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Title: Effect of volatile compounds produced by antagonist Bacillus strains against citrus postharvest decay

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Abstract: The antifungal effects of volatile compounds produced by Bacillus strains; B. subtilis PPCB001 or B. amyloliquefaciens PPCB004 and antagonist combination (PPCB001+ PPCB004) against Penicillium digitatum Sacc., Penicillium italicum Wehmer and Penicillium crustosum Thom isolates were investigated in vitro and in vivo. The antagonists alone or in combination inhibited the radial mycelial growth of Penicillium spp. in vitro. Among the three Penicillium isolates tested P. crustosum showed 73.3% of mycelial growth inhibition in presence of PPCB004. The antifungal effects of volatiles increased with increasing time (days), and PPCB004 showed the highest inhibition of radial mycelial growth in P. crustosum on the 10th day. The EC50 was 2.5 x 105 cfu ml-1 for PPCB001; 9.45 x 106 cfu ml-1 for PPCB004 and 7.76 x 106 cfu ml-1 for PPCB001 + PPCB004. Antagonist PPCB004 incubated at 37 ˚C for 24 h showed higher inhibitory effect on spore germination and germ tube elongation in P. crustosum than all other treatments. 3-Hydroxy-2-butanone (acetoin) was the predominant ketone in PPCB001 (45.98%) and PPCB004 (97.52%). Antagonist PPCB004 showed significant inhibition on decay incidence and severity in soft citrus cv. Valencia, inoculated with P. crustosum and held at 25 ˚C for 12 days. The

observations indicated that Bacillus amyloliquefaciens PPCB004 could be a useful tool to control P. crustosum in postharvest systems.

Dept Microbiology and Plant Pathology
University of Pretoria
Pretoria
0002
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Dear Editor

MANUSCRIPT FOR PUBLICATION IN BIOLOGICAL CONTROL

We herewith submit the manuscript titled: **Effect of volatile compounds produced by antagonist *Bacillus* strains against citrus postharvest decay**

, as a research paper for publication in Biological Control. The manuscript focuses on integrated control of post-harvest decay in citrus while maintaining overall fruit quality using the antagonist, *Bacillus spp* in combination with modified atmosphere packaging to extend the storage life for the organic growers.

The authors of above-mentioned paper are Eva Arrebola, Dharini Sivakumar, Lise Korsten from the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa.

We trust that the manuscript will be reviewed favourably.

Kind regards

Dharini Sivakumar (corresponding author) on behalf of the other co-authors.

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Effect of volatile compounds produced by antagonist *Bacillus* strains against citrus postharvest decay

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ABSTRACT

The antifungal effects of volatile compounds produced by *Bacillus* strains; *B. subtilis* PPCB001 or *B. amyloliquefaciens* PPCB004 and antagonist combination (PPCB001+ PPCB004) against *Penicillium digitatum* Sacc., *Penicillium italicum* Wehmer and *Penicillium crustosum* Thom isolates were investigated *in vitro* and *in vivo*. The antagonists alone or in combination inhibited the radial mycelial growth of *Penicillium spp. in vitro.* Among the three *Penicillium* isolates tested *P. crustosum* showed 73.3% of mycelial growth inhibition in presence of PPCB004. The antifungal effects of volatiles increased with increasing time (days), and PPCB004 showed the highest inhibition of radial mycelial growth in *P. crustosum* on the 10^{th} day. The EC_{50} was 2.5 x 10^5 cfu ml^{-1} for PPCB001; 9.45 x 10^6 cfu ml^{-1} for PPCB004 and 7.76 x 10^6 cfu ml^{-1} for PPCB001 + PPCB004. Antagonist PPCB004 incubated at 37 ˚C for 24 h showed higher inhibitory effect on spore germination and germ tube elongation in *P. crustosum* than all other treatments. 3-Hydroxy-2-butanone (acetoin) was the predominant ketone in PPCB001 (45.98%) and PPCB004 (97.52%). Antagonist PPCB004 showed significant inhibition on decay incidence and severity in soft citrus cv.

Valencia, inoculated with *P. crustosum* and held at 25 ˚C for 12 days. The observations indicated that *Bacillus amyloliquefaciens* PPCB004 could be a useful tool to control *P. crustosum* in postharvest systems.

Keywords antagonists, antifungal effects, *Bacillus* spp

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1. Introduction

The widespread use of chemicals in commercial packinghouse has led to the proliferation of resistant strains of the pathogens (Palou et al., 2002). Continuous use of fungicides such as imazalil, thiabendazole, and *o*-phenylphenol in citrus packing facilities has resulted in the development of *P. digitatum* and *P. italicum* isolates with resistance to various commercially used fungicides (Holmes and Eckert, 1999). *Penicillium crustosum* is an opportunist fungus that is getting more relevance in postharvest practise. Although *P. crustosum* is considered a weak pathogen of certain fruit (Pitt, 1990), it is an ubiquitous fungus that produces a neurotoxin (penitrem A) that is harmful to the consumer (Lewis et al., 2005) and this species has displayed resistance to fungicides such as benomyl and thiabendazole (Iwao, 1999).

Currently, biological control is becoming an increasingly effective alternative control measure to replace postharvest fungicide applications or to be implemented as part of

47 integrated control programs (Conway et al., 1999). Biocontrol applications for postharvest
48 disease control is now directed more towards the use of natural volatile compounds (VCs)
49 produced by microorganisms that are biodegradable, that do not leave toxic residues on the
50 fruit surface and displays as effective disease control as conventional fungicides (Janisiewicz
51 and Roitman, 1988). *Bacillus spp*. were considered as potential biocontrol agents due to their
52 high spore production and the fact that the spores are commonly resistant to desiccation, heat,
53 U.V. irradiation and organic solvents (Huang et al., 1992; Romero et al. 2007). *Bacillus* spp.
54 have been reported to inhibit the growth of a number of plant pathogens through antagonism,
55 with multiple modes of action such as the production of antibiotics (iturin, surfactin,
56 fengycin), enzymes that degrade fungal structural polymers (chitinase, β-1,3 glucanase), and
57 antifungal volatiles (Jiang et al., 2001; Pinchuk et al., 2002; Leelasuphakul et al., 2006).
58 Almenar et al. (2007) have identified 14 different VCs produced by *B. subtilis* spp. that
59 included antifungal VCs such as 2-ethyl-hexanol, 2,4-bis(2-methyipropyl)-phenol, 4-
60 hydroxybenzaldehyde and 2-nonanone. However, the effect of VCs produced by the *Bacillus*
61 spp as a mode of antagonism action for the control of postharvest pathogens is not often
62 described. The objectives of this study were firstly to investigate the effect of VCs generated
63 by two *Bacillus* strains (PPCB001 and PPCB004) alone or in combination on the mycellial
64 growth of *Penicillum* spp, spore production, germination, hyphae morphology and disease
65 incidence and severity of citrus fruit inoculated with *P. crustosum*. Secondly, we aimed at
66 determining the effective concentration of the antagonists applied *in vitro* and the VCs
67 produced by the two *Bacillus* strains were identified using GC/MS.
68
69 **2. Materials and methods**

70

71 *2.1. Bacillus strains*

72 *Bacillus* strains PPCB001 and PPCB004 were isolated from the surface of Valencia
73 and Shamouti oranges respectively (Obagwu and Korsten 2003). Molecular identification of
74 *Bacillus* isolates was based on analysis of the 16S rRNA region after PCR amplification with
75 primer 41F and 1486R-P, specific for Gram-positive bacteria (Stackebrandt and Goodfellow,
76 1991). The sequences for the primers are 41F: (5'-GCT CAG ATT GAA CGC TGG CG-3')
77 and 1486R-P: (5'-GCT ACC TTG TTA CGA CTT CGT CCC-3'. Bacterial DNA was
78 isolated from *Bacillus* strains using the Illustra™ Bacterial genomicPrep Mini Spin kit (GE-
79 Heathcare UK limited). After the PCR reaction was performed, PCR products were cleaned
80 using the MSB Spin PCRapace Kit (Invitek Berlin Germany), and 3 □l of clean PCR product
81 was used for sequencing with the BigDye® Terminator Kit (AB Foster City USA). The
82 resulting PCR products were purified and used directly for sequencing. Homology studies
83 were carried out using the NCBI BLAST.

84

85 *2.2. Postharvest pathogens*

86 *Penicillium digitatum* (Pers.:Fr) Sacc., *P. italicum* Wehmer and *P. crustosum* Thom
87 were obtained from the culture collection of Plant Pathology Laboratories, University of
88 Pretoria, South Africa. The *Penicillium* isolates were maintained on potato dextrose agar
89 (PDA, Merck, Johannesburg, South Africa) and were incubated at 25 ºC for 7-10 days. The
90 spores were harvested and filtered through eight layers of sterile cheese-cloth. Spores were
91 counted using a haemacytometer and adjusted to a final concentration of 6×10^6 spore ml^{-1}.

92

93 *2.3. Antagonism assays in vitro*

94 Inhibition of radial mycelal growth of *Penicillium* isolates: The *Bacillus* strains;
95 PPCB001 and PPCB004 were grown on nutrient agar (NA, Merck) and incubated at 37 ºC for
96 24 h before each assay in order to obtain a fresh culture. Thereafter, the *Bacillus* strains were
97 plated on antibiotic production minimal medium (APM agar) (McKeen et al., 1986) and on
98 nutrient agar (NA) (rich medium) separately. For the antagonist (*Bacillus* strain) combination
99 treatments, a Petri dish sandwich was made and the top Petri dish was plated with each
100 antagonist (upper plate). Thereafter, a 10 □l spore suspension of fungal isolate was
101 inoculated in the centre of the bottom PDA plate (lower plate). The upper and lower plates
102 were sealed together with Parafilm and incubated at 25 ºC up to 10 days. The radial diameter
103 of each antagonist-fungal or combined antagonist-fungal plates and the control was measured
104 daily up to 10 days with a Vernier digital calliper (Digimatic; Mitutoyo Co., Japan) in
105 millimetres and the results were expressed as percentage inhibition of radial mycelial growth.
106 Inhibition of fungal radial mycelial growth was calculated according to the formula: $(R_1$-
107 $R_2/R_1)$ x 100, where R1 is a control value, R_2 is the measurement of the fungus in antagonist-
108 fungal or combined antagonist-fungal set up. Each fungal isolate with single antagonist or
109 antagonist combination had ten replicates. Plates without bacteria served as controls.

110 Effective concentration of antagonist (EC_{50}): Antagonists were grown separately for
111 12-18h in nutrient broth, incubated at 37 ºC and subjected to orbital shaking (Labcon® Marin,
112 California USA) at 150 rpm. Thereafter, antagonists were serially diluted to obtain different
113 concentrations and 100 □l of each dilution was cultured in APM plates (upper plates). For
114 antagonist combination, 50 □l of each antagonist were cultured in the APM plates. A 10 □l
115 fungal spores suspension was inoculated in the centre of PDA plates (lower plates). The

116 antagonist and the fungal plates were sealed together with Parafilm and incubated at 25 ºC up
117 to 10 days. The EC_{50} was defined as antagonist concentration needed to reduce the fungal
118 mycelial growth by 50% in comparison with the control plates (untreated), and it was
119 calculated according to a statistical regression equation.
120 Spore production analysis: After determining the percentage radial mycelial growth
121 from the antagonist-fungal or combined antagonist-fungal plates for each *Penicillium* isolate,
122 three discs each with a 2 mm diameter fungal growth were cut and removed from the plate
123 every day for 10 days. Each disc was soaked in 1 ml of Ringers' solution with 0.02%
124 Tween80, vortexed (LabSytems, Germany Industrial Corp Cannic Inc.USA) for 10 s and the
125 spore count was determined using a haemacytometer. The observation on spore production
126 was carried out from ten replicates of single antagonist or antagonist combination for each
127 fungal isolate.
128
129 *2.4. Antifungal effects of VCs on fungal spore germination, germ tube growth and hyphae*
130 *morphology*
131 Two 100 □l 50% PDA drops were placed on sterile microscopy slides. A fungal spore
132 suspension prepared in Ringers' solution with 0.02% Tween80 was sprayed over the
133 microscope slides. The slides were incubated inside a sterile Petri dish at 100% RH and 25 ºC
134 for 24 h. The antagonists were inoculated on APM plates. For the antagonist combination the
135 upper half Petri dish (as mentioned earlier) was treated with each antagonist. APM plates
136 without bacteria served as control in this study. The plate containing the fungal slide and the
137 bacterial plate were sealed together with Parafilm. At 12 h intervals the germination was
138 stopped and stained with Lacto-phenol cotton blue and observed under the optical light

139 microscope (Zeizz, Germany) with a 40 x magnification. The percentage spore germination
140 was calculated by counting the number of germinated and un-germinated spores out of 200
141 spores. The germ tube lengths were measured using an ocular micrometer disk in the
142 microscope at 40 x magnification. The germ tube measurements of 50 spores were taken
143 from six replicates.
144
145 After 10 days of incubation at 25 ºC, fungal hyphae were taken from areas showing
146 inhibition due to antagonistic activity of the antagonist alone or antagonists combined, stained
147 with Lacto-fuchsin, and subjected to microscopic examination to record structural
148 abnormalities. Samples from control plates without antagonist influence were also stained
149 and observed.
150
151 *2.5. GC/MS identification of VCs produced by Bacillus strains*
152 A purge-and-trap sampling method developed in-house was used to isolate the
153 volatiles from *Bacillus* strains PPCB001 and PPCB004 (Sivakumar et al., 2008). The samples
154 (150 ml) were placed in a water bath at 35 °C. The volatiles were isolated by purging the
155 samples with 500 ml N_2 (g) (5.0, Afrox, Gauteng, South Africa) at 25 ml min^{-1}. Multi-
156 channel open tubular silicone rubber traps (MCTs) were used to collect the volatile
157 compounds from the bacterial strains (Ortner and Rohwer, 1996). The GC column was a
158 Zebron, ZB1 30 m x 250 µm ID x 0.25 µm film thicknesses (Phenomenex, Separations,
159 Randburg, South Africa), the velocity of the carrier gas (helium) was 46 cms^{-1} (1.8 ml min^{-1})
160 and the column head pressure was 65 kPa in the constant pressure mode. The GC oven
161 temperature programme was -20 °C (3 min) at 5 °C min^{-1} to 250 °C (5 min). The GC run

time was 62 min. The GC-MS transfer line was at 280 °C, the mass scan range was 35-450 atomic mass units in full scan mode, the source (EI+) temperature 230 °C, the MS quadruple temperature 150 °C, the ionisation energy 70 eV and the electron multiplier (EM) 1753 V. Tentative identification of organic compounds was performed by a probability based match search of the Wiley spectral library. Matches of the mass spectra of the components to that of the library was ≥80%. The retention indices (RI) of the components were also established. Identification of each individual compound was made by comparison of their retention times with those of pure components, matching mass spectral data with those from NIST mass spectra library software.

2.6. Effects of VCs produced by Bacillus strains PPCB001 and PPCB004 on the control of postharvest decay in inoculated fruit

Freshly harvested citrus fruit cv. Valencia, from Kirkwood (Eastern Cape, South Africa) were washed with 0.5% of NaOCl solution. Thereafter, it was wounded (5 x 5 mm) with a sterile needle. The disinfected, wounded fruit were inoculated with *P. crustosum* by dipping fruit for 3 min in a 3 x 10^6 spore ml^{-1} spore suspension. Spore suspensions were prepared by removing the spores from the sporualting edges of the culture with a sterile glass rod containing water agar (0.1% Bacteriological agar) with 0.02% of Tween80 solution. Five inoculated fruit were packed in a biorientated polypropylene packaging (material perforation 0.00565%, thickness 35µ). The antagonist were inoculated separately on 5 g of APM agar and transferred to a heat sealable grade 126/3 tea bag (Schnabel and Mercier, 2006) which was sealed on both sides with a heat sealer. The tea bags with the antagonists were incubated for 24h at 37 ºC. Thereafter, the antagonist in the tea bag (3 tea bags / modified atmosphere packaging (MAP)) was transferred into the MAP containing five inoculated fruit and the

MAP was heat sealed to create a modified atmosphere around the fruit. The inoculated fruit were held at 25 ºC for 12 days and observations were made regarding the percentage disease incidence and disease severity by measuring the lesion diameter in mm. MAP with inoculated fruit and tea bags without the antagonist served as controls. The treatments namely; PPCB004 + MAP; PPCB001 + MAP, PPCB001 + PPCB004 + MAP and MAP without the antagonist were replicated five times and the experiment was repeated twice.

2.7. Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) using SPSS 8.0 software for Windows (SPSS Inc., Chicago, IL, USA). The mean values were compared using the least significant difference (LSD) test, at $P<0.05$. The EC_{50} values were logarithmically transformed and analysed by regression analysis. The values of EC_{50} were obtained by regression equations where Y represented the fungal mycelial growth in millimetres and x is the logarithms of CFU ml^{-1} of *Bacillus spp.* used.

Results and discussion

3.1. Identification of antagonists used in this study

The species identification of *Bacillus* isolates were confirmed by comparing the results from colony morphology and API test strips with the keys for *Bacillus* genus published in *Bergeys Manual of Determinative Bacteriology* (Holt et al., 1994). Homology DNA studies were carried out with the sequences corresponding to 16S-rDNA from both isolates. The results obtained from sequence comparison of PPCB001 (915bp) with the National Centre for

208 Biotechnology Information (NCBI) data base showed sequence similarity to the *Bacillus*
209 *subtilis* strain NBRC 101239, with 914bp identities from 915bp total (99.89%), and *Bacillus*
210 *subtilis* strain 168, with 913bp identities from 915bp total (99.78%) as second comparison.
211 Thus PPCB001 was identified as a *Bacillus subtilis* strain. Likewise, the results obtained
212 from sequence comparison of PPCB004 (909bp) with the NCBI data base were *Bacillus*
213 *amyloliquefaciens* strain FZB42, with 902pb identities from 909bp total (99.23%) and
214 *Bacillus subtilis* strain 168, 899bp identities from 909bp total (98.9%). Although the two
215 *Bacillus* species were very closely related, the differences obtained between both sequence
216 comparisons, the colony appearance and biochemical tests has proved the identity of
217 PPCB004 as *Bacillus amyloliquefaciens*.
218
219 3.*2 Effect of VCs produced by B. subtilis PPCB001 and B. amyloliquefaciens PPCB004 on*
220 *the radial mycelial growth of Penicillum spp. in vitro*
221 It was evident from the observations that the three tested *Penicillium* spp. failed to
222 show significant difference between the control and the antagonist treatments (*B. subtilis*
223 PPCB001 or *B. amyloliquefaciens* PPBC004) in rich culture medium. However, in minimal
224 medium, *P. crustosum* showed significant ($P<0.05$) inhibition of radial mycelial growth in
225 presence of *B. subtilis* PPCB001 or *B. amyloliquefaciens* PPCB004 (single antagonist
226 treatments) compared to the control. The radial mycelial growth of *P. crustosum* was
227 inhibited by over 50.0%, *P. digitatum* inhibited over 3% and *P. italicum* inhibited over 25% *in*
228 *vitro* in minimal medium, in presence of VCs of antagonists *B. subtilis* PPCB001 or *B.*
229 *amyloliquefaciens* PPCB004. Therefore, the antifungal effects of *Bacillus* spp were
230 confirmed on *P. crustosum* in this study.

231 Furthermore, the percentage inhibition of radial mycelial growth of *P. crustosum*
232 increased in presence of *B. amyloliquefaciens* PPCB004 or *B. subtilis* PPCB001 or in
233 combination with the two antagonists (PPCB004 + PPCB001) compared to the control on the
234 3rd day. However, *B. amyloliquefaciens* PPCB004 alone or in combination with *B. subtilis*
235 PPCB001 (PPCB001 + PPCB004) showed a significant (P<0.05) increase in radial mycelial
236 growth of *P. crustosum in vitro* than *B. subtilis* PPCB001 alone. The antifungal effects of
237 VCs gradually increased with increasing time (days) and the highest inhibition (over 50%) of
238 radial mycelial growth of *P. crustosum* was observed on the 10 th day (Fig. 1).
239 The effective concentration of the antagonist that could inhibit fungal mycellial growth
240 by 50% in comparison to the control (EC_{50}) was estimated as 2.5 x 10^5 CFU ml^{-1} for *B.*
241 *subtilis* PPCB001 according to regression line [Y = 31.9-2.2x, R^2 0.95]; 9.45 x 10^6 CFU ml^{-1}
242 for *B. amyloliquefaciens* PPCB004 [Y = 45.6-3.7x, R^2 0.95] and 7.76 x 10^6 CFU ml^{-1} [Y =
243 31.1-1.6x, R^2 0.91] for the antagonist combination (PPCB001 + PPCB004). The antagonist
244 PPCB001 displayed a lower EC_{50} than PPCB004 and the antagonist combination. This
245 observation indicates that the VCs produced by PPCB001 showed a possible toxic effect at a
246 lower antagonist concentration than that of PPCB004 or the antagonist combination, due to
247 the nature of the volatiles produced (different volatile components and concentrations).
248 Moreover, different dynamics were observed between *B. subtilis* PPCB001 and *B.*
249 *amyloliquefaciens* PPCB004. The antagonist PPCB001 and the antagonist combination
250 showed a logarithmic dynamic. The fungal growth was hardly inhibited at low antagonist
251 concentration, and when the antagonist logarithm was between 3 and 7, the inhibition
252 gradually increased. Further increase in antagonist concentration failed to increase the
253 mycelial growth inhibition in *P. crustosum*. However, the *B. amyloliquefaciens* PPCB004

254 showed a linear dynamic, having a gradual increase in inhibition from the lowest antagonist
255 concentration. This study further showed that the higher bacterial concentrations are needed
256 for antagonist EC_{50} which is relatively higher in comparison to the EC_{50} of antibiotics
257 produced by *Bacillus spp*. (Leelasuphakul et al., 2008). Therefore, it is evident that VCs
258 produced by *B. subtilis* PPCB001 and *B. amyloliquefaciens* PPCB004 are of different nature
259 and its effectiveness differs.
260
261 *3.3 Effect of VCs of Bacillus spp on spore production, germination, germ tube growth and*
262 *hyphal morphology of P. crustosum*
263 It is evident from Fig. 2 that the spore production was affected significantly (P<0.05)
264 when *P. crustosum* was exposed to *B. subtilis* PPCB001 or *B. amyloliquefaciens* PPCB004 or
265 the antagonist combination compared to the control after day 3 of incubation. However, no
266 significant difference in spore production of *P. crustosum* were observed in the presence of
267 VCs produced by *B. subtilis* PPCB001 or *B. amyloliquefaciens* PPCB004 or the antagonist
268 combination from the 2^{nd} to the 10^{th} day of incubation. *P. crustosum* produced an average of
269 $3x10^6$ spores mm^{-2} in absence of *Bacillus* spp. and maintained the spore concentration from
270 the 5^{th} day of incubation onwards. However, *Bacillus* spp. reduced the spore production of *P.*
271 *crustosum* to $3x10^5$ spores mm^{-2} on the 6^{th} day of incubation.
272
273 The effect of VCs on spore germination of *P. crustosum* showed that the percentage
274 inhibition of germination was significantly (P<0.05) higher (77%) in presence of *B.*
275 *amyloliquefaciens* PPCB004 than the other two treatments (32% in presence of PPCB001 and
276 35% in presence of the antagonist combination). Here the antagonists were incubated at 37

˚C for 24 h prior to exposure with *P. crustosum*. VCs of PPCB004 incubated at 37 ºC for 24 h showed a significant effect on the germ tube lengths of *P. crustosum* spores ($P<0.05$; 0.65 µm ± 0.14). However, spores of *P. crustosum* showed germ tube lengths of 4.86 µm ± 0.16 in control paltes; 2.3 µm ± 0.5 in the presence of PPCB001 and 1.02 µm ± 0.2 in the presence of the antagonist combination treatment. Our investigations proved clearly that VCs produced by *B. amyloliquefaciens* PPCB004 affected the radial mycellial growth, the spore production, germination and germ tube elongation of *P. crustosum.* Although the antifungal effects of VCs of *B. subtilis* PPCB001 was improved in the presence of *B. amyloliquefaciens* PPCB004, the findings indicate that there is an absence of synergistic effect during the combined antagonist application. It is also evident from the observations that the antifungal effect of the VCs produced by *Bacillus* spp increased in a closed system after completing its growth. It can be stated that the production of VCs in *Bacillus* spp. takes place at the stationary growth phase similar to secondary metabolites (antifungal compounds) (Romero et al., 2007).

Microscopic observations revealed that the VCs from *Bacillus* spp. induced morphological abnormalities on the conidia of *P. crustosum* (Fig. 3 A-D). The morphological abnormalities in the conidophore and phialide deferred with respect to *Bacillus* spp and combination treatments. The VCs from *B. subtilis* PPCB001 showed abnormalities on conidiophores (Fig. 3 B). However, *B. amyloliquefaciens* PPCB004 alone or in combination with *B. subtilis* PPCB001, showed complete loss of conidiophore structures (Figs. 3 C and D). Also the VCs of PPCB004 reduced the multiple phialide in the end of each hyphae, (Fig. 3 C). The abnormalities included, vacuolation and swelling in hyphae and sporangium. The spores and hyphae of *P. crustosum* affected by the VCs of *Bacillus* spp alone or in combination recovered its growth when it was transferred to the fresh PDA medium (data not presented).

300 Furthermore, these structural abnormalities illustrated the mode of action of VCs produced by
301 the antagonists on spore production
302
303 *3.4 VCs of Bacillus spp PPCB001 and PPCB004 and its application on citrus fruit*
304 *inoculated with P. crustosum*
305
306 The volatile profiles indicated that *Bacillus* spp were diverse, and *B.*
307 *amyloliquefaciens* PPCB004 produced less VCs (8 different VC) than *B. subtilis* PPCB001
308 (21 different VC) (Tabel 1). In common, the VCs of the *Bacillus* spp included alcohols,
309 ketones and N-containing compounds (Table 1). The ketones represented the main fraction in
310 both antagonists. 3-Hydroxy-2-butanone (acetoin) was the predominant ketone in *B. subtilis*
311 PPCB001 (45.98 RA%) and *B. amyloliquefaciens* PPCB004 (97.52 RA%). Production of 3-
312 hydroxy-2-butanone (acetoin), *B. subtilis* and *B. amyloliquefaciens* was reported and by Ryu
313 et al. (2003). The 3-hydroxy-2-butanone (acetoin) showed a significant reduction in
314 symptomatic leaves inoculated with soft rot causing pathogen *Erwinia carotovora* (Ryu et al.,
315 2003).
316 The VCs of PPCB004 significantly (P<0.05) reduced the decay incidence and severity
317 in fruit inoculated with *P. crustosum* in modified atmosphere packaging (Table 2). The
318 PPCB001 alone or antagonist combination treatment (PPCB001+ PPCB004) reduced the
319 decay incidence and severity to a similar extent in the MAP system. Highest decay incidence
320 and severity was observed in control fruit packed in MAP. Very few biological agents have
321 proved the practical application of VCs produced by a microorganism. The best example of
322 biofumigant in controlled atmosphere is *Muscolor albus* (Schotsman et al., 2008). The VCs

produced by *B. amyloliquefaciens* and *B. subtilis PPCB001* showed acetoin, as major compound in this study. The 3-hydroxy-2-butanone (acetoin) showed significant reduction in symptomatic leaves inoculated with soft rot causing pathogen *Erwinia carotovora* (Ryu et al., 2003). According to Choudhary et al. (2008), the *B. subtilis* and *B. amyloliquefaciens*, employ different mechanisms to produce VCs and acetoin was produced under low atmosphere O_2 concentrations to provide an alternative electron sink for the regeneration of NAD+, when the normal respiration was possible via the glycolysis and TCA cycle. This explains the increased antifungal effect with an increased incubation period in closed system (MAP) in this study where the availability of O_2 is limited with increasing time. The gas composition within the MAP was 8 % CO_2 and 2% O_2. These results suggests that this approach with *B. amyloliquefaciens* PPCB004 could offer excellent potential for integrated application with MAP (higher CO_2 concentrations $\leq$10%) for minimising fruit decay caused by *P. crustosum* Furthermore application of biofumigants in a MAP system can be applied for commodities that show limitation for aqueous sanitation.

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Figure captions

Fig. 1. Percentage of inhibition of *Penicillium crustosum* radial mycelial growth in presence of antagonist measured up to 10 days. Bars show the inhibition produced by *Bacillu subtilis* PPCB001 or *Bacillus amyloliquefaciens* PPCB004 or antagonist combination PPCB001 + PPCB004.

Bars with different letters are significantly different ($P<0.05$) according to the least significant difference (LSD) test.

Fig. 2. Spore production of *Penicillium crustosum* in presence of antagonists *Bacillu*s spp. Spore production is expressed as decimal logarithmic concentration per square millimetre of surface. Graph shows the inhibition of spore production produced by *Bacillus subtilis* PPCB001 (■), *Bacillus amyloliquefaciens* PPCB004 (▲), antagonists combination PPCB001 + PPCB004 (□) and absence of antagonist (control) (●).

Means with different letters are significantly different ($P<0.05$) according to the least significant difference (LSD) test.

Fig. 3. Effect of volatile compounds of antagonists *Bacillus* spp on hyphae morphology of *Penicillium crustosum* after 10 days of incubation at 25 ºC. (A) Normal conidia of *P. crustosum* (control). (B) Conidial deformations produced by *Bacillus subtilis* PPCB001. (C)

Conidial deformation produced by *Bacillus amyloliquefaciens* PPCB004. (D) Conidial deformation produced by the combination of PPCB001 + PPCB004.

Table 1

Table 1 GC/MS volatile profile of antagonists *Bacillus* spp

Retention time (min)	Relative peak area (%)	Compound
***Bacillus subtilis* PPCB001**		
Acids		
10.21	0.44	Acetic acid
29.42	0.26	Octanoic acid
32.34	0.79	Nonanoic acid
35.1	1.01	Decanoic acid
40.17	0.48	Dodecanoic acid
44.89	4.33	Tetradecanoic acid
47.04	2.8	Pentadecanoic acid
49.24	31.86	Hexadecanoic acid
51.1	0.52	Heptadecanoic acid
53.02	4.68	Octadecanoic acid
Alcohol		
26.07	0.26	2-Phenyl-2-propanol
Ketones		
7.17	2.1	2,3-Butanedione
11.85	45.98	3-Hydroxy-2-butanone (Acetoin)
22.49	0.22	4-Octanone
N-containing compounds		
14.14	0.09	Pyrrole
17.36	0.22	2-Methyl-1H-pyrrole
20.87	1.57	n-(diphenylmethylene)aminoacetonitrile
S-containing compound		
22.14	0.79	2-Methyl-tetrahydrothiophen-3-one
Esters		
42.76	0.04	n-Hexyl salicylate
46.39	1.57	Tetradecanoic acid, 1-methylethyl ester (Isopropyl myristate)
***Bacillus amyloliquefaciens* PPCB004**		
Alcohol		
13.92	0.18	3-Methyl-1-butanol
Ketones		
11.88	97.52	3-Hydroxy-2-butanone (Acetoin)
19.41	0.36	2-Heptanone
26.56	0.42	2-Nonanone
32.86	0.6	2-Undecanone
34.72	0.12	2-Dodecanone
38.43	0.48	2-Tridecanone
N-containing compound		
20.88	0.3	n-(Diphenylmethylene)aminoacetonitrile

The compounds generated by *Bacillus* strains were tentatively identified by mass spectra comparison to those in the Wiley library (probability based match > 80%).

Table 2.

Percentage of disease incidence and severity measured as average of wound diameter in artificially infected citrus fruit cv Valencia with *Penicillium crustosum*

Treatments	Disease Incidence (%)	Severity wound diameter (mm)
Control (MAP)	36a	17.25±5.2b
PPCB001 + MAP	25b	13.6± 5.2c
PPCB004 + MAP	8.3c	7.2±4.2a
PPCB001+PPCB004 + MAP	24b	12.6±3.7c

Means followed by the same letter within columns for each pathogen for disease or severity inhibition are not significantly different at P <0.05 according to Least significant difference test.

Figure. 1

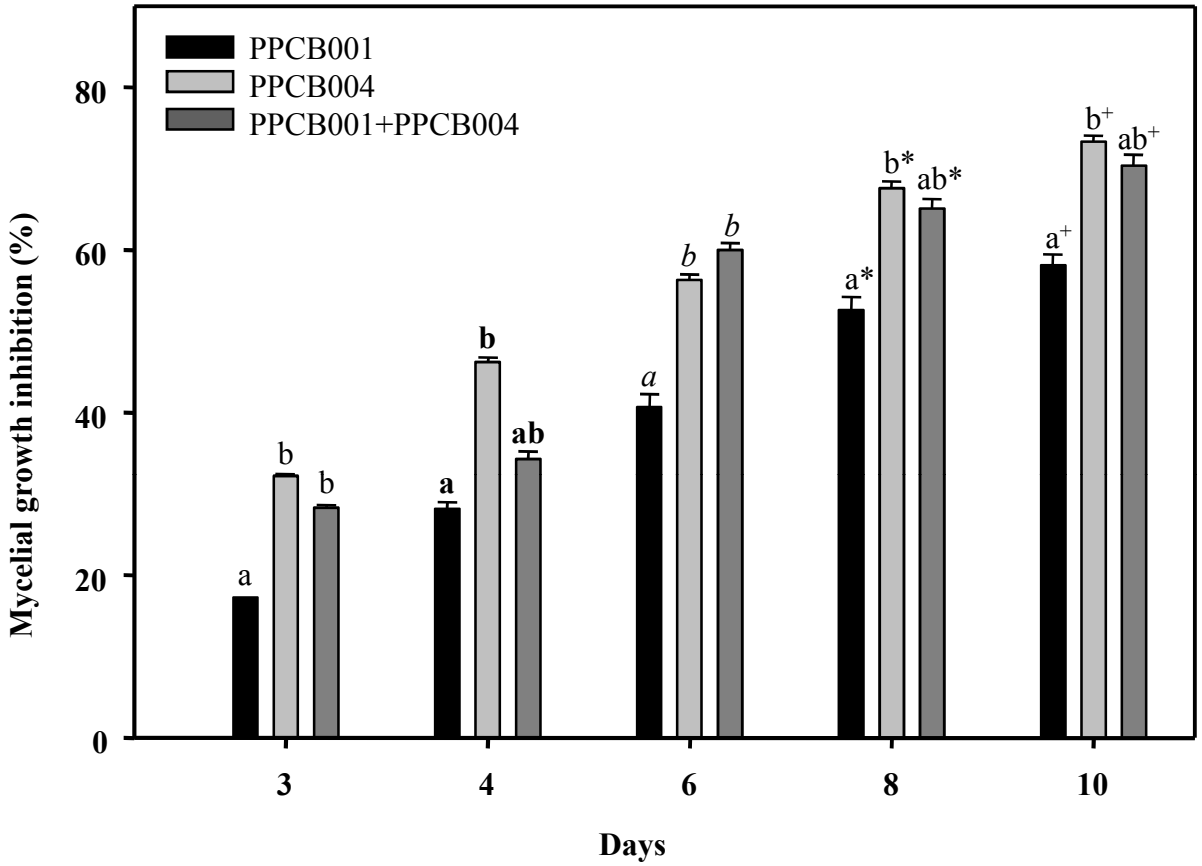

PPCB001
PPCB004
PPCB001+PPCB004
Mycelial growth inhibition (%)
0
20
40
60
80
3
4
6
8
10
Days
a
b
b
a
b
ab
a
b
b
a*
b*
ab*
a+
b+
ab+

Figure. 2

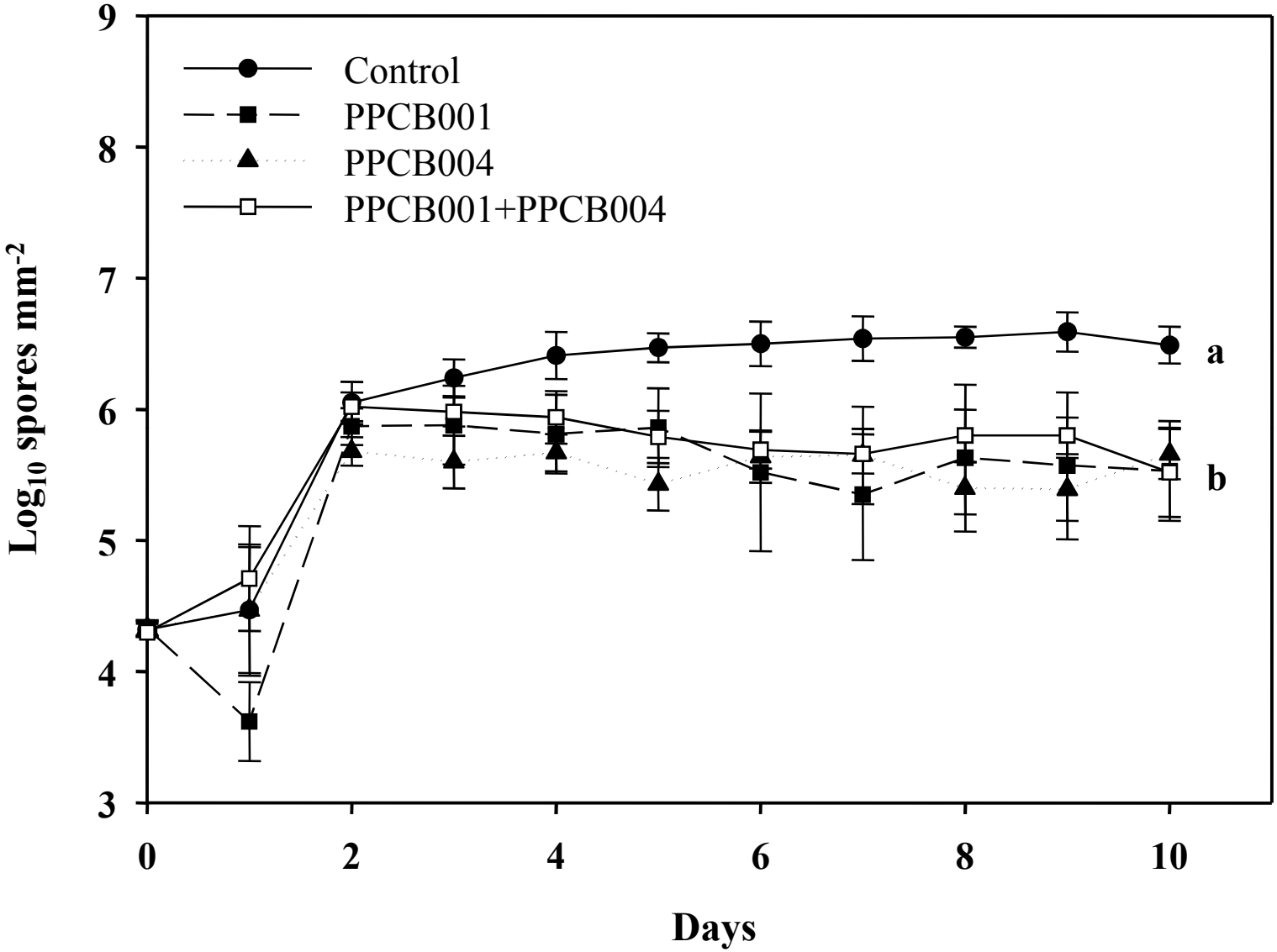
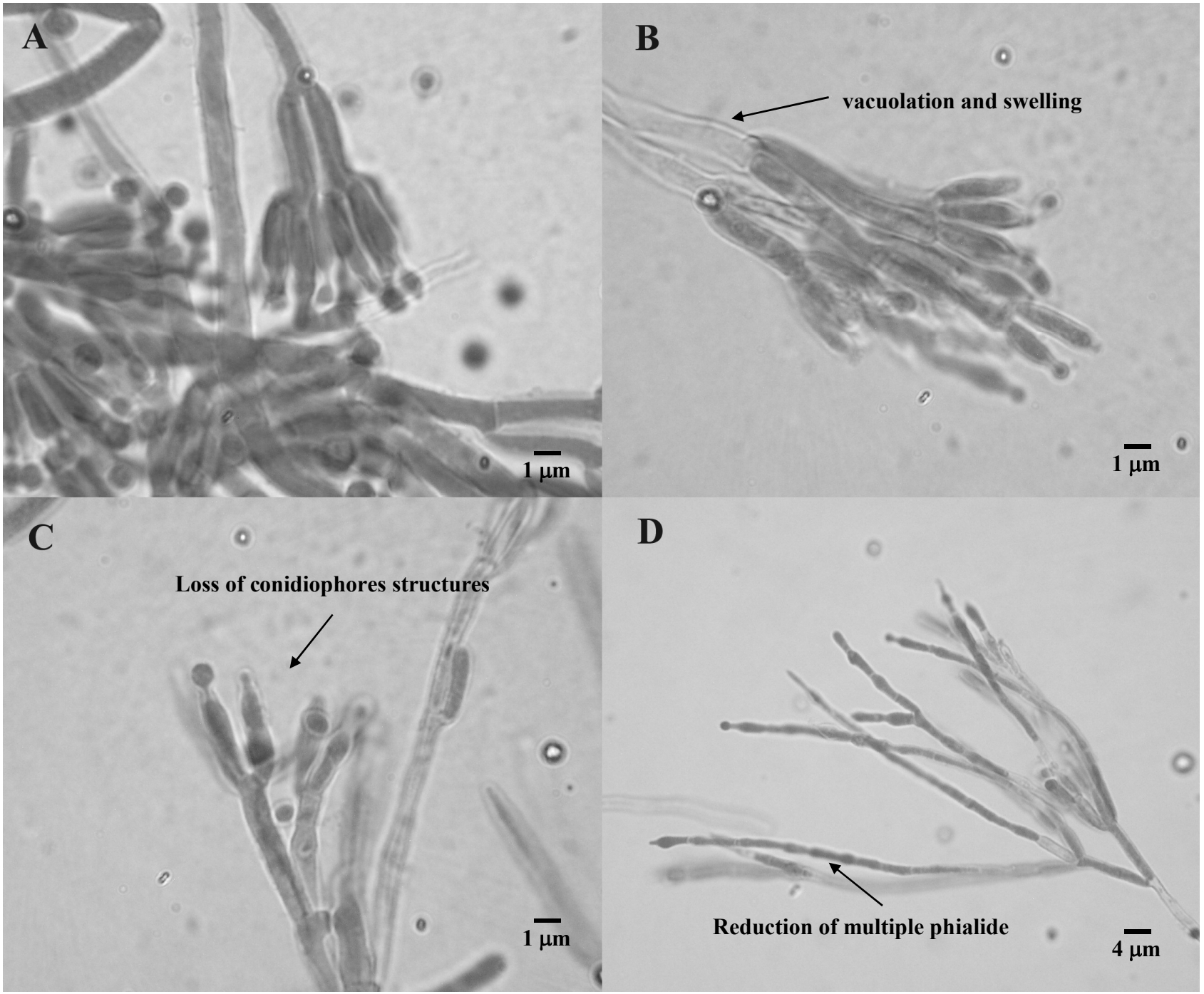

Control
PPCB001
PPCB004
PPCB001+PPCB004
Log10 spores mm-2
Days
a
b
0
2
4
6
8
10
3
4
5
6
7
8
9

Figure. 3


A
1 μm
B
vacuolation and swelling
1 μm
C
Loss of conidiophores structures
1 μm
D
Reduction of multiple phialide
4 μm