Biological Control 114 (2017) 195–200

Contents lists available at ScienceDirect

# Biological Control

journal homepage: www.elsevier.com/locate/ybcon

# Biocontrol activity of *Bacillus subtilis* EA-CB0015 cells and lipopeptides against postharvest fungal pathogens

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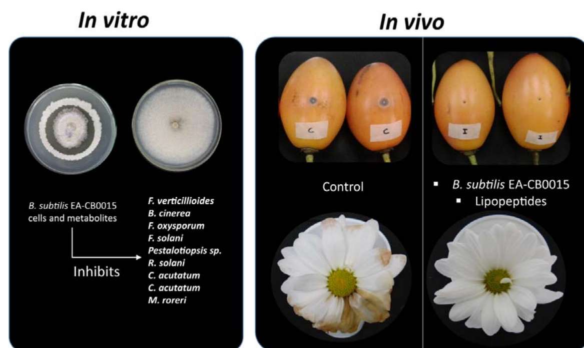
## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Keywords:*
Biological control
Lipopeptides
Fengycin C
Iturin
*Colletotrichum acutatum*
*Botrytis cinerea*

## ABSTRACT

Post-harvest diseases are responsible for significant losses worldwide, especially the plant pathogenic fungi *Botrytis cinerea* and *Colletotrichum* sp. are particularly severe and devastating. In this study, nine fungal pathogens were screened for growth inhibition by *Bacillus subtilis* EA-CB0015 strain and its metabolites. *In vitro* inhibitory assays showed that *B. subtilis* EA-CB0015 cells and the cell free supernatant (CFS) inhibited the growth of the tested fungal pathogens with different susceptibilities. Therefore, the antifungal activities of lipopeptides iturin A and fengycin C contained in the CFS, were tested against *C. acutatum* and *B. cinerea*. *C. acutatum* was more susceptible with minimal inhibitory concentrations (MIC) of 32 ppm (iturin A) and 128 ppm (fengycin C). Fruit and flower trials confirmed that *B. subtilis* EA-CB0015 cells and its lipopeptides reduced postharvest disease development but to differing degrees. Anthracnose symptoms caused by *C. acutatum* in tamarillo fruits were completely abolished by CFS, iturin A and fengycin C and reduced by 76% when treated with *B. subtilis* cells. In contrast, grey mold disease symptoms caused by *B. cinerea* in chrysanthemum flowers were inhibited by 72% when treated with lipopeptides and by 39% when applied with *B. subtilis* EA-CB0015 cells. Our results indicate that lipopeptides and cells of *B. subtilis* EA-CB0015 have a broad antifungal spectrum and control postharvest diseases caused by susceptible fungal pathogens. Our findings open the possibility of incorporating this biological control agent into different disease management programs.

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http://dx.doi.org/10.1016/j.biocontrol.2017.08.014
Received 17 May 2017; Received in revised form 22 August 2017; Accepted 23 August 2017
Available online 24 August 2017
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## 1. Introduction

Plant pathogenic fungi generate losses equivalent to 200 billion dollars each year (Horbach et al., 2011) and without the methods currently available for crop protection, their economic impact would be even higher. In the United States and developing countries, 20%–50% of those losses in revenue come from postharvest diseases (Nunes, 2012), mainly those caused by saprophytic fungi such as *Botrytis cinerea* and *Colletotrichum* sp. These fungi have a wide host range and have been ranked within the ten most devastating plant pathogenic fungi (Dean et al., 2012).

Before infection, these fungi remain on their hosts in a saprophytic phase and once the environmental conditions become suitable (i.e. during post-harvest storage), they switch to a pathogenic phase and generate their characteristic symptoms (Cannon et al., 2012; Williamson et al., 2007). Integrated pest management programs are available; however, growers rely heavily on chemical control for mitigating these diseases. As a consequence, the number of resistant fungal strains as well as the number of prohibited molecules in the exportation policies are increasing, and generates a decrease in the public acceptance (Ecobichon, 2001; Schirra et al., 2011; Sivakumar and Bautista-Baños, 2014; Zhan et al., 2014). Trying to mitigate the negative impacts, growers are starting to incorporate biological control agents into their management programs. However, biological products only account for 2.5% of the agricultural supplies market and are mostly used when the chemical control is not suitable such as in organic crops (Ongena and Jacques, 2008; Mehrotra et al., 2017).

Bacteria from the genius *Bacillus* are known for their ability to produce antimicrobial compounds and resistant endospores that allow them to survive environmental stresses (Emmert and Handelsman, 1999). They inhibit the growth and mitigate the symptoms of plant pathogens both *in vitro* and *in vivo* (Ongena and Jacques, 2008). In particular, lipopeptides contribute to *Bacillus* spp. antimicrobial activity. These antimicrobial compounds are active against a wide range of microorganisms including bacteria, fungi, oomycetes and viruses (Raaijmakers et al., 2010). Out of many examples, lipopeptides reduced the severity of *B. cinerea* on apples (Ongena et al., 2004) and *Alternaria citri*, *C. gloesporoides* and *Peniciliium crustosom* in citrus trees, with the iturin lipopeptide having a major role in preventing disease symptoms (Arrebola et al., 2010).

Within this genus, the bacterial strain Bacillus subtilis EA-CB0015 (Bs EA-CB0015) was isolated from the phyllosphere of banana plants from Uraba (Colombia), selected for its ability to inhibit the growth of Mycosphaerella fijiensis (Ceballos et al., 2012) and for producing high amounts of the lipopeptides fengycin C, iturin A and surfactin (Villegas-Escobar et al., 2013, Mosquera et al., 2014). Furthermore, a formulation containing Bs EA-CB0015 and its lipopeptides was tested in greenhouse and field conditions on banana plants, showing a reduction in Black Sigatoka disease severity comparable to the chemical fungicides chlorotronil and mancozeb (Gutierrez-Monsalve et al., 2015; Villegas-Escobar et al., 2016). Our previous results have suggested that lipopeptides fengycin C and iturin A have a mayor role in controlling the disease, but it has also been shown that cells of *Bs* EA-CB0015 also affected the development of Black Sigatoka.

To determine if pathogenic fungi differ in susceptibilities to the biological control agent *Bs* EA-CB0015, we evaluated its activity *in vitro* against 9 fungal strains and in two post-harvest pathosystems. We found that *Bs* EA-CB0015 and the CFS have differential inhibitory activities on the fungal pathogens *in vitro*, choosing *C. acutatum* as highly susceptible and *B. ci*nerea as more resistant. The difference in susceptibility was also evaluated by determining the MIC of the lipopeptides iturin A and fengycin C, finding lower MIC values for *C. acutatum*. Finally, two different pathosystems were evaluated to determine the effect of *Bs* EA-CB0015 and its lipopeptides to reduce the symptoms caused by *C. acutatum* on tamarillo (*Cyphomandra betacea*) fruits and by *B. cinerea* on chrysanthemum flowers. Tamarillo fruit is the third most important export fruit in Colombia and it has a large local market (Afanador-Kafuri et al., 2003). Chrysanthemum flowers represents 11% of the Colombian flower production (Ascosflores, 2015), which is highly important, as Colombia is one of the world's foremost producers of flowers (https://www.rabobank.com, World Floriculture Map 2015). Therefore, finding new biocontrol strategies to control post-harvest diseases for these agricultural industries is also one of the main objectives of this study.

**Table 1**
Bacterial and fungal strains used in this study.

| Microorganism | Strain | Isolation source | Reference |
|---|---|---|---|
| *B. subtilis* | EA-CB0015 | Banana leaves | GenBank accession number KC006063, Ceballos et al. (2012) |
| *F. verticillioides* | EA-HP013 | Sugar cane | This study |
| *B. cinerea* | IBUN Bc001 | Black berry | Dr. Alba Marina Cotes (Corpoica) |
| *F. oxysporum* | EA-HP005 | Carnation flowers | This study |
| *F. solani* | EA-HP003 | Chrysanthemum flowers | This study |
| *Pestalotiopsis* sp. | EA-HP010 | Avocado | This study |
| *R. solani* | EA-HP002 | Potato | This study |
| *C. acutatum* | EA-HP012 | Tamarillo | This study |
| *C. acutatum* | EA-HP008 | Tamarillo | Afanador-Kafuri et al. (2003) |
| *M. roreri* | EA-HP007 | Cocoa fruits | This study |

## 2. Material and methods

### 2.1. Microorganisms and culture conditions

*Bacillus subtilis* EA-CB0015 (Table 1) was stored at −80 °C in Tryptic Soy Broth (TSB, Oxoid) with 20% glycerol and was activated on half-strength Tryptic Soy Agar (TSA; Oxoid) at 30 °C for 48 h before any experimental use. The fungal strains *Rizoctonia solani* EAHP-002, *Botrytis cinerea* IBUN Bc001 (*Bc* IBUN Bc001), *Fusarium solani* EAHP-003, *F. oxysporum* EAHP-005, *Moniliophthora roreri* EAHP-007, *Pestalotiopsis* sp. EAHP-010, *C. acutatum* EAHP-012, *Colletotricum acutatum* EAHP-008 (*Ca* EAHP-008), *F. verticillioides* EA-HP013 (Table 1) were stored in filter papers at room temperature and activated in Potato Dextrose Agar (PDA, Oxoid) at 20 °C before any experimental use. Fungal strain identification was based in the 5.8S-ITS region sequenced with the ITS1 and ITS4 primers (Tapia-Tussell et al., 2008). When necessary, spores were collected in 0.05% tween 80 (Sigma-Aldrich).

### 2.2. B. subtilis EA-CB0015 biomass, spores, and cell free supernatant production

*Bs* EA-CB0015 biomass was obtained by transferring one bacterial colony into a 500 mL Erlenmeyer containing 200 mL of culture medium D and incubating it for 5 days at 30 °C and 200 rpm. Medium D was composed of 0.042 g/L $MnSO_4*4H_2O$, 0.031 g/L $CaCl_2$, 0.5 g/L $KH_2PO_4$, 0.5 g/L $K_2HPO_4$, 1.0 g/L $(NH_4)2SO_4$, 4.0 g/L $MgSO_4*7H_2O$, 32.5 g/L yeast extract and 33.4 g/L glucose (Mosquera et al., 2014). After 5 days of incubation, cells were recovered by centrifugation at 4500 rpm for 15 min and suspended in water to a final concentration of $6.2 \pm 0.6 \times 10^8$ UFC/mL to obtain the cell suspension (CS). For the spore suspension (SS) an aliquot of the CS was heat treated for 20 min at 80 °C and the concentration calculated ($4.6 \pm 0.6 \times 10^7$ UFC/mL). The supernatant obtained from the centrifugation step was passed through cellulose ester membrane (0.2 µm; Advantec MFS, Inc) to obtain CFS.

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