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

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21 **ABSTRACT**

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

42

43 Keywords: biocontrol, *Monilinia* spp., *P. expansum*, lipopeptides, iturin A.

44

45 **1. Introduction**

46 Nowadays, pesticides and fungicides are the main system used to control biotic agents
47 responsible for pre- and postharvest rot. However, current legislation covering
48 pesticides has become stricter by reducing the MRLs (Maximum Residue Limits) in
49 plants and forbidding numerous effective fungicides because of the development of
50 fungicide-resistant strains of pathogens, the detection of chemical residues in the food
51 chain and their toxicity for human health and the environment. Therefore, new
52 alternatives such as biological control by using natural antagonistic microorganisms are
53 currently being studied in depth (Chung et al., 2008; Zhao et al., 2013). Several wild-
54 type *Bacillus subtilis* and *B. amyloliquefaciens* strains have been reported for their
55 effectiveness in the biocontrol of multiple plant diseases caused by soil borne and
56 postharvest pathogens (Alvarez et al., 2012; Arguelles-Arias et al., 2009; Arrebola et al.,
57 2009; Calvo et al., 2017; Chen et al., 2016; Chen et al., 2009; Chung et al., 2008; Gong
58 et al., 2015; Hinarejos et al., 2016; Romero et al., 2007; Toral et al., 2018; Torres et al.,
59 2017; Touré et al., 2004; Yánez-Mendizábal et al., 2012). Most authors sustain that
60 antibiosis is the main mechanism of action against pathogens since some *Bacillus*
61 strains are capable of producing a substantial number of antifungal secondary
62 metabolites, especially the non-ribosomally synthesised cyclic lipopeptides (Alvarez et
63 al., 2012; Torres et al., 2017; Veras et al., 2016). These substances are divided into three
64 families according to their structure: surfactin, fengycin and iturin. The chemical
65 structure has previously been studied by numerous authors, regarding the type and
66 sequence of amino acid residues, the nature of the peptide and length and branching of
67 the fatty acid chain (Hinarejos et al., 2016; Ongena and Jacques, 2008; Stein, 2005;
68 Torres et al., 2016; Xu et al., 2013). The mechanism of direct antibiosis associated with
69 *Bacillus* strains against foliar and postharvest diseases has been attributed mostly to

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70 iturins and fengycins (Arrebola et al., 2010; Romero et al., 2007; Yánez-Mendizábal et
71 al., 2012) although other metabolites such as polyketides (Chen et al., 2006; 2009),
72 siderophores (Li et al., 2014), bacteriocins (Ayed et al., 2015) and volatile organic
73 compounds (VOCs) (Gotor-Vila et al., 2017) must not be forgotten. These metabolites
74 and compounds can be produced by the biological control agent (BCA) in the
75 formulation prior to the application with the mechanism of action based on a direct
76 antibiosis. Otherwise, these compounds could be produced directly in the plant while
77 the BCA is growing *in situ* (Touré et al., 2004). The latter option would increase the
78 possibility of success because the mechanisms of action such as competition for
79 nutrients or colonization would be taken into account, as well as the fact that the direct
80 inhibition provoked by the metabolites could be stronger. Nevertheless, direct antibiosis
81 has not only been cited as a mechanism for controlling disease since it has also been
82 demonstrated that most of the metabolites produced by *B. subtilis* or *B.*
83 *amyloliquefaciens* strains can induce systemic resistances (ISR) in plants (Cawoy et al.,
84 2014; Choudhary and Johri, 2009; Ryu et al., 2004). The role of lipopeptides from *B.*
85 *subtilis* (Ongena and Jacques, 2008; Ongena et al., 2005; 2007) and from *B.*
86 *amyloliquefaciens* (Chowdhury et al., 2015) has been studied on various plants for
87 inducing immune responses. As an example, fengycins from *B. subtilis* M4 could be
88 involved in the systemic resistance-eliciting effect of this strain, as these molecules may
89 induce the synthesis of plant phenolics involved in or derived from the defence-related
90 phenylpropanoid metabolism (Ongena et al., 2005).

91 The aim of this work was to elucidate the role of the secondary metabolites produced by
92 *B. amyloliquefaciens* BUZ-14 against *B. cinerea*, *M. fructicola*, *M. laxa*, *P. digitatum*,
93 *P. italicum* and *P. expansum* both *in vitro* and *in planta.* For this purpose, 4 steps were
94 addressed (i) characterisation of the *in vitro* and *in vivo* antifungal activity of CFS, LPF

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95 and NBF through the determination of their MICs, (ii) identifying and quantifying the
96 lipopeptides produced (iii) determining the lipopeptide responsible for the antagonistic
97 activity and (iv) quantifying the lipopeptide production on fruit.

98 2. Materials and methods

99 *2.1 Fungal pathogens*

100 The fungal pathogen strains used in this study were *Botrytis cinerea* VG 1, *Monilinia*
101 *fructicola* VG 104, *M. laxa* VG 105, *Penicillium digitatum* VG 20, *P. expansum* CECT
102 20140 and *P. italicum* VG 101. They were obtained from the Plant Food Research
103 Group culture collection (Zaragoza, Spain) and incubated on potato dextrose agar
104 (PDA) (Oxoid Ltd; Basingstoke, Hampshire, England) and potato dextrose broth (PDB).

105 *2.2 Fruit samples*

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

114 *2.3 Bacterial strain identification*

115 *B. amyloliquefaciens* BUZ-14 was identified through a phylogenetic analysis of its 16S
116 rDNA and partial gyrase gene sequences *gyrB*. The genomic DNA of BUZ-14 was
117 extracted using a FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen

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118 Biotech Corporation, Taiwan). The 16S rDNA was amplified by PCR using the
119 universal primers 8F (5′-AGA GTT TGA TCC TGG CTC AG -3′) and 1492R (5′-CGG
120 TTA CCT TGT TAC GAC TT -3′) (Microsynth AG, Switzerland). Part of the *gyrB*
121 sequence was amplified with *gyrB*-BUZ-14F (5′-ACC GGA ACG ATT ACG CAC TT -
122 3′) and *gyrB*-BUZ-14R (5′- AGG GTC CAT TGT CGT TTC CC -3′) (Microsynth AG,
123 Switzerland) primers previously designed with Primer3 v. 0.4.0 software (Untergasser
124 et al., 2007), based on the type strain *Bacillus amyloliquefaciens* FZB42.

125 *2.4 In vitro antifungal activity of the cell-free supernatant (CFS), non-butanolic fraction*
126 *(NBF) and lipopeptide fraction (LPF) from* B. amyloliquefaciens *BUZ-14 against*
127 *fungal pathogens*

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

142 For the *in vitro* inhibition tests, 48-well plates were used. The MIC (Minimal Inhibitory
143 Concentration) of each fraction was found by diluting the antimicrobial into PDB
144 medium. Five hundred μL of the medium were added into the well, followed by 30 μL
145 of each pathogen (10^4 conidia mL^{-1}). Finally, different quantities of LPF, NBF or CFS
146 were transferred until obtaining 1:6, 1:12, 1:24, 1:48, 1:80 and 1:100 dilutions. A
147 positive control without the antimicrobial fraction and a negative control without the
148 pathogen were also included. The plates were incubated at 25 ºC for 7 days. The results
149 were obtained by observing visually the presence (growth) or absence (non-growth) of
150 fungi. Five replicates per pathogen were conducted and the experiment was done in
151 three independent days.

152 *2.5 Antifungal activity of CFS and LPF from BUZ-14 on fruit*

153 In this study, the antimicrobial activity on fruit of the CFS and LPF obtained from 96 h-
154 old cultures was assessed. Fruit were wounded with a sterile tip and 10 μL of the
155 respective pathogen (10^4 conidia mL^{-1}) was inoculated. After 1 hour of absorption of the
156 conidial suspensions, the CFS and LPF were inoculated at 1:6, 1:12, 1:24 and 1:48
157 dilutions. The fruits were stored at 20 ºC for 5 days and 80 % R.H. (relative humidity).
158 The severity was evaluated as a percentage of reduction of the disease by measuring the
159 lesion diameter in treated fruits and the control. Fifteen fruits per pathogen and
160 treatment (one wound per fruit) were disposed. The entire experiment was repeated in
161 three different days.

162 *2.6 Isolation and identification of antifungal lipopeptides*

163 In order to find out the lipopeptides responsible for direct antibiosis, a Thin Layer
164 Chromatography (TLC) followed by bioautography was performed and the active
165 fractions identified by Liquid Chromatography-Electrospray Ionization-Mass

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166 Spectrometry (LC–ESI-MS^{E}) analysis. The lipopeptides present in the LPF were also
167 identified and quantified.

168 *2.6.1 Thin Layer Chromatography (TLC) and bioautography*

169 Ten μL aliquots from the lipopeptide fraction produced by BUZ-14 and the reference
170 strain *B. amyloliquefaciens* FZB42 after 96 h of incubation at 30 ºC were spotted onto
171 TLC aluminium sheets coated with silica gel 60 F254 5 x10 cm (Sigma Aldrich, Spain).
172 The sheets were placed in a separation chamber containing chloroform/methanol/water
173 (65:25:4, v/v/v) as mobile phase, following the protocol proposed by Razafindralambo
174 et al. (1993). The spots were detected under 254 nm UV light and their retention factor
175 (R_f) determined. The strain FZB42, purchased from the DSMZ collection (Germany),
176 was used as a bacillomycin producer since no commercial standard was found.

177 The bioautography was performed as described by Chen et al. (2016) in order to
178 identify the family of lipopeptides responsible for fungal inhibition. Briefly, the TLC
179 plates were placed in 90 x 90 mm Petri plates and covered with melted PDA medium
180 inoculated with pathogens at 1-3 x 10^4 conidia mL^{-1}. The moulds tested were those cited
181 in Section 2.1. The plates were incubated at 25 ºC for 7 days and the R_f of the active
182 fraction was determined. Then, the R_f was compared to that in the previously conducted
183 TLC and the active lipopeptide was established. Moreover, each antifungal compound
184 was scraped from the silica gel and extracted with 2 mL of methanol under magnetic
185 conditions stirred at 300 rpm overnight. Afterwards, the sample was centrifugated at
186 10000 x g and the resulting supernatant was subjected to Liquid Chromatography-Mass
187 Spectrometry analysis as detailed in section 2.6.3. Five replicates were used per
188 pathogen and the experiment was repeated on three different days.

189 *2.6.2 High Performance Liquid Chromatography Diode-Array Detector (HPLC-DAD)*

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190 The lipopeptides were identified and quantified after 24, 48, 72 96 and 120 h of
191 incubation using an Agilent 1100 liquid chromatograph coupled to an Agilent 1200
192 Series DAD detector (Agilent, USA) with a Lichrospher RP18 column, 5µm 250x4 mm
193 (Sigma Aldrich, Spain). The mobile phase A was 0.1% formic acid in acetonitrile, and
194 mobile phase B was 0.1% formic acid in Milli-Q water. The flow rate was 1 mL min^{-1}
195 and the temperature of the column was set at 30 ºC. The gradient started at 35% A and
196 was held for 8 min. From 8 min to 10 min the gradient was raised to 40% A and kept for
197 15 min. Then, the gradient was raised to 80% for 5 min and held at 80% for 15 min.
198 Finally, the gradient reached 100% for 5 min and returned to the initial conditions
199 during 5 min. The total run time was 60 min. Lipopeptides were monitored at 214 nm.
200 The analytical standards were iturin A (purity ≥95%), Chemical Abstract Service
201 identification number: 52229-90-0; surfactin ≥ 98%, 24730-31-2 and fengycin ≥ 90%,
202 102577-03-7. All of them were purchased from Sigma Aldrich, Spain. These analytical
203 standards were used for the identification and quantification of the samples. The
204 calibration curve was determined by using standard solutions containing 20, 100, 400,
205 1000 and 1400 µg mL^{-1} of each compound. In all cases, the solutions were injected in
206 the chromatograph three times and the extraction process was carried out on three
207 separate days.

208 *2.6.3 Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC–ESI-*
209 *MS^E) analysis*

210 The lipopeptide fractions obtained after 96 h of incubation time were also subjected to
211 an LC–ESI-MS^E analysis to identify the different isoforms of each lipopeptide as well
212 as the compounds which exhibited antifungal activity on the TLC plates. For this
213 purpose, an Acquity UPLC H-CLASS system supplied by Waters (Milford, USA) was
214 used. Chromatographic separation was performed on a Waters Acquity UPLC BEH C_{18}

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215 (1.7 µm, 2.1 mm×100 mm) at 40 ºC. Mobile phase A was MilliQ-water with 0.1%
216 formic acid and mobile phase B acetonitrile with 0.1 % formic acid. The flow rate was
217 0.50 ml·min^{-1} and the injection volume 10 µL. The time program for multi-step gradient
218 was 0–5 min, 70% A-30 % B to 5 % A-95 % B, 5–10.10 min, 5 % A-95 % B to 70 %
219 A-30 %. The run time was 12 min and the sample temperature was set at 10 ºC. Mass
220 spectra were acquired using a time-of-flight (TOF) MS Synapt G2 High Definition
221 Mass Spectrometer supplied by Waters (Milford, USA). Nitrogen was used as a
222 desolvation gas at 800 L·h^{-1} flow. The cone gas flow was 40 L·h^{-1}. The ion source
223 parameters were corona current 3 µA, sample cone 40 V and desolvation gas
224 temperature 500 °C. Electrospray ionization mode (ESI) in positive polarity was
225 selected; the source temperature was 120 ºC. The scan time was 1 s and the mass range
226 considered was *m/z* 100–2400. MassLynx v4.1 software supplied by Waters (Milford,
227 USA) was used for the analysis of the results obtained, which considers the isotopic
228 model and the elemental composition.

229 *2.7 BUZ-14 growth and iturin A production on fruit*

230 The growth curves of BUZ-14 and the production of iturin A in fruit were determined.
231 Fruits were wounded as described in section 2.5 and 10 µL of the respective pathogen
232 (10^4 conidia mL^{-1}) was inoculated. After 1 hour at room temperature to favour the
233 absorption of the conidia suspension, a 24 h-old culture (10 µL) of *B. amyloliquefaciens*
234 (10^8 CFU mL^{-1}) was inoculated and the fruit was stored at 20 ºC and 80 % R.H. for 7
235 days. The percentage of reduction of the fungal growth was then recorded. To determine
236 the growth of the BCA, 10 grams of fruit wound was cut through with a sterile scalpel.
237 The fruit plugs were placed in filter blender bags and diluted 1:5 with 0.1% sterile
238 peptone water. The mixture was homogenized in a Stomacher 400 Circulator laboratory
239 blender (Seward Laboratory, London, England) for 120 s at 260 rpm and the resulting

240 suspension was diluted, plated on TSA plates and counted after 24 h at 30 ºC. The
241 extraction of lipopeptides was carried out in the homogenate following the procedure
242 described in section 2.4. The subsequent quantification was conducted with the HPLC-
243 DAD (see section 2.6.2) and the amount of lipopeptides quantified and expressed in µg
244 g^{-1} of fruit tissue. Fifteen fruits per fungal pathogen were used as replicates and the
245 experiment was carried out on 3 separate days.

246 *2.8 Statistical analysis*

247 Data were statistically treated by t-student analysis using SPSS software (SPSS statistics
248 22.0). The statistical differences between means of different treatments were assessed at
249 $P < 0.05$.

250 **3. Results and discussion**

251 *3.1 B. amyloliquefaciens BUZ-14 strain phylogenetic identification*

252 The bacterial strain tested in this study has been previously identified by Calvo et al.
253 (2017) as *Bacillus amyloliquefaciens* and named BUZ-14. However, due to the genetic
254 similarity among species of the *Bacillus* group and the several recently renamed
255 subspecies, we have made a more in-depth study of its identification. *B.*
256 *amyloliquefaciens* BUZ-14 16S rDNA and *gyrB* sequences were obtained and deposited
257 in Genbank with accession numbers MF461174 for 16S rDNA and MF770248 for *gyrB*.
258 The sequences were processed using the BLAST tool provided by Genbank. The
259 information obtained from these results enabled a phylogenetic tree to be constructed
260 (Supplemental files: Figure 1 and 2). In order to achieve a more reliable level of
261 identification, the subunit B protein of DNA gyrase (gyrB) was subjected to sequencing
262 (Hossain et al., 2015; Wang et al., 2007). This strain was designated as *B.*
263 *amyloliquefaciens* subsp. *plantarum* BUZ-14, although recent studies have proposed

264 including this classification within *B. velezensis* together with other *Bacillus* species
265 (Dunlap et al. 2016). Plant-associated *B. amyloliquefaciens* strains belonging to subsp.
266 *plantarum* are distinguished from others such as subsp. *siamensis* or *amyloliquefaciens*
267 by their capacity to stimulate plant growth, to colonize the plant rhizosphere and to
268 suppress competing phytopathogenic bacteria and fungi.

269 *3.2* In vitro *antifungal activity of the cell-free supernatant (CFS), non-butanolic fraction*
270 *(NBF) and lipopeptide fraction (LPF) from* B. amyloliquefaciens *BUZ-14 against*
271 *fungal pathogens*

272 The aim of this assay was to characterize the antifungal activity of BUZ-14 secondary
273 metabolites by establishing differences among the CFS, the NBF and the LPF. The
274 activity of the three fractions was evaluated in liquid medium after 96 h of incubation at
275 30 ºC in 863 broth against the fungal pathogens cited in section 2.1. In this study, we
276 have separated the lipopeptides (LPF) from other compounds (NBF) and we have
277 observed that all the phytopathogens were greatly inhibited by the LPF (Table 1). The
278 results showed that the NBF was barely effective against the six postharvest fungal
279 pathogens tested. However, the LPF achieved the inhibition of all the pathogens in a
280 1:24 dilution for *B. cinerea* and *P. digitatum*, 1:48 for *M. fructicola*, *P. italicum* and *P.*
281 *expansum* and with *M. laxa* as the most susceptible species with a MIC below 1:100.
282 The activity observed *in vitro* in the CFS is related to all the active compounds
283 produced by BUZ-14, so a maximum inhibition was expected. However, the MICs
284 obtained were only slightly higher or even the same as those of the LPF, supporting the
285 idea that the antifungal activity lies with the lipopeptides. Nevertheless, not all the
286 pathogens needed the same concentration, and it was observed that *M. laxa* was much
287 more sensitive to these compounds. It is known that the same substance can exert
288 different antimicrobial activity depending on the target microorganism (Ambrico and

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289 Trupo, 2017; Chen et al., 2009; Ji et al., 2013). Accordingly, Yánez-Mendizábal et al.
290 (2012) identified a fengycin-like lipopeptide as the main metabolite responsible for
291 *Monilinia* spp. inhibition by *B. subtilis* CPA-8. However, Zhao et al. (2013) determined
292 that in the case of the *Bacillus* BH072 strain, iturin A was the main inhibitor of *B.*
293 *cinerea*. Additionally, these kinds of plant-growth-promoting bacteria produce several
294 non-ribosomal polyketides and lipopeptides, apart from iron-siderophores, bacteriocins
295 and volatile compounds with relevant antifungal activity (Alvarez et al., 2012;
296 Arguelles-Arias et al., 2009; Ayed et al., 2015; Borriss et al., 2011; Romero et al.,
297 2007).

298 *3.3 LPF and CFS antifungal activity on fruit*

299 The substantial activity demonstrated by the metabolites produced by BUZ-14 against
300 fungal pathogens *in vitro* does not ensure the same effect on a food matrix. Usually,
301 MICs are significantly higher *in vivo* since the fungi efficiently used the nutrients
302 available in the fruit while the low pH, acids and phenols are all barriers to the
303 development of many bacteria. Therefore, the LPF and CFS were also tested in fruit
304 against the six postharvest pathogens used in the study. In this context, the NBF was
305 discarded due to the poor activity observed in the *in vitro* tests. The results of the LPF
306 and CFS antifungal activity are shown in Table 2. As can be observed, the LPF
307 controlled brown rot caused by *M. fructicola* and *M. laxa* in peaches even by 20-fold
308 dilution (1:24). However, a 10-fold dilution (1:12) was necessary to prevent blue rot in
309 apples. The efficacy against *B. cinerea* was also high, reducing the disease in strawberry
310 by 72% at a 1:6 proportion. However, blue and green rot in mandarins was barely
311 controlled at any concentration. No significant differences were observed between the
312 fractions in most of the pathogens, although the CFS was slightly better at a 1:48 ratio
313 against both *Monilinia* species.

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314 *3.4 Isolation and identification of lipopeptides from* B. amyloliquefaciens *BUZ-14*

315 In order to determine the families of lipopeptides present in the LPF after 96 h of
316 incubation in 863 medium, a TLC analysis of BUZ-14 and FZB42 (as a bacillomycin
317 producer) was performed and their Rf established. Briefly, marks of all the families
318 were observed in the TLC under UV-light. However, no differences were observed
319 between the two BCAs since the same spots and Rf were detected. To identify the
320 active compounds from the lipopeptide fraction of both BCAs, a TLC-bioautography
321 was developed. Only one active fraction was found in BUZ-14 (0.26) resulting in a very
322 strong inhibition against all the pathogens tested (Figure 1). According to the literature,
323 this Rf should belong to mycosubtilin, a typical lipopeptide of the species *B. subtilis*
324 with a large antifungal spectrum range (Duitman et al., 1999). However, the
325 mycosubtilin gene was neither found in BUZ-14 (Supplemental Table 1) nor later
326 detected by HPLC, so this option was discarded. Arrebola et al. (2010) found that iturin
327 A produced by *B. amyloliquefaciens* PPCB004 appeared at a Rf of 0.3 but Yánez-
328 Mendizábal et al. (2012) obtained the same Rf for bacillomycin D. These findings,
329 added to the fact that FZB42 produces another lipopeptide with a Rf of 0.3
330 (bacillomycin), make it impossible to elucidate by TLC whether BUZ-14 was an iturin
331 A or a bacillomycin D producer. Moreover, FZB42 did not show inhibition in the
332 bioautography against any pathogen, indicating that both metabolites developed in the
333 TLC at the same Rf could be different.

334 The lipopeptides from both BCAs were identified by HPLC-DAD. By comparing these
335 metabolites with the analytical standards, three groups of peaks were observed
336 (Supplemental Figure 3A), corresponding to iturin A, fengycin and surfactin,
337 respectively for BUZ-14, although neither bacillomycin or nor mycosubtilin were
338 detected. However, as expected, no iturin A was detected in the FZB42 chromatogram

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339 (Supplemental Figure 3B). Even so, additional information was required in order to
340 confirm the results, so more sensitive chromatographic analyses using LC-MS-MS^E
341 were carried out. The previously obtained LPF containing the lipopeptide families and
342 the active fraction (Rf=0.26) scraped from TLC plates were subjected to mass
343 spectrometry analysis. The molecular masses were monitored in the m/z range of 100–
344 2400 uma. By analysing the lipopeptide profiles after 96 h of BUZ incubation, three
345 isoforms of surfactin (C13, C14 and C15), four of fengycins (C16A, C16B, C17A and
346 C17B) and four of iturins (C13, C14, C15 and C16) were identified (Table 3). The m/z
347 obtained of the active compound scraped from the TLC-bioautography corresponded to
348 iturin A (Hiradate et al., 2002; Yu et al., 2002; Ongena and Jacques, 2008; Athukorala
349 et al., 2009). In addition, this result was confirmed by injecting the iturin A standard in
350 the LC-MS-MS^E. It was observed that both the active fraction and the standard shared
351 the same retention times and masses for the four peaks detected: 1.76 min for m/z
352 1043.5562; 1.98 min for m/z 1057.5704; 2.33 min for m/z 1071.5900 and 2.53 min for
353 m/z 1085.5981. Finally, comparisons of MS^E spectra of the active fraction and the
354 commercial standard of iturin A confirmed its identification (Figure 2) and we could
355 establish iturin A as the main lipopeptide responsible for antifungal activity against
356 these postharvest moulds.

357 *3.4.3 Lipopeptide production curve*

358 The concentration of the compounds during the incubation time in 863 medium was
359 also determined (Figure 3). After 24 hours of incubation, iturin A reached 36.3 µg mL^{-1}
360 but surfactin and fengycin were not detected. The amount of iturin A increased
361 progressively reaching 407 µg mL^{-1} after 96 h of incubation. Fengycin also achieved the
362 highest concentration after 96 h of incubation with 853 µg mL^{-1}. Otherwise, the top
363 peak of surfactin was obtained after 72 h with 658 µg mL^{-1}. After 72 h of incubation of

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364 *B. subtilis* GA1 in a culture medium optimized for lipopeptide production (named Opt),
365 Touré et al. (2004) obtained concentrations of fengycins, iturins and surfactins of 520,
366 460 and 340 $\mu g \cdot mL^{-1}$, respectively. Also, Ambrico and Trupo (2017) in High Medium
367 Broth (HMB) achieved the highest amount of iturin, 422 mg L^{-1}, from *B. subtilis* ET-1
368 after 50 h of incubation. Thus, the incubation time is an essential factor in the
369 production of secondary metabolites together with the culture medium. The production
370 process of biocontrol agents is an essential step for their commercial use as bioproducts.
371 A key factor to consider is the development of an economical culture medium that
372 supports both the production of large commercial amounts of the BCA and its
373 metabolites at a low price. In our case, the 863 medium achieved a notable lipopeptide
374 production and although it cannot really be considered a low cost medium, it is very
375 simple as it has 3 ingredients only. However, considering that *B. amyloliquefaciens* may
376 grow and synthesize its metabolites from cheap substrates, our future research will
377 focus on finding a low cost medium based on commercial products or by-products from
378 the food industry that can provide maximum BUZ-14 growth and lipopeptide
379 production in large scale production processes.

380 *3.5. Correlation between Iturin A concentration and antifungal activity*

381 Finally, relating the production of iturin A in BUZ-14 cultures (Figure 3) with the data
382 of LPF inhibitions *in vitro* (Table 1) and *in vivo* (Table 2) we can establish the MICs of
383 iturin A (Table 4). Thus, the MICs *in vitro* of iturin A ($\mu g\ mL^{-1}$) were as follows: 4.1 for
384 *M. laxa*, 8.5 for *M. fructicola*, *P. italicum* and *P. expansum* and 16.9 for *B. cinerea* and
385 *P. digitatum* (Table 4). These MICs *in vitro* are in partial agreement with those of
386 Ambrico and Trupo (2017) who determined a MIC of iturin A of 6 and 3 $\mu g\ mL^{-1}$ for *P.*
387 *digitatum* and *B. cinerea*, respectively, in a solid medium *in vitro* test. These
388 discrepancies could be explained by several factors such as the different *in vitro* test

389 used (solid and liquid medium), the differences in the type and percentages of the
390 isoforms of iturin A produced by the BCA strains, and the different phytopathogen
391 strains used. However, the MICs of iturin A *in vivo* were only achieved in *Monilinia*
392 spp. and *P. expansum* (16.9 and 33.9 μg mL^{-1} respectively) (Table 4). The growth of the
393 rest of the pathogens were reduced but their respective diseases were manifested (Table
394 2) since their MICs were higher than the concentration tested in this study (67.8 μg mL^{-1}
395).

396 *3.6 BUZ-14 growth and iturin A production in fruit*

397 The results showed that the 24 h-old cultures of BUZ-14 suppressed brown rot by *M.*
398 *fructicola* and *M. laxa* in peaches and also blue rot by *P. expansum* in apples but failed
399 in the rest of the diseases (Table 5). The final concentration of iturin A in peaches
400 achieved 1.6-1.8 μg g^{-1} and BUZ-14 populations reached 10^8 CFU g^{-1} whereas neither
401 growth nor iturin A production was detected in the rest of the fruits. We could deduce
402 that the amount of iturin A brought with the culture (36 ± 14 μg mL^{-1}) could be enough
403 to inhibit the growth of *Monilinia* species in peaches and *P. expansum* in apples, with
404 MICs *in vivo* of 16.9 and 33.9 μg mL^{-1}, respectively. However, this concentration was
405 insufficient to control the rest of the pathogens (*B. cinerea*, *P. digitatum* and *P.*
406 *italicum*). Thus, BUZ-14 cultures drastically prevent *Monilinia* spp. in peach fruit since
407 the microorganism is able to grow and produce significant levels of iturin A but, as in
408 the case of *P. expansum*, a cell free supernatant would also be effective. For the rest of
409 the fruit-pathogen binomials where no growth of the BCA was detected, a more
410 concentrated supernatant in iturin A would need to be tested in order to inhibit the
411 diseases.

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412 Improving our knowledge of the mechanisms of action of BCAs and their behaviour *in*
413 *planta* could help to select the most efficient form of application (BCA cultures, BCA
414 cultures enriched in a specific substance, CFS or iturin A enriched fractions). Finding a
415 BCA able to grow and produce a sufficiently active metabolite *in planta* could be the
416 easiest and cheapest application method but, as our results have shown, this behaviour
417 is highly specific since it could be influenced by many conditions such as pH or
418 temperature, and the available nutrients (Ashis and Kishore, 2005; Monteiro et al.,
419 2016). The use of CFS with the maximum concentration of active metabolites may have
420 more advantages than cultures but the difficulty of determining its complex composition
421 does not allow the innocuousness of its application to be established. On the other hand,
422 formulating a cell free biopesticide enriched in one or various metabolites would require
423 high production costs and yields, but toxicity assays may be easier. Many studies
424 endorse the low toxicity of iturin A (Kim and Lee, 2009; Zhang et al., 2012; Dey et al.,
425 2016; Cao et al., 2017) but strict toxicological studies that guarantee its innocuousness
426 for humans and the environment are essential for any future application.

427 **4. Conclusions**

428 The present study has shown that iturin A produced by *B. amyloliquefaciens* BUZ-14 is
429 the main lipopeptide family responsible for fungal inhibition and which at low levels
430 provides effective control of brown rot in peaches and blue mould in apples. Thus, a
431 BCA that can produce a sufficiently active metabolite *in planta* could be the most
432 suitable pathway for a commercial application. However, BUZ-14 is only able to grow
433 and produce iturin A in peaches, which reveals the complex and specific nature of the
434 BCA-fruit-pathogen interactions. For the rest of the fruit-pathogen binomials in which
435 no growth of the BCA was detected, an iturin A enriched extract could be applied in
436 order to obtain effective disease control. Thus, optimising the production of iturin A by

437 BUZ-14 and testing the antifungal efficacy of these enriched culture extracts will be the
438 focus of our future research.

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635 **Figure captions**

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

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

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

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654 **Table 1.** *In vitro* antifungal activity assayed by the well-dilution method in liquid medium of the Cell-Free Supernatant (CFS), the Lipopeptide
655 Fraction (LPF) and the Non-Butanolic Fraction (NBF) produced by *B. amyloliquefaciens* BUZ-14 after 96 h of incubation at 30 ºC in 863
656 medium against six postharvest fungal pathogens. Five replicates per pathogen and dilution were conducted on 3 different days.

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Fraction	Concentration (Dilution)	*B. cinerea*	*M. fructicola*	*M. laxa*	*P. digitatum*	*P. italicum*	*P. expansum*
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

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671 [a] Non-growth of the fungal pathogen

672 [b] Growth of the fungal pathogen

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673 **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.*
674 *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
675 incubated at 30 ºC in 863 medium. Fruits were stored at 20 ºC and 80 % R.H. for 5 days.

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Fraction	Concentration	Reduction of lesion diameter (%)[a]					
		B. cinerea	*M. fructicola*	*M. laxa*	*P. digitatum*	*P. italicum*	*P. expansum*
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

682 [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,
683 done in 3 separate days, of 15 fruits each.

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

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687 **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 ºC

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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 $[M+H;Na;K]^+$

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697 **Table 4.** Minimum Inhibitory Concentration of iturin A present on the LPF of *B. amyloliquefaciens* BUZ-14 obtained after 96 h of incubation at
698 30 ºC in 863 medium for the six fungal pathogens tested.

Phytopathogenic fungi	Iturin A (μg mL^{-1})[a]	
	In vitro[b]	*In vivo*[c]
B. cinerea	16.9	> 67.8
M. fructicola	8.5	16.9
M. laxa	4.1	16.9
P. digitatum	16.9	> 67.8
P. italicum	8.5	> 67.8
P. expansum	8.5	33.9

699
700 [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 mg mL^{-1} was reached
701 [b]*In vitro* activity was assayed by the well-dilution method in liquid medium
702 [c]*In vivo* activity was tested by the puncture method: fruits were wounded with a sterile tip and 10 μL of the respective pathogen (10^4 conidia mL^{-1}) was inoculated (*B. cinerea*
703 in strawberries, *M. fructicola* and *M. laxa* in peaches, *P. digitatum* and *P. italicum* in mandarins and *P. expansum* in apples).
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715 **Table 5.** Reduction of the fungal growth, BUZ-14 counts and

716 iturin A produced in fruits after 7 days at 20 ºC.

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

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723 Data shown are means ± standard deviation.

724 [a]Inoculated with 10 µL of the respective pathogen (10^4 conidia mL^{-1}) and 10 µL of a BUZ-14 24 h-old culture (10^8 CFU mL^{-1}).

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

726 [c]The initial counts of BUZ-14 in the fruits were 5.0 log CFU g^{-1}.

727 [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.

728 [e]nd: not detected

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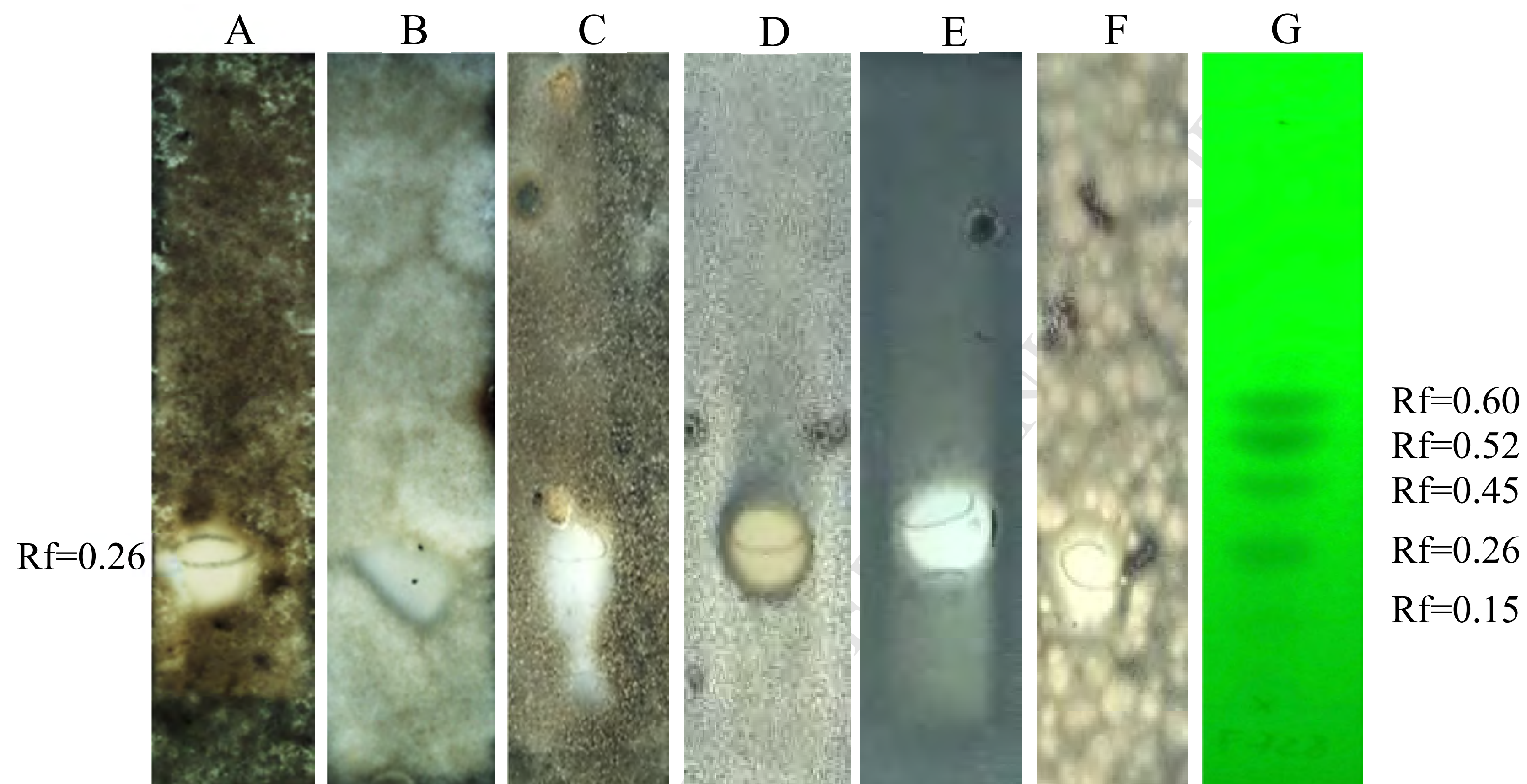

Fig. 1

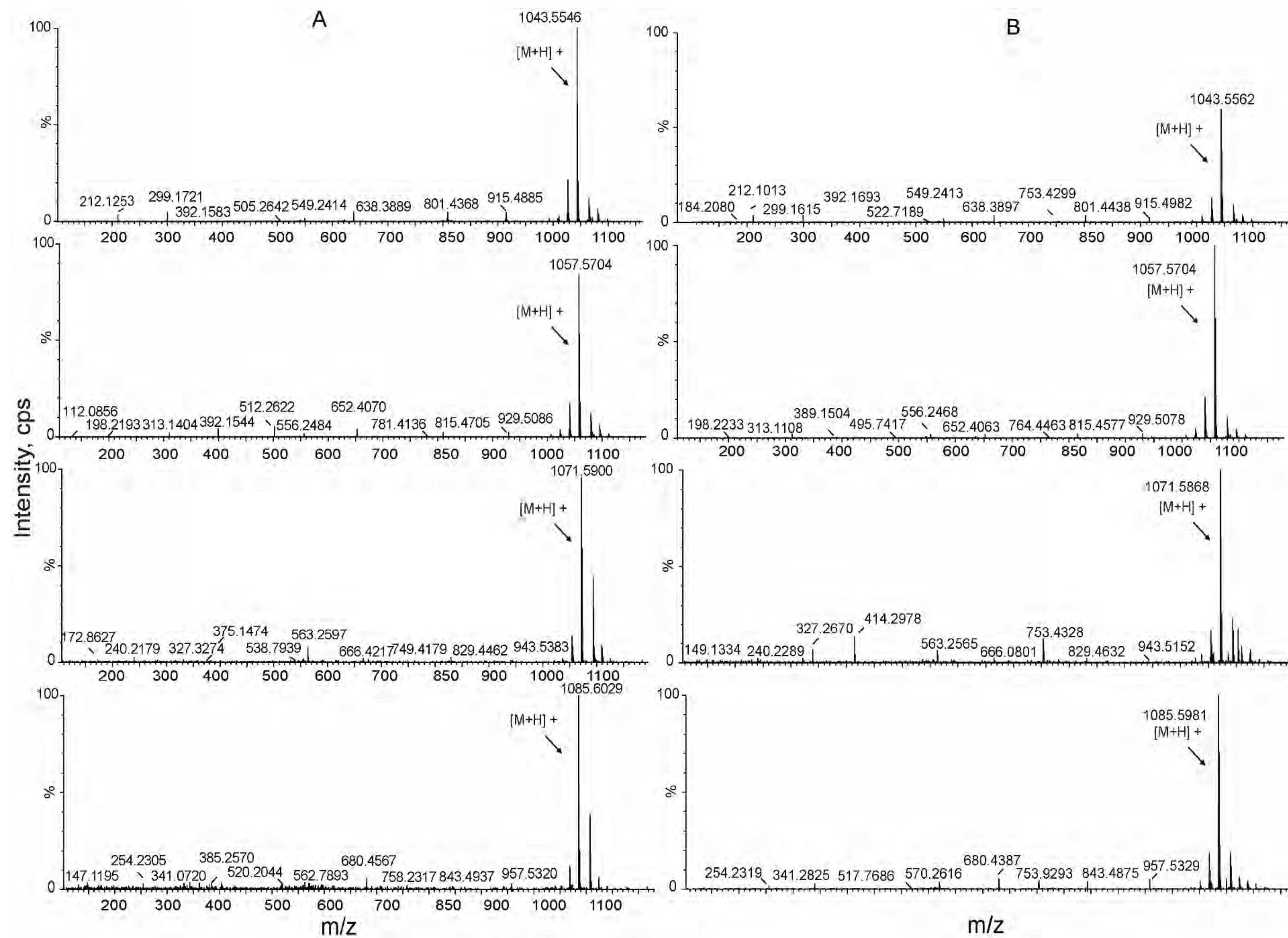

Fig. 2

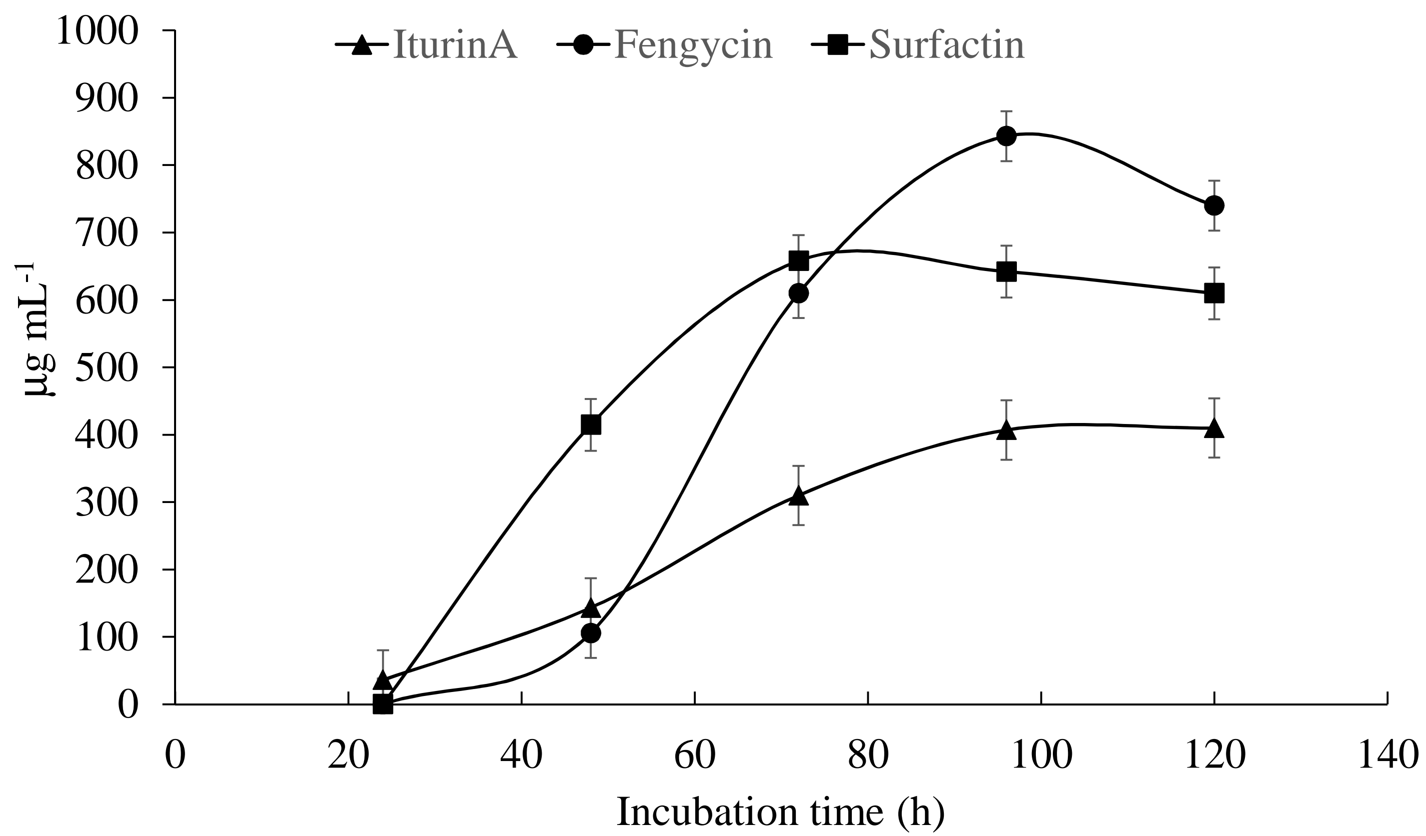

Fig. 3

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1 **Highlights**

2 - Lipopeptides of BUZ-14 are the main metabolites for controlling postharvest
3 rots
4 - Iturin A is the key lipopeptide responsible for direct antibiosis
5 - Only 17 and 34 μg mL^{-1} are necessary to suppress brown and blue rots,
6 respectively
7 - BUZ-14 is able to grow and produce significant iturin A in peaches
8 - First report quantifying the production of iturin A in fruit

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