Kurtzman, C.P.; Buyer, J.S. Yeasts associated with plums and their potential for controlling brown rot after harvest: Plum yeasts. *Yeast* **2014**, *31*, 207–218. [CrossRef]
14. Di Francesco, A.; Ugolini, L.; Lazzeri, L.; Mari, M. Production of volatile organic compounds by *Aureobasidium pullulans* as a potential mechanism of action against postharvest fruit pathogens. *Biol. Control* **2015**, *81*, 8–14. [CrossRef]
15. Meena, M.; Swapnil, P.; Zehra, A.; Dubey, M.K.; Upadhyay, R.S. Antagonistic assessment of *Trichoderma* spp. by producing volatile and non-volatile compounds against different fungal pathogens. *Arch. Phytopathol. Plant Prot.* **2017**, *50*, 629–648. [CrossRef]
16. Moradi, M.; Rohani, M.; Fani, S.R.; Mosavian, M.T.H.; Probst, C.; Khodaygan, P. Biocontrol potential of native yeast strains against *Aspergillus flavus* and aflatoxin production in pistachio. *Food Addit. Contam. Part A* **2020**, *37*, 1963–1973. [CrossRef] [PubMed]
17. Lodhi, M.A.; Ye, G.-N.; Weeden, N.F.; Reisch, B.I. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. *Plant Mol. Biol. Report.* **1994**, *12*, 6–13. [CrossRef]
18. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Elsevier: Amsterdam, The Netherlands, 1990; pp. 315–322. [CrossRef]
19. Carbone, I.; Kohn, L.M. A method for designing primer sets for speciation studies in filamentous Ascomycetes. *Mycologia* **1999**, *91*, 553–556. [CrossRef]
20. Zalar, P.; Gostinčar, C.; De Hoog, G.S.; Uršič, V.; Sudhadham, M.; Gunde-Cimerman, N. Redefinition of *Aureobasidium pullulans* and its varieties. *Stud. Mycol.* **2008**, *61*, 21–38. [CrossRef] [PubMed]
21. Okonechnikov, K.; Golosova, O.; Fursov, M. Unipro UGENE: A unified bioinformatics toolkit. *Bioinformatics* **2012**, *28*, 1166–1167. [CrossRef] [PubMed]
22. Gouy, M.; Tannier, E.; Comte, N.; Parsons, D.P. Seaview Version 5: A multiplatform software for multiple sequence alignment, molecular phylogenetic analyses, and tree reconciliation. In *Multiple Sequence Alignment: Methods and Protocols*; Katoh, K., Ed.; Springer: New York, NY, USA, 2021; Volume 2231, pp. 241–260. [CrossRef]
23. Głos, H.; Bryk, H.; Michalecka, M.; Puławska, J. The recent occurrence of biotic postharvest diseases of apples in Poland. *Agronomy* **2022**, *12*, 399. [CrossRef]
24. Pretscher, J.; Fischkal, T.; Branscheidt, S.; Jäger, L.; Kahl, S.; Schlander, M.; Thines, E.; Claus, H. Yeasts from different habitats and their potential as biocontrol agents. *Fermentation* **2018**, *4*, 31. [CrossRef]
25. Di Canito, A.; Mateo-Vargas, M.A.; Mazzieri, M.; Cantoral, J.; Foschino, R.; Cordero-Bueso, G.; Vigentini, I. The role of yeasts as biocontrol agents for pathogenic fungi on postharvest grapes: A review. *Foods* **2021**, *10*, 1650. [CrossRef] [PubMed]
26. Zhang, D.; Spadaro, D.; Garibaldi, A.; Gullino, M.L. Selection and evaluation of new antagonists for their efficacy against postharvest brown rot of peaches. *Postharvest Biol. Technol.* **2010**, *55*, 174–181. [CrossRef]
27. Gunde-Cimerman, N.; Zalar, P.; Hoog, S.; Plemenitaš, A. Hypersaline waters in salterns—Natural ecological niches for halophilic black yeasts. *FEMS Microbiol. Ecol.* **2000**, *32*, 235–240. [CrossRef]
28. Di Francesco, A.; Zajc, J.; Stenberg, J.A. *Aureobasidium* spp.: Diversity, versatility, and agricultural utility. *Horticulturae* **2023**, *9*, 59. [CrossRef]
29. Gostinčar, C.; Ohm, R.A.; Kogej, T.; Sonjak, S.; Turk, M.; Zajc, J.; Zalar, P.; Grube, M.; Sun, H.; Han, J.; et al. Genome sequencing of four *Aureobasidium pullulans* varieties: Biotechnological potential, stress tolerance, and description of new species. *BMC Genom.* **2014**, *15*, 549. [CrossRef]
30. Öztekin, S.; Karbancioglu-Guler, F. Biological control of green mould on mandarin fruit through the combined use of antagonistic yeasts. *Biol. Control* **2023**, *180*, 105186. [CrossRef]
31. Parafati, L.; Vitale, A.; Restuccia, C.; Cirvilleri, G. Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. *Food Microbiol.* **2015**, *47*, 85–92. [CrossRef]
32. Cignola, R.; Boato, A.; Sadallah, A.; Firrao, G.; Di Francesco, A. Molecular characterization of *Aureobasidium* spp. strains isolated during the cold season. A preliminary efficacy evaluation as novel potential biocontrol agents against postharvest pathogens. *Eur. J. Plant Pathol.* **2023**, *167*, 221–223. [CrossRef]
33. Di Francesco, A.; Di Foggia, M.; Baraldi, E. *Aureobasidium pullulans* volatile organic compounds as alternative postharvest method to control brown rot of stone fruits. *Food Microbiol.* **2020**, *87*, 103395. [CrossRef]
34. Agirman, B.; Erten, H. Biocontrol ability and action mechanisms of *Aureobasidium pullulans* GE17 and *Meyerozyma guilliermondii* KL3 against *Penicillium digitatum* DSM2750 and *Penicillium expansum* DSM62841 causing postharvest diseases. *Yeast* **2020**, *37*, 437–448. [CrossRef] [PubMed]
35. Vero, S.; Mondino, P.; Burgueño, J.; Soubes, M.; Wisniewski, M. Characterization of biocontrol activity of two yeast strains from Uruguay against blue mold of apple. *Postharvest Biol. Technol.* **2002**, *26*, 91–98. [CrossRef]
36. Yalage Don, S.M.; Schmidtke, L.M.; Gambetta, J.M.; Steel, C.C. *Aureobasidium pullulans* volatilome identified by a novel, quantitative approach employing SPME-GC-MS, suppressed *Botrytis cinerea* and *Alternaria alternata* in vitro. *Sci. Rep.* **2020**, *10*, 4498. [CrossRef]
37. Bosqueiro, A.S.; Bizarria, R., Jr.; Rosa-Magri, M.M. Biocontrol of post-harvest tomato rot caused by *Alternaria arborescens* using *Torulaspora indica*. *Biocontrol Sci. Technol.* **2023**, *33*, 115–132. [CrossRef]

38. Maluleke, E.; Jolly, N.P.; Patterton, H.G.; Setati, M.E. Antifungal activity of non-conventional yeasts against *Botrytis cinerea* and non-*Botrytis* grape bunch rot fungi. *Front. Microbiol.* **2022**, *13*, 986229. [CrossRef] [PubMed]
39. Helmy, N.M.; Parang, K. Cyclic peptides with antifungal properties derived from bacteria, fungi, plants, and synthetic sources. *Pharmaceuticals* **2023**, *16*, 892. [CrossRef]