



The role of iturin A from *B. amyloliquefaciens* BUZ-14 in the inhibition of the most common postharvest fruit rots

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ABSTRACT

The aim of this work was to elucidate the role of the secondary metabolites produced by *B. amyloliquefaciens* BUZ-14 against *B. cinerea*, *M. fructicola*, *M. laxa*, *P. digitatum*, *P. italicum* and *P. expansum* both *in vitro* and *in planta*. The entire cell free supernatant (CFS) and the lipopeptide fraction (LPF) showed similar antifungal activities, completely inhibiting all the fungi at dilutions of 1:24 or even lower, whereas the non-butanolic fraction (NBF) barely inhibited the fungi. However, when the LPF and CFS were applied on fruit, only brown rot in peaches and blue rot in apples was totally inhibited. The main families of metabolites in the LPF were iturin A, fengycin and surfactin with maximum concentrations of 407, 853 and 658 $\mu\text{g mL}^{-1}$, respectively. Subsequently, a TLC-bioautography revealed iturin A as the key metabolite in the inhibitions and allowed us to establish *in vivo* MICs of 16.9 and 33.9 $\mu\text{g mL}^{-1}$ for *Monilinia* species and *P. expansum*, respectively. The application of 24 h-old BUZ-14 cultures suppressed brown rot in peaches and also blue rot in apples but failed to inhibit the other diseases. However, BUZ-14 was only able to grow and produce iturin A in peaches so we can deduce that the amount of iturin A brought with the cultures ($36 \pm 14 \mu\text{g mL}^{-1}$) could be enough to control both diseases. The strong antifungal activity of the iturin A present in the BUZ-14 CFS suggests that it could be successfully used for postharvest disease control. However, future research is necessary to maximize the iturin A production by *B. amyloliquefaciens* BUZ-14 in order to optimize a commercial application.

1. Introduction

Nowadays, pesticides and fungicides are the main system used to control biotic agents responsible for pre- and postharvest rot. However, current legislation covering pesticides has become stricter by reducing the MRLs (Maximum Residue Limits) in plants and forbidding numerous effective fungicides because of the development of fungicide-resistant strains of pathogens, the detection of chemical residues in the food chain and their toxicity for human health and the environment. Therefore, new alternatives such as biological control by using natural antagonistic microorganisms are currently being studied in depth (Chung et al., 2008; Zhao et al., 2013). Several wild-type *Bacillus subtilis* and *B. amyloliquefaciens* strains have been reported for their effectiveness in the biocontrol of multiple plant diseases caused by soil borne and postharvest pathogens (Alvarez et al., 2012; Arguelles-Arias et al., 2009; Arrebola et al., 2010; Calvo et al., 2017; Chen et al., 2009, 2016; Chung et al., 2008; Gong et al., 2015; Hinarejos et al., 2016; Romero et al., 2007; Toral et al., 2018; Torres et al., 2017; Touré et al., 2004; Yáñez-Mendizábal et al., 2012). Most authors sustain that antibiosis is the main mechanism of action against pathogens since some *Bacillus*

strains are capable of producing a substantial number of antifungal secondary metabolites, especially the non-ribosomally synthesised cyclic lipopeptides (Alvarez et al., 2012; Torres et al., 2017; Veras et al., 2016). These substances are divided into three families according to their structure: surfactin, fengycin and iturin. The chemical structure has previously been studied by numerous authors, regarding the type and sequence of amino acid residues, the nature of the peptide and length and branching of the fatty acid chain (Hinarejos et al., 2016; Ongena and Jacques, 2008; Stein, 2005; Torres et al., 2016; Xu et al., 2013). The mechanism of direct antibiosis associated with *Bacillus* strains against foliar and postharvest diseases has been attributed mostly to iturins and fengycins (Arrebola et al., 2010; Romero et al., 2007; Yáñez-Mendizábal et al., 2012) although other metabolites such as polyketides (Chen et al., 2006, 2009), siderophores (Li et al., 2014), bacteriocins (Ayed et al., 2015) and volatile organic compounds (VOCs) (Gotor-Vila et al., 2017) must not be forgotten. These metabolites and compounds can be produced by the biological control agent (BCA) in the formulation prior to the application with the mechanism of action based on a direct antibiosis. Otherwise, these compounds could be produced directly in the plant while the BCA is growing *in situ* (Touré

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et al., 2004). The latter option would increase the possibility of success because the mechanisms of action such as competition for nutrients or colonization would be taken into account, as well as the fact that the direct inhibition provoked by the metabolites could be stronger. Nevertheless, direct antibiosis has not only been cited as a mechanism for controlling disease since it has also been demonstrated that most of the metabolites produced by *B. subtilis* or *B. amyloliquefaciens* strains can induce systemic resistances (ISR) in plants (Cawoy et al., 2014; Choudhary and Johri, 2009; Ryu et al., 2004). The role of lipopeptides from *B. subtilis* (Ongena and Jacques, 2008; Ongena et al., 2005, 2007) and from *B. amyloliquefaciens* (Chowdhury et al., 2015) has been studied on various plants for inducing immune responses. As an example, fengycins from *B. subtilis* M4 could be involved in the systemic resistance-eliciting effect of this strain, as these molecules may induce the synthesis of plant phenolics involved in or derived from the defence-related phenylpropanoid metabolism (Ongena et al., 2005).

The aim of this work was to elucidate the role of the secondary metabolites produced by *B. amyloliquefaciens* BUZ-14 against *B. cinerea*, *M. fructicola*, *M. laxa*, *P. digitatum*, *P. italicum* and *P. expansum* both *in vitro* and *in planta*. For this purpose, 4 steps were addressed (i) characterisation of the *in vitro* and *in vivo* antifungal activity of CFS, LPF and NBF through the determination of their MICs, (ii) identifying and quantifying the lipopeptides produced (iii) determining the lipopeptide responsible for the antagonistic activity and (iv) quantifying the lipopeptide production on fruit.

2. Materials and methods

2.1. Fungal pathogens

The fungal pathogen strains used in this study were *Botrytis cinerea* VG 1, *Monilinia fructicola* VG 104, *M. laxa* VG 105, *Penicillium digitatum* VG 20, *P. expansum* CECT 20140 and *P. italicum* VG 101. They were obtained from the Plant Food Research Group culture collection (Zaragoza, Spain) and incubated on potato dextrose agar (PDA) (Oxoid Ltd; Basingstoke, Hampshire, England) and potato dextrose broth (PDB).

2.2. Fruit samples

In this study different fruits were used depending on the diseases tested. Peaches (cv. Calante), strawberries (cv. Fortuna), mandarins (cv. Clementina) and apples (cv. Golden Delicious) were used for *Monilinia* spp., *B. cinerea*, *P. digitatum* and *P. italicum*, and *P. expansum* inoculations, respectively. All fruits were collected from local packing greenhouses and were grown in different areas of Spain (La Almunia de Doña Godina, Tarragona and Teruel). Prior to the experiments, all fruits were surface-disinfected by immersion for 2 min in 1% sodium hypochlorite, rinsed with tap water, and allowed to air-dry at room temperature (20 °C).

2.3. Bacterial strain identification

B. amyloliquefaciens BUZ-14 was identified through a phylogenetic analysis of its 16S rDNA and partial gyrase gene sequences *gyrB*. The genomic DNA of BUZ-14 was extracted using a FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corporation, Taiwan). The 16S rDNA was amplified by PCR using the universal primers 8F (5'-AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'-CGG TTA CCT TGT TAC GAC TT -3') (Microsynth AG, Switzerland). Part of the *gyrB* sequence was amplified with *gyrB*-BUZ-14F (5'-ACC GGA ACG ATT ACG CAC TT -3') and *gyrB*-BUZ-14R (5'-AGG GTC CAT TGT CGT TTC CC -3') (Microsynth AG, Switzerland) primers previously designed with Primer3 v. 0.4.0 software (Untergasser et al., 2007), based on the type strain *Bacillus amyloliquefaciens* FZB42.

2.4. *In vitro* antifungal activity of the cell-free supernatant (CFS), non-butanolic fraction (NBF) and lipopeptide fraction (LPF) from *B. amyloliquefaciens* BUZ-14 against fungal pathogens

The antifungal activity of CFS, NBF and LPF was tested *in vitro* against the fungal pathogens cited in section 2.1. BUZ-14 was incubated in 863 medium (20 g L⁻¹ dextrose, 10 g L⁻¹ peptone, 10 g L⁻¹ yeast extract) at 30 °C for 96 h. The separation of supernatant from cells was done by centrifugation (Megafuge Heraeus 1.0R, Thermo Fisher, UK) at 4000 × g for 10 min in 50 mL falcon tubes containing 25 mL of bacterial culture. The supernatant was decanted and consecutively autoclaved at 121 °C for 15 min. Lipopeptide extraction was carried out following a method described in the bibliography (Yazgan et al., 2001), by which n-butanol was added to the supernatant (ratio 1:4) and the sample was centrifuged at 4000 × g in order to separate the butanolic layer containing all the lipopeptides. On the other hand, the non-butanolic layer contained the rest of the substances produced by BUZ-14 in the conditions explained above. Finally, each fraction was dried using a rotary evaporator. The three fractions were processed in order to maintain the same ratio of concentration throughout the experiment. The fractions were stored at -80 °C until their laboratory use.

For the *in vitro* inhibition tests, 48-well plates were used. The MIC (Minimal Inhibitory Concentration) of each fraction was found by diluting the antimicrobial into PDB medium. Five hundred µL of the medium were added into the well, followed by 30 µL of each pathogen (10⁴ conidia mL⁻¹). Finally, different quantities of LPF, NBF or CFS were transferred until obtaining 1:6, 1:12, 1:24, 1:48, 1:80 and 1:100 dilutions. A positive control without the antimicrobial fraction and a negative control without the pathogen were also included. The plates were incubated at 25 °C for 7 days. The results were obtained by observing visually the presence (growth) or absence (non-growth) of fungi. Five replicates per pathogen were conducted and the experiment was done in three independent days.

2.5. Antifungal activity of CFS and LPF from BUZ-14 on fruit

In this study, the antimicrobial activity on fruit of the CFS and LPF obtained from 96 h-old cultures was assessed. Fruit were wounded with a sterile tip and 10 µL of the respective pathogen (10⁴ conidia mL⁻¹) was inoculated. After 1 h of absorption of the conidial suspensions, the CFS and LPF were inoculated at 1:6, 1:12, 1:24 and 1:48 dilutions. The fruits were stored at 20 °C for 5 days and 80% R.H. (relative humidity). The severity was evaluated as a percentage of reduction of the disease by measuring the lesion diameter in treated fruits and the control. Fifteen fruits per pathogen and treatment (one wound per fruit) were disposed. The entire experiment was repeated in three different days.

2.6. Isolation and identification of antifungal lipopeptides

In order to find out the lipopeptides responsible for direct antibiosis, a Thin Layer Chromatography (TLC) followed by bioautography was performed and the active fractions identified by Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS^E) analysis. The lipopeptides present in the LPF were also identified and quantified.

2.6.1. Thin Layer Chromatography (TLC) and bioautography

Ten µL aliquots from the lipopeptide fraction produced by BUZ-14 and the reference strain *B. amyloliquefaciens* FZB42 after 96 h of incubation at 30 °C were spotted onto TLC aluminium sheets coated with silica gel 60 F254 5 × 10 cm (Sigma Aldrich, Spain). The sheets were placed in a separation chamber containing chloroform/methanol/water (65:25:4, v/v/v) as mobile phase, following the protocol proposed by Razafindralambo et al. (1993). The spots were detected under 254 nm UV light and their retention factor (R_f) determined. The strain FZB42, purchased from the DSMZ collection (Germany), was used as a

bacillomycin producer since no commercial standard was found.

The bioautography was performed as described by Chen et al. (2016) in order to identify the family of lipopeptides responsible for fungal inhibition. Briefly, the TLC plates were placed in 90 × 90 mm Petri plates and covered with melted PDA medium inoculated with pathogens at $1\text{--}3 \times 10^4$ conidia mL⁻¹. The moulds tested were those cited in Section 2.1. The plates were incubated at 25 °C for 7 days and the R_f of the active fraction was determined. Then, the R_f was compared to that in the previously conducted TLC and the active lipopeptide was established. Moreover, each antifungal compound was scraped from the silica gel and extracted with 2 mL of methanol under magnetic conditions stirred at 300 rpm overnight. Afterwards, the sample was centrifuged at 10000 × g and the resulting supernatant was subjected to Liquid Chromatography-Mass Spectrometry analysis as detailed in section 2.6.3. Five replicates were used per pathogen and the experiment was repeated on three different days.

2.6.2. High performance liquid chromatography Diode-Array detector (HPLC-DAD)

The lipopeptides were identified and quantified after 24, 48, 72 96 and 120 h of incubation using an Agilent 1100 liquid chromatograph coupled to an Agilent 1200 Series DAD detector (Agilent, USA) with a Lichrospher RP18 column, 5 μm 250 × 4 mm (Sigma Aldrich, Spain). The mobile phase A was 0.1% formic acid in acetonitrile, and mobile phase B was 0.1% formic acid in Milli-Q water. The flow rate was 1 mL min⁻¹ and the temperature of the column was set at 30 °C. The gradient started at 35% A and was held for 8 min. From 8 min to 10 min the gradient was raised to 40% A and kept for 15 min. Then, the gradient was raised to 80% for 5 min and held at 80% for 15 min. Finally, the gradient reached 100% for 5 min and returned to the initial conditions during 5 min. The total run time was 60 min. Lipopeptides were monitored at 214 nm. The analytical standards were iturin A (purity ≥ 95%), Chemical Abstract Service identification number: 52229-90-0; surfactin ≥ 98%, 24730-31-2 and fengycin ≥ 90%, 102577-03-7. All of them were purchased from Sigma Aldrich, Spain. These analytical standards were used for the identification and quantification of the samples. The calibration curve was determined by using standard solutions containing 20, 100, 400, 1000 and 1400 μg mL⁻¹ of each compound. In all cases, the solutions were injected in the chromatograph three times and the extraction process was carried out on three separate days.

2.6.3. Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS^E) analysis

The lipopeptide fractions obtained after 96 h of incubation time were also subjected to an LC-ESI-MS^E analysis to identify the different isoforms of each lipopeptide as well as the compounds which exhibited antifungal activity on the TLC plates. For this purpose, an Acquity UPLC H-CLASS system supplied by Waters (Milford, USA) was used. Chromatographic separation was performed on a Waters Acquity UPLC BEH C₁₈ (1.7 μm, 2.1 mm × 100 mm) at 40 °C. Mobile phase A was MilliQ-water with 0.1% formic acid and mobile phase B acetonitrile with 0.1% formic acid. The flow rate was 0.50 mL min⁻¹ and the injection volume 10 μL. The time program for multi-step gradient was 0–5 min, 70% A–30% B to 5% A–95% B, 5–10.10 min, 5% A–95% B to 70% A–30%. The run time was 12 min and the sample temperature was set at 10 °C. Mass spectra were acquired using a time-of-flight (TOF) MS Synapt G2 High Definition Mass Spectrometer supplied by Waters (Milford, USA). Nitrogen was used as a desolvation gas at 800 L h⁻¹ flow. The cone gas flow was 40 L h⁻¹. The ion source parameters were corona current 3 μA, sample cone 40 V and desolvation gas temperature 500 °C. Electrospray ionization mode (ESI) in positive polarity was selected; the source temperature was 120 °C. The scan time was 1 s and the mass range considered was *m/z* 100–2400. MassLynx v4.1 software supplied by Waters (Milford, USA) was used for the analysis of the results obtained, which considers the isotopic model and the elemental

composition.

2.7. BUZ-14 growth and iturin A production on fruit

The growth curves of BUZ-14 and the production of iturin A in fruit were determined. Fruits were wounded as described in section 2.5 and 10 μL of the respective pathogen (10^4 conidia mL⁻¹) was inoculated. After 1 h at room temperature to favour the absorption of the conidia suspension, a 24 h-old culture (10 μL) of *B. amyloliquefaciens* (10^8 CFU mL⁻¹) was inoculated and the fruit was stored at 20 °C and 80% R.H. for 7 days. The percentage of reduction of the fungal growth was then recorded. To determine the growth of the BCA, 10 g of fruit wound was cut through with a sterile scalpel. The fruit plugs were placed in filter blender bags and diluted 1:5 with 0.1% sterile peptone water. The mixture was homogenized in a Stomacher 400 Circulator laboratory blender (Seward Laboratory, London, England) for 120 s at 260 rpm and the resulting suspension was diluted, plated on TSA plates and counted after 24 h at 30 °C. The extraction of lipopeptides was carried out in the homogenate following the procedure described in section 2.4. The subsequent quantification was conducted with the HPLC-DAD (see section 2.6.2) and the amount of lipopeptides quantified and expressed in μg g⁻¹ of fruit tissue. Fifteen fruits per fungal pathogen were used as replicates and the experiment was carried out on 3 separate days.

2.8. Statistical analysis

Data were statistically treated by t-student analysis using SPSS software (SPSS statistics 22.0). The statistical differences between means of different treatments were assessed at *P* < 0.05.

3. Results and discussion

3.1. *B. amyloliquefaciens* BUZ-14 strain phylogenetic identification

The bacterial strain tested in this study has been previously identified by Calvo et al. (2017) as *Bacillus amyloliquefaciens* and named BUZ-14. However, due to the genetic similarity among species of the *Bacillus* group and the several recently renamed subspecies, we have made a more in-depth study of its identification. *B. amyloliquefaciens* BUZ-14 16S rDNA and *gyrB* sequences were obtained and deposited in Genbank with accession numbers MF461174 for 16S rDNA and MF770248 for *gyrB*. The sequences were processed using the BLAST tool provided by Genbank. The information obtained from these results enabled a phylogenetic tree to be constructed (Supplemental files: Figs. 1 and 2). In order to achieve a more reliable level of identification, the subunit B protein of DNA gyrase (*gyrB*) was subjected to sequencing (Hossain et al., 2015; Wang et al., 2007). This strain was designated as *B. amyloliquefaciens* subsp. *plantarum* BUZ-14, although recent studies have proposed including this classification within *B. velezensis* together with other *Bacillus* species (Dunlap et al., 2016). Plant-associated *B. amyloliquefaciens* strains belonging to subsp. *plantarum* are distinguished from others such as subsp. *siamensis* or *amyloliquefaciens* by their capacity to stimulate plant growth, to colonize the plant rhizosphere and to suppress competing phytopathogenic bacteria and fungi.

3.2. *In vitro* antifungal activity of the cell-free supernatant (CFS), non-butanolic fraction (NBF) and lipopeptide fraction (LPF) from *B. amyloliquefaciens* BUZ-14 against fungal pathogens

The aim of this assay was to characterize the antifungal activity of BUZ-14 secondary metabolites by establishing differences among the CFS, the NBF and the LPF. The activity of the three fractions was evaluated in liquid medium after 96 h of incubation at 30 °C in 863 broth against the fungal pathogens cited in section 2.1. In this study, we have separated the lipopeptides (LPF) from other compounds (NBF) and

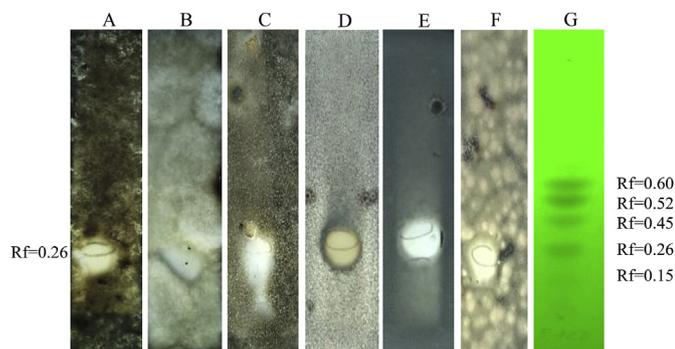


Fig. 1. Thin layer chromatography (TLC)-bioautography analysis of the lipopeptide fraction (LPF) of BUZ-14 after 96 h of incubation in 863 medium at 30 °C. TLC plates A; B; C; D; E and F were covered with PDA inoculated with a fungal spore suspension at a concentration of $1-3 \times 10^5$ conidia mL^{-1} and incubated for 7 days at 25 °C. A- *M. fructicola*; B- *M. laxa*; C- *B. cinerea*; D- *P. italicum*; E- *P. expansum*; F- *P. digitatum*. View in a fluorescence chamber at 254 nm of TLC three family spots (fengycin, iturin and surfactin). The inhibition zone for A, B, C, D, E and F can be observed at $R_f = 0.26$.

we have observed that all the phytopathogens were greatly inhibited by the LPF (Table 1). The results showed that the NBF was barely effective against the six postharvest fungal pathogens tested. However, the LPF achieved the inhibition of all the pathogens in a 1:24 dilution for *B. cinerea* and *P. digitatum*, 1:48 for *M. fructicola*, *P. italicum* and *P. expansum* and with *M. laxa* as the most susceptible species with a MIC

below 1:100. The activity observed *in vitro* in the CFS is related to all the active compounds produced by BUZ-14, so a maximum inhibition was expected. However, the MICs obtained were only slightly higher or even the same as those of the LPF, supporting the idea that the antifungal activity lies with the lipopeptides. Nevertheless, not all the pathogens needed the same concentration, and it was observed that *M. laxa* was much more sensitive to these compounds. It is known that the same substance can exert different antimicrobial activity depending on the target microorganism (Ambrico and Trupo, 2017; Chen et al., 2009; Ji et al., 2013). Accordingly, Yáñez-Mendizábal et al. (2012) identified a fengycin-like lipopeptide as the main metabolite responsible for *Monilinia* spp. inhibition by *B. subtilis* CPA-8. However, Zhao et al. (2013) determined that in the case of the *Bacillus* BH072 strain, iturin A was the main inhibitor of *B. cinerea*. Additionally, these kinds of plant-growth-promoting bacteria produce several non-ribosomal polyketides and lipopeptides, apart from iron-siderophores, bacteriocins and volatile compounds with relevant antifungal activity (Alvarez et al., 2012; Arguelles-Arias et al., 2009; Ayed et al., 2015; Borriss et al., 2011; Romero et al., 2007).

3.3. LPF and CFS antifungal activity on fruit

The substantial activity demonstrated by the metabolites produced by BUZ-14 against fungal pathogens *in vitro* does not ensure the same effect on a food matrix. Usually, MICs are significantly higher *in vivo* since the fungi efficiently used the nutrients available in the fruit while the low pH, acids and phenols are all barriers to the development of

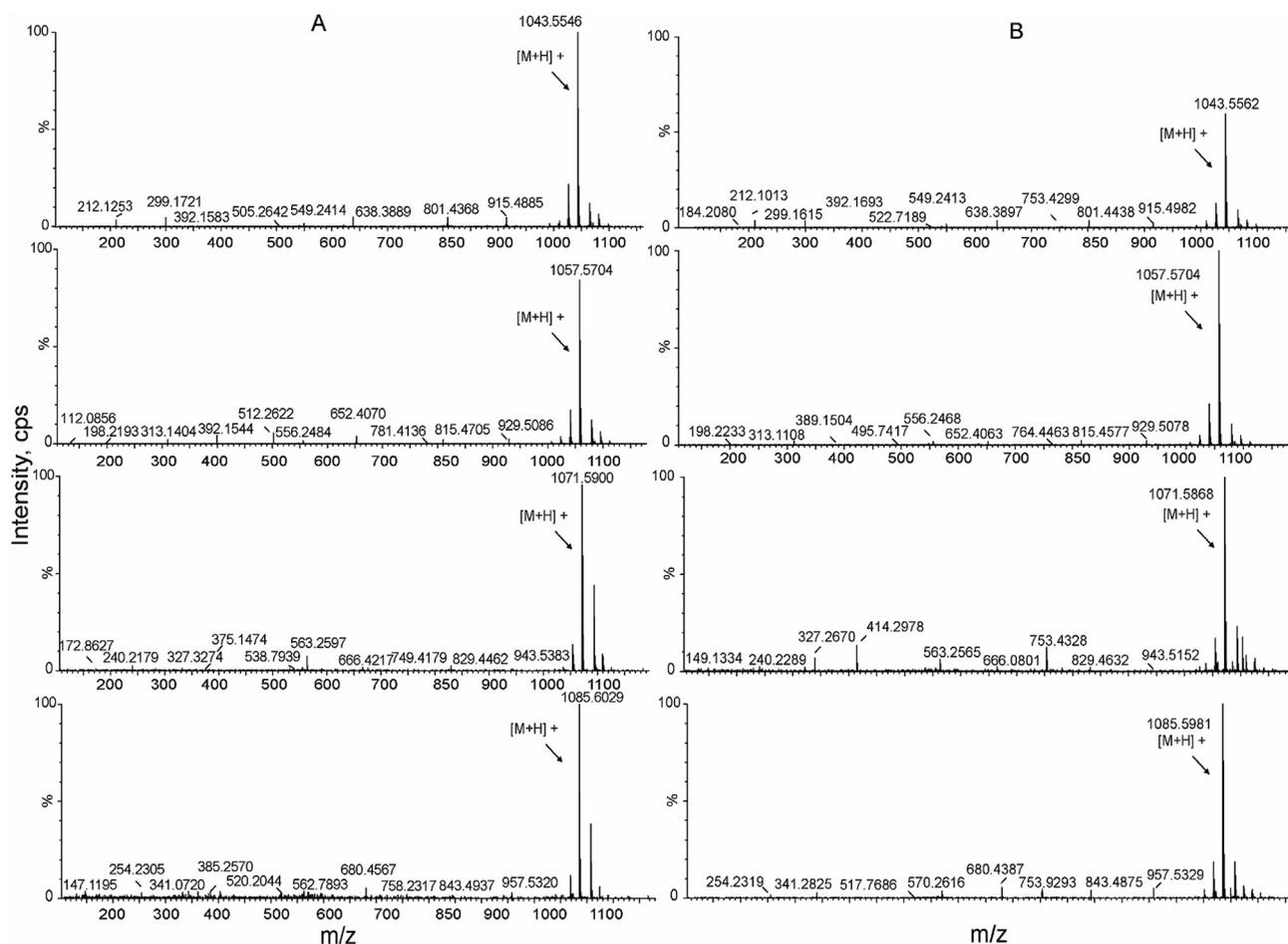


Fig. 2. Comparison between MS^E spectra from commercial Iturin (A) and the active compound from *B. amyloliquefaciens* BUZ 14 (B). (A) MS^E spectra of the four peaks detected for m/z (1043.5562; 1057.5704; 1071.5900; 1085.5981). (B). MS^E spectra obtained after scraping the active compound from TLC plates at 96 h of incubation time.

Table 1

In vitro antifungal activity assayed by the well-dilution method in liquid medium of the Cell-Free Supernatant (CFS), the Lipopeptide Fraction (LPF) and the Non-Butanolic Fraction (NBF) produced by *B. amyloliquefaciens* BUZ-14 after 96 h of incubation at 30 °C in 863 medium against six postharvest fungal pathogens. Five replicates per pathogen and dilution were conducted on 3 different days.

Fraction	Concentration (Dilution)	<i>B. cinerea</i>	<i>M. fructicola</i>	<i>M. laxa</i>	<i>P. digitatum</i>	<i>P. italicum</i>	<i>P. expansum</i>
Lipopeptide fraction (LPF)	1:6	NG ^a	NG	NG	NG	NG	NG
	1:12	NG	NG	NG	NG	NG	NG
	1:24	NG	NG	NG	NG	NG	NG
	1:48	G ^b	NG	NG	G	NG	NG
	1:80	G	G	NG	G	G	G
	1:100	G	G	NG	G	G	G
Non-butanolic fraction (NBF)	1:6	NG	NG	NG	NG	G	G
	1:12	G	NG	G	G	G	G
	1:24	G	G	G	G	G	G
	1:48	G	G	G	G	G	G
	1:80	G	G	G	G	G	G
	1:100	G	G	G	G	G	G
Cell-free supernatant (CFS)	1:6	NG	NG	NG	NG	NG	NG
	1:12	NG	NG	NG	NG	NG	NG
	1:24	NG	NG	NG	NG	G	G
	1:48	NG	G	NG	G	G	G
	1:80	G	G	G	G	G	G
	1:100	G	G	G	G	G	G

^a Non-growth of the fungal pathogen.

^b Growth of the fungal pathogen.

Table 2

Percentage of reduction of the lesion diameter of *B. cinerea* in strawberries, *M. fructicola* and *M. laxa* in peaches, *P. digitatum* and *P. italicum* in mandarins and *P. expansum* in apples. The LPF and CFS were obtained from a 96 h-old culture of *B. amyloliquefaciens* BUZ-14 incubated at 30 °C in 863 medium. Fruits were stored at 20 °C and 80% R.H. for 5 days.

Fraction	Concentration	Reduction of lesion diameter (%) ^a					
		<i>B. cinerea</i>	<i>M. fructicola</i>	<i>M. laxa</i>	<i>P. digitatum</i>	<i>P. italicum</i>	<i>P. expansum</i>
Lipopeptide fraction (LPF)	1:6	72.6 ± 5.7 ^b	100.0 ± 0.0	100.0 ± 0.0	9.5 ± 3.3	7.1 ± 2.1	100.0 ± 0.0
	1:12	31.6 ± 9.6	100.0 ± 0.0	100.0 ± 0.0	8.1 ± 3.1	6.4 ± 5.0	100.0 ± 0.0
	1:24	11.1 ± 4.8	100.0 ± 0.0	100.0 ± 0.0	7.1 ± 2.6	5.1 ± 2.5	16.0 ± 5.3
	1:48	8.3 ± 3.5	75.3 ± 5.2a ^b	80.1 ± 3.9a	5.3 ± 2.4	4.2 ± 2.0	10.1 ± 4.1
Cell-free supernatant (CFS)	1:6	76.6 ± 4.4	100.0 ± 0.0	100.0 ± 0.0	13.2 ± 4.2	9.1 ± 4.1	100.0 ± 0.0
	1:12	24.0 ± 13.7	100.0 ± 0.0	100.0 ± 0.0	10.3 ± 3.5	8.3 ± 3.0	100.0 ± 0.0
	1:24	9.1 ± 5.4	100.0 ± 0.0	100.0 ± 0.0	8.6 ± 3.2a	7.9 ± 3.4	20.6 ± 4.1
	1:48	6.5 ± 2.6	90.3 ± 4.6b	92.1 ± 2.5b	5.2 ± 1.6	6.1 ± 1.9	16.3 ± 3.4

^a Data are expressed as percentages of fungal growth inhibition compared with control fruits without treatment. Each value is the mean ± standard deviation of three replicates, done in 3 separate days, of 15 fruits each.

^b No letter means no significant differences between fractions for the same phytopathogen and the same concentration. Different letters indicate significant differences at $P < 0.05$ according to the Tukey test.

many bacteria. Therefore, the LPF and CFS were also tested in fruit against the six postharvest pathogens used in the study. In this context, the NBF was discarded due to the poor activity observed in the *in vitro* tests. The results of the LPF and CFS antifungal activity are shown in Table 2. As can be observed, the LPF controlled brown rot caused by *M. fructicola* and *M. laxa* in peaches even by 20-fold dilution (1:24). However, a 10-fold dilution (1:12) was necessary to prevent blue rot in apples. The efficacy against *B. cinerea* was also high, reducing the disease in strawberry by 72% at a 1:6 proportion. However, blue and green rot in mandarins was barely controlled at any concentration. No significant differences were observed between the fractions in most of the pathogens, although the CFS was slightly better at a 1:48 ratio against both *Monilinia* species.

3.4. Isolation and identification of lipopeptides from *B. amyloliquefaciens* BUZ-14

In order to determine the families of lipopeptides present in the LPF after 96 h of incubation in 863 medium, a TLC analysis of BUZ-14 and FZB42 (as a bacillomycin producer) was performed and their Rf established. Briefly, marks of all the families were observed in the TLC under UV-light. However, no differences were observed between the

two BCAs since the same spots and Rf were detected. To identify the active compounds from the lipopeptide fraction of both BCAs, a TLC-bioautography was developed. Only one active fraction was found in BUZ-14 (0.26) resulting in a very strong inhibition against all the pathogens tested (Fig. 1). According to the literature, this Rf should belong to mycosubtilin, a typical lipopeptide of the species *B. subtilis* with a large antifungal spectrum range (Duitman et al., 1999). However, the mycosubtilin gene was neither found in BUZ-14 (Supplemental Table 1) nor later detected by HPLC, so this option was discarded. Arrebola et al. (2010) found that iturin A produced by *B. amyloliquefaciens* PPCB004 appeared at a Rf of 0.3 but Yáñez-Mendizábal et al. (2012) obtained the same Rf for bacillomycin D. These findings, added to the fact that FZB42 produces another lipopeptide with a Rf of 0.3 (bacillomycin), make it impossible to elucidate by TLC whether BUZ-14 was an iturin A or a bacillomycin D producer. Moreover, FZB42 did not show inhibition in the bioautography against any pathogen, indicating that both metabolites developed in the TLC at the same Rf could be different.

The lipopeptides from both BCAs were identified by HPLC-DAD. By comparing these metabolites with the analytical standards, three groups of peaks were observed (Supplemental Fig. 3A), corresponding to iturin A, fengycin and surfactin, respectively for BUZ-14, although neither bacillomycin nor mycosubtilin were detected. However, as

Table 3
Lipopeptides and isoforms of *B. amyloliquefaciens* BUZ-14 detected by LC-MS-MS^E in 863 medium after 96 h of incubation at 30 °C.

Lipopeptide	Product and m/z observed	Assignment
Surfactin	1008.660; 1030.642; 1046.642	C13-surfactin [M + H,Na,K] ⁺
	1022.676; 1044.660; 1060.660	C14-surfactin [M + H,Na,K] ⁺
	1036.691; 1058.676; 1074.676	C15-surfactin [M + H,Na,K] ⁺
Fengycin	1463.804; 1485.804	C16-fengycin A [M + H,Na] ⁺
	1477.820; 1499.799; 1515.822	C17-fengycin A [M + H,Na,K] ⁺
	1491.804; 1529.804	C16-fengycin B [M + H,K] ⁺
	1505.814; 1527.804; 1543.804	C17-fengycin B [M + H,Na,K] ⁺
Iturin	1043.5562; 1065.5403; 1082.4514	C14-iturin A [M + H,Na,K] ⁺
	1057.5704; 1079.5516; 1095.5531	C15-iturin A [M + H,Na,K] ⁺
	1071.5900; 1093.5695; 1110.5402	C16-iturin A [M + H,Na; K] ⁺
	1085.5981; 1107.5704; 1124.5570	C17-iturin A [M + H; Na; K] ⁺

expected, no iturin A was detected in the FZB42 chromatogram (Supplemental Fig. 3B). Even so, additional information was required in order to confirm the results, so more sensitive chromatographic analyses using LC-MS-MS^E were carried out. The previously obtained LPF containing the lipopeptide families and the active fraction (R_f = 0.26) scraped from TLC plates were subjected to mass spectrometry analysis. The molecular masses were monitored in the m/z range of 100–2400 u.m.a. By analysing the lipopeptide profiles after 96 h of BUZ incubation, three isoforms of surfactin (C13, C14 and C15), four of fengycins (C16A, C16B, C17A and C17B) and four of iturins (C13, C14, C15 and C16) were identified (Table 3). The m/z obtained of the active compound scraped from the TLC-bioautography corresponded to iturin A (Hiradate et al., 2002; Yu et al., 2002; Ongena and Jacques, 2008; Athukorala et al., 2009). In addition, this result was confirmed by injecting the iturin A standard in the LC-MS-MS^E. It was observed that both the active fraction and the standard shared the same retention times and masses for the four peaks detected: 1.76 min for m/z 1043.5562; 1.98 min for m/z 1057.5704; 2.33 min for m/z 1071.5900 and 2.53 min for m/z 1085.5981. Finally, comparisons of MS^E spectra of the active fraction and the commercial standard of iturin A confirmed its identification (Fig. 2) and we could establish iturin A as the main lipopeptide responsible for antifungal activity against these postharvest moulds.

3.4.1. Lipopeptide production curve

The concentration of the compounds during the incubation time in 863 medium was also determined (Fig. 3). After 24 h of incubation, iturin A reached 36.3 µg mL⁻¹ but surfactin and fengycin were not detected. The amount of iturin A increased progressively reaching 407 µg mL⁻¹ after 96 h of incubation. Fengycin also achieved the highest concentration after 96 h of incubation with 853 µg mL⁻¹. Otherwise, the top peak of surfactin was obtained after 72 h with 658 µg mL⁻¹. After 72 h of incubation of *B. subtilis* GA1 in a culture medium optimized for lipopeptide production (named Opt), Touré et al. (2004) obtained concentrations of fengycins, iturins and surfactins of 520, 460 and 340 µg mL⁻¹, respectively. Also, Ambrico and Trupo (2017) in High Medium Broth (HMB) achieved the highest amount of iturin, 422 mg L⁻¹, from *B. subtilis* ET-1 after 50 h of incubation. Thus, the incubation time is an essential factor in the production of secondary metabolites together with the culture medium. The production process of biocontrol agents is an essential step for their commercial use as bioproducts. A key factor to consider is the development of an economical culture medium that supports both the production of large commercial amounts of the BCA and its metabolites at a low price. In our case, the 863 medium achieved a notable lipopeptide production and although it cannot really be considered a low cost medium, it is

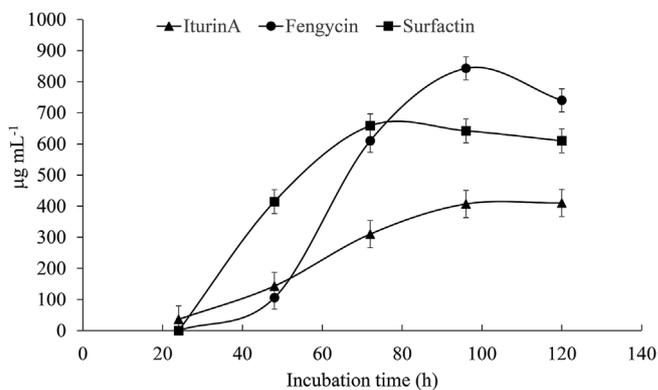


Fig. 3. Quantification of fengycin, surfactin and iturin produced by BUZ-14 in 863 medium during the incubation time. Measurements of concentration were carried out following the lipopeptide extraction protocol at 24 h, 48 h, 72 h, 96 h and 120 h. Analytical standards were used as reference compounds. Data represent the average of three samples extracted on three different days ± SD.

very simple as it has 3 ingredients only. However, considering that *B. amyloliquefaciens* may grow and synthesize its metabolites from cheap substrates, our future research will focus on finding a low cost medium based on commercial products or by-products from the food industry that can provide maximum BUZ-14 growth and lipopeptide production in large scale production processes.

3.5. Correlation between iturin A concentration and antifungal activity

Finally, relating the production of iturin A in BUZ-14 cultures (Fig. 3) with the data of LPF inhibitions *in vitro* (Table 1) and *in vivo* (Table 2) we can establish the MICs of iturin A (Table 4). Thus, the MICs *in vitro* of iturin A (µg mL⁻¹) were as follows: 4.1 for *M. laxa*, 8.5 for *M. fructicola*, *P. italicum* and *P. expansum* and 16.9 for *B. cinerea* and *P. digitatum* (Table 4). These MICs *in vitro* are in partial agreement with those of Ambrico and Trupo (2017) who determined a MIC of iturin A of 6 and 3 µg mL⁻¹ for *P. digitatum* and *B. cinerea*, respectively, in a solid medium *in vitro* test. These discrepancies could be explained by several factors such as the different *in vitro* test used (solid and liquid medium), the differences in the type and percentages of the isoforms of iturin A produced by the BCA strains, and the different phytopathogen strains used. However, the MICs of iturin A *in vivo* were only achieved in *Monilinia* spp. and *P. expansum* (16.9 and 33.9 µg mL⁻¹ respectively) (Table 4). The growth of the rest of the pathogens were reduced but their respective diseases were manifested (Table 2) since their MICs

Table 4

Minimum Inhibitory Concentration of iturin A present on the LPF of *B. amyloliquefaciens* BUZ-14 obtained after 96 h of incubation at 30 °C in 863 medium for the six fungal pathogens tested.

Phytopathogenic fungi	Iturin A (µg mL ⁻¹) ^a	
	<i>In vitro</i> ^b	<i>In vivo</i> ^c
<i>B. cinerea</i>	16.9	> 67.8
<i>M. fructicola</i>	8.5	16.9
<i>M. laxa</i>	4.1	16.9
<i>P. digitatum</i>	16.9	> 67.8
<i>P. italicum</i>	8.5	> 67.8
<i>P. expansum</i>	8.5	33.9

^a LPF started at an iturin concentration of 407 µg mL⁻¹ and was serially diluted by a factor of two until a concentration of 4.1 µg mL⁻¹ was reached.

^b *In vitro* activity was assayed by the well-dilution method in liquid medium.

^c *In vivo* activity was tested by the puncture method: fruits were wounded with a sterile tip and 10 µL of the respective pathogen (10⁴ conidia mL⁻¹) was inoculated (*B. cinerea* in strawberries, *M. fructicola* and *M. laxa* in peaches, *P. digitatum* and *P. italicum* in mandarins and *P. expansum* in apples).

Table 5
Reduction of the fungal growth, BUZ-14 counts and iturin A produced in fruits after 7 days at 20 °C.

Fruit (fungal pathogen) ^a	% inhibition ^b	BUZ-14 counts ^c (log CFU g ⁻¹)	Iturin A ^d (µg g ⁻¹)
Strawberries (<i>B. cinerea</i>)	15.4 ± 3.4	4.9 ± 0.5	nd ^e
Peach (<i>M. fructicola</i>)	100.0 ± 0.0	8.3 ± 0.7	1.8 ± 0.3
Peach (<i>M. laxa</i>)	100.0 ± 0.0	8.6 ± 0.6	1.6 ± 0.5
Mandarins (<i>P. digitatum</i>)	8.4 ± 2.3	5.3 ± 0.4	nd
Mandarins (<i>P. italicum</i>)	7.2 ± 2.4	5.1 ± 0.5	nd
Apple (<i>P. expansum</i>)	100.0 ± 0.0	5.2 ± 0.4	nd

Data shown are means ± standard deviation.

^a Inoculated with 10 µL of the respective pathogen (10⁴ conidia mL⁻¹) and 10 µL of a BUZ-14 24 h-old culture (10⁸ CFU mL⁻¹).

^b The percentage of inhibition of the disease was determined by measuring the lesion diameter in treated fruits and the control.

^c The initial counts of BUZ-14 in the fruits were 5.0 log CFU g⁻¹.

^d The initial concentration of iturin A in the fruit tissue was 0.036 ± 0.014 µg g⁻¹. These data were calculated from the iturin A concentration quantified in the 24 h-old cultures.

^e nd: not detected.

were higher than the maximum concentration tested in this study (67.8 µg mL⁻¹).

3.6. BUZ-14 growth and iturin A production in fruit

The results showed that the 24 h-old cultures of BUZ-14 suppressed brown rot by *M. fructicola* and *M. laxa* in peaches and also blue rot by *P. expansum* in apples but failed in the rest of the diseases (Table 5). The final concentration of iturin A in peaches achieved 1.6–1.8 µg g⁻¹ and BUZ-14 populations reached 10⁸ CFU g⁻¹ whereas neither growth nor iturin A production was detected in the rest of the fruits. We could deduce that the amount of iturin A brought with the culture (36 ± 14 µg mL⁻¹) could be enough to inhibit the growth of *Monilinia* species in peaches and *P. expansum* in apples, with MICs *in vivo* of 16.9 and 33.9 µg mL⁻¹, respectively. However, this concentration was insufficient to control the rest of the pathogens (*B. cinerea*, *P. digitatum* and *P. italicum*). Thus, BUZ-14 cultures drastically prevent *Monilinia* spp. in peach fruit since the microorganism is able to grow and produce significant levels of iturin A but, as in the case of *P. expansum*, a cell free supernatant would also be effective. For the rest of the fruit-pathogen binomials where no growth of the BCA was detected, a more concentrated supernatant in iturin A would need to be tested in order to inhibit the diseases.

Improving our knowledge of the mechanisms of action of BCAs and their behaviour *in planta* could help to select the most efficient form of application (BCA cultures, BCA cultures enriched in a specific substance, CFS or iturin A enriched fractions). Finding a BCA able to grow and produce a sufficiently active metabolite *in planta* could be the easiest and cheapest application method but, as our results have shown, this behaviour is highly specific since it could be influenced by many conditions such as pH or temperature, and the available nutrients (Ashis and Kishore, 2005; Monteiro et al., 2016). The use of CFS with the maximum concentration of active metabolites may have more advantages than cultures but the difficulty of determining its complex composition does not allow the innocuousness of its application to be established. On the other hand, formulating a cell free biopesticide enriched in one or various metabolites would require high production costs and yields, but toxicity assays may be easier. Many studies endorse the low toxicity of iturin A (Kim and Lee, 2009; Zhang et al., 2012; Dey et al., 2016; Cao et al., 2017) but strict toxicological studies that guarantee its innocuousness for humans and the environment are essential for any future application.

4. Conclusions

The present study has shown that iturin A produced by *B. amyloliquefaciens* BUZ-14 is the main lipopeptide family responsible for fungal inhibition and which at low levels provides effective control of brown

rot in peaches and blue mould in apples. Thus, a BCA that can produce a sufficiently active metabolite *in planta* could be the most suitable pathway for a commercial application. However, BUZ-14 is only able to grow and produce iturin A in peaches, which reveals the complex and specific nature of the BCA-fruit-pathogen interactions. For the rest of the fruit-pathogen binomials in which no growth of the BCA was detected, an iturin A enriched extract could be applied in order to obtain effective disease control. Thus, optimising the production of iturin A by BUZ-14 and testing the antifungal efficacy of these enriched culture extracts will be the focus of our future research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fm.2019.01.010>.

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