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Antifungal activity of the volatile organic compounds produced by *Bacillus velezensis* strains against postharvest fungal pathogens

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ABSTRACT

It is known that Volatile Organic Compounds (VOCs), among several other mechanisms, are responsible for the antagonistic activity produced by microorganisms. In this work the volatilome of three biocontrol *Bacillus velezensis* strains (BUZ-14, I3 and I5) was tested *in vitro* and on fruit against *Botrytis cinerea*, *Monilinia fructicola*, *M. laxa*, *Penicillium italicum*, *P. digitatum* and *P. expansum. In vitro,* pathogens growth was significantly inhibited, in particular *M. laxa, M. fructicola* and *P. italicum* (66, 72 and 80 %, respectively) by BUZ-14 and *B. cinerea* (100 %) by I3 and I5, compared to the control. *In vivo* tests also showed significant inhibitions since volatile metabolites of I3 reduced grey mould in grapes by 50 % and those of BUZ-14 decreased brown rot severity in apricots, especially by *M. fructicola,* from 60 to 4 mm. VOCs were identified by solid phase microextraction (SPME) coupled with Gas Chromatography-Mass Spectrometry (GC–MS) and the antifungal activity of some of them was tested both *in vitro* and *in vivo* against the fungal pathogens. The main volatiles identified ranged from 12 to 15 compounds including 2-nonanone, 2-undecanone, 2-heptanone, 1-butanol, acetoin, benzaldehyde, butyl formate, diacetyl, nonane, or pyrazine, among others. Benzaldehyde and diacetyl obtained the lowest minimum inhibitory concentrations *in vitro*, ranging from 0.005 to 0.125 mL L^{-1} depending on the pathogen tested. Moreover, diacetyl was able to control grey mould caused by *B. cinerea* in table grapes with only 0.02 mL L^{-1} and to reduce blue rot in mandarins at the same dose up to 60 %. In this study, diacetyl and benzaldehyde have been identified as promising compounds to apply in active packaging during the postharvest commercialization of fruit. However, prior to the application, it is crucial to determine not only the active dose but also the phytotoxic, since some fruit species such as apples and apricots have proven to be highly sensitive.

1. Introduction

The use of antagonistic microorganisms as an alternative control to chemical pesticides has gained importance in recent years. The prohibition of numerous active substances by legislation and the easier commercialization of biopesticides based on biological agents (BCAs) has led to an increase in research on this subject. The bacterial genus *Bacillus* has been studied for its high capacity to produce metabolites, and antibiosis has been reported as the main mode of action against pathogens (Alvarez et al., 2012; Chen et al., 2009; Romero et al., 2007). Normally, these metabolites are soluble compounds such as lipopeptides (iturin A, fengycin, surfactin), polyketides (bacilysin, bacillobactin, difficidin…), lytic enzymes (chitinase, galactanase, isoamylase, lipase…), siderophores (hydroxymate, catecholet or carboxylate) (Kesaulya et al., 2018) or bacteriocins (lantibiotics, pediocin or thuricin) (Abriouel et al., 2011). However, the gaseous metabolites from *Bacillus* species have also been recently studied as potential antifungals (Asari et al., 2016; Raza et al., 2016; Yuan et al., 2012). These metabolites are commonly denominated as Volatile Organic Compounds (VOCs) and can act directly against the pathogen (direct antibiosis) by destroying the cell wall or indirectly, inducing systemic resistance to the plant (Chen et al., 2008; Zheng et al., 2013). Chemically, these lipophilic compounds (normally alcohols, alkanes, acids or ketones), have low molecular weight and are derived from microorganism biosynthetic pathways as part of their primary and secondary metabolism (Gotor-Vila et al., 2017). They are used for different purposes such as increasing productivity in agriculture or plant protection from fungal diseases (Kai et al., 2007). Numerous bacterial agents belong to the genus *Streptomyces* or *Pseudomonas* (Karimi et al., 2012; Paulitz et al., 2000; Zhang et al., 2019) and several yeasts such as

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Wickerhamomyces, *Metschnikowia*, *Aureobasidium* or *Saccharomyces* (Contarino et al., 2019; Di Francesco et al., 2015) are able to produce VOCs. However, these compounds synthetized by the genus *Bacillus* have demonstrated high antifungal activity (Chaurasia et al., 2005). This bacterial group produces a wide range of VOCs, also denominated volatilome, including aldehydes, ketones, hydrocarbons, alcohols, *etc.* (Gotor-Vila et al., 2017). The most relevant factor which influences the production of these metabolites is the BCA strain. There are many differences in the number of VOCs and their concentration, depending on the bacteria or the yeast, the type of *Bacillus* species or even differences within strains of the same species. Moreover, the substrate where the antagonist is grown also significantly influences the production due to the differences in the nitrogen or carbon sources (Di Francesco et al., 2020; Fiddaman and Rossall, 1994; Gotor-Vila et al., 2017; Raza et al., 2016).

Volatiles are increasingly used as a potential sustainable alternative against phytopathogens. Their application is still uncertain, but considerable progress has been made in research into biofumigation in the preharvest and active packaging in the postharvest phases (Di Francesco and Mari, 2014; Di Francesco et al., 2016; Mari et al., 2016). The easy volatilization of these compounds would allow their displacement through the entire orchard and cultivars, facilitating their scattering and dispersion. In the postharvest stage, VOCs could be considered as ideal antimicrobials since their activity does not require contact between BCA and pathogen or between BCA and food. However, in order to accomplish a reliable application, the study of the modes of action of the antagonist and the pathogen is crucial for understanding their interaction and biology. The plant or the fruit as a third factor must also be taken into account (Parafati et al., 2015). The integration of VOCs as a different strategy to achieve higher levels of disease control will contribute to a more successful handling of postharvest diseases. Moreover, the pooling of research results and the identification of the most significant modes of action will result in more effective and productive formulation to apply in field and postharvest managements.

This study assesses the *in vitro* and *in vivo* antifungal activity of the volatilome produced by three strains of *B. velezensis*, BUZ-14, I3 and I5. The identification of the most predominant gaseous metabolites and their antifungal activity *in vitro* and on fruit has also been tested.

2. Materials and methods

2.1. Fungal pathogens

The fungal pathogen strains used in this study were *Botrytis cinerea* VG1, *Monilinia fructicola* VG 104, *M. laxa* VG 105, *Penicillium digitatum* VG 20, *P. expansum* CECT 20140 and *P. italicum* VG 101. Isolates from the Plant Food Research Group (Zaragoza, Spain) culture collection (VG1, VG 20, VG 101, VG 104 and VG 105) grown on Potato Dextrose Agar (PDA) (Oxoid Ltd; Hampshire, England) at 25 °C for 10 days had been identified by morphological analysis and sequencing of ribosomal DNA ITS (internal transcribed spacer) regions. Conidia suspensions were obtained from PDA by scraping and suspending spores in 0.1 % peptone water. Broth was centrifuged at 16,000 × *g* for 10 min at 4 °C and the cell pellet was resuspended in PrepMan(R) Ultra Sample Preparation (Applied Biosystem, Foster City, USA), following the protocol recommended by the manufacturer for DNA extraction. The quantity and quality of the isolated DNA was evaluated by spectrophotometry with Nanodrop 2000 (Thermo Scientific, Waltham, USA) previously calibrated (2 µL of water sterile Mili-Q). The DNA concentration (ng/µL) was determined from absorbance at 260 nm using the formula: [ADN] $ng\,\mu L^{-1}$ = A260 × 50 × Dilution factor. DNA purity was evaluated by the ratio of the absorbance at 260/280 nm. It was considered that the purity was optimal when the relationship of the absorbance at 260/280 nm was between 1.7 and 2. Amplification of ITS region by ITS1 (GCCGTAGGTGAACCTGCGG) and ITS4 (GCCTCCGCT TATTGATATGC) primers was carried out with a thermocycler Mini Opticon™ Real-Time PCR detection System (Bio-Rad Laboratories, Hercules, USA), using the following thermal cycling conditions: initial denaturation at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s and DNA extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. Amplicons were visualized by electrophoresis through 1.5 % agarose gels (w/v) (Bioron, Ludwigshafen, Germany) in 1X TBE (Bio-Rad, Hercules, USA) and staining with 5 µL of Midori green (Nippon Genetics, Düren, Germany). After electrophoresis, gels were visualized and captured using a Bio-Rad Gel Doc 1000 under UV light and photographed with the software Quantity One v.4.4.0 (Bio-Rad, Hercules, USA). The quality and size of the amplicons were evaluated in the presence of a 100 bp ladder (Bioron, Ludwigshafen, Germany). Amplified products were purified using FavorPrepTM Gel/PCR Purification (Favorgen, Ping-Tung, Taiwan), following the protocol recommended by the manufacturer and subsequently sequenced using external services (STAB VIDA, Lisbon, Portugal). The DNA sequences obtained from both regions were compared to those of the GenBank database at the National Centre for Biotechnology Information (NCBI) using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All the fungal strains showed 100 % of homology with the respective pathogen found in the database.

Isolates were grown and maintained on PDA at 25 °C and periodically transferred to fresh fruit to induce infection and spore production for their subsequent use as primary inoculum. Conidia suspensions were obtained from 7 days-old colonies grown on PDA by scraping and suspending spores in 0.1 % peptone water with 0.05 % (v/v) of Tween 80 and counted through a haemocytometer. The spore solution was adjusted to the concentration relating to the experiments through serial dilutions in potato dextrose broth (PDB) (Oxoid).

2.2. Biocontrol agents: identification and inoculum production

BUZ-14 was previously identified by Calvo et al. (2019), and I3 and I5 identification followed the same protocol as used by those authors. In short, 16S rDNA was amplified using universal primers (8F and 1492R) and the *gyr*B gene using previously designed specific primers (Calvo et al., 2019). The phylogenetic trees were constructed and the three antagonistic bacteria were identified as *B. velezensis* (formerly named *B. amyloliquefaciens* subsp. *plantarum*) (GenBank accession numbers for 16 S rRNA genes were MF461174, MH464903 and MH464958 for BUZ-14, I3 and I5, respectively).

The strains were grown in 250 mL conical flasks containing 50 mL of 863 medium (10 $g\,L^{-1}$ peptone, 10 $g\,L^{-1}$ yeast extract and 20 $g\,L^{-1}$ glucose; pH 7) (Yánez-Mendizábal et al., 2011). A 24h-old culture on Tryptone Soy Agar (TSA, Oxoid, Basingstoke, Hampshire, U.K.) was transferred to 7 mL of Tryptose Soy Broth (TSB, Oxoid, Basingstoke, Hampshire, U.K.) to prepare fresh cell suspensions of the strains. The suspensions were incubated for 24 h at 30 °C and adjusted to 40 ± 5 % transmittance at 700 nm with a spectrophotometer, corresponding to 2×10^8 colony forming units (CFU) per milliliter. These were adjusted to the concentration used in the experiments through serial dilutions in TSB and verified by plating on TSA for 24 h at 30 °C.

2.3. Fruit

Different fruit were used for this study according to the pathogen tested. Apricots (cv. Pinkcot), grapes (cv. Thomson Seedless), apples (cv. Golden Delicious) and mandarins (cv. Clemenules) were used for *Monilinia* spp., *B. cinerea*, *P. expansum, P. digitatum* and *P. italicum,* respectively. All fruit were collected from local packing greenhouses at commercial maturity stage, having been grown in different areas of Spain (La Almunia de Doña Godina, Castellon and Murcia). All fruit were surface-disinfected by immersion in 1 % sodium hypochlorite, rinsed with tap water, and allowed to air-dry at room temperature (20 °C).

2.4. In vitro antifungal activity of the volatile fraction or volatilome of BUZ-14, I3 and I5 strains

Two solid culture media, MOLPA (casein peptone, 30 g L^{-1}; saccharose, 20 g L^{-1}; yeast extract, 7 g L^{-1}; K_2HPO_4, 1.9 g L^{-1}; $MgSO_4$, 0.45 g L^{-1}; citric acid, 0.01 g L^{-1}; $CuSO_4$, 0.001 mg L^{-1}; $FeCl_3$, 0.005 mg L^{-1}; $NaMoO_4$, 0.004 mg L^{-1}; KCl, 0.002 mg L^{-1}; $MnSO_4$, 3.6 mg L^{-1}; $ZnSO_4$, 0.14 mg L^{-1}; H_3BO_3, 0.05 mg L^{-1}; agar-agar, 15 g L^{-1}) (Ahimou et al., 2000) and 863A (casein peptone, 10 g L^{-1}; yeast extract, 10 g L^{-1}; D-glucose, 20 g L^{-1}; agar-agar, 15 g L^{-1}) were selected for this study since the nutrients present in the medium could significantly influence the BCA metabolite production (Di Francesco et al., 2020; Gotor-Vila et al., 2017; Raza et al., 2016). The double Petri dish assay (Rouissi et al., 2013) was used to determine *in vitro* antifungal activity of volatiles of BUZ-14, I3 and I5 against the fungal pathogens cited in Section 2.1. For *Monilinia* spp. inoculation, an agar plug (5 mm diameter) from actively-growing margins of fungal colonies (7 days-old culture on PDA) was placed at the center of PDA dishes. For *B. cinerea*, *P. expansum* and *P. italicum*, the conidia suspension (10^5 conidia mL^{-1}) was inoculated at the center of PDA dishes using a sterile needle. In parallel, MOLPA and 863 plates were spread with 100 µL of each strain of BCA (10^8 CFU mL^{-1}) and the lid of the plates was replaced by a base plate of PDA of each pathogen. The two base plates were sealed with Parafilm and incubated at 25 °C for 5 d. Afterwards, the fungal diameter was measured and compared with control plates (without BCA). The results were expressed as the percentage of inhibition (%) in comparison with the control. Five plates were used per pathogen and BCA, and the experiment was repeated in two separated days.

2.5. Antifungal activity of the volatilome produced by BUZ-14 and I3 strains on fruit

Although *in vitro* tests showed that the inhibitory effect of I3 and I5 volatilome was very similar, I3· resulted slightly more active, especially against *B. cinerea*. These results also showed that the volatiles of BUZ-14 highly inhibited *Monilinia* spp. and *P. italicum*. Thus, *in vivo* assays were conducted on mandarins, table grapes and apricots that were inoculated-treated with *P. italicum*-BUZ-14, *B. cinerea*-I3, and *M. fructicola* and *M. laxa*-BUZ-14, respectively.

The fruits were wounded with a sterile micropipette tip and subsequently inoculated with 10 µL of pathogen spore suspension (10^4 conidia mL^{-1}) before arranging for 30 min at ambient temperature to allow the absorption of the inoculum. Afterwards, the incubation chambers were prepared. Cylindrical plastic boxes (15 × 9.5 cm, height × diameter, 1,000 mL capacity, disinfected with isopropanol 70 % for 3 min under UV-C) containing 50 mL of MOLPA at the bottom were surface-spread with 250 µL of BCA suspension (10^7 CFU mL^{-1}). The fruits, previously inoculated with the pathogen, were suspended in the lid of the plastic box onto sterile gauze to assure a separation distance of at least 8 cm from the bottom substrate. In the case of the grapes, 3 fruit were placed per box and 3 boxes were used per treatment. Otherwise, one mandarin, apple and apricot were placed individually per box and 6 boxes were used per treatment. In addition, one wound per grape and two wounds in the rest of fruit were made. The experiment was repeated in 3 separated days. The boxes were closed, sealed with Parafilm and incubated at 25 °C for 5 d. Boxes without BCAs inoculation in the agar were considered as control. After incubation, incidence (percentage of infected fruit) and severity (as lesion diameter in mm using a caliper) were determined. In grapes, a severity scale according to the infected area (0 = no symptoms; 1 = 1–25 %; 2 = 25–50 %; 3 = 50–75 %; 4 > 75 %) was used due to the difficulty of measuring the diameter. Thus, the infection index (McKinney, 1923) was calculated, which expresses the weighted average of the disease as a percentage of the maximum possible level (100 %):

$$DS\ (\%) = \Sigma\ (dxf)NxDx100$$

Where d = scale of fruit infection; f = frequency of fruit with the infection scale; N = number of fruit examined; D = highest scale found.

After that, inhibition percentages were calculated as follows:

$$PI\ (\%) = \frac{C-T}{C}x100 \text{ (for apricots, mandarins and apples)}$$

Where C = lesion diameter (mm) of control; T = lesion diameter (mm) of treated fruit

$$PI\ (\%) = DS_c - DS_t \text{ (for grapes)}$$

Where DS = disease severity (%) of control; DS_t = disease severity (%) of treated fruit

2.6. Identification of VOCs

The volatiles produced by the bacterial strains were identified. One hundred microliters of a 24 h-old 863 liquid culture (10^8 CFU mL^{-1}) of each strain was inoculated in vials containing 20 mL of MOLP liquid medium. Samples were hermetically closed and incubated at 30 °C for 24 h. Vials without bacterial inoculation were used as controls.

The detection of volatile compounds was carried out using solid-phase microextraction (SPME) coupled with gas-chromatography tandem mass spectrometry analysis, following the method previously described by Chaves-López et al. (2015). Briefly, a SPME fibre (75 mm, carboxen/polydimethylsiloxane) was exposed to the headspace of the vials while maintaining the sample at 35 °C for 40 min. Compounds were then desorbed for 10 min in the injection port of the gas chromatograph at 220 °C for 10 min with the purge valve off (split-less mode). An Agilent Hewlett-Packard 6890 GC gas-chromatograph equipped with a MS detector 5970 MSD (Hewlett-Packard, Geneva, Switzerland) was used for peak separation and detection. A fused silica capillary column was used: a CP-Wax 52 CB (50 m x0.32 mm – Chrompack – Middelburg, Netherlands) coated with polyethylenglycol. The injector and FID temperature was 250 °C; the detector temperature 220 °C; and the carrier gas (He) flow rate, 1 mL min^{-1}.

Volatile peak identification was carried out by computer matching of mass spectral data with those of the compounds contained in the chromatograph and the Wiley ver. 6 mass spectral database (probability set at > 90 %). Positive identification of each chemical constituent was performed whenever possible by comparison with that of authentic standards (Sigma-Aldrich, Germany).

2.7. Antifungal activity of single VOCs

The minimum inhibitory concentrations (MICs) of previously detected individual volatile compounds were determined both *in vitro* and *in vivo*.

2.7.1. In vitro

The 11 standards (Table 3) used during the assay were purchased from Sigma-Aldrich (Germany). For *in vitro* test, the double sealed method (Rouissi et al., 2013) was used similar to that described in Section 2.4, although commercial volatiles were used instead of the antagonist. Thus, 100 µL of each pathogen suspension (10^4 conidia mL^{-1}) was spread onto the PDA surface facing one 13 mm diameter disc of cellulose placed inside the base of another Petri dish and impregnated with different aliquots of pure compounds, from 0.4–150 µL, in order to find the MIC for all the pathogens tested. Thus, the concentrations of pure compounds inside the Petri dishes varied from 0.005 to 1.875 mL L^{-1} headspace. Both base plates were sealed with Parafilm and incubated for 5 d at 25 °C. Plates with a non-spread paper filter were used as control. The results were expressed as the MIC needed to suppress the growth of the fungal pathogen. Five plates per pathogen and compound concentration were used and the experiment was

repeated in two separated days.

2.7.2. In vivo

In this case, the methodology was similar to that described in Section 2.5, although the volatile compound (instead of the antagonist) was impregnated in a 90 mm diameter Whatmam nº4 paper disc (Whatman paper Ltd, Springfield Mill, Maidstone, UK) at the bottom of the box. The single volatile used in this assay was benzaldehyde, diacetyl, and 3-methyl-1-butanol (isoamyl alcohol), following the *in vitro* results (lower concentrations were taken into account). The aliquots introduced in the boxes were calculated based on the volume of the container (1000 mL) and corresponded to 0.006, 0.01, 0.02, 0.025, 0.05, 0.1 and 0.2 mL L^{-1} headspace. Mandarins were inoculated with 10 µL of a suspension of *P. italicum* (10^4 conidia mL^{-1}) and treated with diacetyl (0.01, 0.025 and 0.05 mL L^{-1} headspace), benzaldehyde (0.025, 0.05 and 0.1 mL L^{-1} headspace) and isoamyl alcohol (0.1 and 0.2 mL L^{-1} headspace). Grapes were inoculated with 10 µL of a suspension of *B. cinerea* (10^4 conidia mL^{-1}) and treated with diacetyl (0.01, 0.02 and 0.05 mL L^{-1} headspace), benzaldehyde (0.01, 0.025, 0.05 and 0.1 mL L^{-1} headspace) and isoamyl alcohol (0.025, 0.05 and 0.1 mL L^{-1} headspace). Apples were inoculated with 10 µL of a suspension of *P. expansum* (10^4 conidia mL^{-1}) and treated with diacetyl (0.01, 0.025 and 0.05 mL L^{-1} headspace), benzaldehyde (0.01, 0.025, 0.05 and 0.1 mL L^{-1} headspace) and isoamyl alcohol (0.1 mL L^{-1} headspace). Finally, apricots were inoculated with 10 µL of a suspension of *M. laxa* or *M. fructicola* (10^4 conidia mL^{-1}) and treated with diacetyl (0.006, 0.02 mL L^{-1} headspace), benzaldehyde (0.02 mL L^{-1} headspace) and isoamyl alcohol (0.02 mL L^{-1} headspace). The incubation chambers were stored at 25 °C for 120 h and the diseases evaluated as described in Section 2.5. The same replication as described in Section 2.5 was also carried out.

2.8. Statistical analysis

Student *t*-test and one-way analysis of variance (ANOVA) followed by a Tukey's HSD (honestly significant difference) post hoc test were used to analyse the results using SPSS statistics software (version 22.0) was used for statistical analysis. A $p < 0.05$ was considered to be significantly different.

3. Results and discussion

3.1. In vitro antifungal activity of the volatilome produced by BUZ-14, I3 and I5 strains

The objective of this assay was to determine the most suitable medium for the production of active antifungal volatiles. The results shown in Table 1 revealed significant differences between the two media tested (863A and MOLPA), but also among the bacterial strains.

In general, the volatilome produced in MOLPA was more active than that produced in 863A. For the species *M. laxa*, *M. fructicola*, *P. italicum* and *P. digitatum,* BUZ-14 showed the highest percentages of inhibition (65.8, 72.0, 80.6 and 20.3 %, respectively), although I3 and I5 were more effective against *B. cinerea* (100 % of inhibition). I5 strain was the least effective against *Penicillium* spp. and *M. fructicola*. Therefore, and given that its activity was lower or similar to I3, the strain I5 was omitted from the subsequent studies. These results provided an indication of the different spectrum of volatiles produced by the 3 biocontrol agents.

Some studies have been published concerning the importance of the nutrients used by the bacteria for the production of antibiotics. Normally, these studies have been conducted with lipopeptides (Fernando et al., 2016; Singh et al., 2014), although there are other studies focused on VOCs. For instance, Gotor-Vila et al. (2017) observed differences in VOCs production depending on the culture medium. The antifungal activity against *M. laxa*, *M. fructicola* and *B. cinerea* was significantly higher when *B. amyloliquefaciens* CPA-8 was grown in TSA instead of NYDA (Nutrient Yeast Dextrose Agar) or NAglu20 (Nutrient Agar supplemented with glucose). Moreover, Raza et al. (2016) tested the influence of two organic fertilizers (BOF) in the production of VOCs by using two *B. amyloliquefaciens* strains against *Ralstonia solanaceum* in tomato, relating the kind of nutrients and also their concentration in the medium with the antimicrobial activity. Fiddaman and Rossall (1994) pointed out the variation in the production of these volatiles depending on the carbohydrate used as a source of carbon. For example, they observed that a strain of *B. subtilis* grown in basic nutrient agar did not provide high levels of volatile production. However, the addition of *D*-glucose or complex substrates such as cellulose gum or carboxymethylcellulose to the medium significantly increased the antifungal activity. Similar to the case of carbohydrates, the same authors found significantly different results depending on the type of peptone. Bacteriological and protease peptones showed the lowest antifungal activity, although this activity was significant in comparison with the control and nutrient agar, reducing the growth of *Rhizoctonia solani* PJF1 by about 50 %. Nevertheless, the use of mycological peptone and tryptone achieved up to 80 % of reduction of the disease.

Normally, volatiles production is merely associated with the bacterium growth. However, Fiddaman and Rossall (1994) suggested that this question required some reconsideration. They observed that nutrient agar supplemented with *L*-glucose was not able to enhance the growth of *B. subtilis*, but was able to increase the activity of volatiles. The stimulation of volatile production by nutritional amendments to an inactive basal medium probably reflects the specific metabolism of the components, rather than a simple stimulation of overall biological activity. Our study has shown that MOLPA medium provides greater antifungal activity than 863A against most of the fungal pathogens tested. MOLPA is a richer medium which, as well as having a greater number of ingredients, including minerals, contains a 3-fold higher concentration

Table 1
In vitro percentage of inhibition (%) of the volatilome produced by *B. velezensis* BUZ-14, I3 and I5 against postharvest fungal pathogens.

Culture medium	BCA	Percentage of inhibition (%)[a]					
		M. laxa	*M. fructicola*	*B. cinerea*	*P. italicum*	*P. expansum*	*P. digitatum*
863A	BUZ-14	2.1 ± 0.6aAv	58.3 ± 3.1aAw	3.7 ± 1.1aAx	54.0 ± 4.5aAy	0.0 ± 0.0aAz	0.0 ± 0.0aAz
	I3	42.8 ± 9.0aBv	5.3 ± 2.2aBw	77.1 ± 8.5aBx	41.1 ± 19.7aAv	5.1 ± 1.0aBw	28.0 ± 9.5aBv
	I5	37.4 ± 5.7aBv	60.6 ± 12.2aAw	47.3 ± 3.9aCw	35.8 ± 15.2aAvw	19.4 ± 4.1aCx	8.2 ± 7.2aCx
MOLPA	BUZ-14	65.8 ± 10.4bAvw	72.0 ± 3.3bAv	54.1 ± 9.9bAw	80.6 ± 2.2bAx	6.0 ± 1.2bAy	20.3 ± 5.6bAz
	I3	38.9 ± 15.1aBv	19.3 ± 2.4bBw	100.0 ± 0.0bBx	70.1 ± 4.7bBy	13.5 ± 5.6bBwz	15.3 ± 0.9bAz
	I5	39.3 ± 0.8aBv	0.0 ± 0.0bCw	100.0 ± 0.0bBx	34.4 ± 11.9aCv	0.0 ± 0.0bCw	12.0 ± 9.2aAx

[a] Each value is the mean of two replicates (5 plates each) ± standard deviation per pathogen, BCA and medium. Different lowercase letters between culture media mean significant differences ($p < 0.05$) for the same BCA and pathogen. Within the same pathogen and medium different uppercase letters represent significant differences ($p < 0.05$) between the inhibitions of BCAs. Different letters (v-z) for the same BCA and medium indicate significant differences ($p < 0.05$) between the inhibition of the pathogens.

of peptone.

The importance of the medium can also be observed in fruit. Calvo et al. (2019) studied the production of lipopeptides and the growth capacity of BUZ-14 in different fruit such as peach, orange and apple. BUZ-14 was only able to grow in peaches, probably influenced by the pH, the kind of organic acids and the nutrients available. In addition, the bacterium was able to produce enough metabolites *in planta* to prevent the brown rot caused by *Monilinia* spp, but it was unable to do so in apples and oranges. Initial *in vitro* tests might help to improve the screening of microorganisms and to identify the appropriate formulation in order to produce a bioformulate rich in the essential nutrients required by the BCAs to produce the desirable antifungal VOCs.

3.2. Antifungal activity of the volatilome produced by BUZ-14 and I3 strains on fruit

The VOCs produced by BUZ-14 grown on MOLPA were the most effective against *Monilinia* spp. and *P. italicum* whereas those of I3 and I5 totally inhibited *in vitro* growth of *B. cinerea*. Thus, BUZ-14 volatilome was tested in apricots against *Monilinia* spp. and in mandarins against blue rot and I3 strain was selected to study the effect against grey mold in table grapes (Fig. 1). For grapes, the severity was reported as percentage of rotten surface, due to the fruit small size, and for apricots and mandarins as millimetres (mm) of the lesion. In both cases the incidence was expressed as percentage of rotten wounds compared with the control.

In contrast to *in vitro* results, BUZ-14 was unable to reduce either the incidence or the severity of *P. italicum* in mandarins (data not shown). However, the inhibitions correlated more closely with *in vitro* results in the case of apricots and grapes. As can be observed in Fig. 1A, I3 strain reduced the incidence and severity of *B. cinerea* from 80 to 45 % and from 60 to 35 %, respectively. This is approximately a 50 % reduction, suggesting that volatiles are another kind of relevant substances with which to continue working and testing. Similar results were obtained against *Monilinia* spp., where both the incidence and the severity were significantly reduced, especially in the case of *M. fructicola* where the severity (lesion diameter) decreased from 60 mm in control fruit to 4 mm in the treated ones.

Gotor-Vila et al. (2017) also studied the antifungal activity of *B. amyloliquefaciens* CPA-8 against *M. fructicola*, *M. laxa* and *B. cinerea* in sweet cherry fruit, although they only reduced significantly the incidence against *M. fructicola*. These results are in agreement with the present study since *M. fructicola* was the most susceptible to the VOCs produced by BUZ-14. On the other hand, an only BCA would not be able to exert an inhibitory effect against several pathogens and on several cultivars. As many studies show, BCAs have more affinity, in terms of antifungal activity, against some specific pathogens and cultivars (Calvo et al., 2017; Gao et al., 2016; Yánez-Mendizábal et al., 2011). The strain I3 has been used against *B. cinerea* because the *in vitro* results were remarkedly better than with BUZ-14. This preliminary screening might be useful in order to obtain better subsequent *in vivo* inhibitions. Other studies such as those conducted by Arrebola et al. (2010), Gao et al. (2018) or Lim et al. (2017) agree on the importance of the biocontrol agent chosen. The results can be notably different depending on the interaction between the BCA and the pathogen, making the commercial product more or less effective during the final application.

3.3. Identification of VOCs produced by B. velezensis BUZ-14 and I3

Extraction from the vial headspace containing MOLP medium was carried out to identify volatile compounds. Table 2 shows the qualitative analysis of VOCs produced by *B. velezensis* BUZ-14 and I3.

Some compounds were detected both in the medium with the bacteria and in the medium alone (blank). These volatiles were 2-heptanone, 2-ethylhexanol, 1-phenylethanol, isovaleric acid, 2,3-dimethylpyrazine and tetramethylpyrazine. However, there were others

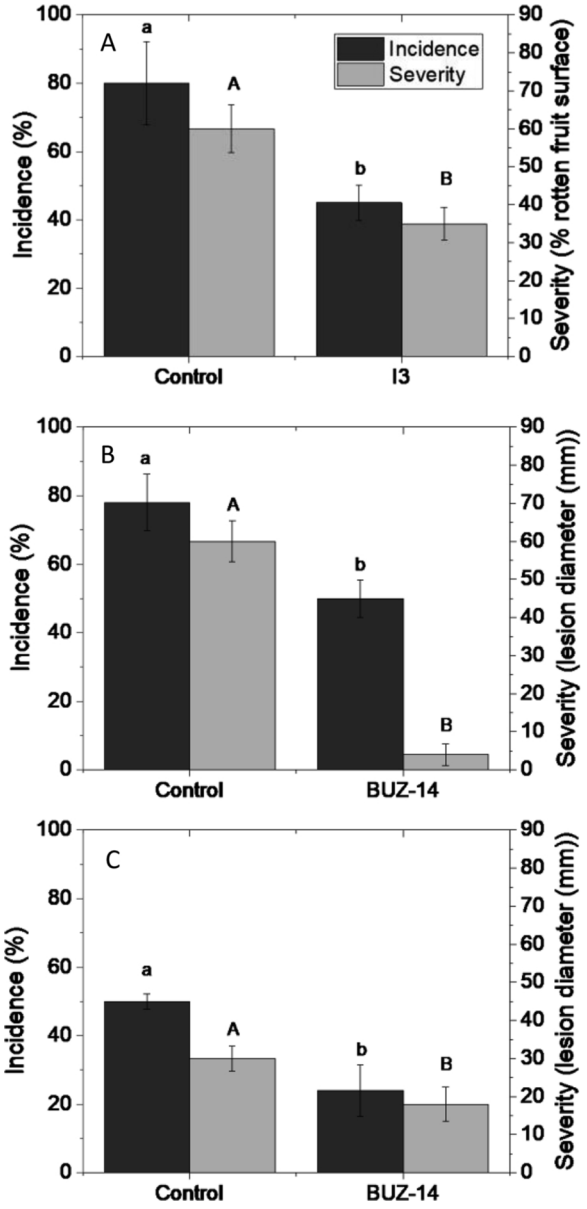

Fig. 1. Antifungal efficacy of the volatilome produced by *B. velezensis*. Incidence was reported as % of rotted wounds and severity as % rotten fruit surface for (A) *B. cinerea* inoculated in table grapes and treated with I3 strain and as lesion diameter (mm) for (B) *M. fructicola* and (C) *M. laxa* inoculated in apricots and treated with BUZ-14. Fruit were incubated at 25 °C for 5 d. Each value is the mean ± standard deviation of three replicates of 9 and 6 fruit each for grapes and apricots, respectively. Within the same figure, incidence (lowercase) and severity (uppercase) different letters represent significant differences ($p < 0.05$) among the control and the BCA strain.

detected in both antagonistic strains or only produced by one of them, *i.e.* benzaldehyde, butyl formate, nonane and pyrazine or 8-methylheptadecane and 2-undecanone. Although the analysis was exclusively qualitative, a preliminary classification with the areas of the compounds obtained during the analysis might be estimated. Hence, the highest areas and therefore the highest concentration of the compounds produced by BUZ-14 were identified in the following order (absolute areas are shown inside the parentheses): isoamyl alcohol (129,541,598) > benzaldehyde (30,792,534) > 1-butanol (19,886,911) > 2-nonanone (14,086,585) > acetoin (13,375,747) > pyrazine (1,831,981); whereas those produced by I3 were as follows: isoamyl alcohol (102,456,847) > 1-butanol (18,236,851) > 2-nonanone (17,102,563) > acetoin (11,854,752) > 2-undecanone (7,432,

Table 2
Identification of the volatiles produced by *B. velezensis* BUZ-14 and I3 in MOLP medium after 24 h of incubation at 30 °C.

Nº	Family	Identified compound	Molecular formula	CAS Number	Retention time	Blank	BUZ-14	I3
1	Aldehydes	Benzaldehyde	C_7H_6O	100-52-7	16.3	nd[a]	x	nd
2	Ketones	Diacetyl/Butane-2,3-dione	$C_4H_6O_2$	431-03-8	7.0	nd	x	x
3		2-heptanone	$C_7H_{14}O$	110-43-0	13.25	x[b]	x	x
4		Acetoin/3-hydroxy-2 butanone	$C_4H_8O_2$	513-86-0	17.31	nd	x	x
5		2-nonanone	$C_9H_{18}O$	821-55-6	19.98	nd	x	x
6		2-undecanone	$C_{11}H_{22}O$	112-12-9	25.26	nd	nd	x
7	Alcohols	1-butanol	$C_4H_{10}O$	71-36-3	12.08	nd	x	x
8		Isoamyl alcohol/3-methyl-1-butanol	$C_5H_{12}O$	123-51-3	14.29	nd	x	x
9		2-ethylhexanol	$C_8H_{18}O$	104-76-7	22.75	x	x	x
10		2,3-butanediol	$C_4H_{10}O_2$	513-85-9	24.21	nd	x	x
11		1-phenylethanol	$C_8H_{10}O$	98-85-1	33.06	x	x	x
12	Esters	Butyl formate	$C_5H_{10}O_2$	589-40-2	12.00	nd	x	nd
13	Hydrocarbures	Nonane	C_9H_2O	111-84-2	8.55	nd	x	nd
14		8-methylheptadecane	$C_{18}H_{38}$	4390-04-9	14.63	nd	nd	x
15	Acids	Isovaleric acid	$C_5H_{10}O_2$	503-74-2	27.55	x	x	x
16	Pyrazines	2,3-dimethylpyrazine	$C_6H_8N_2$	5910-89-4	18.04	x	x	x
17		Pyrazine	$C_4H_4N_2$	290-37-9	14.50	nd	x	nd
18		Tetramethylpyrazine	$C_8H_{12}N_2$	1124-11-4	22.38	x	x	x

[a] nd: not detected.
[b] x: detected.

154) > pyrazine (1,226,521). These metabolites have been partially found in other *Bacillus* strains reported in the bibliography. For example, Arrebola et al. (2010) compared the volatiles produced by two strains of *B. subtilis* and *B. amyloliquefaciens* and their effect on the reduction of *Penicillium* spp. in oranges. They observed significant differences in the volatile production: 21 compounds were detected in *B. subtilis* PPCB001 and only 8 were identified in *B. amyloliquefaciens* PPCB004. However, acetoin was the predominant compound in both BCAs (45.98 and 97.52 %, respectively), but only PPCB004 strain was able to suppress the rot caused by *P. crustosum* in oranges. They also showed that the difference in volatile production depended on the strain used and suggested that the number of volatiles is not the most important factor given that the prevalence of the active volatile might be the crucial factor in the prevention of postharvest diseases. Another study conducted by Chaves-López et al. (2015) also with *B. subtilis* and *B. amyloliquefaciens* strains showed about 30 compounds produced by each strain. They also detected volatiles such as benzaldehyde, 2-heptanone, isoamyl alcohol and pyrazines. The study demonstrated that isoamyl alcohol, 2-methyl propanoic acid, 3-methyl butanoic acid and carbon disulphide reduced the growth of the fungal pathogens *Moniliophthora perniciosa* and *Fusarium oxysporum* in *in vitro* tests.

On the other hand, it is important to point out that the methodology applied to collect and detect VOCs might strongly influence the results and confuse the comparison between studies (Gotor-Vila et al., 2017). Therefore, the variation in volatile identification among studies is to be expected due to the use of different types of equipment and material.

3.4. Antifungal activity of single volatile compound

After the identification of the volatiles produced by the biocontrol agents, the objective was to identify those with antifungal activity. Both *in vitro* and *in vivo* studies as described in Section 2.7 were carried out and the MICs were established.

3.4.1. In vitro tests

In vitro results corresponding to the single volatile MICs can be observed in Table 3. Some compounds were removed from these assays due to their toxicity, availability or extremely bad odour (2-ethylhexanol, butyl formate, nonane, 8-methylheptadecane, isovaleric acid and tetramethylpirazyne). Other volatiles such as acetoin, 2,3-butanediol or 2,3-dimethylpyrazine only exerted a partial inhibition (PI), meaning that the MIC was not achieved and above 1.875 mL L^{-1} headspace, the highest concentration tested. 2-nonanone and 2-undecanone inhibited the growth of *B. cinerea* and *Monilinia* spp. with MICs equal or below 0.125 mL L^{-1} headspace, but at the highest concentration tested had no effect on the growth of the three *Penicillium* strains. Isoamyl alcohol also showed a notable antifungal activity against *B. cinerea* and *Monilinia* spp., with MICs of 0.125 mL L^{-1} for *B. cinerea* and *M. laxa*, and even better for *M. fructicola* with a MIC of 0.038 mL L^{-1}. However, for *Penicilium* spp. the MICs were significantly higher, inoculating 0.938 mL L^{-1} to suppress *P. italicum* and *P. digitatum* and 1.250 mL L^{-1} for *P. expansum*. The most active volatiles were benzaldehyde and diacetyl, obtaining the lowest MICs for all the pathogens tested. For example, only 0.006 mL L^{-1} of diacetyl was necessary to inhibit *Monilinia* spp. in Petri plates and 0.025 mL L^{-1} in the case of *P. digitatum* and *P. italicum*. Benzaldehyde inhibited the growth of *B. cinerea* and *P. expansum* with only 0.063 mL L^{-1} and *M. fructicola* with 0.038 mL L^{-1}.

Other studies have reported the single antifungal activity *in vitro* of VOCs produced by various antagonistic strains. Zheng et al. (2013) used single volatiles identified from *B. pumilus* and *B. thuringiensis* strains and observed that 2-nonanone, β-benzeneethanamine and 2-decanone completely inhibited the growth *in vitro* of *Colletotrichum gloeosporioides* (a causal agent of Anthracnose in mango fruit) at only 0.1 mL L^{-1}, while thymol required between 50 and 100 mg L^{-1} for its total inhibition. Our study has demonstrated that compounds such as 2-nonanone or 2-undecanone also needed low concentrations to completely inhibit *Monilinia* spp. or *B. cinerea*, ranging between 0.025 and 0.125 mL L^{-1}. However, the MICs were higher to inhibit *Penicillium* spp. (> 1.875 mL L^{-1}). Gotor-Vila et al. (2017) established that thiophene produced by *B. amyloliquefaciens* CPA-8 was the volatile responsible for the inhibition of *Monilinia* spp and *B. cinerea*. The thiazole family has also been described as powerfully antifungal, as Yuan et al. (2012) reported, the benzothiazole phenol or 2,3,6 trimethylphenol from *B. amyloliquefaciens* exerted a significant effect against *F. oxysporum*.

The current study demonstrates that diacetyl has the highest antifungal capacity against the fungal pathogens. Antimicrobial properties of diacetyl have been studied over many years (Jay, 1982). However, most of the studies have focussed on its antibacterial activity. Jay (1982) reported its bactericidal capacity against gram negative bacteria and the bacteriostatic effect against gram positives with doses ranging from 200 – 300 mg L^{-1}. On the other hand, there are fewer references to its antifungal activity in the literature. Major studies have investigated the diacetyl produced by *Lactobacillus* strains during fermentation, such as Aunsbjerg et al. (2015) who reported this volatile as the main inhibitor of *Penicillium* spp. strains isolated from fermented

Table 3
In vitro Minimum Inhibitory Concentrations (MICs) of several pure volatiles identified in the volatilome produced by *B. velezensis* BUZ-14 against CFU growth of tested fungal postharvest pathogens.

Volatile	MIC (mL L^{-1} of head space) *in vitro*					
	B. cinerea	*M. fructicola*	*M. laxa*	*P. italicum*	*P. expansum*	*P. digitatum*
Benzaldehyde	0.062	0.038	0.125	0.125	0.062	0.125
Diacetyl	0.006	0.006	0.006	0.025	0.030	0.025
2-Heptanone	0.25	0.038	0.375	0.875	0.625	1.0
Acetoin	PI[a]	PI	PI	PI	PI	PI
2-nonanone	0.062	0.125	0.125	PI	PI	PI
2-Undecanone	0.025	0.125	0.038	PI	PI	PI
1-Butanol	0.625	0.075	1.0	0.150	PI	PI
Isoamyl alcohol	0.125	0.038	0.125	0.938	1.25	0.938
2,3-Butanediol	PI	PI	PI	PI	PI	PI
1-Phenylethanol	0.938	PI	0.938	PI	PI	PI
2,3-Dimethylpyrazine	PI	PI	PI	PI	PI	PI

[a] Partial inhibition at 1.875 mL L^{-1} (maximum concentration tested).

dairy products.

Benzaldehyde is the second most powerful single volatile produced by BUZ-14 after diacetyl (Table 3), its MIC being very low for all the fungal pathogens. Other studies such as that by Gao et al. (2018) have observed a positive correlation between this VOC and the inhibition of *M. fructicola in vitro*. However, it was unable to reduce the disease in peaches. Rajer et al. (2017) demonstrated the effect of benzaldehyde against *Clavibacter michiganensis* subsp. *sepedonicus,* the bacterium responsible for ring rot in potato. It was able to reduce the microbial counts up to 6 log units. Benzaldehyde has also been reported as a promising alternative against *Colletotrichum acutatum in vitro* (Che et al., 2017).

This information provides clues to the importance of testing the volatiles individually since they are mould-specific. To the best of our knowledge, this is the first study showing such low MICs of benzaldehyde and diacetyl against *M. fructicola*, *M. laxa*, *B. cinerea* and *Penicillium* spp. (see Table 3). In addition, 2-nonanone and 2-undecanone have shown low concentrations for *in vitro* inhibition of *Monilinia* spp. and *B. cinerea*. In our opinion, single tests are mandatory in order to identify the most relevant volatiles, but further studies will be required to quantify VOCs produced and to find out the relationship amongst all of them.

3.4.2. *In vivo tests*

Following the *in vitro* results, three pure volatiles (diacetyl, benzaldehyde and isoamyl alcohol) were tested against some postharvest fungal rots in different fruit: *P. italicum* in mandarins, *B. cinerea* in table grapes, *P. expansum* in apples and *M. fructicola* and *M. laxa* in apricots. The results can be observed in Table 4 as well as the standards used and the fruit tested. Moreover, some of the most representative inhibitions are shown in Fig. 2.

P. italicum was suppressed from mandarins up to 80 % when treated with 0.025 mL L^{-1} of diacetyl. However, phytotoxicity (as browning of the fruit skin) caused by the volatile appeared when the dose was increased up to 0.05 mL L^{-1}. Benzaldehyde was not able to totally inhibit blue rot in this fruit even at 0.1 mL L^{-1} (37 % of inhibition). Isoamyl alcohol at 0.1 mLn L^{-1} did not show any inhibition although it caused phytotoxicity at 0.2 mL L^{-1}. Interestingly, *B. cinerea* in grapes showed the best results *in vivo*. Diacetyl at 0.02 mL L^{-1} was enough to control the grey mould in grapes and no phytotoxicity occurred even at 0.05 mL L^{-1}. Benzaldehyde also inhibited the growth of this mould at 0.05 mL L^{-1} but by doubling the dose phytotoxic damage appeared. This fact could generate uncertainty during the development of new applications due to the proximity between the active and the phytotoxic doses. Isoamyl alcohol was also able to inhibit the pathogen, although at higher doses (0.1 mL L^{-1}). The apple showed significant sensitivity to the volatiles used in this study since most of the compounds, except benzaldehyde, caused phytotoxicity to the fruit at the doses employed. The phytotoxic dose of diacetyl was 0.025 mL L^{-1} and that of isoamyl alcohol was 0.1 mL L^{-1}. An example of diacetyl phytotoxicity in apples can be observed in Fig. 2. On the other hand, benzaldehyde was able to inhibit blue rot up to 22 % by treating the fruit with 0.05 mL L^{-1}, but even doubling the dose did not improve the inhibition (Table 4). Finally, the apricots showed the highest sensitivity to diacetyl and benzaldehyde since doses as low as 0.02 mL L^{-1} caused phytotoxicity. However, significant differences were found between both species when isoamyl alcohol was applied (Table 4). *Monilinia fructicola* did not suffer any antifungal effect and *M. laxa* was completely inhibited with 0.02 mL L^{-1} isoamyl alcohol.

Phytotoxicity in the fruit used during the study was manifested as browning skin, rendering the product completely uncommercial. The fruit with phytotoxicity did not showed any fungal disease. This could be due to the excessive doses of the volatiles, which might directly inhibit the growth of the pathogens, or because of the negative correlation between the phytotoxicity and the fungi. It could be that the effect on the plant physiology caused by the volatiles also has an influence over the hyphal or spore development. More studies are required in order to obtain clearer conclusions about this issue. These results demonstrated the importance of the type of cultivar when these volatiles were applied. The phytotoxic doses shown by apricots and also apples differ completely from that shown by grapes or mandarins. This study also showed that the doses of the volatile must be regulated depending on the cultivar and the pathogen. As can be observed in Table 4, the suppression of *B. cinerea* in grapes was very successful. The three compounds were able to control the grey mould and at low doses, mainly in the case of diacetyl and benzaldehyde, but in the latter compound it is important to highlight the close proximity between the MIC (0.05 mL L^{-1}) and the phytotoxic dose (0.1 mL L^{-1}).

To the best of our knowledge, *in vivo* studies regarding the antifungal activity of volatiles produced by microorganisms and mainly by bacteria are scarce. Most of the published reports have focused on the identification of the volatiles produced by the strains and subsequent *in vitro* tests (Lim et al., 2017; Morita et al., 2019; Wu et al., 2019). Probably many authors have decided to reject the tests *in planta* due to the difficulties in their implementation. However, we are optimistic about active packaging with these compounds, either including the bacteria or the VOCs as pure compounds. Zhou et al. (2019) have recently studied the antifungal activity of VOCs produced by *B. subtilis* CF-3 against *M. fructicola* in peaches. In this study, benzothiazole was the compound responsible for the inhibition. It was suggested that their study should serve as a theoretical basis for future applications but toxicological risks were not mentioned. However, Ginsberg et al. (2011) had already reported the possible toxic effects in rats when they are exposed to this compound, but also conclude that more studies would

Table 4
Percentage of inhibition of pure volatiles identified in the volatilome produced by *B. velezensis* BUZ-14 against fungal postharvest pathogens inoculated on fruit.

Pathogen	Fruit	Volatile	Concentration (mL L^{-1})	% Inhibition[a]
P. italicum	Mandarin	Diacetyl	0.01	60.5 ± 12.8a
			0.025	80.6 ± 14.3a
			0.05	100b (phytotoxicity)
		Benzaldehyde	0.025	10.3 ± 3.6a
			0.05	16.5 ± 5.2a
			0.1	37.1 ± 6.7b
		Isoamyl alcohol	0.1	NIa[b]
			0.2	100b (phytotoxicity)
B. cinerea	Grape	Diacetyl	0.01	80.7 ± 9.3a
			0.02	100b
			0.05	100b
		Benzaldehyde	0.01	9.0 ± 2.5a
			0.025	12.6 ± 4.3a
			0.05	100b
			0.1	100b (phytotoxicity)
		Isoamyl alcohol	0.025	20.0 ± 6.4a
			0.05	60.5 ± 7.4b
			0.1	100c
P. expansum	Apple	Diacetyl	0.01	25.6 ± 5.2a
			0.025	64.3 ± 7.8b
			0.05	100c (phytotoxicity)
		benzaldehyde	0.01	NIa
			0.025	15.1 ± 3.3b
			0.05	22.6 ± 12.2b
			0.1	24.2 ± 10.1b
		Isoamyl alcohol	0.1	100 (phytotoxicity)
M. fructicola	Apricot	Diacetyl	0.006	NIa
			0.02	100b (phytotoxicity)
		Benzaldehyde	0.02	100 (phytotoxicity)
		Isoamyl alcohol	0.02	4.2 ± 2.4
M. laxa	Apricot	Diacetyl	0.006	NIa
			0.02	100b (phytotoxicity)
		Benzaldehyde	0.02	100 (phytotoxicity)
		Isoamyl alcohol	0.02	100

[a] Each value is the mean ± standard deviation of three replicates of 9 fruit each for grapes (one wound per fruit) and 6 fruit each for mandarins, apples and apricots (two wounds per fruit). Different letters between media mean significant differences ($p < 0.05$) for the same pathogen, fruit and volatile compound.
[b] NI: no inhibition.

be needed in order to support their preliminary study. This means that there remain many gaps in our knowledge of this compound and many others. Thus, the residue remaining in the fruit must also be studied in order to test for any possible undesirable odour remaining in the product but also to ensure safety for human health since some of these compounds could have a certain degree of toxicity.

Diacetyl has hardly any toxicity and has been classified as GRAS (Generally Recognized As Safe) by the American Food and Drug Administration (FDA). Moreover, no scientific reports examining the antifungal activity of diacetyl against spoilage postharvest moulds have been found in the bibliography. Most reports address its antibacterial activity so, to the best of our knowledge, this is the first article showing the antifungal activity of this VOC. Benzaldehyde is another volatile produced by BUZ-14 whose *in vitro* antifungal activity has been widely demonstrated (Table 3). This compound has been classified as a GRAS substance by the FDA and as it is used as a flavouring substance (CoE 101) to confer almond flavour to foods in the European Union. The oral LD_{50} of benzaldehyde in rats and mice ranged from 800 to 2850 mg kg^{-1} (Andersen, 2006). As previously mentioned, there are numerous publications in the literature about *in vitro* VOCs effectiveness against many diseases. However, the activity of this VOC against postharvest rots in fruit has not been studied neither in depth in the scientific literature.

4. Conclusions

This study has assessed a mode of action based on the volatilome produced by different strains of *B. velezensis* species, mainly by BUZ-14 and I3 which have been especially active against brown and grey mould, respectively. The identification of the VOCs has shown at least 12 compounds for which *in vitro* tests have highlighted diacetyl, benzaldehyde, 2-heptanone and isoamyl alcohol with MICs lower than those previously reported in the scientific literature. The study in fruit has demonstrated the potential of compounds such as diacetyl or benzaldehyde, especially the first one, as promising antifungal volatiles against *B. cinerea* in grapes. Thus, an active packaging with these GRAS substances might be a good alternative to control grey mould during transportation and commercialization. However, prior to application it is crucial to determine the possible detrimental effects in the flavour and also the safe doses to avoid phytotoxic effects.

CRediT authorship contribution statement

Héctor Calvo: Investigation, Writing - original draft, Data curation, Formal analysis. **Isabel Mendiara:** Investigation, Methodology. **Esther Arias:** Conceptualization, Visualization, Supervision. **Ana Pilar Gracia:** Investigation. **Domingo Blanco:** Visualization, Supervision. **María Eugenia Venturini:** Conceptualization, Visualization, Writing - review & editing, Supervision, Funding acquisition.

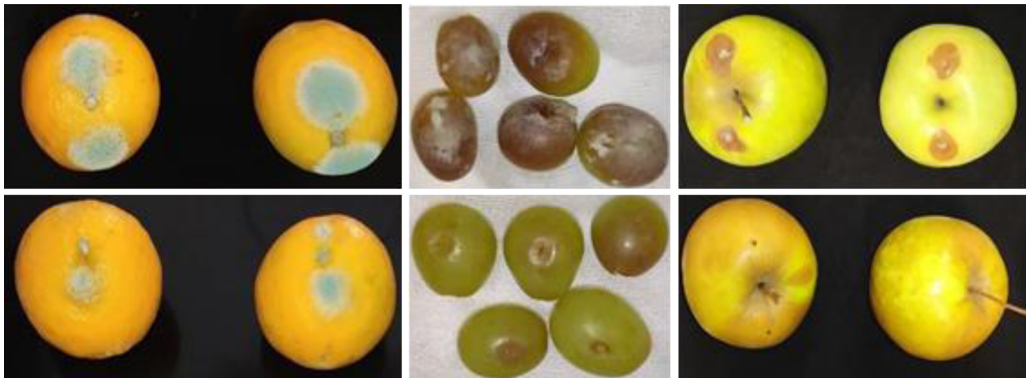

Fig. 2. Mandarins (left) grapes (centre) and apples (right) treated with diacetyl 0.01 mL L^{-1}, benzaldehyde 0.05 mL L^{-1} and diacetyl 0.05 mL L^{-1}, respectively. The pictures located above are the control and those below correspond to the treated fruit.

Declaration of competing interests

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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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2020.111208.

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