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# Endophytic Yeasts for the Biocontrol of *Phlyctema vagabunda* in Apples

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**Abstract:** Bull's-eye rot, produced by *Phlyctema vagabunda*, is an important postharvest disease in apples. Current measures to control infection include synthetic fungicides, in addition to the application of copper hydroxide and potassium phosphite. However, growing public concern regarding fungicide residues in food has generated interest in developing non-chemical alternative control methods; biological control is one of the most promising alternatives. In this research, native endophytic yeasts were isolated and evaluated for the biocontrol of *P. vagabunda* in apples. The mechanisms of action involved were also determined. Our research found 2 isolates, *Vishniacozyma victoriae* EPL4.5 and EPL29.5, which exhibited biocontrol activity against *P. vagabunda* at 20 °C in apples, the incidence of bull's-eye rot was reduced by 39% and 61%, respectively, and the severity of the disease was decreased by 67% and 70%, respectively, when apples were inoculated with these yeasts 24 h before applying the pathogen. The main mechanisms that could be involved in the observed biocontrol activity are the ability to form biofilms and the production of volatile organic compounds.

**Keywords:** fruit; *Malus domestica*; mode of action; preharvest



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## 1. Introduction

*Phlyctema vagabunda* (Guthrie) Verkley (syn. *Neofabraea vagabunda* (E.J. Guthrie) Verkley), the causal agent of bull's-eye rot, has become an important and frequent postharvest disease in apples [1]. It has caused great economic losses in Europe [2], the Pacific Northwest of the United States [3], and Chile [1], and is considered a quarantine disease in some fruit destinations, such as China [4]. In Chile, *P. vagabunda* was first reported in 2005 [5]. Late-harvest cultivars like 'Cripps Pink' are the most affected varieties, with disease incidences reaching 60%, depending on the season and locality [1]. In organic production, incidences in these cultivars can be as high as 80–90%. Fruit infection occurs in the orchard [2] throughout the season [3] and is favored by abundant rainfall [6]. Infection can become established between petal fall and harvest, increasing susceptibility gradually during fruit development [7]. However, symptoms appear only a few months after harvest (usually 3–4 months in cold storage), when numerous lesions can develop on a single fruit [8]. This is, therefore, a major limitation for the late harvest variety 'Cripps Pink' when apples are held under prolonged cold storage [9].

Current management practices to control *P. vagabunda* in Chile include pre- and postharvest treatments with fungicides [10]. However, increasing concerns regarding chemical residues in food [11] have generated interest in developing non-chemical control methods, with biological control being one of the most promising and explored alternatives [12].

The use of antagonistic microorganisms has been recognized as one of the most promising alternatives to fungicides [11]. Among these microorganisms, antagonistic yeasts have proven to effectively control numerous postharvest diseases in apples [13–23]. Nonetheless, no studies have yet been carried out on *P. vagabunda*. Yeasts may be a viable control alternative for this pathogen because they have the ability to colonize the surface of fruits for long periods of time under dry conditions, produce extracellular polysaccharides that enhance their ability to survive, and rapidly use available nutrients. Yeasts are also minimally affected by pesticides [24], do not produce allergenic spores or mycotoxins [25,26], are genetically stable [19], and have simple nutritional requirements [19,26]. In addition, endophytic yeasts grow in fruit tissues under the same growth conditions as the pathogen *P. vagabunda* and, therefore, are already colonizing these tissues, which gives them an advantage over the pathogen [27].

An effective biocontrol agent requires multiple modes of action to antagonize a pathogen [28]; a combination of different mechanisms thus provides yeast with its antagonistic capacity [29] and reduces the risk of pathogen resistance [30]. The reported modes of action in yeasts include the ability to compete for nutrients and space, oxidative stress tolerance, parasitism, secretion of hydrolytic enzymes, and the ability to produce siderophores, volatile organic compounds, and biofilms, in addition to the induction of resistance [27,31–37].

In this research, we selected a native endophytic yeast for the biocontrol of *P. vagabunda* in apples. The mechanisms of action of the yeast strains involved in the biocontrol activity against the pathogen were also determined. This research could generate beneficial knowledge regarding the application of yeasts for food production and security.

## 2. Materials and Methods

### *2.1. Endophyte Isolation and Preparation of Inocula*

Endophytic yeasts from 'Cripps Pink' apples from organic orchards in the Ñuble and Bío Bío Regions of central Chile were isolated according to the methodology described by Glushakova and Kachalkin [38], with some modifications. The fruits were treated according to the following scheme: 70% ethanol for 30 min; 2% sodium hypochlorite for 30 min; 70% ethanol for 30 s, followed by sterile water for 10 min. The exocarp was then removed and macerated in 5 mL of saline solution (0.9% NaCl); 100 µL of the suspension obtained was spread on Yeast Peptone Dextrose Agar (YPD) containing 0.05 g $L^{-1}$ of streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA). Petri dishes were then incubated at 4 °C to observe the development of the colonies (between 30 and 45 days).

Inocula of the antagonists for all of the experiments were prepared in a flask containing 20 mL of yeast dextrose broth with a loop of yeast inoculum. The liquid culture was incubated on a rotary shaker (150 rpm) for 72 h at 25 °C. Antagonist cells were then collected by centrifugation at 1914× *g* for 10 min, washed, and re-suspended in sterile distilled water. The concentration of the suspensions was adjusted to $1 \times 10^9$ cells $mL^{-1}$ by means of a Neubauer's chamber.

### *2.2. Fruit*

'Cripps Pink' apples with no visible wounds were harvested from organic orchards in the Maule Region of central Chile. Apples were superficially disinfected with 0.5% sodium hypochlorite for 5 min, rinsed 3 times with distilled water, and air-dried.

### *2.3. Pathogen Inoculum*

*P. vagabunda* was obtained from apples affected by bull's-eye rot and identified by sequencing the β-tubulin gene (GenBank ID: OL450471) as described by Cao et al. [39]. Conidia suspensions were attained according to the methodology described by Cameldi et al. [2]. Briefly, a mycelial plug was transferred to Petri dishes with Tomato Agar and incubated in darkness at 5 °C; after 14 days of incubation, pathogen conidia suspensions were pre-

pared by scraping and suspending conidia in sterile distilled water and adjusted to a concentration of $5 \times 10^5$ conidia $mL^{-1}$, using a Neubauer's chamber.

### *2.4. Selection and Identification of Yeasts as a Potential Biocontrol of P. vagabunda*

Nine isolates of the most frequently isolated yeast were evaluated as biocontrol agents against *P. vagabunda*. Apples were wounded in the equatorial axis (3 mm diameter and 3 mm deep) using a sterile pipette tip and inoculated with 20 µL of a yeast suspension ($1 \times 10^9$ cells $mL^{-1}$). After 24 h, 20 µL of a *P. vagabunda* suspension ($5 \times 10^5$ conidia $mL^{-1}$) was inoculated [8]. In the control treatment, the yeast cell suspension was substituted for sterile distilled water. After 20 days at 20 °C, rot incidence and severity were recorded according to Vero et al. [40].

Yeast strains were identified by phylogenetic analyses of the D1/D2 domain of the 26S LSU of rRNA using the primers NL1 (5′-GCA TAT CAA TAA GCG GAGGAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAGACG G-3′) in MEGA version 11. DNA sequences were aligned, together with sequences of homologous regions of closely related species retrieved from the GenBank. Evolutionary distances were computed using the Jukes–Cantor method, and phylogenetic trees were obtained by neighbor-joining. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. The stability of clades was assessed with 1000 bootstrap replications.

Strain identification was confirmed by sequencing the 5.8S-ITS rDNA region using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) in Macrogen Inc. (Seoul, Korea). These sequences were then compared with those published in the GenBank database with the BLAST program [19].

### *2.5. Antagonistic Activity of Yeast on the Fruit*

The isolates that inhibited the fungus during the selection ($n$ = 2) were evaluated for biocontrol activity, apples were wounded as described above, and 20 µL of a yeast suspension ($1 \times 10^9$ cells $mL^{-1}$) was inoculated into each wound. After 24 h, the wounds were inoculated with 20 µL of a *P. vagabunda* suspension ($5 \times 10^5$ conidia $mL^{-1}$) [8]. In the control treatment, the yeast cell suspension was substituted for sterile distilled water. After 20 days at 20 °C, rot incidence and severity were recorded according to Vero et al. [40].

Three replicates were established for each treatment in a completely randomized design in which each replicate was made up of eight apples. The entire experiment was also repeated twice.

### *2.6. Biofilm Formation by Yeast*

The crystal violet (CV) methodology [41] was used to quantify the biofilm formation by yeast. Polystyrene tissue culture multi dishes (Nunclon) with 1800 µL of sterile apple juice were inoculated with a yeast suspension ($1 \times 10^9$ cells $mL^{-1}$). The yeast suspension in the control treatment was substituted with distilled sterile water. After 2 days of incubation at 25 °C, the wells were emptied and washed with 2 mL of distilled water using a pipette. This step was carried out 3 times. The biofilm layer on the wall of the wells was fixed by air-drying and stained with 2 mL of 1% crystal violet for 20 min; the cells were then washed and dried again, after which 2 mL of ethanol was added. The absorbance of the eluate was determined at 620 nm with a spectrophotometer (Epoch™ Microplate Spectrophotometer, BioTek, Winooski, VT, USA). Biofilm formation was considered to be positive when absorbance was equal to or higher than that of the control, plus three times the standard deviation [41]. Four replicates were performed, and the experiment was repeated twice.

### *2.7. Production of Volatile Antifungal Compounds*

The antifungal effect of the volatile organic compounds (VOCs) produced by the yeast strains was assayed by the double Petri dish assay according to Di Francesco et al. [42]. For this purpose, plates with YPD or an apple juice agar medium (AJA) were inoculated with

100 µL of a yeast suspension ($1 \times 10^8$ cells $mL^{-1}$). Then, 48 h later, 100 µL of a *P. vagabunda* suspension ($1 \times 10^4$ conidia $mL^{-1}$) was inoculated in plates with Potato Dextrose Agar (PDA). Subsequently, the plates with the pathogen were individually covered mouth-to-mouth with the plates containing the yeasts, sealed with parafilm, and incubated at 20 °C. The control corresponded to plates containing YPD or AJA without yeast. The inhibition of the colony-forming unit (CFU) and radial growth of the pathogen was calculated after 10 days of incubation using the equation:

$$\text{CFU inhibition } (\%) = \frac{d1 - d2}{d1} \times 100 \quad (1)$$

where d1 is the number of CFU in the control and d2 is the number of CFU in the treated.

$$\text{Micelial growth inhibition } (\%) = \frac{d1 - d2}{d1} \times 100 \quad (2)$$

where d1 is the radial growth (mm of the colony diameter) in the control and d2 is the radial growth (mm of the colony diameter) in the treated.

Three replicates were used for each treatment, and the experiment was repeated twice.

### *2.8. Chemical Characterization of Volatile Organic Compounds*

The analysis of the composition of VOCs produced by yeast was carried out as reported by Zhou et al. [37]. Volatile compounds were collected from yeast samples using Headspace Solid Phase Micro Extraction (HS-SPME), which was identified by gas chromatography–mass spectroscopy (GC–MS; QP2010 Ultra, Shimadzu, Kyoto, Japan). HS-SPME was performed with a 2 cm fiber coated with 50/30 µm DVB/CAR/PDMS. Briefly, we cultured the yeast in a 50 mL Erlenmeyer flask sealed with parafilm and sampled the volatile yeast by inserting the SPME fiber into the head-space of the culture in an Erlenmeyer flask for 5 min at 30 °C. The fiber was injected into a gas chromatograph (GC–MS; QP2010 Ultra, Shimadzu, Kyoto, Japan) containing a 30 m × 0.25 mm fused silica Rxi-5ms column. The chromatographic conditions used were inlet 280 °C; column 40 °C for 2 min followed by ramping at 5 °C $min^{-1}$ to 280 °C. Mass spectral analyses were carried out with gas chromatography–mass spectroscopy (GC–MS; QP2010 Ultra, Shimadzu, Kyoto, Japan). The scan mass range extended from $m/z$ 35 to 500. Mass spectra of VOCs were compared with those obtained from the NIST05 library, and comparison qualities higher than 90% were considered (Standard Reference Data, NIST, Gaithersburg, MD, USA).

### *2.9. Statistical Analysis*

Statistical analyses of incidence and severity of bull's-eye rot were subjected to one-way analysis of variance (ANOVA), and comparison of means was performed by Tukey's with a probability level of 5%. Mycelial growth inhibition (%) was subjected to Student's *t*-test mean comparison method with a probability level of 5%. All the data were analyzed by statistical software InfoStat (InfoStat® 2011).

## 3. Results

A total of 29 endophytic yeasts were obtained from 'Cripps Pink' apples, and then 9 isolates of the most frequent yeasts, according to their morphological and phenotypical characteristics, were evaluated against bull's-eye rot in apples at 20 °C in a preliminary assay. Two native yeasts had biocontrol activity against *P. vagabunda* and were identified as *Vishniacozyma victoriae* (EPL4.5 GenBank ID: OL453201, EPL29.5 GenBank ID: OL453202) based on the sequencing of the 5.8S-ITS rDNA region and D1/D2 domain of the 26S LSU of rRNA (Figure 1).

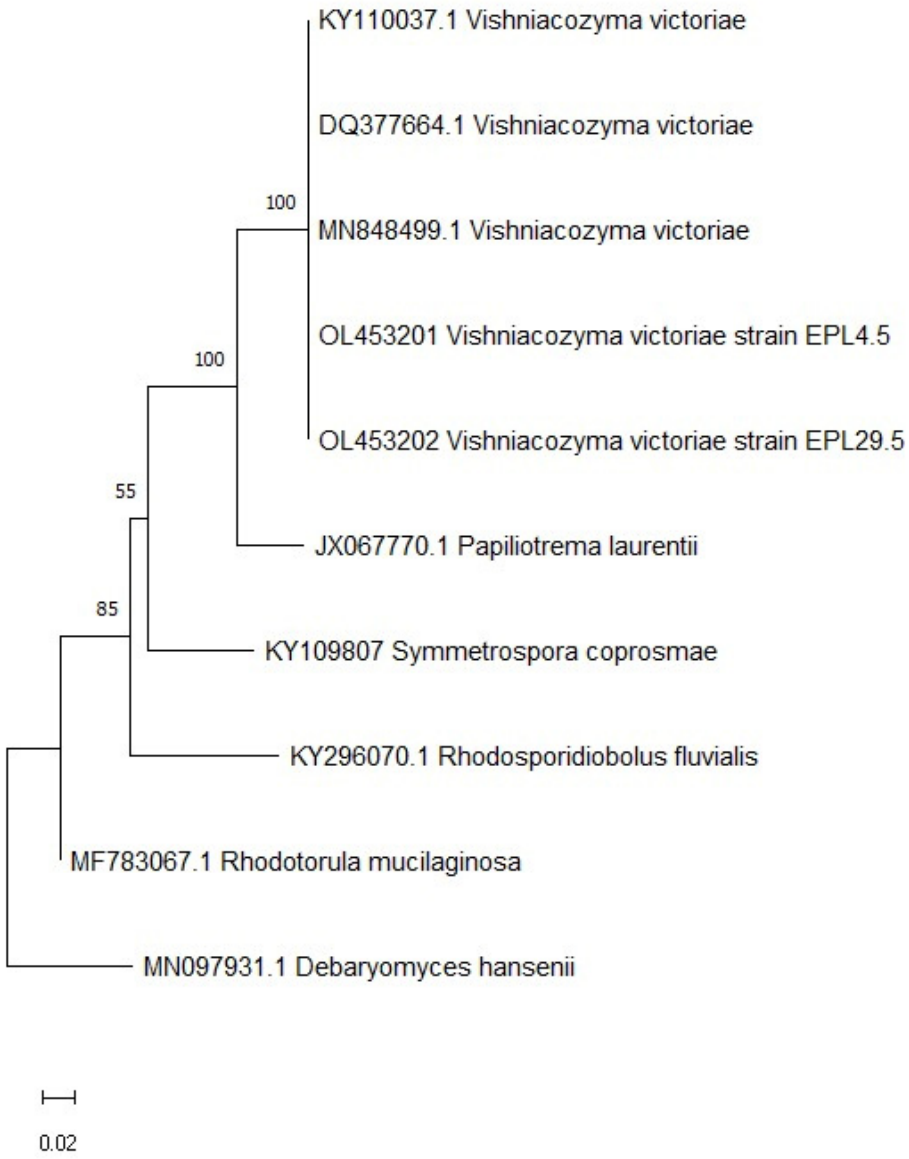


**Figure 1.** Phylogenetic analysis of yeast *Vishniacozyma victoriae* strains EPL4.5 and *Vishniacozyma victoriae* EPL29.5 with large subunit ribosomal gene nucleotide sequences. This analysis was inferred using the neighbor-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The scale bar (0.02) indicates 0.2% divergence. This analysis involved 26 nucleotide sequences.

### *3.1. Antagonistic Activity of Yeast on Fruit*

Yeast strains of *V. victoriae* (EPL4.5 and EPL29.5) inhibited bull's-eye rot on apples at 20 °C after apple wounds were inoculated with a yeast suspension ($1 \times 10^9$ cells $mL^{-1}$), 24 h before inoculation with a *P. vagabunda* suspension ($5 \times 10^5$ conidia $mL^{-1}$) (Figure 2). Yeast strain EPL4.5 reduced the incidence of bull's-eye disease by 58.3% and the severity by 67.4%, whereas yeast strain EPL29.5 decreased the disease incidence by 37.5% and the severity by 70.3% compared to the control.

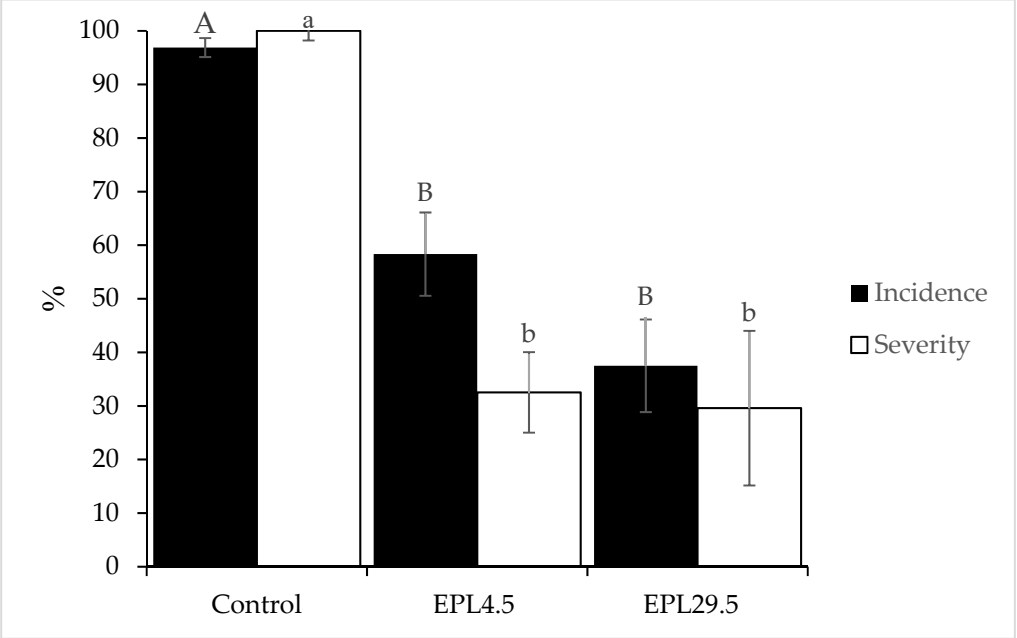


**Figure 2.** Incidence and severity of bull's-eye rot in 'Cripps Pink' apples treated with endophytic yeasts. Fruits were wounded and treated with a yeast suspension ($1 \times 10^9$ cells $mL^{-1}$). After 24 h, a spore suspension of pathogen fungus ($5 \times 10^5$ spores $mL^{-1}$) was applied, and the apples were then stored at 20 °C for 20 days. Mean values of incidence or severity linked by the same letter (upper or lower case, respectively) are not significantly different according to the Tukey's test (Incidence dF = 2; F= 10.16; $p$ = 0.0119; Severity dF = 2; F = 32.25; $p$ = 0.0006).

### *3.2. Biofilm Formation*

The ability to produce biofilm was evaluated in tissue culture multi dishes (Table 1). One of the two evaluated yeast strains (EPL29.5) was able to produce biofilm, and the optical density value (A620) was found to be higher than the cut-off criterion (0.04).

**Table 1.** Biofilm formation in polystyrene tissue culture multi dishes by *Vishniacozyma victoriae* yeast strains EPL4.5 and EPL29.5 at 20 °C. Biofilm formation was considered to be positive when absorbance was equal to or higher than that of the control, plus three times the standard deviation.

| Yeast Strain | Biofilm Formation | |
|---|---|---|
| | Absorbance ($A_{620}$) | |
| EPL4.5 | 0.023 ± 0.003 | − |
| EPL29.5 | 0.061 ± 0.006 | + |

+ Presence of biofilm. − Absence of biofilm. Cut-off value of biofilm formation = 0.04. Data on biofilm formation is expressed as mean ± standard error.

### *3.3. Production of Volatile Antifungal Compounds*

In the double Petri dish assay system (Figure 3), the VOCs produced by yeast strains EPL4.5 and EPL29.5 on the YPD medium inhibited pathogen colony-forming unit (CFU) by 54.8% and 50.6%, respectively, and mycelial growth was reduced around 69.1% and 66.1%, compared to the control. When the yeast isolates were grown on the AJA medium, strain EPL29.5 reduced CFU by 19.4% and inhibited mycelial growth by a maximum of 43.6%. Strain EPL4.5 inhibited CFU by 10.4% and mycelial growth by 42% (Table 2). These results indicate that the production of volatile organic compounds with biological activity was influenced by the culture medium.

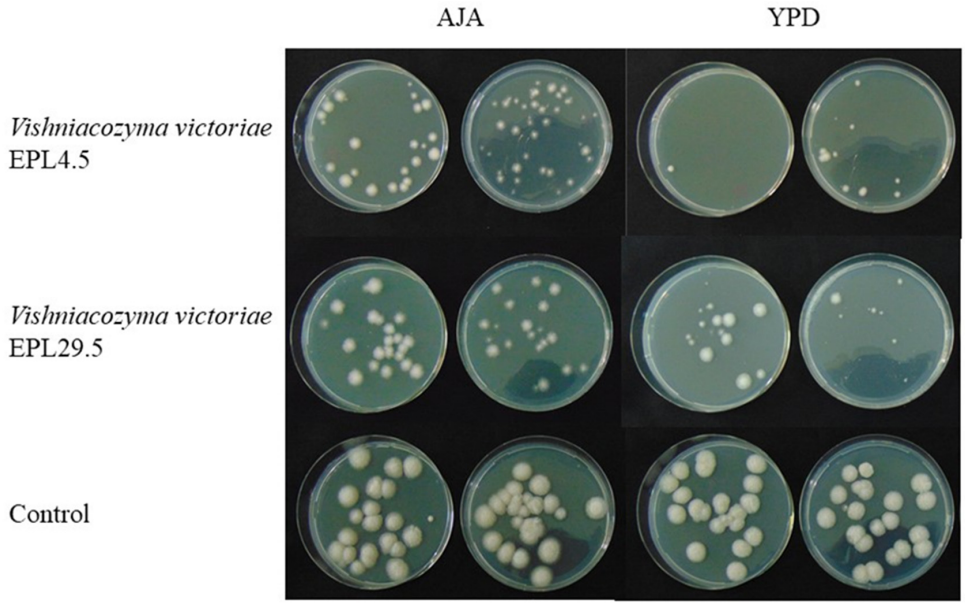


**Figure 3.** Inhibition of *Phlyctema vagabunda* growth by *Vishniacozyma victoriae* strains EPL4.5 and EPL29.5 antifungal volatile compounds activity on double Petri dish. Pathogen colony-forming unit and mycelial growth were inhibited in the presence of the yeast compared to the control.

**Table 2.** Effect of VOCs produced by *Vishniacozyma victoriae* yeast strains EPL4.5 and EPL29.5 in Yeast Peptone Dextrose Agar (YPD) and apple juice agar (AJA) mediums on colony-forming unit and mycelial growth of *Phlyctema vagabunda* at 20 °C.

| Yeast Strain | Colony Forming Unit Inhibition (%) | | Mycelial Growth Inhibition (%) | |
|---|---|---|---|---|
| | YPD | AJA | YPD | AJA |
| EPL4.5 | 54.84 ± 27.4 | 10.38 ± 21.9 | 69.14 ± 3.6 * | 42.1 ± 7.4 |
| EPL29.5 | 50.63 ± 7.6 | 19.44 ± 7.3 | 66.10 ± 12.1 | 43.62 ± 2.4 |

Data expressed as mean ± standard error. Asterisks denote a significant difference among treatments for the same yeast strain (EPL4.5 dF = 3, $p = 0.046$; EPL29.5 dF = 3, $p = 0.0997$), according to Student's *t*-test.

### *3.4. Chemical Characterization of Volatile Organic Compounds*

The VOCs produced by yeast strains EPL4.5 and EPL29.5 on the mediums AJA and YPD were analyzed by GC-MS (Figure 4). The VOCs present in just AJA or YPD medium without any yeast were not considered to be produced by *V. victoriae* EPL4.5 and EPL29.5. A total of 10 compounds were detected, including 5 alcohols, 2 ketones, and 3 hydrocarbons.

**Table 3.** Chemical characterization and relative abundance (%) of volatile fraction of *Vishniacozyma victoriae* yeast strains EPL4.5 and EPL29.5 in YPD and AJA media.

| Peak Number | RT(min) | Possible Compound | Molecular Formula | *m/z* | Relative Abundance (%) | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | EPL4.5 | | EPL29.5 | | |
| | | | | | AJA | YPD | AJA | YPD | |
| 1 | 2.14 | Hexane # | $C_6H_{14}$ | 41 | 0.49 | N.D. | 2.15 | N.D. | |
| 2 | 2.32 | Isobutyl chloride | $C_4H_9Cl$ | 43 | 0.65 | 1.83 | N.D. | N.D. | |
| 3 | 2.41 | 1-Propanol, 2-methyl | $C_4H_{10}O$ | 43 | 0.46 | 0.53 | 2.19 | 1.97 | [43,44] |
| 4 | 2.77 | 1-Butanol | $C_4H_{10}O$ | 56 | 3.98 | 46.34 | 5.25 | 38.7 | |
| 5 | 3.18 | Silanediol, dimethy l # | $C_2H_8O_2Si$ | 77 | 3.97 | 2.35 | 7.82 | 2.69 | |
| 6 | 3.43 | 2,5-Dimethylfuran | $C_6H_8O$ | 45 | 1.08 | N.D. | N.D. | N.D. | |
| 7 | 3.87 | 1-Butanol, 3-methyl | $C_5H_{12}O$ | 56 | 1.94 | 8.18 | N.D. | 17.63 | [32,35,43] |
| 8 | 3.98 | 1-Butanol, 2-methyl-, (S)- | $C_5H_{12}O$ | 41 | 19.06 | 1.33 | 13.27 | 2.97 | [32,43,44] |
| 9 | 4.13 | Disulfide, dimethyl | $C_2H_6S_2$ | 94 | N.D. | 2.47 | N.D. | N.D. | [45] |

**Table 3.** *Cont.*

| Peak Number | RT(min) | Possible Compound | Molecular Formula | *m/z* | Relative Abundance (%) EPL4.5 AJA | EPL4.5 YPD | EPL29.5 AJA | EPL29.5 YPD | Reference |
|---|---|---|---|---|---|---|---|---|---|
| 10 | 5.95 | Cyclotrisiloxane, hexamethyl- # | $C_6H_{18}O_3Si_3$ | 207 | 5.55 | 3.25 | N.D. | N.D. | |
| 11 | 7.32 | 1-Hexanol | $C_6H_{14}O$ | 56 | 3.39 | N.D. | 3.27 | N.D. | [35,46] |
| 12 | 7.99 | 2-Heptanone | $C_7H_{14}O$ | 43 | N.D. | N.D. | 1.65 | N.D. | [45] |
| 13 | 14.51 | 2-Nonanone | $C_9H_{18}O$ | 43 | N.D. | N.D. | 2.04 | 0.49 | [35] |
| 14 | 16.45 | Cyclopentasiloxane, decamethyl # | $C_{10}H_{30}O_5Si_5$ | 73 | N.D. | N.D. | 3.99 | 1.34 | |
| 15 | 20.35 | Cyclohexasiloxane, dodecamethyl # | $C_{12}H_{36}O_6Si_6$ | 88 | N.D. | N.D. | 2.94 | N.D. | |

Not detected is referred to as N.D. # Putative compounds from the column and fiber.

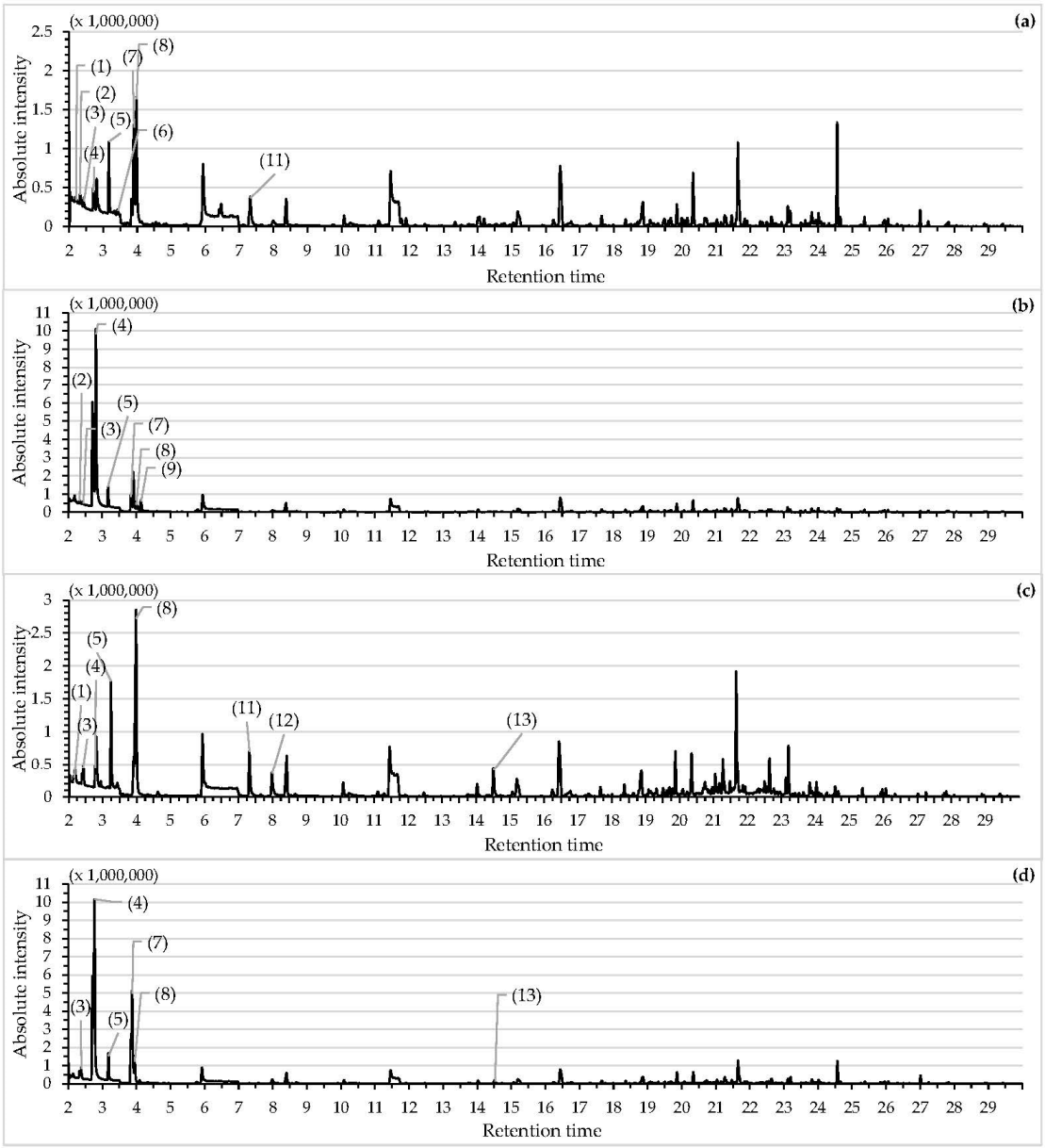


**Figure 4.** Gas Chromatography–Mass Spectrometry traces (total ion chromatograms) of VOCs collected in SPME. (**a**) *Vishniacozyma victoriae* EPL.4.5 on AJA medium, (**b**) *Vishniacozyma victoriae* EPL.4.5 on YPD medium, (**c**) *Vishniacozyma victoriae* EPL.29.5 on AJA medium, (**d**) *Vishniacozyma victoriae* EPL.29.5 on YPD medium. Peak top numbers refer to compounds listed in Table 3.

On the YPD medium, the most abundant compound was 1-Butanol; the abundance relative area (RA) of this compound was 46.34% and 38.7% for strains EPL4.5 and EPL29.5, respectively. The rest of the components produced by the strain EPL4.5 on YPD medium mainly corresponded to 1-Butanol, 3-methyl and Disulfide, dimethyl representing an RA of 8.18% and 2.47%. The EPL29.5 strain on YPD medium produced mainly corresponded to 1-Butanol, 3-methyl; 1-Butanol, 2-methyl-, (S)- and 1-Propanol, 2-methyl with an RA between 17.63% and 1.97% (Table 3).

The most abundant compound produced by both yeast strains on the AJA medium was 1-Butanol, 2-methyl-, (S)-; the abundance relative area (RA) of this compound was 19.6% and 13.27% for strains EPL4.5 and EPL29.5, respectively. The rest of the components produced by the strain EPL4.5 on AJA medium mainly corresponded to 1-Butanol; 1-Hexanol and 1-Butanol, 3-methyl with a RA between 3.98% and 1.94%. The EPL29.5 strain on AJA medium produced were 1-Butanol; 1-Hexanol; 1-Propanol, 2-methyl; 2-Nonanone, and 2-Heptanone with a RA between 5.25% and 1.65% (Table 3).

## 4. Discussion

In this study, we screened endophytic yeasts isolated from organic 'Cripps Pink' apples for the control of *P. vagabunda*. The most effective yeast that significantly decreased the incidence of bull's-eye rot in apples was the EPL29.5 strain which was identified as *Vishniacozyma victoriae*. Previously, the yeast *V. victoriae* had been reported to be an effective biocontrol agent for *Botrytis cinerea* and *Penicillium expansum* on pears [47] and *Penicillium crustosum* and *Mucor piriformis* on cherries [48]. To the best of our knowledge, no study on *P. vagabunda* has yet been recorded, making this the first report of a yeast controlling bull's-eye rot in apples.

*P. vagabunda* fruit infection occurs in the orchard [2], so the presence of the antagonist prior to or during the initial phases of the disease cycle is of crucial importance, considering the difficulties in controlling previously established infections [48]. We tested that the application of *V. victoriae* EPL29.5 24 h before the pathogen reduces the incidence of *P. vagabunda* by 58.3%. The pre-harvest application of antagonistic yeasts could protect fruits against pathogen infection in the orchard, but for the biological control to be successful, the yeast needs to possess effective mechanisms to cope with the abiotic stresses to which they are exposed [49]. In this sense, endophytic yeasts could be a very promising new source of biological control agents because they can grow and develop inside plants, thus avoiding the negative influences of environmental factors such as solar radiation and desiccation [38].

It has been suggested that biological control agents utilize different strategies depending on the pathogen, host, and environment [50]. Understanding these diverse mechanisms is essential to determine how a combination of different yeasts affects pathogen control in order to take advantage of their multiple means of action [51]. This is also an important topic in the development process of bio fungicide formulations because it permits an increase in the performance of biocontrol agents [52].

In this study, we evaluated different mechanisms of action of *V. victoriae* (EPL4.5 and EPL29.5), including antibiosis, pathogen hyphal adhesion, and siderophore production (Figures S1–S3 Supplementary Material), biofilm-forming capacity and production of volatile organic compounds.

Only *V. victoriae* EPL29.5 formed biofilms in sterile apple juice at 20 °C. This mechanism has been demonstrated by Lutz et al. [53] at 0 ± 1 °C in pear juice with glucose peptone yeasts extract as a culture medium. Biofilms are a network of cells and extracellular polysaccharides that form a gel that holds microorganisms together [54], creating a mechanical barrier between the wound and the pathogen surface [55], thereby preventing the onset of the infection process.

Several studies have shown that the production of VOCs by yeasts has a significant role in their antagonistic activities [56–58]. In this study, VOCs emitted by the *V. victoriae* strains EPL4.5 and EPL29.5 reduced colony-forming unit (CFU) and mycelial growth of *P. vagabunda*. Strain EPL4.5 reduced CFU by 54.8% and mycelial growth by 69.1% when

grown in a YPD medium, while strain EPL29.5 reduced CFU by 50.63% and mycelial growth by 66.1%.

The analyses of the VOC profiles produced by the evaluated yeast strains were conducted with HS-SMPE coupled with GC-MS, which indicated that the VOCs were mainly alcohols, with the main components being 1-Butanol, 2-methyl-, (S)- in the AJA medium and 1-Butanol in the YPD medium (Table 3). These compounds have previously been observed in the yeasts *Aureobasidium pullulans* and *Meyerozyma caribbica* when grown on a Nutrient Broth, Yeast Extract, Dextrose Agar, and PDA [32,43]. Arrate et al. [56] found that the main VOCs produced by *C. sake* in an AJA medium were 3-Methylbutyl hexanoate, 3-Methylbutyl pentanoate, and 2-Methylpropyl hexanoate; however, none of the compounds found in this research were detected in the current study. This difference may be due to the fact that the production of such volatiles is strongly influenced by the ability of yeast to assimilate and ferment carbohydrates [59].

The production of VOCs characterized as effective biofumigants for disease control in plants [60] may represent an important biological control mechanism for a wide range of postharvest pathogens. In this study, the main components produced by the yeast strains in the media evaluated were alcohols, which damage the plasma membrane and rapidly denature proteins, producing a subsequent interference with metabolism and cell lysis [32] and could explain the observed decreases in colony-forming unit and mycelial growth of *P. vagabunda*.

Our results suggest that strains EPL4.5 and EPL29.5 of the endophytic yeast *V. victoriae* are potential biocontrol agents of *P. vagabunda* in apples and could be used in preharvest applications. Due to the fact that these yeasts are endophytes, they could colonize fruit tissues, including wounds and lenticels, through rapid cell proliferation, thus allowing them to compete with the pathogen. Further research is needed to demonstrate the biocontrol activity of these isolates against bull's eye rot in orchards.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8060535/s1, Figure S1: No production of diffusible antifungal compounds of (a) *Vishniacozyma victoriae* EPL4.5 and (b) *Vishniacozyma victoriae* EPL29.5 on Potato Dextrose Agar medium at 20 °C. Lawns on plate *Phlyctema vagabunda*.; Figure S2: Lack of attachment of (a) *Vishniacozyma victoriae* EPL4.5 and (b) *Vishniacozyma victoriae* EPL29.5 to the hyphae of *Phlyctema vagabunda*, after 24 h incubation at 20 °C; Figure S3: No detection of siderophore production by (a) *Vishniacozyma victoriae* EPL4.5 and (b) *Vishniacozyma victoriae* EPL29.5 after 15 days of incubation at 20 °C on CAS agar medium.

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