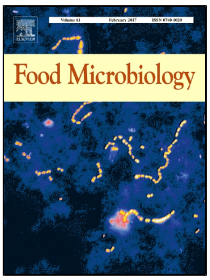
# Accepted Manuscript




Potential of a new strain of Bacillus amyloliquefaciens BUZ-14 as a biocontrol agent of postharvest fruit diseases

H. Calvo, P. Marco, D. Blanco, R. Oria, M.E. Venturini

| | |
|---|---|
| PII: | S0740-0020(16)30169-1 |
| DOI: | 10.1016/j.fm.2016.11.004 |
| Reference: | YFMIC 2666 |
| To appear in: | *Food Microbiology* |
| Received Date: | 02 March 2016 |
| Revised Date: | 28 October 2016 |
| Accepted Date: | 07 November 2016 |

Please cite this article as: H. Calvo, P. Marco, D. Blanco, R. Oria, M.E. Venturini, Potential of a new strain of Bacillus amyloliquefaciens BUZ-14 as a biocontrol agent of postharvest fruit diseases, *Food Microbiology* (2016), doi: 10.1016/j.fm.2016.11.004

**Highlights**

- *Bacillus amyloliquefaciens* BUZ-14 inhibited major postharvest rots in fruits
- Preventive treatments were effective against *Penicillium* spp. in oranges and apples
- *B. amyloliquefaciens* exhibited a curative effect against brown rot in stone fruits
- BUZ-14 survived at cool temperatures making it suitable for postharvest treatment

# Potential of a new strain of *Bacillus amyloliquefaciens* BUZ-14 as a biocontrol agent of postharvest fruit diseases

Calvo, H., Marco, P., Blanco, D., Oria, R. and Venturini, M.E*

Plant Food Research Group. Instituto Agroalimentario de Aragón– IA2 -(Universidad de Zaragoza-CITA), Miguel Servet, 177. 50013, Spain.

***Corresponding author:**

Maria E Venturini, Plant Food Research Group. Instituto Agroalimentario de Aragón– IA2 - (Universidad de Zaragoza-CITA), Miguel Servet, 177. 50013, Spain.

Tlf: +34976762683; Fax: +34976761590;

E-mail address: ugeventu@unizar.es (M.E. Venturini)

## ABSTRACT

The biocontrol potential of the *Bacillus amyloliquefaciens* strain BUZ-14 was tested against the main postharvest diseases of orange, apple, grape and stone fruit. After characterizing the temperature and pH growth curves of strain BUZ-14, its *in vitro* antifungal activity was determined against *Botrytis cinerea*, *Monilinia fructicola*, *M. laxa, Penicillium digitatum, P. expansum* and *P. italicum*. Subsequently, *in vivo* activity was tested against these pathogens by treating fruit with cells, endospores and cell-free supernatants. The *in vitro* results showed that BUZ-14 inhibited the growth of all the pathogens tested corresponding to the least susceptible species, *P. italicum*, and the most susceptible, *M. laxa*. *In vivo* tests corroborated these results as most of the treatments decreased the incidence of brown rot in stone fruit from 100 % to 0 %, establishing $10^7$ CFU $mL^{-1}$ as the minimum inhibitory concentration. For the *Penicillium* species a preventive treatment inhibited *P. digitatum* and *P. italicum* growth in oranges and reduced *P. expansum* incidence in apples from 100 % to 20 %. Finally, it has been demonstrated that BUZ-14 was able to survive and to control brown rot in peaches stored at cool temperatures, making it a very suitable biocontrol agent for application during the post-harvest storage and marketing of horticultural products.


*Keywords*: biocontrol, *Botrytis cinerea*, *Monilinia* spp., *Penicillium* spp.

## 1. Introduction

It has been estimated that about 20-25 % of harvested fruits and vegetables decay by filamentous fungi during postharvest handling even in developed countries, so control of fungal infections at this stage is critical (Droby, 2006; Singh and Sharma, 2007). Currently control measures of phytopathogens during pre- and post-harvest practices are primarily based on synthetic chemicals (El-Ghaouth et al., 2004; Ismail and Zhang, 2004; Droby, 2006; Korsten, 2006; Zhu, 2006; Singh and Sharma, 2007). However, the use of such chemicals is becoming increasingly problematic due to stricter legislation (Dir. 91/414/CEE) and growing public pressure resulting from their toxicological risks to human health (Dir. 2009/128/CE; U.S. National Research Council, 1987), especially children´s health (U.S. National Research Council, 1993) and possible environmental pollution. A further limiting factor for chemical application is the development of fungicide-resistant strains for the main phytopathogenic fungi (Kinay et al., 2007; Zhao et al., 2010; Chen et al., 2013; Panebianco et al., 2015; Vitale et al., 2016). Consequently, researchers are currently looking for alternative methods to control postharvest diseases (Romero et al., 2007; Dimkic et al., 2013; Oro et al., 2014; Jiang et al., 2015; Parafati et al., 2015).

Biological control, which includes the use of antagonist microorganisms (BCAs-biological control agents) such as yeast and bacteria, has been a promising alternative to synthetic fungicides since it is safer for both human health and the ecosystem (Wilson and Wisniewski, 1994; Janisiewicz and Korsten, 2002; Korsten, 2006). During the last 30 years, over one thousand articles on postharvest biocontrol have been published and several microorganisms have been tested against various postharvest fungal pathogens on fruit. However, only few biologicals are commercially available for control of fruit diseases during the postharvest phase. These include, Bio-Save® 10 LP (*Pseudomonas*

*syringae*; Jet Harvest Solutions, USA) (Janisiewicz and Jeffers, 1997), registered in the USA by the EPA (Environmental Protection Agency) and used mostly for the control of sweet potato and potato diseases, BoniProtect® (*Aureobasidium pullulans*; Bio-Protect GmbH, Germany) for the control of apple storage diseases and Candifruit (*Candida sake* CPA-1, Sipcam Inagra S.A.) (Viñas et al., 1998), commercialized in Spain and recommended against the major postharvest diseases of pome and citrus fruit.

*Bacillus* species are widely distributed in the rhizosphere. They have high thermal tolerance, grow rapidly in liquid cultures, readily form spores and are not phytopathogenic. Their spores are resistant to physical and chemical treatments such as desiccation, heat, UV irradiation or organic solvents (Leelasuphakul et al., 2008), and some species are able to produce biofilms. Several strains of the *Bacillus* genus have been studied for their production of anti-fungal metabolites, which are potential BCAs against a wide range of fungal pathogens (Touré et al., 2004; Ongena et al., 2005; Chung et al., 2008; Arguelles-Arias et al., 2009; Torres et al., 2016). *B. subtilis* is the species most extensively used against plant diseases and 4-5 % of its genome is dedicated to antibiotics´ production (Stein 2005). Some strains have been employed to develop commercial products such as Subtilex® (*B. subtilis* MBI 600; BASF), registered in the USA by the EPA and used for the control of fruit, herb and vegetable diseases, and Serenade® Max (*B. subtilis* QST 713, AgraQuest Inc., California, USA and BASF, Ludwigshafen, Germany), currently registered for the use against *B. cinerea*, *Pseudomonas* spp., *Sclerotinia* spp. *Venturia* spp. and *Monilinia* spp. in more than 20 countries in Europe, Africa, Middle East, Asia and Latin America. *B. amyloliquefaciens*, closely related to *B. subtilis*, dedicates 8.5 % of its genome to produce several bioactive compounds with high antifungal activity such as lipopeptides including surfactin (Ahimou et al., 2000; Hsieh et al., 2004) iturin (Hsieh et al., 2008;

Yu et al., 2002) or fengycin (Lin et al., 1999; Ongena et al., 2007) and several polyketides such as macrolactin, bacillaene, bacilysin, bacillomycin and difficidin (Chen et al., 2009). Furthermore, other antibiotics such as plantazolicin or chlorotetaine, all of which have substantial antimicrobial and antifungal activity, are also secreted (Scholz et al., 2011; Mudgal et al., 2013; Wang et al., 2016). Recently, Taegro® *(B. amyloliquefaciens* FZB24); Novozymes (Copenhagen, Denmark) and Syngenta (Basel, Switzerland) (Lecomte et al., 2016), recommended for suppressing selected soil-borne and foliar diseases of fruits and leafy vegetables, cucurbits, and ornamentals, have been registered in the USA by the EPA.

The main objective of this study is to evaluate the potential of the *B. amyloliquefaciens* strain BUZ-14 for controlling postharvest diseases in orange, apple, grape and stone fruit. Our study has four phases: (1) to characterize the strain growth at different temperature and pH values; (2) to determine its *in vitro* antifungal activity against several postharvest pathogens; (3) to establish the efficacy of cells, endospores and cell-free supernatants against the main postharvest pathogens in fruit models; and (4) to assess its survival rate and antifungal activity under cold storage temperatures.

## 2. Materials and methods

### *2.1. Pathogens*

*Penicillium expansum* CECT 20140 and *Monilinia fructicola* ATCC 44557 were obtained from the respective culture collections. *Botrytis cinerea* VG 1, *Penicillium digitatum* VG 20*, Penicillium italicum* VG 101*,* and *Monilinia laxa* VG 105 were obtained from the Plant Food Research Group culture collection at Zaragoza University (Spain). All pathogens were incubated on potato dextrose agar (PDA) (Oxoid Ltd; Basingstoke, Hampshire, England) Petri dishes supplemented with 1 % acetone in the

case of *M. fructicola* and *M. laxa* to induce conidia production (Pascual et al., 1990). Periodically, the strains were transferred to fresh fruit to induce infection and spore production.

### *2.2.* Bacillus *strain and inoculum production*

The strain BUZ-14 used in this study was obtained from the Plant Food Research Group Collection at Zaragoza University. It was isolated from the surface of peach fruit from an orchard in Zaragoza and tested for its preliminary antifungal potential against *M. fructicola* (data not shown). A partial 16s rRNA sequence from the BUZ-14 strain was conducted by the Spanish Type Culture Collection (CECT). It was identified as a member of the *B. amyloliquefaciens* species complex. Cultures were stored at 5 ºC and subcultured on tryptose soy agar (TSA, Oxoid Ltd) supplemented with 0.6% yeast extract at 30 ºC for 24 h when required. Criobilles (Deltalab, Barcelona, Spain) were used for long-term storage at -80 ºC. To prepare the initial fresh cell suspension of BUZ-14, the 24 h-old culture on TSA was transferred to 7 mL of tryptose soy broth (TSB, Oxoid Ltd). This initial suspension was incubated for 24 h at 30 ºC and adjusted to 40 ± 5 % transmittance at 700 nm with a spectrophotometer, corresponding to 2 x $10^8$ colony forming units (CFU) per milliliter. 100 µL of this suspension was transferred to 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., 2010) to obtain the initial cultures. Cultures were always incubated on a rotary shaker at 150 rpm.

### *2.3. BUZ-14 growth temperature, pH curves and endospore production*

Three Erlenmeyer flasks containing 50 mL of 863 medium inoculated with 100 µL of the initial suspension were disposed per time, temperature and pH on a rotary

shaker at 150 rpm.

BUZ-14 strain growth ($log_{10}$ CFU $mL^{-1}$) at 4, 10, 20, 30 and 37 ºC at 150 rpm was determined by subsampling bacterial cultures every 2 h from 0 to 24 h, and at 36, 48, 72, 96 and 120 h. Besides, its growth at different pH values at 30 ºC was studied (3.0, 4.0, 4.5, 5.0 and 7.0) using citric acid (Merck) to adjust the medium. In this case BUZ-14 growth was determined by subsampling bacterial cultures at 0, 10, 24, 48 and 120 h. Endospore production at 30 ºC was determined from 24, 48, 72, 96 and 120 h old BUZ-14 strain cultures. Bacterial cells (vegetative cells + endospores) were separated from the supernatant (antifungal metabolites) by centrifugation for 10 min at 9000 x *g* at 10 ºC (Beckman Coulter™). The pellet obtained was resuspended in buffered peptone water and vegetative cells were killed by heat treatment at 80 ºC for 12 min. The entire experiment was repeated three times. Bacteria and endospores counting (CFU $mL^{-1}$) were done by dilution and plated on TSA.

*2.4. In vitro antifungal activity*

Cultures (vegetative cells + endospores + supernatant), endospores and cell-free supernatant from the *B. amyloliquefaciens* strain BUZ-14 were tested against *B. cinerea, M. fructicola, M. laxa, P. digitatum, P. expansum,* and *P. italicum*. An agar plug (5 mm diameter) from actively-growing margins of fungal colonies (7 day-old culture on PDA) was placed at the center of PDA dishes for each *M. fructicola* and *M. laxa*. For *B. cinerea*, *P. digitatum*, *P. expansum* and *P. italicum*, the inoculum was obtained from a 7-day old culture in potato dextrose broth (PDB) (Oxoid Ltd). The concentrations of CFU units were determined using a haemocytometer and a Leica microscope (Leica Microsystems, Germany) and the suspensions were adjusted to $10^5$ CFU $mL^{-1}$ and inoculated at the center of PDA dishes using a sterile needle. Then,

BUZ-14 strain obtained from a 24 h-old culture on 863 broth ($10^9$ CFU $mL^{-1}$) was inoculated using a sterile needle in three equidistant parts from the center of the plate (3 cm) where the fungal pathogens was previously placed. The fungal pathogens with slow growth (*M. fructicola* and *M. laxa*) were inoculated 24 h prior to the bacteria. To test the efficacy of the endospores 30 µL aliquots of endospore suspension from 72, 96 and 120 h-old cultures in 863 medium, adjusted to $10^7$ endospores $mL^{-1}$ by decimal dilution, were dispensed in 3 wells (6 mm diameter) made in the gel medium with a sterile scalpel before placing the fungal inoculum. Supernatant samples obtained by centrifugation at 9000 x *g* from 72, 96 and 120 h-old BUZ-14 cultures in 863 medium at 30 ºC were mixed with PDA in proportions of 1:1, 1:4 and 1:10. Each mixture was then sterilized at 121 ºC for 15 min and poured into 90 mm diameter Petri plates. After solidification, single agar plugs of *M. fructicola* and *M. laxa* 7 day-old cultures were placed on each plate, whereas *B. cinerea*, *P. digitatum, P. expansum* and *P. italicum* inocula from PDB liquid culture ($10^5$ conidia $mL^{-1}$) were punctured at three equidistant places from each other and at 3 cm from the center of the plate. The control samples consisted of PDA Petri dishes with only the fungal inoculum. The PDA Petri dishes were incubated for 7 days at 25 ºC under aerobic conditions. Fungal growth inhibition was evaluated by measuring the diameter of the fungal colony and expressed as the percentage of fungal growth inhibition compared to that of the control plates. Mean values and standard errors of the mean were calculated from five PDA dishes for each pathogen and treatment.

### *2.5. Antifungal activity of* B. amyloliquefaciens *BUZ-14 strain on fruit*

#### *2.5.1 Fruit*

The fruit used in this study were obtained from local packinghouses and were

grown in different areas of Spain (La Almunia de Doña Godina, Tarragona, and Teruel). Fruit free of visible wounds and rot and homogeneous in size and maturity were stored at 1 ºC and used for experiments within 3 days of collection. Prior to the experiments, all fruit 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 ºC).

*2.5.2 Phytopathogenic fungal and bacteria inocula preparation*

The pathogenic fungal inocula consisted of aqueous conidial suspensions prepared from 7 day-old cultures of *B. cinerea*, *P. digitatum, P. expansum* and *P. italicum* grown on PDA at 25 ºC and 80 % RH. *M. fructicola* and *M. laxa* isolates were inoculated onto peaches or nectarines in order to obtain a high conidia production (Casals et al., 2010). Fruit were wounded with a sterilized steel rod (1 x 2 mm) and conidia and mycelium were transferred to the wound site with a sterile pipette tip. Fruit were then incubated at 25 °C and 80 % RH for 7 days. Conidia were loop-washed from the PDA plates or from the surface of infected fruits, filtrated through four layers of sterile cheesecloth and transferred to a test tube with 9 mL of sterile distilled water with 0.01 % Tween 80. The suspensions were adjusted at $10^5$ conidia (CFU) mL $^{-1}$ for *B. cinerea*, *P. digitatum, P. expansum* and *P. italicum* and at $10^4$ CFU mL $^{-1}$ for *Monilinia* spp.

Cultures (vegetative cells + endospores + supernatant), cells (vegetative cells + endospores), at different concentrations ($10^8$,$10^7$, $10^6$ CFU mL$^{-1}$), endospores ($10^7$ CFU mL$^{-1}$) and cell-free supernatant undiluted and 1:10 diluted were prepared from cultures at different incubation times (24, 28, 72, 96 and 120 h). To obtain the cell suspensions, the supernatant was separated by centrifugation at 9000 x *g* for 10 min at 10 ºC and the pellet obtained was resuspended in buffered peptone water. Endospores and cell-free

supernatants were obtained as described in sections 2.3. and 2.4, respectively. Cultures and cells were adjusted by spectrophotometry to $10^8$ CFU $mL^{-1}$ and subsequent concentrations were obtained by dilution and verified via viable counts on TSA.

*2.5.3. Efficacy of cultures, endospores and cell-free supernatant curative treatments against the main postharvest pathogens*

The efficacy of cultures ($10^8$ CFU $mL^{-1}$), endospores ($10^7$ CFU $mL^{-1}$), and cell-free supernatant (undiluted and 1:10 diluted) from 72, 96 and 120 h-old cultures were determined on fruit. For this purpose, oranges (cv. Valencia) were inoculated with *P. digitatum* and *P. italicum,* apples (cv. Golden Delicious) with *P. expansum*, grapes (cv. Sultanina) with *B. cinerea* and cherries (cv. Lapins) with *M. fructicola* and *M. laxa*. Apples and oranges were inoculated by making two wounds (3 x 3 mm width and depth) on the fruit surface with a sterile micropipette tip, but only one wound was made in the case of cherries and grapes. Each wound was inoculated with 10 µL of $10^5$ conidia $mL^{-1}$ for *B. cinerea, P. digitatum, P. expansum* and *P. italicum* and 10 µL of $10^4$ conidia $mL^{-1}$ for *M. fructicola* and *M. laxa* prior to bacterial treatment. The fruits were stored for 1 hour at room temperature to permit the absorption of the conidia suspension. After that, 10 µL of bacterial treatment was inoculated in each wound. Fruit with only fungal inocula served as control treatments. All the samples were stored at 20 ºC and 80 % RH for 7 days. Incidence (percentage of rotted wounds) and severity were measured to compare the treatments. In the case of small fruits (grapes and cherries) the severity rating scale of infected wounds was: 0 = no symptoms; 1 = 1-25 % of the fruit infected; 2 = 25-50% of the area infected; 3 = sporulation cover 50-75 %; 4 > 75% of the fruit infected. In addition, the lesion diameter (mm) around the wound was measured for the rest of the fruits to determine the severity. Ten fruits were used for

each treatment and pathogen. The entire experiment was repeated three times.

*2.5.4. Efficacy of BUZ-14 curative and preventive treatments against* B. cinerea *and* Penicillium *species*

In the case of *B. cinerea* and *Penicillium* species, the effects of curative and preventive treatments using 24 h-old cultures were tested. Oranges (cv. Valencia) were inoculated with *P. digitatum* and *P. italicum,* apples (cv. Golden Delicious) with *P. expansum* and grapes (cv. Sultanina) with *B. cinerea* as previously described. For the preventive treatments, the strain BUZ-14 was first inoculated. Thus, 10 µL from a 24 h-old culture in 863 medium ($10^8$ CFU $mL^{-1}$) were transferred to each wound and fruits were placed at 20 ºC and 80 % RH for 24 h. Afterwards, 10 µL from $10^5$ conidia $mL^{-1}$ suspension of the pathogen was inoculated to the fruits and stored at 20 ºC and 80 % RH for 7 days. For the curative treatment, fruits were inoculated with 10 µL of conidia suspensions ($10^5$ conidia $mL^{-1}$) 1 h prior to the bacterial treatment (10 µL from a 24 h-culture at $10^8$ CFU $mL^{-1}$) and incubated at 20 ºC and 80 % RH for 7 days. In both cases, a control treatment with only pathogen conidia (controls) was included. Incidence and severity were measured to compare the treatments as described above. Ten fruits were used for each treatment and pathogen. The entire experiment was repeated three times.

*2.5.5. Efficacy of different concentrations of culture, cell and cell-free supernatant treatments against* M. fructicola *and* M. laxa *on wounded stone fruit*

Cultures (vegetative cells + endospores + supernatant in 863 medium) adjusted to $10^8$, $10^7$ and $10^6$ CFU $mL^{-1}$, cells (vegetative cells + endospores resuspended in buffered peptone water) adjusted to $10^8$, $10^7$ and $10^6$ CFU $mL^{-1}$ and cell-free supernatant treatments (undiluted and 1:10 diluted) from 24, 48 and 72 h-old culture of the *B. amyloliquefaciens* strain BUZ-14 were tested against both *Monilinia* species on stone

252 fruit (peaches cv. Calante). Treatment with Serenade® Max at the commercial dose of
253 2.5 g $L^{-1}$ (approximately 1.8 x $10^7$ CFU $mL^{-1}$), with the fungicide Scholar at the
254 recommended dose of 2 mL $L^{-1}$, and a control treatment with only distilled water were
255 also included. Bacterial concentrations were verified after the application by plate count
256 on TSA. Peaches were inoculated by making two wounds (3 x 3 mm width and depth)
257 on the fruit surface with a sterile micropipette tip. Each wound was inoculated with 10
258 µL of *M. fructicola* and *M. laxa* ($10^4$ conidia $mL^{-1}$) 2 h prior to bacterial treatment. After
259 that, 10 µL of bacterial treatments was inoculated in each wound. Fruits with only
260 fungal inocula served as control treatments. Treated peaches were stored at 20 ºC, 80 %
261 RH for 7 days. Incidence (percentage of rotted wounds) and severity (lesion diameter
262 (mm)) were measured to compare the treatments. Ten peaches were used for each
263 treatment and pathogen. The entire experiment was repeated three times.

264 *2.5.6. Survival and efficacy against brown rot of* B. amyloliquefaciens *in wounded fruit*
265 *at cool and room temperatures*

266 Peaches cv. Calante inoculated with *M. fructicola* or *M. laxa* and treated with *B.*
267 *amyloliquefaciens* BUZ-14 were used to determine bacterial survival and efficacy
268 against brown rot at cool and room temperatures.

269 For the bacterial survival experiment, peaches were inoculated by making two
270 wounds (3 x 3 mm width and depth) on the fruit surface with a sterile micropipette tip.
271 Each wound was inoculated with 10 µL of $10^4$ conidia $mL^{-1}$ of *M. fructicola* and *M. laxa*
272 prior to bacterial treatment. The fruits were stored for 2 hours at room temperature to
273 favour the absorption of the conidia suspension. After that, 10 µL ($10^7$ CFU $mL^{-1}$)
274 obtained from a 24 h-old culture (vegetative cells + endospores + supernatant) was
275 inoculated in each wound. Then, one batch of fruits were stored for 10 days at 1 ºC

276 followed by a shelf-life period of 3 days at 20 ºC and another batch was disposed at
277 room temperature (4 days at 20 ºC). BUZ-14 counts were determined on days 0, 5 and
278 10 at cool temperature and daily during the shelf-life period. At room temperature the
279 counts were conducted daily during the four days of the experiment. A plug sample (10
280 x 5 mm, diameter and depth) of the wound previously inoculated with bacterial
281 inoculum was removed with a sterile scalpel. The peach plugs were placed in filter
282 blender bags with 0.1 % sterile peptone water. The mixture was homogenized in a
283 laboratory blender Stomacher 400 Circulator (Seward Laboratory, London, England) for
284 120 s at 260 rpm and the resulting suspension was diluted, plated on TSA plates and
285 counted after 24 h at 30 ºC. Three peaches (2 wounds per peach) were analyzed at each
286 sampling point and the results were expressed as CFU *Bacillus* per wound (CFU wound$^{-}$
287 $^{1}$). The entire experiment was repeated three times.

288 The antifungal activity was studied in parallel to the BCA survival experiments.
289 For that purpose, ten peaches for each *Monilinia* species and storage conditions were
290 wounded and inoculated retracing the steps described above, and stored at cool and
291 room temperatures. The entire experiment was repeated three times.

292 *2.6. Statistical analysis*

293 The data were statistically analyzed using an SPSS software package for
294 Windows version 19.0 (SPSS Inc., Chicago, IL, USA). Differences in the mean values
295 of parameters were tested by one-way analysis of variance and separated by Tukey's
296 honestly significant difference test ($P < 0.05$).

297 **3. Results and discussion**

298 *3.1. Characterization of* B. amyloliquefaciens *strain BUZ-14 growth*

Characterization of the growth of the *B. amyloliquefaciens* strain BUZ-14 at different temperatures and pH values is necessary to ascertain whether this strain will be able to survive in the intrinsically acidic conditions of the fruit and at the cool temperatures employed during postharvest storage, distribution and commercialization. These tests were conducted previously on laboratory media and subsequently on fruit as described in section 2.5.6.

*3.1.1. Growth temperature and pH curves and endospore production*

The growth temperature curve of *B. amyloliquefaciens* (Figure 1A) showed that the population increased from 4.1 to 5.0 $\log_{10}$ CFU mL $^{-1}$ in only 2 h at 37 ºC. At this temperature, the lag phase was almost inexistent and the maximum population of 9.1 $\log_{10}$ CFU mL $^{-1}$ was reached after 16 h. At 20 ºC the lag phase was prolonged during 8 h of incubation, after which logarithmic growth was observed to reach the stationary phase after 48 h. This means that the BUZ-14 strain at 20 ºC needs around 40 hours to attain the stationary phase, keeping large differences with higher temperatures. It can also be observed that the population decreased 1-1.5 log units after 120 h of incubation at cool temperatures (4 and 10 ºC).

The growth curves of the *B. amyloliquefaciens* strain BUZ-14 at different pH values are shown in Figure 1B. At pH 7, used as control, the population increased from 4.8 to 9.0 $\log_{10}$ CFU mL $^{-1}$ in the first 24 h whereas at pH 5 the same maximum concentration was achieved after 48 h of incubation following a lag phase of 24 h. At lower pH values (4.5, 4.0 and 3.5) the counts decreased by 2 log units in just 12 h of incubation, the final counts after 120 h being about 2-2.5 $\log_{10}$ CFU mL$^{-1}$. It is clear that a pH below 5 has an inhibitory effect on BUZ-14 growth, decreasing the initial populations by almost 3 log units. Although it would be expected that the concentration

of BUZ-14 in acid fruits such as strawberry or oranges should be significantly reduced, its survival was observed instead. However, its growth can be assured in fruits with higher pH such as such as ripe peaches used in this study (section 3.4).

Endospore production (Figure 1A) reached 4.3 log units after 24 h and increased by more than 1 log unit per day until the fourth day of incubation, achieving 8.5 log units after 120 h of incubation. The BUZ-14 spore production (3.2 x $10^8$ spores $mL^{-1}$) and sporulation efficiency (33 %) on 683 culture media were in the range reported for other *Bacillus* strains, with densities from 1.0 x $10^5$ spores $mL^{-1}$ (Cayuela et al., 1993) to 3.0 x $10^9$ spores $mL^{-1}$ (Warriner and Aites, 1999) and typical efficiencies of 30-100% (Nicholson and Setlow, 1990). Industrial exploitation of spores requires high cell density bioreaction and good sporulation efficiency. For that purpose the cultivation parameters (pH, dissolved oxygen concentration, and media composition) have to be optimized. Monterio et al. (2005) for *B. subtilis* strain MB24 established an optimal pH value of 7.5, no significative influence of the dissolved oxygen concentration within the studied range (10-50% of the oxygen saturation concentration) and that as nutrient depletion is the main stimulus for sporulation, it is very important to achieve glucose depletion at the end of the exponential growth phase.

### *3.2. In vitro antifungal activity of* B. amyloliquefaciens *strain BUZ-14*

The strain BUZ-14 was able to inhibit mycelium growth of all the tested postharvest fungal pathogens *in vitro* (Table 1). Vegetative cells (24 h-old cultures) reduced fungal growth between 39 %, for *P. italicum*, and 73 %, for *M. laxa*, in comparison with the untreated pathogen control. Endospores also showed strong antifungal activity, those obtained from 96 and 120 h-old cultures being the most effective. BUZ-14 cell-free supernatants obtained after 72, 96 and 120 h of incubation completely inhibited the growth of all tested pathogens, except for *P. digitatum* and *P.*

348 *italicum*. Total inhibition of *P. digitatum* was obtained with metabolites 1:1 and 1:4
349 diluted obtained after 72 h, and at any concentration in the case of 96 and 120 h of
350 incubation. The worst results were observed for *P. italicum* as total reduction was only
351 achieved with 1:1 and 1:4 supernatant dilutions obtained from 96 and 120 h cultures.

352 Vegetative cells, endospores and cell-free supernatants of BUZ-14 have shown
353 strong *in vitro* antifungal activity against *B. cinerea, M. fructicola, M. laxa, P.*
354 *digitatum, P. expansum,* and *P. italicum.* The activity of cells and endospores was
355 associated with white precipitates surrounding the bacterial colonies inwards from the
356 zone of mycelium inhibition. Touré et al. (2004) observed these white precipitates when
357 testing the *in vitro* activity of *B. subtilis* GA1 endospores against *B. cinerea* and
358 suggested that they were related with the excretion of fungitoxic compounds which
359 precipitate in contact with the acidified medium induced by the mold growth. This
360 hypothesis was exemplified by testing filter-sterilized crude supernatants and verifying
361 their high antifungal activity. Several studies with different strains of *B. subtilis*
362 suggested that antibiosis could be the principal mode of action in postharvest disease
363 suppression and some of them have identified the presence of powerful antifungal
364 metabolites, in special lipopeptides of surfactin, iturin and fengycin families (Touré at
365 al., 2004; Ongena et al. 2005; Stein 2005; Chung et al., 2008; Joshi et al., 2008;
366 Waewthomgrak et al., 2015; Torres et al., 2016). The ability to produce a wide range of
367 antifungal compounds has also been reported for *B. amyloliquefaciens* strains (Yoshida
368 et al., 2001; Arguelles-Arras et al., 2009; Arrebola et al., 2010; Hao et al., 2011; Ben
369 Ayed et al., 2015; Torres et al., 2016). Chen et al. (2006) revealed the capacity of *B.*
370 *amyloliquefaciens* FZB42 to produce several lipopeptides such as surfactin,
371 bacillomycin, fengycin and bacillibactin with antifungal, antibacterial and even
372 nematocidal activity. Subsequently, Schneider et al. (2007) detected that this strain was

373 also able to produce some polyketides with high antifungal activity such as difficidin
374 and bacylisin. The *in vitro* antifungal activity of BUZ-14 cell-free supernatants, that
375 equal or better that of cells and endospores, supported the role of these antifungal
376 compounds in the biocontrol activity of the strain. However, there are some bacteria that
377 produce great amounts of antibiotics *in vitro*, but cannot always do so in fruit (Droby et
378 al. 1992, Bull et al., 1997; Touré et al. 2004; Kim et al., 2007; Lai et al., 2012).
379 Consequently, *in vitro* assays should be followed by *in vivo* assays to check that the
380 mechanism of action does not change.

381 *3.3 In vivo assays of* B. amyloliquefaciens *strain BUZ-14 to control postharvest*
382 *incidence and severity of representative postharvest diseases*

383 *3.3.1 Efficacy of cells, endospores and cell-free supernatant curative treatments against*
384 *postharvest pathogen molds*

385 The efficacy of cultures (vegetative cells + endospores + cell-free supernatant),
386 endospores and cell-free supernatant treatments at 72, 96 and 120 h of incubation
387 against postharvest pathogenic fungi is shown in Figure 2. *B. amyloliquefaciens*
388 treatments did not significantly reduce the incidence (% of rotted wounds) or severity
389 (lesion diameter) of the diseases caused by *Penicillium* species, neither by *P. digitatum*
390 or *P. italicum* in oranges neither by *P. expansum* in apples. Nevertheless, *P. expansum*
391 decreased its growth from 23 mm to 17 mm with undiluted cell-free supernatant
392 obtained after 120 h of incubation. Similar results were obtained against gray mold on
393 grapes in terms of incidence, although the severity reduction was higher. The best
394 reduction was provided by undiluted cell-free supernatants, since the severity decreased
395 from 2.5 (untreated samples) to 1.2. These data show that BUZ-14 treatments were not
396 effective in controlling previous Penicilia and Botrytis fruit infections. Our results are
397 consistent with those found by Yánez-Mendizábal et al. (2011) that testing the activity

of *B. subtilis* CPA-8 against *P. digitatum* and *P. italicum* found minimal or no efficient control of green and blue mold decay on orange, despite the good results achieved in the *in vitro* assays. In this study the inoculation of the pathogens was prior to that of the BCA. It seems that in the case of *Bacillus* species the preventive application of the BCA is crucial to achieving a good control of Penicillia and Botrytis infections. Leelasuphakul et al. (2008) with *B. subtilis* 155 endospores applied prior to *P. digitatum* spores in citrus fruit obtained better results in terms of decay incidence and severity than when the two microorganisms were inoculated together. Hang et al. (2005) detected that a pre-application of *Bacillus subtilis* S1-0210 before *B. cinerea* inoculation was more effective in controlling gray mold in strawberry than a post-application, concluding that the pre-colonization of antagonistic agents on host plants can be a critical factor in protecting the host from infection of fungal pathogens. Lai et al. (2012) also detected that the control of postharvest green mold on citrus fruit improved when the period between the treatment with *Paenibacillus polymyxa* strain SG-6 and the inoculation with the fungal pathogen was increased. In view of these results, the effect of preventive treatments with BUZ-14 cultures, $10^8$ CFU $mL^{-1}$ applied 24 h prior to *Penicillium* species and *B. cinerea,* was investigated (section 3.3.2).

*M. fructicola* and *M. laxa* were the most susceptible species as the incidence obtained with undiluted cell-free supernatant at any incubation times and 72 and 96 h-old cultures treatments was reduced to 0 % in comparison with 100 % for the untreated sample. For endospores, the best result was achieved with those collected after 96 h of incubation as *M. fructicola* growth was totally inhibited and the incidence and severity of *M. laxa* was reduced to 10 % and 1.2, respectively. These results confirm the potential of cultures, endospores and cell-free supernatants of BUZ-14 to control decay by *M. fructicola* and *M. laxa* already reported in previous studies for other *Bacillus*

423 strains (Pusey and Wilson, 1984; Altindag et al., 2006; Yañez-Mendizabal et al., 2010;
424 Liu et al., 2011; Rungjindamai et al., 2013; Gao et al., 2016). The high efficacy against
425 brown rot of BUZ-14 endospores is very interesting as they are more stable than
426 vegetative cells maintaining their viability for years and far more resistant to drying
427 processes for powder formulations (Brannen and Kenney, 1997), all of them being
428 crucial aspects to develop a commercial formulation. In addition, the cell free
429 supernatant, composed by bioactive compounds, such as lipopeptides and polyketides,
430 with low toxicity, high biodegradability and environmentally friendly characteristics,
431 show great potential for future applications, being an effective alternative to the
432 chemical pesticides usually applied to control brown rot. In section 3.3.3. the
433 relationship between the number of cells and the protection level is elucidated and
434 compared to those of BUZ-14 cell-free supernatants, fungicide Scholar® and Serenade®
435 Max.

436 *3.3.2 Efficacy of curative and preventive treatments against* B. cinerea and Penicillium
437 *species*

438 The results of preventive and curative treatments from 24 h-old cultures against
439 *B. cinerea* and *Penicillium* species are shown in Figure 3. Preventive inoculation
440 avoided *P. digitatum* and *P. italicum* growth in oranges and reduced *P. expansum*
441 incidence in apples from 100 % to 20 %. For *B. cinerea*, only a 20 % of incidence
442 reduction was observed, although the severity decreased by 40 %. Curative treatments
443 using 24 h-old cultures had no effect on the incidence and slighty reduced the severity,
444 showing similar results to those obtained with 72, 96 and 120 h-old cultures, except in
445 the gray mold severity that was greater (Fig. 2). These data confirmed that BUZ-14
446 cultures have a limited curative effect on *P. expansum*, *P. digitatum* and *P. italicum*
447 infections and, as has been discussed in the previous section, preventive inoculation of

448 the BCA is crucial to achieve a good disease control. For these species, the bacterial
449 tissue colonization and a certain level of antifungal compounds production that protect
450 the fruit against the pathogen infection could be necessary (Arrebola et al., 2010, Hang
451 et al., 2005).

452 However, Arrebola et al. (2010) demonstrated that the effectiveness of the
453 treatments with the antagonist *B. amyloliquefaciens* PPCB004 applied 1 day after or 1
454 day before pathogen application depends on the pathogen tested. For example, to
455 control *Alternaria citri* or *Penicillium crustosum* the antagonist treatment 1 day before
456 showed promise; however, for *Colletotrichum gloeosporioides* the antagonist
457 application 1 day after was more effective so the efficacy of curative and preventive
458 treatments should be determined for each antagonism and pathogen. Hao et al. (2011)
459 and Hong et al. (2014) obtained good control of citrus green and blue mold and sour rot
460 inoculating the pathogens almost immediately after the BCA (*B. amyloliquefaciens* HF-
461 01) and three hours later, respectively. So, another aspect to study is the determination
462 of the optimal interval of time between the BCA and the pathogen inoculation (also in
463 reverse order of inoculation) to obtain the maximum postharvest disease control.

464 *3.3.3 Efficacy of different concentrations of culture, cell and cell-free supernatant*
465 *treatments against* M. fructicola *and* M. laxa *on wounded stone fruit*

466 Since our previous data show that *M. fructicola* and *M. laxa* are most susceptible
467 to BUZ-14 applications, these fungi were chosen for subsequent bioassays in Calante
468 peaches. The activity of 24, 48 and 72 h-old cultures were tested in this assay to shorten
469 incubation times, which are always preferred when obtaining BCAs. The results of the
470 culture, cells and cell-free supernatant treatments are shown in Figure 4. Cultures and
471 cells at $10^8$ and $10^7$ CFU $mL^{-1}$ and cell free supernatant obtained from 24 h-old cultures

472 reduced the incidence of brown rot to 0 %, compared to 100 % in the untreated control.
473 However, $10^6$ CFU $mL^{-1}$ treatments were ineffective since the disease incidence was
474 similar to that of untreated samples although the lesion diameter was reduced. A notable
475 decrease of efficacy was detected with treatments obtained from 48 h-old cultures since
476 only cultures and cells at $10^8$ CFU $mL^{-1}$ and undiluted cell free supernatant reduced
477 disease incidence to 0% for both species. Cultures and cells obtained from a 72 h-
478 culture were also unable to control the disease and only the cell free supernatant
479 obtained a total inhibition of *M. laxa* growth. The BUZ-14 treatments were compared
480 with two commercial standards, Serenade® Max, a biocontrol product formulated with
481 *Bacillus subtilis* QST-713, and Scholar®, a postharvest treatment based on fludioxonil.
482 No evidence of disease was detected with Scholar®. Serenade® Max ($1.7 \times 10^7$ CFU
483 $mL^{-1}$) significantly reduced the incidence and severity compared to untreated control
484 samples but did not fully control the disease.

485 For both species the experimental data show that concentrations of $10^8$ and $10^7$
486 CFU $mL^{-1}$ of cultures and cells obtained after 24 h of incubation of the BCA provided a
487 total control of brown rot in peaches. Data reported by Zhang and Dou (2002), Touré et
488 al. (2004) and Yánez-Mendizábal et al. (2010) using *B. subtilis* to green mold, brown
489 rot and gray mold control indicated that $>10^7$ CFU $mL^{-1}$ was the appropriate
490 concentration for bacterial treatments. An evident decrease in efficacy was detected
491 with the reduction of the number of cells in the treatment and with the increasing of
492 previous incubation times of the BUZ-14 cultures. Touré at al. (2004) suggested that
493 antibiosis could play a major role in the inhibition of the disease since the protection
494 level by the bacterium increased proportionally with the number of bacterial cells used
495 for treatment although competition for nutrients cannot obviously be ruled out.
496 Treatment of fruits with BUZ-14 cell free supernatants also provided a strong protective

497 effect against brown rot that was similar to, or higher than the one observed with live
498 cells. This indicated the important role of the antifungal compounds in the biocontrol
499 activity of BUZ-14. However, the maximum inhibition of supernatants (that is, no
500 evidence of disease detected with undiluted and 1:10 diluted treatments) was obtained
501 with extracts from different times of incubation, 48 h-old cultures for *M. fructicola* and
502 72 h-old cultures for *M. laxa*. This could be due to the different amounts and types of
503 antifungal compounds in the supernatant and the different susceptibility of each species.
504 Although several authors have revealed the major role of fengycnin-like lipopeptides in
505 the biological control of *Bacillus* species against fungal pathogens (Ongena et al., 2005;
506 Romero et al., 2006; Hu et al., 2007; Alvarez et al., 2012) including *M. fructicola* (Lui
507 et al., 2011; Yánez-Mendizábal et al., 2012), the activity of other lipopeptides such as
508 iturins (Yu et al., 2002; Arrebola et al., 2010) or other antimicrobial compounds such as
509 plantazolicin (Scholz et al., 2010) or chlorotetaine (Wang et al., 2016) and the presence
510 of synergistic effects (Maget-Dana et al., 1992; Lui et al., 2011; Tao et al., 2011) could
511 not be ruled out. Thus, the characterization of the bioactive compounds produced by
512 BUZ-14 in terms of identity, antifungal activity using non-producing mutants, total and
513 individual amounts, relative proportions and progressive accumulation both in culture
514 media and in fruit are key aspects to understand the mechanism of action of this BCA
515 against both fungal pathogens.

516 *3.4.* B. amyloliquefaciens *survival and efficacy against brown rot on wounded stone*
517 *fruit stored at room and cool temperatures*

518 Our experimental data show that BUZ-14 was more effective than Serenade®
519 Max in reducing brown rot caused by *M. laxa* and *M. fructicola* in peaches, providing
520 opportunities for the use of a new *Bacillus* strain to develop commercial formulations.
521 However, given that fruits are usually stored and distributed at cool temperatures (1-10

ºC), the BCA strain must be able to grow in this temperature range or at least not decrease during the postharvest storage period. Thus, it is important to establish the survival and antifungal activity of BUZ-14 in fruit stored at cool temperatures. These data were compared with those obtained at room temperature. Bacterial populations of *B. amyloliquefaciens* BUZ-14 on wound inoculated peaches (pH 5.1) at 20 ºC was characterized by a 24 h lag-phase followed by a constant increase of cell population from 3.2 x $10^5$ CFU wound$^{-1}$ to values up to $10^8$ CFU wound$^{-1}$ from the 48 h and onwards (Figure 5) indicating a good bacterial survival and growth. BUZ-14 bacterial concentration after 10 days of storage at 1 ºC and 80 % RH decreased from 1.3 x $10^5$ to 2.5 x $10^4$ CFU wound$^{-1}$. When the fruits were transferred to room temperature conditions (20 ºC) in order to simulate a period of commercialization, the populations recovered after 48 h reached 4 x $10^6$ CFU wound$^{-1}$ in 72 h (Figure 5). Compared with the initial concentration ($10^5$ CFU wound$^{-1}$), the bacterial population only decreased by 0.7 log units after 10 days at cool temperature. These data coincide with those obtained previously in 863 medium at 4 ºC, where bacterial growth decreased by around 0.8 log units. No symptoms of decay were detected in any of the inoculated and treated peaches so we can be sure that BUZ-14 survived and colonized the injured fruit tissue and maintained its antifungal activity even after a cool storage period of 10 days.

## 4. Conclusions

The results obtained in this study have demonstrated that *B. amyloliquefaciens* BUZ-14 is a promising BCA for major postharvest fruit diseases. Its relevance in curative treatments against *M. fructicola* and *M. laxa* and preventive treatments against *P. expansum, P. digitatum* and *P. italicum* should be highlighted. In addition, the BUZ-14 strain survives at cool temperatures, as low as 1 ºC, which make it suitable for postharvest application. Its mode of action, antibiotics´ production and activity, and

biotechnological issues of production and formulation are the subjects of ongoing research.

**Acknowledgements**

This work was supported by the European Project Life +12 ENV/ES/000902 "ZERO RESIDUES", the Department of Industry and Innovation of the Aragón Government and the European Social Fund: Project 229402/1 (Plant Food Research Group). H. Calvo is the beneficiary of a predoctoral grant from the Aragón Regional Government (Spain).

**References**

Ahimou, F., Jacques, P., Deleu, M., 2000. Surfactin iturin A effects on *Bacillus subtilis* surface hydrophobicity. Enzyme Microb. Technol. 27, 749-754.

Altindag, M., Sahin, M., Esitken, A., Ercisli, S., Guleryuz, M., Donmez, M.F., Sahin, F., 2006. Biological control of brown rot (*Moniliana laxa* Ehr.) on apricot (*Prunus armeniaca* L. cv. Hacihaliloglu) by *Bacillus*, *Burkholdria*, and *Pseudomonas* application under *in vitro* and *in vivo* conditions. Biol. Control 38, 369-372.

Álvarez, F., Castro, M., Principe, A., Borioli, G., Fischer, S., Mori, G., Jofre, E., 2012. The plant-associated *Bacillus amyloliquefaciens* strains MEP218 and ARP23 capable of producing the cyclic lipopeptides iturin or surfactin and fengycin are effective in biocontrol of sclerotinia stem rot disease. J. Appl. Microbiol. 112, 159-174.

Arguelles-Arias, A., Ongena, M., Halimi, B., Lara, Y., Brans, A., Joris, B., Fickers, P., 2009. *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microb. Cell Fact. 8:63.

571 Arrebola, E., Sivakumar, D., Bacigalupo, R., Korsten, L., 2010. Combined application
572 of antagonist *Bacillus amyloliquefaciens* and essential oils for the control of
573 peach postharvest diseases. Crop Prot. 29, 369-377.
574 Ben Ayed, H., Maalej, H., Hmidet, N., Nasri, M., 2015. Isolation and biochemical
575 characterisation of a bacteriocin-like substance produced by *Bacillus*
576 *amyloliquefaciens* An6. J. Glob. Antimicrob. Resist. 3, 255-261.
577 Brannen, P.M., Kenney, D.S., 1997. 'Kodiak® - A successful biological-control product
578 for suppression of soil-borne plant pathogens of cotton. J. Ind. Microbiol.
579 Biotechnol. 19, 169-171.
580 Bull, C.T., Stack, J.P., Smilanick, J.L., 1997. *Pseudomonas syringae* strains ESC-10
581 and ESC-11 survive in wounds on citrus and control green and blue molds of
582 citrus. Biol. Control 8, 81-88.
583 Casals, C., Vinas, I., Landl, A., Picouet, P., Torres, R., Usall, J., 2010. Application of
584 radio frequency heating to control brown rot on peaches and nectarines.
585 Postharvest Biol. Technol. 58, 218-224.
586 Cayuela, C., Kai, K., Park, Y.S., Iijima, S., Kobayashi, T., 1993. Insecticide production
587 by recombinant *Bacillus subtilis*-1a96 in fed-batch culture with control of
588 glucose-concentration. J. Ferment. Bioeng. 75, 383-386.
589 Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, L.,
590 Morgenstem, B., Voss, B., Hess, W.R., Reva, O., Junge, H., Voigt, B., Jungblut,
591 P.-J., Vater, J., Süssmuth, R., Liesegang, H., Strittmatter, A., Gottschalk, G.,
592 Borriss, R., 2006. Comparative analysis of the complete genome sequence of the
593 plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat.
594 Biotechnol. 25, 1007-1014.
595 Chen, X.H., Koumoutsi, A., Scholz, R., Borriss, R., 2009. More than anticipated

596 production of antibiotics and other secondary metabolites by *Bacillus*
597 *amyloliquefaciens* FZB42. J. Mol. Microb. Biotech. 16, 14-24.
598 Chen, F., Liu, X., Schnabel, G., 2013. Field strains of *Monilinia fructicola* resistant to
599 both MBC and DMI fungicides isolated from stone fruit orchards in the eastern
600 United States. Plant Dis. 97, 1063-1068.
601 Chung, S., Kong, H., Buyer, J.S., Lakshman, D.K., Lydon, J., Kim, S.D., Roberts, D.P.,
602 2008. Isolation and partial characterization of *Bacillus subtilis* ME488 for
603 suppression of soilborne pathogens of cucumber and pepper. Appl. Microbiol.
604 Biotechnol. 80, 115-123.
605 Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant
606 protection products on the market. Official Journal of the European
607 Communities. Nº L230/1 published 19.8.91.
608 Dimkić, I., Živković, S., Berić, T., Ivanović, Ž., Gavrilović, V., 2013. Characterization
609 and evaluation of two *Bacillus* strains, SS-12.6 and SS-13.1, as potential agents
610 for the control of phytopathogenic bacteria and fungi. Biol. Control 65, 312-321.
611 Directive 2009/128/EC of the European Parliament and of the Council of 21 October
612 2009 establishing a framework for Community action to achieve the sustainable
613 use of pesticides. Official Journal of the European Communities. Nº L309/71
614 published 24.11.2009.
615 Droby, S., 2006. Improving quality and safety of fresh fruit and vegetables after harvest
616 by the use of biocontrol agents and natural materials. Acta Hortic. 709, 45-51.
617 Droby, S., Chalutz, E., Wilson, C. L., Wisniewski, M. E., 1992. Biological control of
618 postharvest diseases: a promising alternative to the use of synthetic fungicides.
619 Phytoparasitica 20, 149-153.

El-Ghaouth, A., Wilson, C.L., Wisniewski, M.E., 2004. Biologically based alternatives to synthetic fungicides for the postharvest diseases of fruit and vegetables. In: Naqvi, S.A.M.H. (Ed.), Diseases of Fruit and Vegetables, vol. 2. Kluwer Academic Publishers, The Netherlands, pp. 511–535.

Gao, H.Y., Xu, X.X., Dai, Y.W., He, H.X., 2016. Isolation, identification and characterization of *Bacillus subtilis* CF-3, a bacterium from fermented bean curd for controlling postharvest diseases of peach fruit. Food Sci. Technol. Res. 22, 377-385.

Hang, N., Oh, S., Kim, G., Hur, J., Koh, Y., 2005. *Bacillus subtilis* S1-0210 as a biocontrol agent against *Botrytis cinerea* in strawberries. Plant Pathol. J. 21, 59-63.

Hao, W., Li, H., Meiying H., Liu Y., Muhammad, R., 2011. Integrated control of citrus green and blue mold and sour rot by *Bacillus amyloliquefaciens* in combination with tea saponin. Postharvest Biol. Technol. 59, 316-323.

Hong, P., Hao, W.N., Luo, J.J., Chen, S.H., Hu, M.Y., Zhong, G.H., 2014. Combination of hot water, *Bacillus amyloliquefaciens* HF-01 and sodium bicarbonate treatments to control postharvest decay of mandarin fruit. Postharvest Biol. Technol. 88, 96-102.

Hsieh, F.C., Li, M.C., Lin, T.C., Kao, S.S., 2004. Rapid detection and characterization of surfactin-producing *Bacillus subtilis* and closely related species based on PCR. Curr. Microbiol. 49, 186-191.

Hsieh, F.C., Lin, T.C., Meng, M., Kao, S.S., 2008. Comparing methods for identifying *Bacillus* strains capable of producing the antifungal lipopeptide iturin A. Curr. Microbiol. 56, 1-5.

644 Hu, L.B., Shi, Z.Q., Zhang, T., Yang, Z.M., 2007. Fengycin antibiotics isolated from B-
645 FS01 culture inhibit the growth of *Fusarium moniliforme* Sheldon ATCC 38932.
646 FEMS Microbiol. Lett. 272, 91-98.
647 Ismail, M., Zhang, J., 2004. Post-harvest citrus diseases and their control. Outlooks Pest
648 Manage. 15, 29–35.
649 Janisiewicz, W., Korsten, L., 2002. Biological control of postharvest diseases of fruit.
650 Annu. Rev. Phytopathol. 40, 411–441.
651 Janisiewicz, W.J., Jeffers, S.N., 1997. Efficacy of commercial formulation of two
652 biofungicides for control of blue mold and gray mold of apples in cold storage.
653 Crop Prot. 16, 629-633.
654 Jiang, C., Wu, F., Yu, Z., Xie, P., Ke, H., Li, H., Yu, Y., Guo, J., 2015. Study on
655 screening and antagonistic mechanisms of *Bacillus amyloliquefaciens* 54 against
656 bacterial fruit blotch (BFB) caused by *Acidovorax avenae* subsp. *citrulli.*
657 Microbiol. Res. 170, 95-104.
658 Joshi, S., Bharucha, C., Desai, A.J., 2008. Production of biosurfactant and antifungal
659 compound by fermented food isolate *Bacillus subtilis* 20B. Bioresource Technol.
660 99, 4603-4608.
661 Kim, S.Y., Kim, J.Y., Kim, S.H., Bae, H.J., Yi, H., Yoon, S.H., Koo, B.S., Kwon, M.,
662 Cho, J.Y., Lee, C.E., Hong, S., 2007. Surfactin from *Bacillus subtilis* displays
663 anti-proliferative effect via apoptosis induction, cell cycle arrest and survival
664 signaling suppression. FEBS Lett. 581, 865-871.
665 Kinay, P., Mansour, M.F., Gabler, F.M., Margosan, D.A., Smilanick, J.L., 2007.
666 Characterization of fungicide-resistant isolates of *Penicillium digitatum*
667 collected in California. Crop Prot. 26, 647-656.
668 Korsten, L., 2006. Advances in control of postharvest diseases in tropical fresh produce.

669 Int. J. Postharvest Technol. Innov. 1, 48.

670 Lai, K.P., Chen, S.H., Hu, M.Y., Hu, Q.B., Geng, P., Weng, Q.F., Jia, J.W., 2012.
671 Control of postharvest green mold of citrus fruit by application of endophytic
672 *Paenibacillus polymyxa* strain SG-6. Postharvest Biol. Technol. 69, 40-48.

673 Lecomte, C., Alabouvette, C., Edel-Hermann, V., Robert, F., Steinberg, C., 2016.
674 Biological control of ornamental plant diseases caused by *Fusarium oxysporum*:
675 a review. Biol. Control 101, 17-30.

676 Leelasuphakul, W., Hemmanee, P., Chuenchitt, S., 2008. Growth inhibitory properties
677 of *Bacillus subtilis* strains and their metabolites against the green mold
678 pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. Postharvest Biol. Technol.
679 48, 113-121.

680 Lin, T.P., Chen, C.L., Chang, L.K., Tschen, J.S.M., Liu, S.T., 1999. Functional and
681 transcriptional analyses of a fengycin synthetase gene, fenC, from *Bacillus*
682 *subtilis*. J. Bacteriol. 181, 5060-5067.

683 Liu, J., Zhou, T., He, D., Li, X.Z., Wu, H.J., Liu, W.Z., Gao, X.W., 2011. Functions of
684 lipopeptides bacillomycin D and fengycin in antagonism of *Bacillus*
685 *amyloliquefaciens* C06 towards *Monilinia fructicola*. J. Mol. Microbiol.
686 Biotechnol. 20, 43-52.

687 Maget-Dana, R., Thimon, L., Peypoux, F., Ptak, M., 1992. Surfactin iturin-A
688 interactions may explain the synergistic effect of surfactin on the biological
689 properties of iturin-A. Biochimie. 74, 1047-1051.

690 Monteiro, S.M., Clemente, J.J., Henriques, A.O., Gomes, R.J., Carrondo, M.J., Cunha,
691 A.E., 2005. A procedure for high-yield spore production by *Bacillus subtilis*.
692 Biotechnol. Progr. 21, 1026-1031.

693 Mudgal, S., De Toni, A., Tostivint, C., Hokkanen, H., Chandler, D., 2013. Scientific

694 support, literature review and data collection and analysis for risk assessment on
695 microbial organisms used as active substance in plant protection products –Lot 1
696 Environmental Risk characterization. EFSA supporting publications 2013:EN-
697 518. [149 pp.].
698 Nicholson, W. L., Setlow, P., 1990. Sporulation, germination and outgrowth. In:
699 Harwood CR, Cutting SM (Eds), Molecular biological methods for *Bacillus*.
700 John Wiley and Sons, Chichester, United Kingdom, pp. 391-450.
701 Ongena, M., Jacques, P., Toure, Y., Destain, J., Jabrane, A., Thonart, P., 2005.
702 Involvement of fengycin-type lipopeptides in the multifaceted biocontrol
703 potential of *Bacillus subtilis*. Appl. Microbiol. Biotechnol. 69, 29-38.
704 Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., Arpingny, J.L.,
705 Thonart, P., 2007. Surfactin and fengycin lipopeptides of *Bacillus subtilis* 194 as
706 elicitors of induced systemic resistance in plants. Environ. Microbiol. 9, 1084–
707 1090.
708 Oro, L., Feliziani, E., Ciani, M., Romanazzi, G., 2014. Biocontrol of postharvest brown
709 rot of sweet cherries by *Saccharomyces cerevisiae* Disva 599, *Metschnikowia*
710 *pulcherrima* Disva 267 and *Wickerhamomyces*. Postharvest Biol. Technol. 96,
711 64-68.
712 Panebianco, A., Castello, I., Cirvilleri, G., Perrone, G., Epifani, F., Ferrara, M., Polizzi,
713 G., Walters, D.R., Vitale, A., 2015. Detection of *Botrytis cinerea* field isolates
714 with multiple fungicide resistance from table grape in Sicily. Crop Prot. 77, 65-
715 71.
716 Parafati, L., Vitale, A., Restuccia, C., Cirvilleri, G., 2015. Biocontrol ability and action
717 mechanism of food-isolated yeast strains against *Botrytis cinerea* causing
718 postharvest bunch rot of table grape. Food Microbiol. 47, 85-92.

719 Pascual, S., De Cal, A., Melgarejo, P., 1990. Induction of conidia production by
720 *Monilinia* laxa on agar media by acetone. Phytopathol. 80, 494–496
721 Pusey, P.L., Wilson, C.L., 1984. Postharvest biological-control of stone fruit brown rot
722 by *Bacillus-subtilis*. Plant Dis. 68, 753-756.
723 Romero, D., Pérez-García, A., Veening, J.W., de Vicente, A., Kuipers, O.P., 2006.
724 Transformation of undomesticated strains of *Bacillus subtilis* by protoplast
725 electroporation. J. Microbiol. Methods 66, 556-559.
726 Romero, D., de Vicente, A., Rakotoaly, R., Dufour, S., Veening, J.W., Arrebola, E.,
727 Cazorla, F., Kuipers, O., Paquot, M., Pérez-García, A., 2007. The iturin and
728 fengycin families of lipopeptides are key factors in antagonism of *Bacillus*
729 *subtilis* toward *Podosphaera fusca*. Mol. Plant Microbe In. 20, 430-440.
730 Rungjindamai, N., Xu, X-M., Jeffries, P., 2013. Identification and characterisation of
731 new microbial antagonists for biocontrol of *Monilinia laxa*, the causal agent of
732 brown rot on stone fruit. Agronomy 3, 685-703.
733 Schneider, K., Chen, X., Vater, J., Franke, P., Nicholson, G., Borriss, R., Süssmuth, R.
734 D., 2007. Macrolactin is the polyketide biosynthesis product of the pks2 cluster
735 of *Bacillus amyloliquefaciens* FZB42. J. Nat. Prod. 70, 1417-1423.
736 Scholz, R., Molohon, K.J., Nachtigall, J., Vater, J., Markley, A.L., Sussmuth, R.D.,
737 Mitchell, D.A., Borriss, R., 2011. Plantazolicin, a novel microcin
738 B17/streptolysin S-like natural product from *Bacillus amyloliquefaciens* FZB42.
739 J. Bacteriol. 193, 215-224.
740 Singh, D., Sharma, R.R., 2007. Postharvest diseases of fruits and vegetables and their
741 management. In: Prasad, D. (Ed.), Sustainable Pest Management. Data
742 Publishing House, New Delhi, India.
743 Stein, T., 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific

functions. Mol. Microbiol. 56, 845-857.

Tao, Y., Bie, X. M., Lv, F. X., Zhao, H. Z., Lu, Z. X., 2011. Antifungal activity and mechanism of fengycin in the presence and absence of commercial surfactin against *Rhizopus stolonifer*. J. Microbiol. 49, 146−150.

Torres, M.J., Brandan, C.P., Petroselli, G., Erra-Balsells, R., Audisio, M.C., 2016. Antagonistic effects of *Bacillus subtilis* subsp *subtilis* and *B. amyloliquefaciens* against *Macrophomina phaseolina*: SEM study of fungal changes and UV-MALDI-TOF MS analysis of their bioactive compounds. Microbiol. Res. 182, 31-39.

Toure, Y., Ongena, M., Jacques, P., 2004. Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. J. Appl. Microbiol. 96, 1151-1160.

United States National Research Council, Board Agric., 1987. Regulating pesticides in food—The delaney paradox. Nat. Acad. Press,Washington DC, 272 pp.

United States National Research Council, Board Agric., 1993. Pesticides in the diets of infants and children. Nat. Acad. Press,Washington DC, 408 pp.

Viñas, I., Usall, J., Teixidó, N., Sanchis, V., 1998. Biological control of major postharvest pathogens on apple with *Candida sake*. Int. J. Food Microbiol. 40, 9-16.

Vitale, A., Panebianco, A., Polizzi, G., 2016. Baseline sensitivity and efficacy of fluopyram against *Botrytis cinerea* from table grapes in Italy. Ann. Appl. Biol. 169, doi: 10.1111/aab.12277.

Waewthongrak, W., Pisuchpen, S., Leelasuphakul, W., 2015. Effect of *Bacillus subtilis* and chitosan applications on green mold (*Penicilium digitatum* Sacc.) decay in citrus fruit. Postharvest Biol. Technol. 99, 44–49.

769 Wang, T., Wu, M.B., Chen, Z.J., Lin, J.P., Yang, L.R., 2016. Separation, determination
770 and antifungal activity test of the products from a new *Bacillus*
771 *amyloliquefaciens*. Nat. Prod. Res. 30, 1215-1218.
772 Warriner, K., Waites, W.M., 1999. Enhanced sporulation in *Bacillus subtilis* grown on
773 medium containing glucose: ribose. Lett. Appl. Microbiol. 29, 97-102.
774 Wilson, C.L., Wisniewski, M. (Eds.), 1994. Biological control of postharvest diseases:
775 Theory and practice. CRC Press, Boca Raton, FL, 182 pp.
776 Yánez-Mendizábal, V., Usall, J., Viñas, I., Casals, C., Solsona, S., Teixidó, N., 2010.
777 Potential of a new strain of *Bacillus subtilis* CPA-8 to control the major
778 postharvest diseases of fruit. Biol. Control 21, 409-426.
779 Yánez-Mendizábal, V., Zeriouh, H., Viñas, I., Torres, R., Usall, J., de Vicente, A.,
780 Pérez-García, A., Teixido, N., 2012. Biological control of peach brown rot
781 (*Monilinia* spp.) by *Bacillus subtilis* CPA-8 is based on production of fengycin-
782 like lipopeptides. Eur. J. Plant. Pathol. 132, 609-619.
783 Yoshida, S., Hiradate, S., Tsukamoto, T., Hatakeda, K., Shirata, A., 2001. Antimicrobial
784 activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from
785 mulberry leaves. Phytopathology 91, 181-187.
786 Yu, G.Y., Sinclair, J.B., Hartman, G.L., Bertagnolli, B.L., 2002. Production of iturin A
787 by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biol.
788 Biochem. 34, 955-963.
789 Zhang, J., Dou, H., 2002. Evaluation of *Bacillus subtilis* as potential biocontrol agent
790 for postharvest green mold control on 'Valencia' Orange. Proceedings of the
791 Florida State Horticultural Society. 115, 60-64.

792 Zhao, Z., Wang, Q., Wang, K., Brian, K., Liu, C., Gu, Y., 2010. Study of the antifungal
793 activity of *Bacillus vallismortis* ZZ185 *in vitro* and identification of its
794 antifungal components. Bioresour. Technol. 101, 292–297.
795 Zhu, S.J., 2006. Non-chemical approaches to decay control in postharvest fruit. In:
796 Noureddine, B., Norio, S. (Eds.), Advances in Postharvest Technologies for
797 Horticultural Crops. Research Signpost, Trivandrum, India, pp. 297–313.

798

799 **Figure Captions**

800

801 **Fig. 1. Growth of *B. amyloliquefaciens* BUZ-14 in 863 medium.** Effect of the
802 temperature: 4 (▲), 10 (△), 20 (■), 30 (□) and 37 (●) ºC and endospore production at
803 30 ºC (×) (**A**). Effect of the pH: 3.0 (▲), 4.0 (△), 4.5 (■), 5.0 (□) and 7.0 (●) (**B**). Each
804 value is the mean of three separate replicates of three Erlenmeyer flasks each and
805 vertical bars represent the standard error of the mean.

806 **Fig. 2. Effect of *B. amyloliquefaciens* BUZ-14 curative treatments from 72, 96 and**
807 **120 h-old cultures against fruit postharvest rots caused by several mold pathogens**.
808 Incidence as % of rotted wounds (□) and severity as lesion diameter (mm) for apples
809 and oranges and % of the single fruit rotted referred to 0-to-4 scale for grapes and
810 cherries (■). Golden delicious apples infected with *P. expansum* (A); Sultanina
811 seedless grapes infected with *B. cinerea* (B); Valencia oranges infected with *P.*
812 *digitatum* (C) and *P. italicum* (D); Lapins cherries infected with *M. fructicola* (E) and
813 *M. laxa* (F). Fruit were stored at 20 ºC for 7 d. Cont: untreated pathogen control; Cul:
814 vegetative cells + endospores + supernatant ($10^8$ CFU $mL^{-1}$); End: endospores at $10^7$
815 CFU $mL^{-1}$; CF Sup.: cell-free supernatant undiluted and 1:10 diluted. Each value is the

816 mean of three replicates of ten fruits each, 30 fruits per treatment. For each pathogen,
817 different letters above the columns (capital letters for rot incidences and lower-case
818 letters for lesion severities) indicate statistical difference at $P < 0.05$. Incidence columns
819 for A, B, C and D are omitted since their values are similar (about 100%) without
820 statistical differences.

821 **Fig. 3: Curative and preventive effect of *B. amyloliquefaciens* BUZ-14 treatments**
822 **from 24 h-old cultures on fruit.** Golden delicious apples infected with *P. expansum*
823 (A); Sultanina seedless grapes infected with *B. cinerea* (B) and Valencia oranges
824 infected with *P. digitatum* (C) and *P. italicum* (D). Incidence as % of rotted wounds (□)
825 and severity as lesion diameter (mm) for apples and oranges and % of the single fruit
826 rotted referred to 0-to-4 scale for grapes (■). Fruit were stored at 20 ºC for 7 d. Control:
827 untreated pathogen control; Cur 24 h: 24 h-old culture (vegetative cells + endospores +
828 supernatant ($10^8$ CFU $mL^{-1}$)) inoculated 1 h after pathogen infection; Prev 24 h: 24 h-
829 old culture inoculated 1 day prior to pathogen infection. Each value is the mean of three
830 replicates of ten fruits each, 30 fruits per treatment. For each pathogen, different letters
831 above the columns (capital letters for rot incidences and lower-case letters for lesion
832 severities) indicate statistical difference at $P < 0.05$.

833 **Fig. 4: Efficacy of several concentrations of culture, cell and cell-free supernatant**
834 **curative treatments of *B. amyloliquefaciens* BUZ-14 from 24, 48 and 72 h-old**
835 **cultures against *Monilinia* species in Calante peaches.** Incidence (% of rotted
836 wounds): □ and severity (lesion diameter (mm)): ■. *M. fructicola* (A) and *M. laxa* (B).
837 Peaches were stored at 20 ºC for 7 d. Cont: untreated pathogen control ($10^4$ conidia $mL^{-1}$);
838 Cul: vegetative cells + endospores + supernatant at $10^8$, $10^7$ and $10^6$ CFU $mL^{-1}$; Cel:
839 Cell suspensions at $10^8$, $10^7$ and $10^6$ CFU $mL^{-1}$; CF sup: Cell-free supernatant; CF sup
840 1:10: CF sup 1:10 diluted; Com. Stand.: Commercial standards (Ser: Serenade® Max

841 (1.8 x $10^7$ CFU mL $^{-1}$), Scho: Scholar® (2 mL $L^{-1}$)). Each value is the mean of three
842 replicates of ten fruits each, 30 fruits per treatment. For each pathogen, different letters
843 above the columns (capital letters for rot incidences and lower-case letters for lesion
844 diameters) indicate statistical difference at $P < 0.05$.

845 **Fig. 5: *B. amyloliquefaciens* BUZ-14 growth in wounded Calante peaches during**
846 **cold storage and at ambient temperature.** 10 days at 1 ºC followed by a shelf life
847 period of 3 days at 20 ºC (●) and 4 days at 20 ºC (○). Each value is the mean of three
848 replicates (3 fruits with 2 wounds per peach per replicate) and vertical bars correspond
849 to standard deviation.

## Tables

### Table 1

Antifungal activity of *B. amyloliquefaciens* strain BUZ14 on PDA plates against several postharvest mold pathogens.

| Treatment | Percentage of inhibition[a] against: | | | | | |
|---|---|---|---|---|---|---|
| | *Botrytis cinerea* | *Monilinia fructicola* | *Monilinia laxa* | *Penicillium digitatum* | *Penicillium expansum* | *Penicillium italicum* |
| Veg. cells[b] 24 h | 52.0 ± 10.9ab,AB[e] | 66.6 ± 0.7a,AB | 73.1 ± 4.2a,B | 54.5 ± 2.5a,AB | 56.8 ±6.3a,AB | 39.3 ± 7.5a,A |
| End[c] 72 h | 65.4 ± 7.2b,A | 66.4 ± 2.2a,AB | 81.5 ± 2.1b,C | 76.5 ± 1.8bcd,BC | 59.5 ± 1.3a,A | 36.3 ± 1.2a,D |
| End 96 h | 97.0 ± 2.4c,A | 60.0 ± 3.9b,B | 89.6 ± 1.2c,AC | 81.9 ± 0.7d,CD | 79.2 ± 2.0b,D | 68.5 ± 1.2bc,B |
| End 120 h | 92.1 ± 4.7c,A | 57.0 ± 1.2b,BC | 88.2 ± 0.9c,AD | 80.9 ± 4.4cd,D | 51.6 ± 1.7a,B | 63.8 ± 2.8b,C |
| CF Sup[d] 1:1 24 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 77.4 ± 6.6bcd,B | 32.11 ± 0.82c,C | 84.8 ± 7.6de,D |
| CF Sup 1:4 24 h | 62.0 ± 6.1ab,A | 80.9 ± 1.7d,B | 100.0 ± 0.0d,C | 73.2 ± 7.7bc,B | 8.65 ± 0.78d,D | 79.7 ± 10.2cd,B |
| CF Sup 1:10 24 h | 50.3 ± 6.1a,A | 76.0 ± 2.5e,B | 45.5 ± 6.7e,A | 70.9 ± 8.4b,B | 1.29 ± 0.46d,C | 2.8 ± 2.4f,C |
| CF Sup 1:1 72 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 100.0 ± 0.0e,A | 100.0 ± 0.0e,A | 96.6 ± 0.8eg,B |
| CF Sup 1:4 72 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 100.0 ± 0.0e,A | 100.0 ± 0.0e,A | 88.4 ± 1.9de,B |
| CF Sup 1:10 72 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 92.4 ± 0.8e,B | 100.0 ± 0.0e,A | 84.3 ± 1.4de,C |
| CF Sup 1:1 96 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 100.0 ± 0.0e,A | 100.0 ± 0.0e,A | 100.0 ± 0.0g,A |
| CF Sup 1:4 96 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 100.0 ± 0.0e,A | 100.0 ± 0.0e,A | 100.0 ± 0.0g,A |
| CF Sup 1:10 96 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 100.0 ± 0.0e,A | 100.0 ± 0.0e,A | 81.7 ± 0.8de,B |
| CF Sup 1:1 120 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 100.0 ± 0.0e,A | 100.0 ± 0.0e,A | 100.0 ± 0.0g,A |
| CF Sup 1:4 120 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 100.0 ± 0.0e,A | 100.0 ± 0.0e,A | 100.0 ± 0.0g,A |
| CF Sup 1:10 120 h | 100.0 ± 0.0c,A | 100.0 ± 0.0c,A | 100.0 ± 0.0d,A | 100.0 ± 0.0e,A | 100.0 ± 0.0e,A | 92.6 ± 1.6eg,B |

[a] Data are expressed as percentage of fungal growth inhibition compared with control plates without bacteria or supernatant and represent mean values of five plates (replicates) ± standard errors.

856 [b] Cells at $10^9$ CFU $mL^{-1}$.

857 [c] Endospores at $10^7$ CFU $mL^{-1}$.

858 [d] Cell-free supernatant.

859 [e] Values in the same colum followed by different lower-case letters show significant differences between treatments for the same fungal pathogen ($P < 0.05$). Values in the

860 same line followed by different capital letters show significant differences between fungal pathogens for the same treatment ($P < 0.05$).

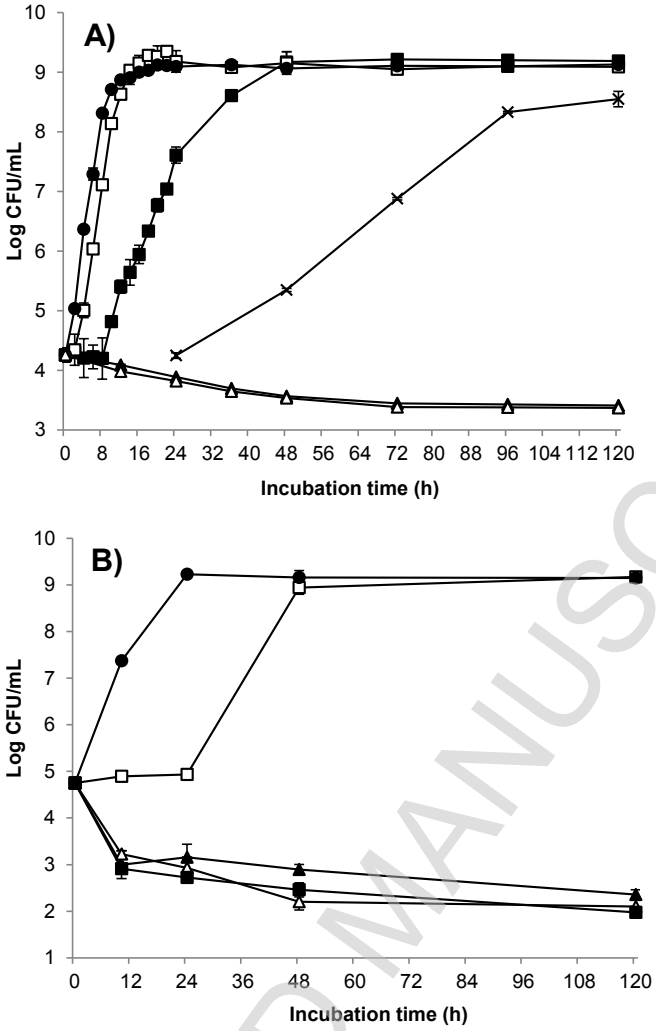





**Fig. 1**

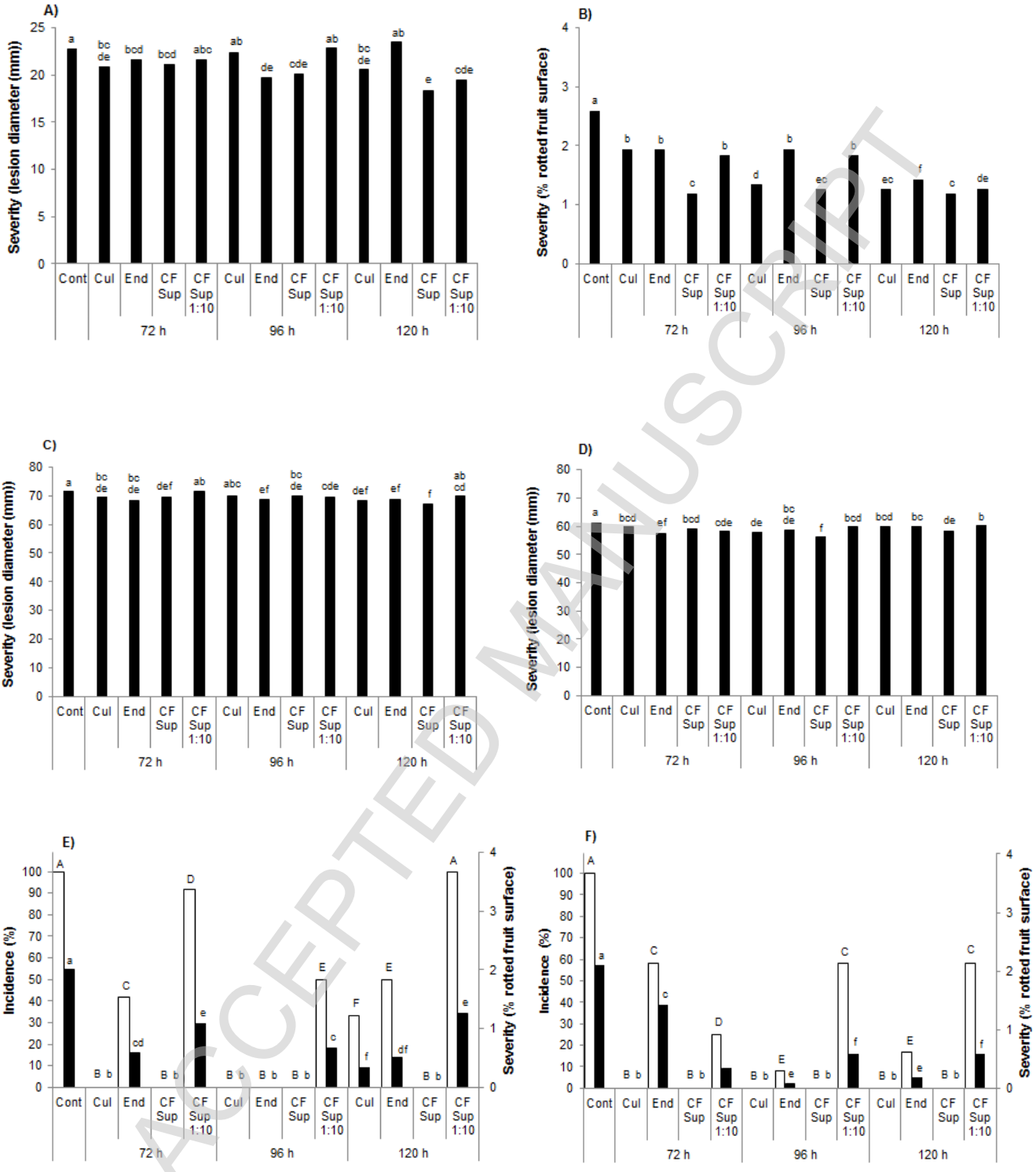





**Fig. 2**

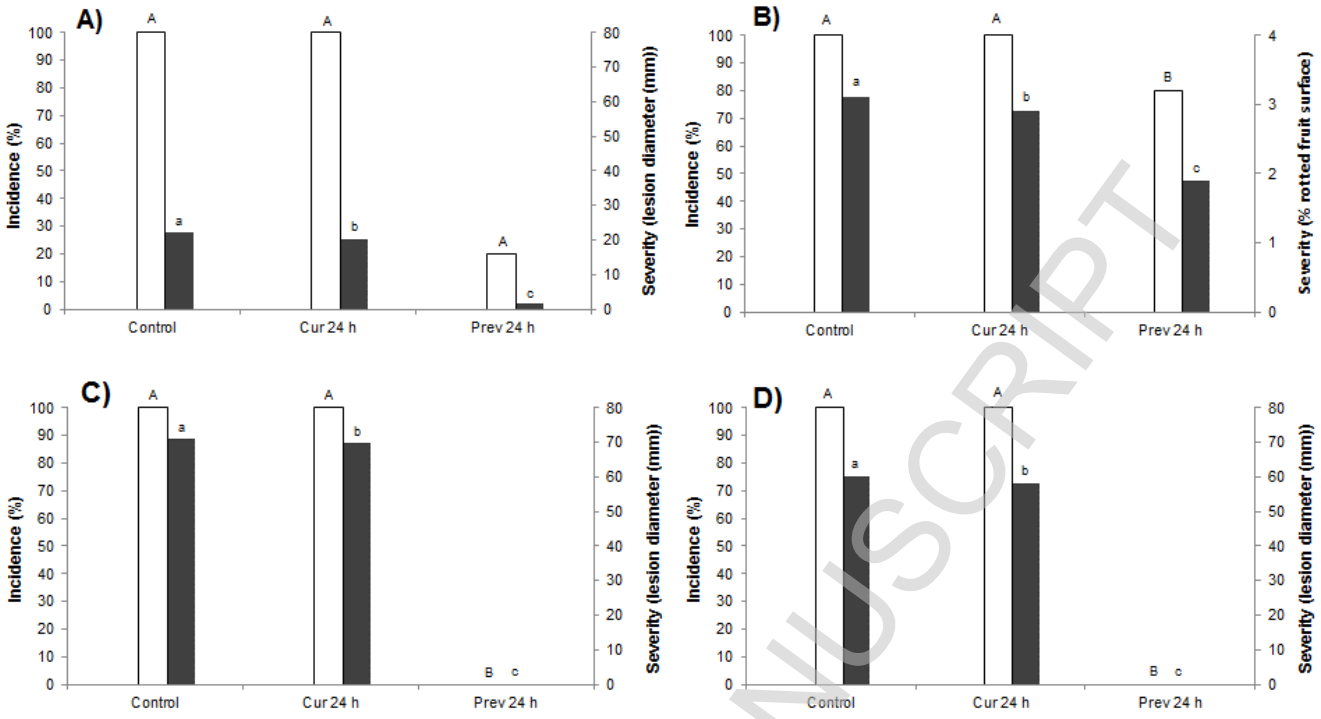





**Fig. 3**

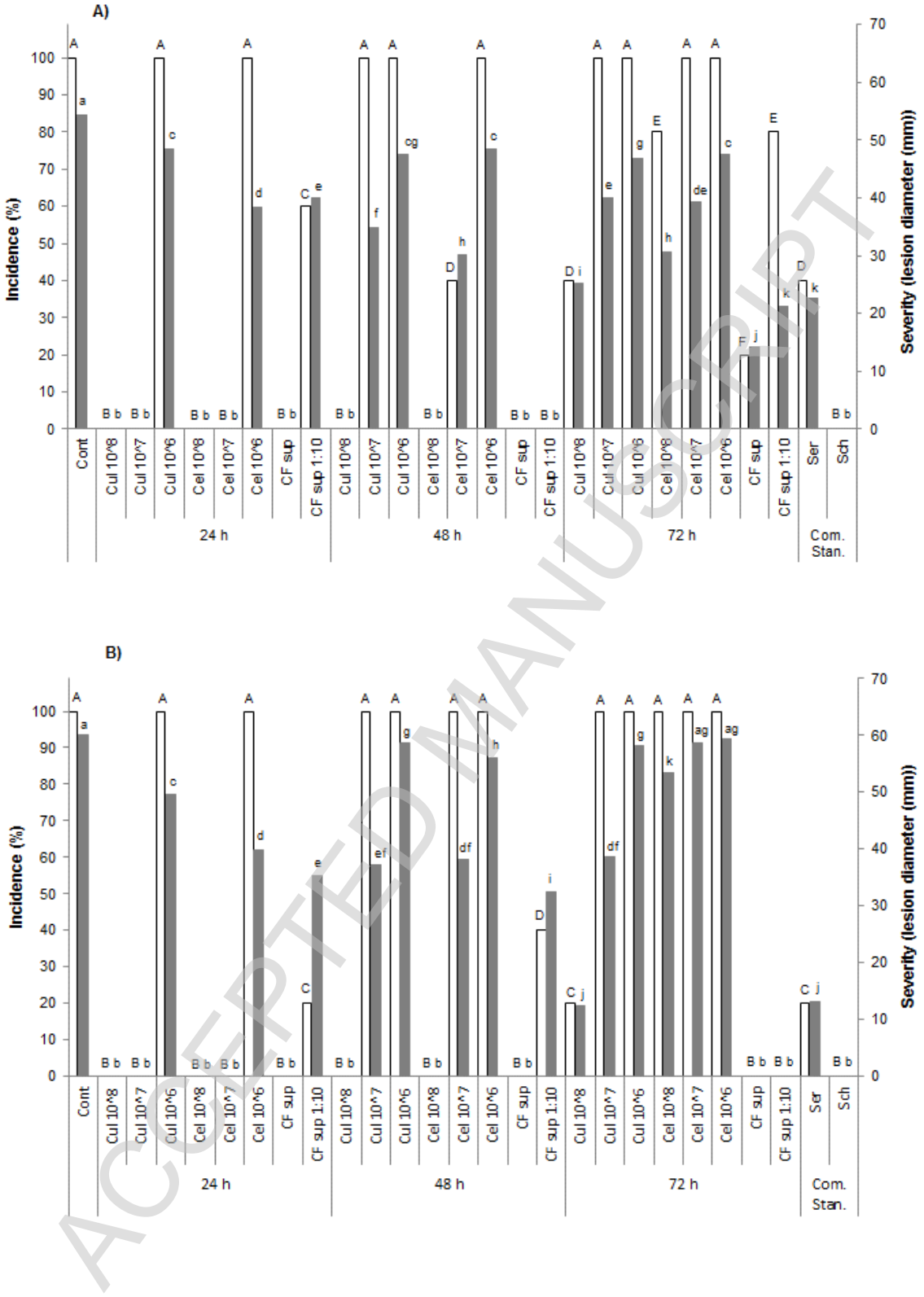



**Fig. 4**

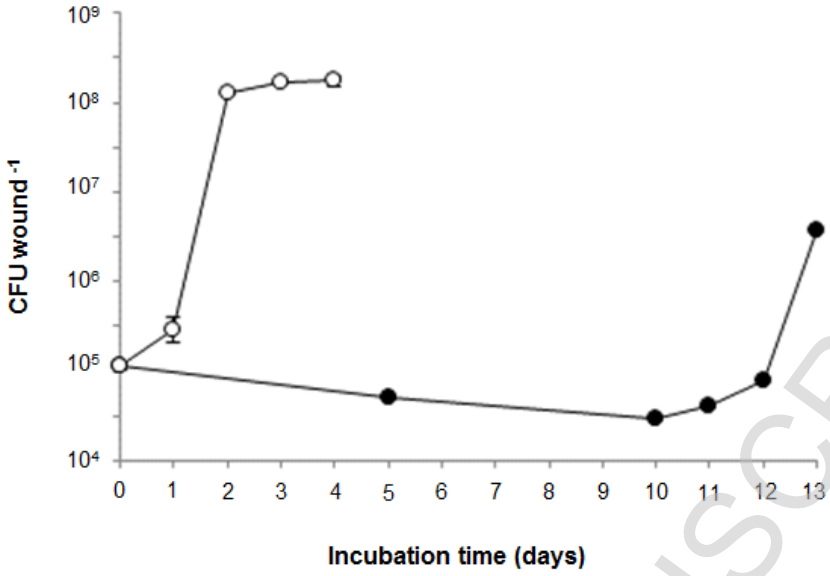



**Fig. 5**