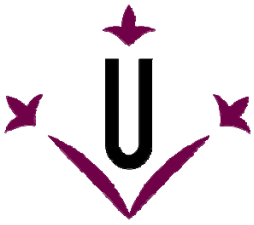

Chapter III

Antifungal effect of volatile organic compounds produced by *Bacillus amyloliquefaciens* CPA-8 against fruit pathogen decays of cherry

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Food Microbiology (2017) 64, pp: 219–225 doi: 10.1016/j.fm.2017.01.006

ABSTRACT

The present work focuses on the antifungal effect of volatile organic compounds (VOCs) produced by *Bacillus amyloliquefaciens* CPA-8 against *Monilinia laxa*, *Monilinia fructicola* and *Botrytis cinerea*, three postharvest fruit pathogens of sweet cherry fruit. VOCs were evaluated with a double petri dish assay against mycelial and colony growth of target pathogens. For this purpose, CPA-8 was grown on different media and cultured for 24 and 48 h at 30 ^{o}C before assays. Data showed that mycelial growth inhibition was higher when CPA-8 was grown on Tryptone Soya Agar (TSA) while no differences were generally observed when CPA-8 was cultured for either, 24 or 48 h. Moreover, no effects were observed on colony growth. The main volatile compounds emitted by CPA-8 were identified by solid-phase microextraction (SPME)-gas chromatography as 1,3 pentadiene, acetoin (3-hydroxy-2-butanone) and thiophene. Pure compounds were also tested *in vitro* on mycelial growth inhibition and their EC_{50} values against the three pathogens were estimated. Thiophene was the most effective VOC, showing more than 82 % suppression of mycelial growth at the highest concentration (1.35 μL mL^{-1} headspace) and EC_{50} values ranging from 0.06 to 6.67 μL mL^{-1} headspace. Finally, the effectiveness of thiophene and CPA-8 VOCs was evaluated against artificially inoculated cherry fruit. Among the target pathogens, *M. fructicola* was clearly controlled by CPA-8 with less than 25 % of rotten fruits compared to the control (65 % disease incidence) and for all pathogens, less than 37.5 % of CPA-8 treated decayed fruit produced spores (disease sporulation). Otherwise, pure thiophene showed no effect against any pathogen on disease incidence and disease sporulation. The results indicated that VOCs produced by *B. amyloliquefaciens* CPA-8 could develop an additive antifungal effect against postharvest fruit pathogens in stone fruit.

Keywords: Biocontrol; *Bacillus* spp.; *Monilinia* spp.; *Botrytis* spp.; VOCs; cherry.

INTRODUCTION

Postharvest decay of fruit presents a major factor causing postharvest losses and limits the duration of storage and shelf-life of produce. Numerous fungal pathogens infect stone fruit after harvest, including the wound-invading fungi *Monilinia* spp. and *Botrytis* spp., and cause economically important diseases of stone fruit worldwide (Mari *et al.*, 2016; Usall *et al.*, 2015). *Monilinia* rot is specially responsible for substantial postharvest losses, reaching even as high as 80 % in years when the climate conditions are favorable for the development of the disease, especially in late-ripening varieties (Usall *et al.*, 2015).

Traditionally, synthetic fungicides have been used to control postharvest decays; however, the appearance of fungicide-resistant population of pathogens and the concerns of the consumers about the possible toxicological risks of the residues have resulted in the need of developing other methods that involve a reduction in the number of field chemical applications (Droby *et al.*, 2016; Sharma *et al.*, 2009; Usall *et al.*, 2016). The biological control of postharvest pathogens using microbial antagonists has been the focus of considerable research over the last 30 years by many scientists and several commercial companies worldwide (Droby *et al.*, 2016) although it is not already routinely applied in fruit industry.

Antagonists can display a wide range of modes of action, at different stages of their activity, relating to different hosts and pathogens. Sometimes, different modes act simultaneously and it is therefore difficult to establish which individual mechanism has contributed to a specific antifungal function (Di Francesco *et al.*, 2016). To clarify the mechanism of action, as well as the understanding of biocontrol systems, it is crucial to know the interactions among environment, pathogen, and biocontrol agent (BCA) and, therefore, the expected biocontrol efficacy (Parafati *et al.*, 2015).

Bacillus strains exhibit various cytological traits, such as stress-resistant endospore formation and the synthesis of extracellular enzymes, to compete for niches (Nihorimbere *et al.*, 2011). Other mechanisms of action, such as volatile organic compounds (VOCs), siderophore production and the induction of reactive oxygen species (ROS) in the host, are under investigation (Asari *et al.*, 2016; Elshakh *et al.*, 2016; Zheng *et al.*, 2013).

Chapter III

Among the substances produced by BCAs, VOCs are frequently involved in the biological control of several fungal diseases of fruit. These compounds typically constitute a complex mixture of low-molecular weight lipophilic compounds derived from different biosynthetic pathways by many microorganisms as part of their metabolism. Some of these secondary products could be potentially employed with success as gaseous treatments in a process defined by the term biofumigation (Di Francesco *et al.*, 2016; Mari *et al.*, 2016). The microbial activity of VOCs produced by *Bacillus* strains has been widely studied. In the nineties, Fiddaman & Rossall (1994) reported the importance of substrate on the production of antifungal volatiles from *Bacillus subtilis* and the response of fruit pathogens to the volatile organic compounds produced by *Bacillus amyloliquefaciens* strains has been described during recent years (Asari *et al.*, 2016; Raza *et al.*, 2016; Yuan *et al.*, 2012). Integration of VOCs as a different strategy to achieve higher levels of disease control will contribute to a successful handling of postharvest diseases (Mari *et al.*, 2016).

B. amyloliquefaciens CPA-8 -formerly *B. subtilis*-, has been previously reported as BCA due to its effectiveness against postharvest diseases caused by *Monilinia* spp. and *Botrytis* spp. (Casals *et al.*, 2012; Yánez-Mendizábal *et al.*, 2011). Regarding its mode of action, the work conducted by Yánez-Mendizábal *et al.*, (2012b) provided experimental evidence about the strong antifungal effect against *Monilinia* species, mainly based on fengycin-like lipopeptides production. However, the synthesis of this product could not be considered as the only mechanism of action.

The objective of the present work was to investigate the antifungal effect of the VOCs produced by CPA-8 against three postharvest fruit pathogens on cherries. In order to do this, (i) an *in vitro* approach was used to evaluate the antifungal effect of CPA-8 on colony and mycelial growth against *Monilinia laxa*, *Monilinia fructicola* and *Botrytis cinerea*; (ii) compounds emitted by CPA-8 were identified by using the SPME-gas chromatographic technique, (iii) the effect of pure compounds on target pathogens was tested *in vitro* and (iv) the antifungal activity of CPA-8 VOCs and pure thiophene was assayed on cherries artificially inoculated with *M. laxa*, *M. fructicola* and *B. cinerea*.

MATERIALS AND METHODS

Microorganisms and culture media

The antagonist CPA-8 was isolated from a nectarine surface by the Postharvest Pathology Group of IRTA (Lleida, Catalonia, Spain) and has been recently reclassified as member of *B. amyloliquefaciens* species (Gotor-Vila *et al.*, 2016). Stock cultures were stored at 4 °C and subcultured on Nutrient Yeast Dextrose Agar (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar) at 30 °C for 24 h when required. Fresh bacteria were suspended in potassium phosphate buffer (PB, 70 mL KH_2PO_4 0.2 mol L^{-1}; 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionized water v/v/v pH 6·5) and adjusted by hemocytometer to a final concentration of 10^7 CFU mL^{-1}.

As pathogens, *M. laxa* (co33), *M. fructicola* (McL4), isolated from decayed stone fruit, and *B. cinerea*, isolated from decayed kiwi fruit, were obtained in Bologna (Italy) and identified by the Department of Agricultural Sciences CRIOF-DipSA (Bologna, Italy). They were maintained on Potato Dextrose Agar (PDA, Sigma-Aldrich, St. Louis, MO, USA, 39 g L^{-1}) plates at 25 °C for a maximum of 15 days. Conidia from the strains were collected and suspended in sterile distilled water containing 0.05 % (v/v) Tween 80. The suspension was adjusted by hemocytometer to a final concentration of 10^3 conidia mL^{-1}.

In vitro antagonistic activity of VOCs produced by CPA-8

The efficacy of the VOCs produced by CPA-8 on the mycelium and colony growth of the target pathogens was tested by the double petri dish assay. For this purpose, 100 µL of CPA-8 cell suspension (10^7 CFU mL^{-1}) were sprayed in petri dishes containing three different growth media, parafilmed, and incubated for 24 and 48 h at 30 °C. The culture media used in this study were NYDA, Nutrient Agar (NA, Oxoid, Cambridge, UK, 31 g L^{-1}) supplemented with glucose 20 g L^{-1} (NAglu20) and Triptone Soya Agar (TSA, Oxoid, Cambridge, UK, 40 g L^{-1}). After CPA-8 incubation time, two different trials were done. (i) For colony growth trials, the lid of the plate was removed and replaced by a base plate inoculated with 100 µL of a conidia suspension of each pathogen (10^3 conidia mL^{-1}). For *M. laxa* and *M. fructicola* conidia suspension, PDA base plates where used. In case of *B. cinerea*, the suspension was sprayed on Malt Extra Agar (MEA, Oxoid, Cambridge, UK,

33.6 g L^{-1}) base plates. The two base plates were sealed immediately with double layer of parafilm and incubated at 25 °C. After two days, the number of colonies were counted. (ii) For mycelial growth trials, mycelial agar discs (5-mm square plug) of every fungus were placed in the centre of the PDA base plate when either *M. laxa* or *M. fructicola* was used or MEA base plates for *B.cinerea*. Every double petri dish was double sealed as previously described and incubated at 25 °C for 3 days. The diameter of the colony was measured and expressed in millimetres. The percentage of inhibition of the colony and mycelial growth was calculated on the basis of the difference between treatment and control according to the formula: ((C-T)/C) x 100, where C is the control value and T is the measurement of the fungus in each antagonist-fungal set-up. The sample unit was represented by nine plates (replicates) for each pathogen and antagonist interaction in every condition mentioned. Plates without CPA-8 served as control.

Analysis of the VOCs produced by CPA-8

A qualitative evaluation of CPA-8 VOCs composition was done using HeadSpace Solid Phase MicroExtraction (HS)-SPME coupled with gas chromatography tandem mass spectrometry analysis (GC-MS) according to the method previously described by Di Francesco *et al.*, (2015) with modifications. SPME fibre (2cm- 50/30 µm DVB/CAR/PDMS, Supelco Inc, Bellefonte, PA, USA) was preconditioned according to manufacturer's recommendations and exposed to the headspace of CPA-8 petri dishes for 5 min at 30 °C. Analysis were performed in CPA-8 plates grown in the optimum media tested above for 24 and 48 h.

Trapped compounds were then thermally desorbed from the fibre for 2 min in the GC injection port at 250 °C in the split-less injection mode. For peak separation and detection, a Bruker GC 451 gas chromatograph equipped with a HP-5 fused silica capillary column (30 m by 0.25 mm inside diameter; 0.25 µm film thickness, J&W Scientific Inc, Folsom, CA, USA) connected to a quadrupole mass detector Bruker Scion SQ Premium (Bruker Daltonics, Macerata, Italy) was used. The transfer line was heated at 250 °C, the ion source at 220 °C, and carrier gas (He) flow rate was 1 mL min^{-1}. The mass spectrometer was operated in electron impact mode at 70 eV, scanning the range of 35/500 m/z in a full scan acquisition mode. The GC oven temperature was set at 40 °C for 4 min and then programed to rise from 40 to 90 °C at 10 °C min^{-1}, from 90 to 160 °C at 5 °C min^{-1}, and from 160 to 280 °C at 40 °C min^{-1}. The tentative identification of VOCs was

done by comparing the mass spectra and the retention times with the data system library (NIST 11 MS Library) and GC peak data were used to estimate the relative abundance (relative peak area, RA) of each volatile compound. Blank sample analysis (growth medium not inoculated with CPA-8) was performed under the same conditions in order to exclude interfering substances. All measurements were made with three replicates, each replicate representing the analysis of a different petri dish.

Antifungal activity of selected synthetic VOCs

Pure standards of the most representative VOCs produced by CPA-8 and identified by GC-MS analysis (Table 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and individually tested for suppressing mycelial growth of target pathogens. For this purpose, mycelial agar discs (5-mm square plug) were placed in the centre of petri dishes containing PDA or MEA, depending on the pathogen. Then, a paper filter (90 mm diameter) with different aliquots of pure compounds: 15, 30 and 60 µL were positioned inside the cover of the dishes. The aliquots of pure compounds introduced in the petri dishes corresponded to 0.34, 0.67 and 1.35 µL mL^{-1} headspace, respectively. The dishes were immediately doubled sealed with parafilm and incubated at 25 ^{o}C for three days. The sample unit was represented by three replicates for each dose and pathogen and plates with a non-spread paper filter were used as control. The percentage of inhibition of mycelial growth was calculated according to the formula described above and EC_{50} values (expressed as µL mL^{-1}) were calculated as the effective headspace concentration that inhibit fungal mycelial growth by 50 % in comparison to the control.

***In vivo* test of disease control by CPA-8 and pure thiophene**

Two different trials were conducted with 'Skeena' cherries to evaluate the antagonistic activity of both, the volatiles produced by CPA-8 and the pure compound thiophene, to control brown rot caused by *M. laxa* and *M. fructicola* and grey mold caused by *B. cinerea*. Fruit selected without visible injuries and rots and homogeneous in maturity and size was wounded in the equator with a sterile nail (3 mm wide and 3 mm deep) and inoculated with 15 µL of *M. laxa, M. fructicola* or *B. cinerea* conidial suspension (10^3 conidia mL^{-1}).

In the CPA-8 trials, sterile plastic boxes (30 x 19 x 12 cm. LxWxH) containing in the bottom a thin layer of TSA (400 mL) were inoculated 24 h before with 2 mL of a CPA-8 suspension adjusted at 10^7 CFU mL^{-1} and incubated at 30 ºC. For thiophene evaluation, six paper filters (90 mm diameter) were spread with 60 µL of the pure volatile compound each and placed in the bottom of sterile plastic boxes. Then, the inoculated cherries were placed inside the box. To avoid the contact between fruit and substrate, a sterile grid was used. Boxes non-inoculated with the antagonist or non-spread with thiophene were used as control. The boxes were closed with plastic lids and double sealed with parafilm. For each pathogen and trial (natural VOCs or pure thiophene), five cherries constituted a single replicate and each treatment was replicated four times. The percentage of rotten fruit (disease incidence) and the percentage of rotten fruit with spores on the surface (disease sporulation) were determined after four days of storage at 20 ºC and 85 % relative humidity, RH.

Data analysis

Data on the percentage of colony and mycelial growth inhibition was calculated according to the formula described before. Disease incidence and sporulation were also analysed and expressed as percentage. Differences in mycelial growth inhibitions as well as differences in disease incidence and sporulation data were evaluated using analysis of variance (ANOVA) with the JMP®8 statistical software (SAS Institute, Cary, NC, USA). Statistical significance was judged at the level $P<0.05$. When the analysis was statistically significant, the Tukey's HSD Test was used for separation of the means. EC_{50} of each substance was calculated using the probit analysis applied to the percentage of mycelial growth inhibition (Lesaffre & Molenberghs, 1991).

RESULTS

***In vitro* antagonistic activity of VOCs produced by CPA-8**

Data from the double petri dish assays indicated that VOCs produced by CPA-8 inhibited the mycelial growth of all tested pathogens (*M. laxa*, *M. fructicola* and *B. cinerea*) with variable efficacy depending on the growth media of CPA-8 (Fig. 1). On the whole, mycelial growth inhibition was higher when CPA-8 was cultured on TSA medium than when NYDA or NAglu20 were used. Otherwise, the antagonistic activity was the same

(data no significant) when CPA-8 was cultured for either, 24 and 48 h. In detail, higher values of mycelial growth inhibition were observed for *M. laxa* when CPA-8 grew on TSA medium (>78.6 %) compared to NYDA and NAglu20 (no more than 53.4 %). Similar results were obtained for *B. cinerea*, showing the highest mycelial growth inhibition when CPA-8 was grown on TSA medium for 24 h (86.8 %). When *M. fructicola* was tested, CPA- 8 grown on TSA for 24 h also showed the best results (68.6 %) and no differences were observed compared to CPA-8 grown on NAglu20 for 24 h (48.5 %). Regarding the colony growth, no differences were observed between the control and CPA-8 in all conditions tested. However, the size of the colony of each pathogen was meaningfully smaller when CPA-8 was co-incubated (data not shown).

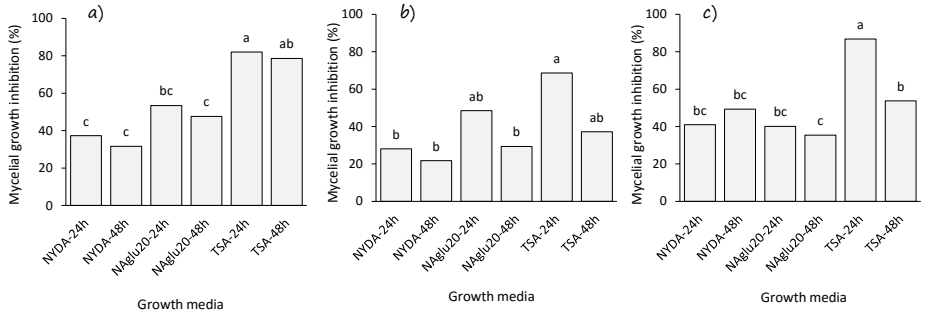

Figure 1. Effects of volatile organic compounds from *B. amyloliquefaciens* CPA-8 grown on NYDA, NAGlu20 and TSA medium for 24 and 48 h at 30 ºC on mycelial growth inhibition of a) *M. laxa*, b) *M. fructicola* and, c) *B. cinerea*, previously incubated at 25 ºC for three days on PDA or MEA medium. Within the same figure, different letters indicate significant differences (P<0.05) according to Tukey's HSD test.

SPME GC-MS analysis of VOCs produced by CPA-8

The headspace analysis indicated that CPA-8 grown on TSA produced a diverse volatile profile including ketones, aromatic compounds, hydrocarbons, and esters. Table 1 shows the tentative identification of the most representative VOCs emitted by CPA-8 plates but not by control plates. No differences were observed between CPA-8 cultured for 24 and 48 h. The compound 1,3 pentadiene was the most abundant VOC produced by CPA-8 (highest RA) followed by acetoin (3-hydroxy-2-butanone), thiophene, and ethylacetate. Compounds such as 2 butanone, 1-butanol 3-methyl and 1-butanol 2-methyl were also found in both tests, CPA-8 and control (data not shown), probably due to the protein source of the growth medium used. For this reason, these compounds have not been considered.

Table 1. Most representative volatile organic compounds detected and identified by SMPE-GC-MS analysis in the headspace of *B. amyloliquefaciens* CPA-8 plates grown in TSA medium for 24 h at 30 ºC. Retention Time (RT), Molecular Weight (MW) and GC peak Relative Area (RA) are shown.

RT (min)	Compound	MW	RA (%)
1.8	1,3 Pentadiene	68.12	61.01
2.3	Ethylacetate	88.11	6.61
3.0	Thiophene	84.14	11.32
6.8	Acetoin	88.11	21.06

Antifungal activity of selected synthetic VOCs

The pure VOCs 1,3 pentadiene, acetoin, and thiophene were tested for antifungal activity against *M. laxa*, *M. fructicola*, and *B. cinerea*. Results showed that thiophene was the most effective one in mycelial growth inhibition, showing over 82 % suppression when the highest concentration was used (1.35 µL mL^{-1} headspace). For this compound, EC_{50} values ranging from 0.06 to 6.67 µL mL^{-1} headspace were obtained for the target pathogens (Table 2). No inhibition was observed when 1,3 pentadiene was used against *M. laxa* and *M. fructicola* at any concentration tested; however, approximately 50 % of mycelial growth inhibition was observed against *B. cinerea* at the two highest concentrations (0.67 and 1.35 µL mL^{-1} headspace) with EC_{50} values over 10^5 µL mL^{-1} headspace (Table 2). Acetoin showed poor antifungal activity against *M. laxa* and *B. cinerea* (<50 %) and no mycelial growth inhibition was observed against *M. fructicola* (Table 2).

Table 2. Antifungal activity of pure volatile organic compounds on the *in vitro* mycelial growth inhibition (%) tests against *M. laxa*, *M. fructicola* and *B. cinerea*. Three different concentrations of each compound were used (0.34, 0.67, and 1.35 µL mL^{-1} headspace). When possible, EC_{50} values were represented (µL mL^{-1} headspace).

Pathogen	Compound	Concentration	Mycelial growth inhibition	EC_{50}
M. laxa	Acetoin	0.34	9.7	-
		0.67	8.7	
		1.35	23.2	
	1,3 Pentadiene	0.34	ni	-
		0.67	ni	
		1.35	ni	
	Thiophene	0.34	44.0	2.62
		0.67	68.9	
		1.35	95.1	
M. fructicola	Acetoin	0.34	ni	-
		0.67	ni	
		1.35	ni	
	1,3 Pentadiene	0.34	ni	-
		0.67	ni	
		1.35	ni	
	Thiophene	0.34	50.7	0.06
		0.67	86.4	
		1.35	100.0	
B. cinerea	Acetoin	0.34	34.3	-
		0.67	41.4	
		1.35	47.1	
	1,3 Pentadiene	0.34	30.0	$2.20 \cdot 10^5$
		0.67	62.9	
		1.35	57.1	
	Thiophene	0.34	27.6	6.67
		0.67	48.6	
		1.35	82.9	

ni: no mycelial growth inhibition observed
-: < 50 % mycelial growth inhibition. Insufficient data to calculate EC_{50} values

In vivo test of disease control by CPA-8 and thiophene

Results obtained from the *in vivo* tests are shown in Figures 2 and 3. Regarding the percentage of disease incidence, the pathogen *M. fructicola* was clearly susceptible to VOCs produced by CPA-8 (Fig. 2). In this case, less than 25 % of rotten fruit (compared to 65 % in the control) was observed. Otherwise, no significant differences (P<0.05) were detected between treatments and control in *M. laxa* and *B. cinerea*. Concerning the presence of spores on the surface of rotten fruit (Fig. 3) it could be observed that for all pathogens, less than 37.5 % of rotten fruit showed spores on the surface when CPA-8 VOCs were used. Furthermore, the quantity of the spores observed was small and

restricted to the wound (Fig. 4). The VOCs produced by CPA-8 against *M. laxa* and *M. fructicola* showed more than 41 % reduction of the percentage of disease sporulation compared with the control but no statistically differences were found between treatments due to the variability of the data (Fig. 3). However, results for *B. cinerea* revealed a complete disease sporulation reduction (100 %) as no spores were observed over the decayed fruit (Fig. 3). Otherwise, when thiophene was tested, no effect was observed in either disease incidence or disease sporulation against any of the target pathogens (Fig. 2 and Fig. 3).

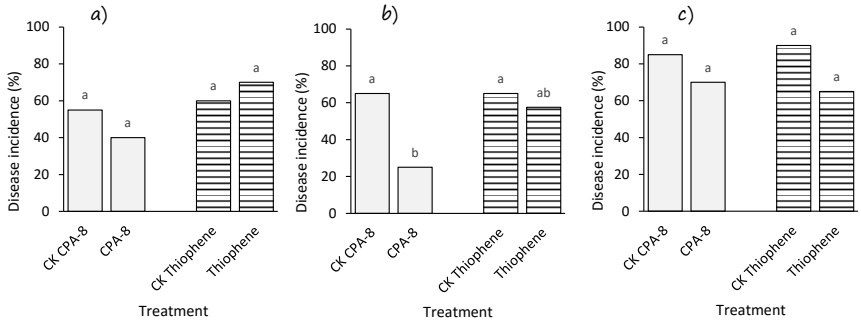

Figure 2. *In vivo* antagonistic activity of volatile organic compounds from *B. amyloliquefaciens* CPA-8 grown on TSA ( ) and the pure compound thiophene ( ). The figure shows the disease incidence (percentage of rotten fruit) of cherry fruit artificially inoculated with a) *M. laxa*, b) *M. fructicola,* and c) *B. cinerea* and incubated for four days at 20 ºC and 85 % RH. CK means control treatment, without CPA-8 or thiophene, respectively. Within the same figure, different letters in the same column pattern indicate significant differences (P<0.05) according to Tukey's HSD test.

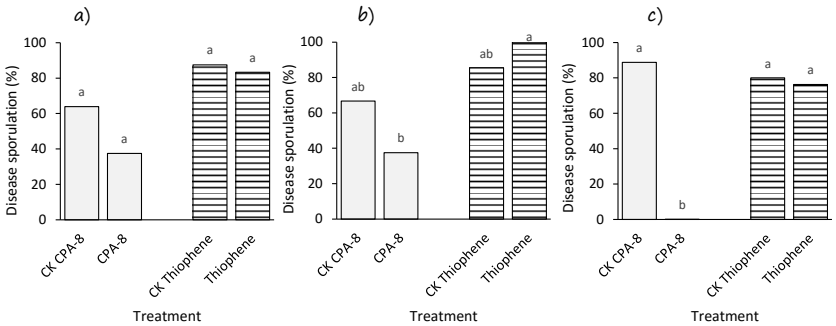

Figure 3. *In vivo* antagonistic activity of volatile organic compounds from *B. amyloliquefaciens* CPA-8 grown on TSA ( ) and the pure compound thiophene ( ). The figure shows the disease sporulation (percentage of rotten fruit with spores on the surface) of cherry fruit artificially inoculated with a) *M. laxa*, b) *M. fructicola* and c) *B. cinerea* and incubated for four days at 20 ºC and 85 % RH. CK means control treatment, without CPA-8 or thiophene, respectively. Within the same figure, different letters in the same column pattern indicate significant differences (P<0.05) according to Tukey's HSD test.

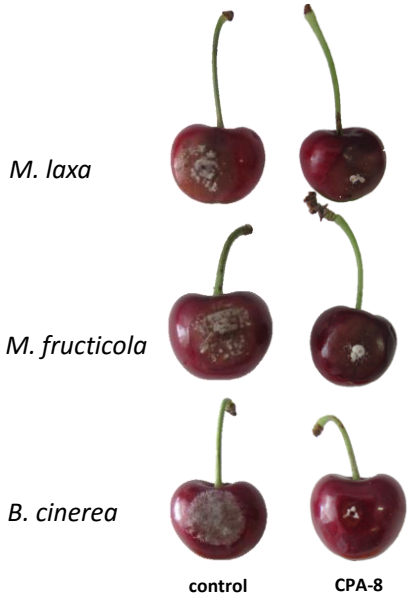

Figure 4. Effect of the volatile organic compounds from TSA cultures of *B. amyloliquefaciens* CPA-8 on sporulated cherry fruit artificially inoculated with *M. laxa*, *M. Fructícola*, and *B.cinerea*.

DISCUSSION

In this study, the antifungal effects of VOCs produced by *B. amyloliquefaciens* CPA-8 against the postharvest fruit pathogens *M. laxa*, *M. fructicola*, and *B. cinera* were determined. The results of the antagonistic activity in the preliminary *in vitro* assays demonstrated that VOCs emitted by CPA-8 were able to supress the mycelial growth of all target pathogens. Regarding the raw data obtained, the diameter of the colonies of all pathogens ranged from 18.7 to 64.7 mm in case of the control plates and from 0 to 7.8 mm when the pathogens were co-incubated with CPA-8. Results also indicated that the antifungal activity of CPA-8 volatiles was variable, depending on the growth media, with TSA probing to be the most effective. Therefore, this results are in agreement with the development of effective formulations for CPA-8, in which the low-cost media used for the production of this bacterium contained defatted soya flour as nitrogen source (Yánez-Mendizábal *et al.*, 2012a). The works previously conducted by Fiddaman and Rossall (1933, 1994) also reported the importance of the substrate on the production of antifungal volatiles; however, they indicated that NA and diluted TSA media were poor substrates for *B. subtilis* volatile production.

Whereas our results demonstrated a clear reduction in the mycelial growth, no differences (except the size of the colony) were observed in the colony growth when comparing the control and CPA-8, indicating that the volatiles from this bacterium have a fungistatic effect rather than a fungicidal action towards the target pathogens. Furthermore, VOCs produced by CPA-8 did not show selective effect against any target pathogen.

The work conducted by Arrebola *et al.* (2010) previously revealed microscopic observations in which VOCs from *Bacillus* strains induced morphological abnormalities on the conidia of *Penicillium crustosum*. Other authors have also reported the ability of volatiles produced by different *Bacillus* species to inhibit not only mycelial growth but also spore germination and tube elongation of *B. cinerea*, *Penicillium* spp. and *Fusarium oxyxporum* (Arrebola *et al.*, 2010; Chen *et al.*, 2008; Yuan *et al.*, 2012). These data suggest that VOCs produced by CPA-8 could represent an effective tool in the biocontrol of postharvest diseases caused by fungi.

In order to understand the nature of VOCs produced by CPA-8, SPME coupled with GC-MS was used. This simple and rapid technology for sampling volatile compounds at low concentrations in headspace analysis has been successfully used to characterise the VOCs profile produced by fungi, bacteria and yeasts (Chaves-Lopez *et al.*, 2015; Di Francesco *et al.*, 2015; Strobel *et al.*, 2001). The fibre used in this analysis was previously optimised by Di Francesco *et al.* (2015). The dual coated fibre selected allowed to extract a great number of compounds and also adsorb volatile molecules at low concentration in a wide molecular weight range due to its physico-chemical characteristics suitable for complex mixtures. CPA-8 VOCs were detected after 24 and 48 h of bacteria incubation and no different peak areas were observed between different culture times. As expected, the relative percentage of compounds detected in the headspace analyses did not vary due to the physiological state of the bacterium after being cultured 24 h. However, these results differ from the work conducted by Di Francesco *et al.* (2015) on yeasts in which different VOCs were observed starting from 48 h of yeast incubation. The main volatile compounds produced by CPA-8 were identified as 1,3 pentadiene, acetoin (3-hydroxy-2-butanone), and thiophene. Although most of the compounds detected have already been reported to be produced by different *Bacillus* strains (Arrebola *et al.*, 2010; Chaves-Lopez *et al.*, 2015), it should be taken into account that the methodologies applied to collect and detect VOCs can strongly influence the results and often confuse the comparison between

different studies. Moreover, RA values should not be extrapolated as quantitative considerations because these data not only depend on compound concentration but also on the fibre affinity and the detector sensing to the different analytes.

Pure synthetic compounds were purchased to determine the EC_{50} values against the fungal pathogens in *in vitro* mycelial growth inhibition tests. The compound thiophene resulted the most effective, showing over 82 % suppression of mycelial growth at the highest concentration (1.35 µL mL^{-1} headspace) and EC_{50} values ranging from 0.06 to 6.67 µL mL^{-1} headspace depending on the pathogen. Fokialakis et al., (2006) reported that constituents from bioactive extracts from different species of the genus *Echinops* resulted in the isolation of eight thiophenes possessing varying degrees of termiticidal activity against the Formosan subterranean termite, whose ability to damage wood and trees is widely known. However, to the best of our knowledge, little is known about thiophene production from BCAs and the ecological significance of sulphur compounds is still poorly understood. Regarding the use of either acetoin or 1,3 pentadiene, poor results were observed, suggesting that higher concentrations were needed. Nevertheless, VOCs produced by microorganisms are commonly found at very low concentrations and their effect is supposed to be due to synergic or additive action and not to a single component activity (Mercier & Jimenez, 2004; Strobel *et al*., 2001). Therefore, more research would be necessary to determine lethal concentrations of the VOCs produced by CPA-8 and whether their combined action against fungal growth is additive or synergistic.

The inhibitory effect of VOCs from TSA cultures of CPA-8 on wounded cherry fruit artificially inoculated with *M. laxa*, *M. fructicola* and *B. cinerea* was also demonstrated, providing the best results for *M. fructicola* disease incidence. Furthermore, CPA-8 reduced and minimised the presence of spores on the surface of decayed fruit and soft rot symptoms were generally observed in all target pathogens. *B. cinerea* reached a complete suppression in the percentage of sporulation disease, indicating that VOCs from CPA- 8 are able to fit in well with the wound environment of artificially inoculated cherry fruit. These data meaningfully differ from the results obtained under *in vitro* conditions, suggesting that the high biocontrol efficacy observed when CPA-8 was applied against fungal mycelial growth *in vitro* is not always enough to explain the accumulative effects of several control mechanisms occurred in *in vivo* assays. It is known, for instance, that

each volatile compound has its own production or release dynamic depending on the available volume/headspace (Mercier & Jimenez, 2004).

Even though some studies reported *in vitro* analysis of VOCs produced by *Bacillus* species, little is known about volatiles produced by *Bacillus* spp. in controlling postharvest disease of fruit. Chen *et al.* (2008) demonstrated that volatiles generated by *B. subtilis* JA had significant effect on *B. cinerea* inhibition. Furthermore, the volatiles produced by *B. subtilis* and *B. amyloliqueaciens* also had a significant reduction in the decay incidence of citrus diseases in *in vitro* and *in vivo* trials in oranges (Arrebola *et al.*, 2010).

This work, investigates for the first time the capability of the volatiles produced by CPA-8 as an effective mechanism of action against postharvest fruit pathogens. In this context, this study provides experimental evidence about the antifungal effect and biological control ability of CPA-8 to reduce cherry rot caused by *M. laxa*, *M. fructicola* and *B. cinerea*. Although Yánez-Mendizábal *et al.* (2012b) have previously described the main mode of action of CPA-8 based on lipopeptide production, this works proves that this mechanism is not the only factor that needs to be considered in a biological control program for fruit disease. Future research would be needed on designing agriculturally acceptable and practical ways for an efficient use of *B. amyloliquefaciens* CPA-8 antifungal volatiles to keep stone fruit quality during postharvest storage, distribution, and marketing period.

Acknowledgments

This research was supported by the European project BIOCOMES FP7-612713 and by the Catalan government (Generalitat de Catalunya) for the PhD grant 2016-FI-B2 00143 (Amparo M. Gotor).

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