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Study of the efficacy of bacterial antagonists against *Cadophora luteo-olivacea* of kiwifruit

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1 **Research article**

2

3 **Study of the efficacy of bacterial antagonists against *Cadophora luteo-olivacea* of kiwifruit**

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6

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11

12 **Abstract**

13 Skin pitting currently represents one of the major postharvest diseases of kiwifruit and one of the  
14 most difficult to manage in packinghouses due to its latent behavior and the difficulty in predicting  
15 its emergence. Our research demonstrates the potential to use different bacterial strains (*Pseudomonas*  
16 *synxantha* and *Bacillus* spp.) instead of synthetic compounds to preserve kiwifruit from the  
17 development of postharvest skin pitting symptoms, following the momentum towards sustainable  
18 strategies. The antagonists tested with in vitro assays showed different efficacy rates against *C. luteo-*  
19 *olivacea* (strain Cad21) mycelial growth by producing non-volatile metabolites. The biochemical  
20 composition of the most active bacterial non-volatile secondary metabolites was described through  
21 FT-IR (Fourier-Transform Infrared) spectroscopy. *Pseudomonas synxantha* strain 117-2b emerged as  
22 the most active strain in in vivo experiments, both as a curative and preventive treatment (63% and  
23 84.7% of inhibition, respectively). In addition to its ability to reduce disease incidence, the biological  
24 antagonism exerted by *P. synxantha* strain 117-2b was further demonstrated by qPCR analysis as a  
25 reduction in the pathogen's abundance. In view of these results, alternative solutions in the field and  
26 during postharvest storage could be considered to control *C. luteo-olivacea* of kiwifruit.

27 **Keywords:** Storage; Biological control; Skin pitting; Antibiosis; FT-IR

28

## 29 **1. Introduction**

30 Kiwifruit is a recently domesticated plant belonging to the genus *Actinidia*, which contains  $\pm$  60  
31 species (Zhang et al., 2020). However, two species, *A. deliciosa* and *A. chinensis*, dominate the  
32 kiwifruit industry (Huang et al., 2004). In particular, *Actinidia deliciosa* (A. Chev.) C.F. Liang &  
33 A.R. Ferguson) cv. “Hayward”, which is planted worldwide in temperate-zone countries (Michaelidis  
34 and Elmer, 2000), remains one of the most commercially important cultivars (Choi et al., 2022). The  
35 consumption of kiwifruit has been increasing steadily; kiwifruit is one of the most popular and widely  
36 consumed fruits due to its flavor and remarkable nutrients (Wang et al., 2022a). Kiwifruit can be  
37 stored for about 4–5 months at 0 °C with relative humidity (R.H.) of 92–95% using standard  
38 refrigeration or for a longer time in a controlled atmosphere (CA) (Taş et al., 2022). Nevertheless, the  
39 fruit is susceptible to different fungal pathogens, among which the best known is *Botrytis cinerea*  
40 (Pers: Fr). It has always been considered one of the most important pathogens for stored kiwifruit  
41 (Costa et al., 1991), becoming a serious problem over the last 15 years in kiwifruit production  
42 (Michaelidis and Elmer, 2000, Di Francesco et al., 2018). During the last few years, the re-emerging  
43 pathogen *Cadophora luteo-olivacea*, which causes the skin-pitting disease of kiwifruit, has been  
44 detected in Italian and Chilean packaging houses (Di Francesco et al., 2022, Auger et al., 2018). Skin  
45 pitting symptoms (oval dark brown lesions) appear after several months of cold storage (Spadaro et  
46 al., 2010, Gilardi et al., 2007). *Cadophora luteo-olivacea* is a fungal pathogen that infects fruits during  
47 the developing period, remaining inactive for up to 4–5 months of cold storage (Di Francesco et al.,  
48 2022) and can appear immediately during the shelf-life period.

49 Usually, the risk of fungal postharvest diseases is mitigated with fungicide treatments before and after  
50 harvest (Palm and Kruse, 2012). However, issues related to chemical residues in the fruit have  
51 recently made these treatments challenging to justify, and this has stimulated the exploration of  
52 alternative strategies (Chowdhury et al., 2022). Microorganisms used as biocontrol agents (BCAs)

53 could represent one of the sustainable alternative methods (Wang et al., 2022b) to apply. Among  
54 them, bacteria usually show interesting antagonistic mechanisms of action toward fungal pathogens,  
55 making them attractive for biological control strategies. Sometimes, different mechanisms can act  
56 simultaneously, and it is not easy to establish which individual mechanism contributes the most to  
57 the inhibition of fungal pathogen growth (Di Francesco et al., 2016). Among the different species of  
58 bacteria used as effective BCAs, *Bacillus*, and *Pseudomonas* spp. have been mainly exploited for  
59 their synthesis of extracellular enzymes (Nihorimbere et al., 2011), production of volatile organic  
60 compounds (VOCs) (Gotor-Vila et al., 2017, Ni et al., 2022), and induction of resistance responses  
61 in the host (Carmona-Hernandez et al., 2019). All these characteristics could represent a useful  
62 starting point for selecting active and effective BCAs.

63 The objective of the present work was to investigate the antifungal effect of four different bacterial  
64 strains belonging to *Bacillus* and *Pseudomonas* spp. against *C. luteo-olivacea* by: i) in vitro assays  
65 through a co-culturing method at different growth temperatures and using agar infusion of bacterial  
66 cell filtrates from different growing times; ii) by their biochemical characterization (FT-IR); and iii)  
67 via in vivo assays on kiwifruit to verify their effectiveness as a preventive or curative treatment.

68

## 69 **2. Materials and methods**

### 70 **2.1 Fruit**

71 Kiwifruit cv 'Hayward' (*Actinidia deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson) were obtained  
72 from orchards cultivated under integrated pest management (IPM) production system located in  
73 Zoppola (Pordenone province, Google: 45°56'56.91" N, 12°47'15.83" E) and Sedegliano (Udine  
74 province: 46°02'11.02" N, 12°57'46.51" E). Fruits of uniform size and free from lesions were  
75 collected and immediately stored at 4 °C with RH 92% until use.

76

### 77 **2.2 Microorganisms**

78 *Cadophora luteo-olivacea* strain Cad21 was isolated from the tissue of symptomatic kiwifruit; its  
79 fresh mycelium was used for genomic DNA extraction following a procedure by Lecellier and Silar  
80 (1994), modified by Martini et al. (2009). The extracted and quantified DNA was diluted in sterile  
81 water to obtain a concentration of about 2–5 ng/μl. The fungal strain was molecularly identified by  
82 using the specific primers Cad (KSDA)-f2 5'-GTG GCG GGG CTA CCC TAC-3'/Cad (KSDA)-r1  
83 5'-GCT GGC AAG TAG ACC TAC CG-3') (Martini et al., unpublished) to amplify a DNA fragment  
84 of 485 bp in 25 μL reactions containing 200 μM of each of the four dNTPs, 0.4 μM of each primer,  
85 1.5 mM MgCl<sub>2</sub>, 0.625 units of GoTaq Flexi DNA Polymerase (Promega, WI, USA) and 1 μL of  
86 diluted DNA. The amplification reactions were carried out in a T gradient thermal cycler (MiniAmp  
87 Plus, Thermo Fisher Scientific) using the following program: initial denaturation at 94 °C for 2 min;  
88 35 cycles at 94 °C for 40 s, 58 °C for 30 s, 72 °C for 40 s; final extension at 72 °C for 7 min. PCR  
89 products were run on 1% agarose gels, stained with ethidium bromide, and visualized under UV light.  
90 The amplified DNA was purified with a Nucleospin Extracts II Kit (Macherey–Nagel, Germany) and,  
91 finally, Sanger sequenced by BMR Genomics (Padova, Italy). The sequences were compared to those  
92 of the GenBank database (<https://www.ncbi.nlm.nih.gov/BLAST>) using the BLASTN search. The  
93 fungal colony was maintained on potato dextrose agar (PDA, 39 g L<sup>-1</sup>, Oxoid - UK) at 4 °C until use.  
94 Regarding the antagonists, the *Pseudomonas synxantha* 117-2b strain belonging to the  
95 microbiological collection of DI4A-University of Udine, was originally isolated from the surface of  
96 kiwifruits and subsequently molecularly characterized (Di Lenarda et al., 2010). The *Bacillus*  
97 *amyloliquefaciens* FZB24 strain was acquired from the DSM-Deutsche Sammlung von  
98 Mikroorganismen collection. The *Bacillus subtilis* QST713 and *Bacillus pumilus* QST2808 strains  
99 were purchased from NRRL (Northern Regional Research Laboratory, IL, USA). Two weeks before  
100 the experiments, *C. luteo-olivacea* strain Cad21 was grown on PDA (potato dextrose agar, 39 g L<sup>-1</sup>;  
101 Oxoid - UK) at 25 °C. The conidial suspension was prepared by scraping and suspending conidia in  
102 sterile distilled water with 0.05% (v/v) Tween 80 and adjusted to the final required concentration (10<sup>5</sup>  
103 conidia mL<sup>-1</sup>) with a hemocytometer. All the bacterial strains were maintained on nutrient agar (NA,

104 13 g L<sup>-1</sup>, Oxoid - UK) at 4 °C until use. For the assays, bacterial cells from two-day-old plates were  
105 suspended in potassium phosphate buffer (PPB, 70 mL KH<sub>2</sub>PO<sub>4</sub> 0.2 M; 30 mL K<sub>2</sub>HPO<sub>4</sub> 0.2 M and  
106 300 mL deionized water v/v/v pH 6.5) and adjusted to a final concentration of 10<sup>6</sup> cells mL<sup>-1</sup> and  
107 10<sup>8</sup> cells mL<sup>-1</sup> according to the experiment.

108

### 109 **2.3 *In vitro* assays**

110 Two different experiments were performed to test the antagonism of the four bacterial strains. The  
111 first, a co-culture assay, was conducted using bacterial cells grown in nutrient broth (NB, 13 g L<sup>-1</sup>,  
112 Oxoid - UK) for 24 h. A mycelial plug (6 mm diameter) of the fungal strain Cad21 derived from 14-  
113 day-old colonies was placed in the center of the PDA plate. A loopful (1 µL) of each bacterial culture  
114 was deposited at four equidistant peripheral locations on the dish. Plates inoculated only with the  
115 fungal pathogen were considered as control. Plates were incubated at 1 °C and 25 °C in dark  
116 conditions, and the colony diameters were measured with a ruler after 8 and 4 weeks, respectively.  
117 Five replicates for each condition were used, and the experiment was conducted twice.

118 The second assay tested the efficacy of bacterial cell filtrates collected at different growing times. A  
119 loop of cells of each strain was grown in 100 mL of NB broth at 25 °C in a rotary shaker (250 rpm)  
120 for 4 days. A flask was prepared for each sampling time (1 h, 24 h, 48 h, 72 h, 96 h). An aliquot of  
121 50 mL of each sampling time was centrifuged (10,000 rpm at 4 °C for 30 min), and the supernatants  
122 were filtered using 0.20 µm Millipore filters (Sigma Aldrich, USA). An aliquot of each collected cell  
123 filtrate (50 mL) was infused into 50 mL of PDA and plated onto Petri dishes. Dishes were inoculated  
124 with Cad21 strain plugs (6 mm diameter) and kept at 25 °C. After 2 weeks, the colony diameters were  
125 measured using a digital caliper (Borletti, Italy). Plates inoculated with the fungal pathogen plug on  
126 simple PDA were considered as control. Eight replicates were used for each condition, and the  
127 experiment was conducted twice.

128

### 129 **2.4 FT-IR analysis of bacterial cell filtrates**

130 The most active bacterial cell filtrates were analyzed with FT-IR (Fourier-Transform Infrared)  
131 spectroscopy to obtain a rapid and non-destructive characterization of their main antifungal  
132 components. Filtrates were obtained as described above in paragraph 2.3. An aliquot (1.5 mL) of each  
133 treated sample was collected in sterile tubes (2 mL), stored at  $-80\text{ }^{\circ}\text{C}$ , and lyophilized. Infrared spectra  
134 were recorded with a Bruker ALPHA series FT-IR spectrophotometer (Bruker, Ettlingen, Germany)  
135 equipped with an attenuated total reflectance (ATR-Diamond crystal) apparatus at the standard  
136 resolution of  $4\text{ cm}^{-1}$ . Three spectra (averaged over 64 measures) were measured for each cell filtrate.  
137 The spectra were collected as described by Di Francesco et al. (2021).

138

### 139 **2.5 *In vivo* assay**

140 For the *in vivo* assay, kiwifruits (6.5° Brix) were divided into two groups according to the applied  
141 treatment: curative or preventive. For both treatments, fruits were surface sterilized with sodium  
142 hypochlorite (0.1% v/v), rinsed with distilled water for one minute, and air dried at room temperature.  
143 Fruits were artificially wounded once at the equatorial line by using a sterile steel nail (2 mm  $\times$  2 mm  
144  $\times$  2 mm). For curative treatment, 20  $\mu\text{L}$  of pathogen conidial suspension ( $10^5$  conidia  $\text{mL}^{-1}$ ) were  
145 pipetted into each wound. After 24 h, the same wounds were inoculated with 20  $\mu\text{L}$  of *P. synxantha*  
146 (117-2b), *B. amyloliquefaciens* (FZB24), *B. subtilis* (QST713), and *B. pumilus* (QST2808)  
147 suspensions ( $10^8$  cells  $\text{mL}^{-1}$ ).

148 For preventive treatment, 20  $\mu\text{L}$  of each bacterial cell suspension were inoculated first, and after 24  
149 h, the same aliquot of the conidial suspension was added as described above. Treated fruits were kept  
150 in plastic trays at  $1\text{ }^{\circ}\text{C}$  and 92% relative humidity (R.H.) for 4 months. After the cold storage, fruits  
151 were maintained at  $20\text{ }^{\circ}\text{C}$  (shelf life) for 1 week. Kiwifruits inoculated with sterile water represented  
152 the negative control, while fruits treated with Scholar® (active ingredient: Fludioxonil; Syngenta,  
153 Basel, Switzerland) ( $1.3\text{ mL L}^{-1}$ ) represented the positive one. The experiment consisted of three  
154 replicates of 15 fruits for each treatment, and it was performed twice.

155

## 156 **2.6 DNA Extraction and quantification of the amount of fungal DNA by qPCR**

157 The total genomic DNA was extracted from artificially wounded-inoculated kiwifruits (as described  
158 above in paragraph 2.5) to quantify the amount of fungal pathogen DNA using a qPCR assay. DNA  
159 was extracted from 10 portions of kiwifruit tissue (150 mg) for each condition, collected at 1 cm from  
160 the inoculation wound following a Doyle and Doyle (1990) protocol modified by Martini et al. (2009).  
161 The quality and quantity of extracted DNA were assessed using a NanoDrop 1000 Spectrophotometer  
162 (Thermo Scientific, Wilmington, DE, USA), and each sample concentration was adjusted to 20 ng/ $\mu$ L  
163 by dilution in nuclease-free water. Qualitative qPCR was performed using ITS as a target using the  
164 *C. luteo-olivacea* specific primers Cad (KSDA)-f2 5'-GTG GCG GGG CTA CCC TAC-3'/Cad  
165 (KSDA)-r2 5'-CGC CAA AGC AAC AAA GGT AGT-3' (fragment 105 bp long). qPCRs were  
166 performed in 15  $\mu$ L per reaction in a 96-well Bio-Rad CFX96 RealTime PCR System (Bio-Rad Inc.,  
167 Hercules, CA, USA). Reaction mixtures contained 0.3  $\mu$ M of each primer, 1X SsoFast™ EvaGreen  
168 ® Supermix (Bio-Rad Inc., Hercules, CA, USA), molecular grade H<sub>2</sub>O; 2  $\mu$ L of DNA solution  
169 containing 20 ng/ $\mu$ L of extracted DNA as a template. Cycling conditions were as follows: initial  
170 denaturation at 98 °C for 2 min; 50 cycles of 5 sec at 98 °C; 5 sec at 58 °C. A low-resolution melting  
171 curve (ramp from 65 °C to 95 °C with 0.5 °C increments and holding times of 5 s) was programmed  
172 at the end of the cycling reaction.

173 In all positive samples, the amount of pathogen DNA was assessed by qPCR as pg of *C. luteo-*  
174 *olivacea* DNA/mg of kiwifruit tissue to normalize the data. To quantify *C. luteo-olivacea* DNA, a  
175 standard curve was prepared with 10-fold serial dilutions of total DNA extracted from a pure culture  
176 of the Cad21 strain (quantified by using Qubit® 2.0 Fluorimeter), starting at 2 ng/ $\mu$ L and up to 2  
177 pg/ $\mu$ L. PCR mixtures and cycling conditions were performed as described above.

178 The amount of fluorescence for each sample was measured at the end of each cycle and analyzed via  
179 CFX-Manager Software v. 2.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The baseline was  
180 automatically determined, and the fluorescence threshold was set manually to maximize the standard  
181 curve efficiency. Each diluted sample and each standard were replicated three times in the



182 experiment. Fungal DNA quantity was expressed as pg of *C. luteo-olivacea* DNA/mg of kiwifruit  
183 tissue.

184

## 185 **2.7 Population dynamics of *Pseudomonas synxantha* strain 117-2b on kiwifruit**

186 To analyze the ability of *P. synxantha* (117-2b) to colonize and proliferate on kiwifruit wound sites,  
187 fruits were surface sterilized, as reported in section 2.5. Fruits were wounded at three equidistant  
188 points by using a sterile needle. Aliquots of 20  $\mu\text{L}$  of *P. synxantha* strain 117-2b suspension (106  
189 cells  $\text{mL}^{-1}$ ) were pipetted into each wound. Fruits were kept at 0 °C for 4 months. BCA growth was  
190 determined at 0 (2 h from the inoculation), 1, 2, 3, and 4 months of storage. Plugs (6 mm,  $\emptyset$ ) of fruit  
191 tissue from each wound site were removed with the help of a sterile cork borer. Fruit plugs were  
192 transferred into a sterile stomacher bag containing 5 mL of sterile distilled water and Tween 80  
193 (0.05%). The bag was stomached for 20 min (Bag Mixer 400; Interscience, St Nom, France). The  
194 juice (100  $\mu\text{L}$ ), diluted in sterile distilled water, was surface-plated on NA, and the Petri dishes were  
195 incubated at 25 °C for 2 days. Three fruits representing three replicates were set up for each sampling  
196 time, and the experiment was performed twice.

197

## 198 **2.8 Statistical analysis**

199 All the experiments were analyzed by one-way analysis of variance (ANOVA). Statistical means  
200 were compared by using *Tukey's* test ( $\alpha = 0.05$ ). Data were reported as mean values  $\pm$  SE of two  
201 independent experiments. All analyses were performed with the software MiniTab.16.

202

## 203 **3. Results**

### 204 **3.1 *In vitro* assays**

205 Different inhibition values of the fungal pathogen were observed with the *in vitro* assays after the  
206 direct application of the bacterial strain or their secondary metabolites. Furthermore, the colony  
207 diameter of the control observed at 1 °C showed values that support the ability of the pathogen to

208 grow at low temperatures. Regarding the results of the co-culture assay (Fig. 1), at 1 °C, *P. synxantha*  
209 (117-2b) showed the best antagonistic activity by reducing the growth of the fungal pathogen strain  
210 Cad21 by 74.7%. *Bacillus amyloliquefaciens* (FZB24) and *B. subtilis* (QST713) reduced the colony  
211 diameter of the *C. luteo-olivacea* strain by 35.9% and 42.1%, respectively. Conversely, *B. pumilus*  
212 (QST2808) was the least active strain against fungal pathogen growth, showing a reduction of only  
213 15.1%. Compared to the lower temperature, the BCAs tested have shown a higher antagonism  
214 potential at 25 °C. Plates inoculated with *P. synxantha* (117-2b), *B. amyloliquefaciens* (FZB24), and  
215 *B. subtilis* (QST713) showed an inhibition of 73.3%, 72.1%, and 70.2%, respectively. *Bacillus*  
216 *pumilus* (QST2808) was the least active strain, with a 53.5% reduction in fungal mycelial growth.  
217 The culture filtrates of the tested bacterial strains were assayed against the growth of the fungal  
218 pathogen strain Cad21 and indicated a variable inhibition activity (Fig. 2). The effectiveness of *B.*  
219 *amyloliquefaciens* (FZB24) culture filtrates was significantly higher at growing times of 48 h, 72 h,  
220 and 96 h, inhibiting fungal growth by 43.6% on average, compared to the control. *Pseudomonas*  
221 *synxantha* (117-2b) filtrates showed a higher inhibition rate at 72 h and 96 h (9.2% and 24.2%,  
222 respectively). Culture filtrates of *B. subtilis* (QST713) had their highest activity at 48 h, 72 h, and 96  
223 h, inhibiting the growth of the fungal mycelium by 31.7%, 41.7%, and 20%, respectively, compared  
224 to the control. The tested culture filtrates seemed to be, for all strains, most effective immediately  
225 after 48 h of growth. Nevertheless, the activity of *B. pumilus* (QST2808) culture filtrates was  
226 confirmed to be ineffective in inhibiting the growth of *C. luteo-olivacea* strain Cad21.

227

### 228 **3.2 Biochemical analysis of bacterial cell filtrates**

229 The spectroscopic measurements were carried out to determine the main non-volatile metabolites  
230 produced by the different bacterial antagonists. Fig. 3 shows the infrared difference spectra between  
231 cell-filtrate and the nutrient broth (the spectrum of nutrient broth is reported for comparison in Figure  
232 S1, supplementary material). Difference spectra were considered more valuable for discriminating  
233 the metabolites produced by bacteria (positive peaks) and the medium itself (negative peaks). The

234 bands of the nutrient broth medium were attributed thanks to previous data generated from cultures  
235 grown on peptone-containing medium (Trivedi et al., 2015).

236 The prominent positive peaks were attributed to peptidic compounds: 1699, 1664, and 1625  $\text{cm}^{-1}$   
237 bands are located in the amide I spectral region, arising from the C=O and N-H vibration of peptide  
238 (and protein) backbones (Besson et al., 1996) and sensitive to their secondary structure. A positive  
239 peak in the amide II spectral region at 1546  $\text{cm}^{-1}$  (N-H and C-N vibrations) further confirms the  
240 peptidic nature of the compounds in the cell filtrate. More specifically, the 1625  $\text{cm}^{-1}$  band was  
241 attributed to asparagine residues as observed in iturin A, a lipopeptide produced by *Bacillus subtilis*  
242 (Besson et al., 1996), while the same authors attributed the 1699 and 1664  $\text{cm}^{-1}$  bands to a type II  $\beta$ -  
243 turn structure adopted by the lipopeptide. This secondary structure is characterized by the presence  
244 of selected amino acids such as asparagine and proline (band at 1625  $\text{cm}^{-1}$ ), glycine (band at 698  
245  $\text{cm}^{-1}$ ), aspartic acid (bands at 1218, 1069, 910, and 871  $\text{cm}^{-1}$ ) and serine (band at 988  $\text{cm}^{-1}$ ) (Barth,  
246 2007).

247 1441, 1355, 1314, and 832  $\text{cm}^{-1}$  bands were attributed to C-H vibrations; therefore, they may refer to  
248 the hydrophobic side chains of amino acids (i.e., alanine, leucine, etc.) or a lipidic component of an  
249 antimicrobial compound as observed in *Bacillus paralicheniformis* (Ahire et al., 2020). The bands at  
250 1218 and 618  $\text{cm}^{-1}$  have been previously observed in the IR spectra of linear lipopeptides produced  
251 by *Paenibacillus polymixa* (Deng et al., 2011). The 1092 and 698  $\text{cm}^{-1}$  peaks were associated with  
252 the C-O and C-S bond vibrations in bacitracin, an antibiotic polypeptide produced by *B. subtilis* and  
253 *B. licheniformis* (Li et al., 2017). Finally, the 1920  $\text{cm}^{-1}$  band could be attributed to the O-N-O  
254 vibration in an antibiotic peptide produced by *B. brevis* (Muhammad et al., 2016).

255 The presence of antibiotic peptides was further supported by the appearance of other bands already  
256 reported for *Bacillus megaterius*: the O-N-O band at 1920  $\text{cm}^{-1}$  and the C-S band at 698  $\text{cm}^{-1}$  are  
257 typical of bacitracin, a polypeptide rich in cysteine, glutamic acid, histidine, phenylalanine, lysine,  
258 isoleucine, leucine, ornithine, and aspartic acid (Al-Thubiani et al., 2018).

259

260 IR spectra reported in Fig. 3 show many similarities. However, the differences between the different  
261 cell filtrates may reflect a different chemical composition: 1625 (attributed to Asn and Pro amino  
262 acids), and the 618  $\text{cm}^{-1}$  bands were more intense in the QST2808 filtrate, which had a different  
263 profile in the 1080–920  $\text{cm}^{-1}$  spectral region. The *P. synxantha* strain 117-2b filtrate was  
264 characterized by the most intense peaks, mainly in the 1400–800  $\text{cm}^{-1}$  region, with a typical peak at  
265 988  $\text{cm}^{-1}$  attributed to Ser (Barth, 2007).

266

### 267 **3.3 *In vivo* assays**

268 The efficacy of all tested bacterial strains as curative and preventive applications was demonstrated  
269 by *in vivo* assays.

270 The wounded fruits treated with *P. synxantha* (117-2b) showed the highest inhibition rate against  
271 fungal pathogen strain Cad21 on kiwifruits (63% and 84.3%) in the case of both curative and  
272 preventive application, respectively, compared to the other strains and relative to the control (Fig. 4).  
273 However, *B. amyloliquefaciens* (FZB24) and *B. subtilis* (QST713) showed low curative activity  
274 against the pathogen with reductions of only 13% and 18.5%, respectively, relative to the control.  
275 *Bacillus pumilus* (QST2808) had the lowest efficacy against skin pitting disease, with no reduction  
276 as a curative treatment and only a 16.3% reduction as a preventive treatment. The synthetic product  
277 (Scholar®) was confirmed in both experiments as the most effective treatment. However, the  
278 effectiveness of *P. synxantha* (117-2b) was very close to the performance of the chemical treatment,  
279 particularly in the preventive application.

280

### 281 **3.4 qPCR analysis**

282 qPCR analysis quantified the amount of fungal pathogen DNA in each portion of the kiwifruit tissue  
283 analyzed. The standards were used to construct a standard curve, which presented an optimal  
284 efficiency of 94.8%, with  $y = -3.453x + 16.578$  and  $R^2 = 0.999$  (Fig. 5a).

285 The real-time amplification reaction gave positive results, especially with all DNA samples obtained  
286 by processing the portions of untreated fruits (negative controls) and with the majority of DNA  
287 samples obtained from *P. synxantha* (117-2b) treated fruits, thus allowing their quantification. The  
288 samples' melting temperatures (Figure 3.2.4) corresponded to 82.5 °C, which was the same as the  
289 standards (Fig. 5b) and indicated that the amplification was specific.

290 The quantification of *C. luteo-olivacea* in infected samples, measured after 4 months after the  
291 artificial inoculation of fruits, was obtained by extrapolating from the standard curve the pg of *C.*  
292 *luteo-olivacea* DNA and normalizing these data with mg of kiwifruit tissue sampled at 1 cm from the  
293 inoculation wound. The results of the quantification are summarized in Table 1. The amount of fungal  
294 DNA in the negative control (fungus and water) was estimated at around 30.02 pg/mg. In kiwifruits  
295 treated with *P. synxantha* strain 117-2b, selected as the best bacterial strain for controlling the  
296 development of skin pitting symptoms, and Scholar®, the amounts of DNA were drastically reduced  
297 to 0.13 pg/mg and 0.03 pg/mg, respectively (Table 1).

298

### 299 **3.5 *Pseudomonas synxantha* strain 117-2b population dynamics**

300 The *Pseudomonas synxantha* (117-2b) population dynamics on artificially wounded-inoculated  
301 kiwifruit were determined during the 4-month cold storage period (Fig. 6). During the first three  
302 months of storage, a slight but significant increase in the CFU of the strain was registered, reaching  
303 almost  $4.9 \times 10^3$  CFU wound<sup>-1</sup>. After the third month of storage, the strain showed a slight but  
304 significant decrease with respect to the previous months.

305

## 306 **4. Discussion**

307 *Cadophora luteo-olivacea* has presented a critical problem in recent years in Italian packaging houses,  
308 and the connection between its incidence during the postharvest phase and field conditions at the time  
309 of fruit production has made it difficult to manage (Di Francesco et al., 2021). The possible  
310 appearance of skin pitting during storage is a challenge for kiwifruit marketing operators and often

311 prevents them from waiting for the best market combination of demand and more profitable prices.  
312 This study aimed to find a sustainable solution to preserve kiwifruit from skin pitting losses by using  
313 BCAs as an alternative o fungicides. The most intensively studied bacteria for use as BCAs belong  
314 to the genera of *Pseudomonas*, *Bacillus*, and *Streptomyces* spp. (Bonaterra et al., 2022). Moreover,  
315 many of these have already been registered and marketed as biopesticides.

316 Among the most interesting, *Pseudomonas* spp. have been studied as potential antagonists against a  
317 wide range of fungal pathogens, mainly for their ability to produce active antifungal compounds  
318 (Rojas-Solis et al., 2020, Sang and Kim, 2014, Aiello et al., 2019). For example, *Pseudomonas*  
319 *synxantha* produces bioactive compounds such as a biosurfactant effective against several  
320 microorganisms (Mukherjee et al., 2014). For this reason, the strain *P. synxantha* 117-2b belonging  
321 to our microbiological collection has been studied and tested in the present work as an alternative  
322 method to control kiwifruit skin pitting, together with other known bacterial strains.

323 Among the tested BCAs, *B. amyloliquefaciens* (FZB24), *B. pumilus* (QST2808), and *B. subtilis*  
324 (QST713) were assayed for their effectiveness against a broad range of plant pathogens. In fact, these  
325 strains are the active components of the bio-products Taegro®, Sonata®, and Serenade®,  
326 respectively, whose effectiveness we also wanted to test against *C. luteo-olivacea* for a possible and  
327 valuable field-ready treatment.

328 It is known that bacterial antagonists can produce bioactive compounds such as hydrolytic enzymes,  
329 phytohormones, and volatile and non-volatile metabolites or induce systemic resistance in plants and  
330 promote plant growth (Calderón et al., 2015, De Vleeschauwer et al., 2008, Lo Cantore et al., 2015,  
331 Raza et al., 2016, Rojas-Solis et al., 2020). In this regard, non-volatile metabolites, selected as the  
332 most active against *C. luteo-olivacea* by in vitro assay, were analyzed after different growing times.  
333 As noted, all the tested strains, except for *B. pumilus* QST2808, produced active metabolites against  
334 *C. luteo-olivacea*, starting from the stationary phase.

335 The biochemical analysis conducted using infrared spectroscopy on bacterial cell filtrate revealed the  
336 presence of peptidic compounds, mainly from the appearance of amide I and amide II bands in 1699–

337 1625 cm<sup>-1</sup> and 1546 cm<sup>-1</sup> spectral regions (Fig. 3). These compounds could be related to the well-  
338 known production of antibiotic peptides and lipopeptides by several bacteria, such as *B. subtilis*,  
339 which produces iturin A, a heptapeptide closed in a ring with an amino fatty acid, whose antifungal  
340 activity is related to its ability to induce a massive leakage of K<sup>+</sup> and other cell components from the  
341 cytosol (Besson et al., 1996). The different profiles observed in the IR spectra could also explain the  
342 behavior of the antagonists in the in vivo assays. The corresponding in vivo effects of the QST713  
343 and FZB24 strains could be related to their similar chemical composition due to the analogous  
344 positions and intensities of their peaks (Fig. 3). At the same time, the different spectral profile of the  
345 QST2808 strain may reflect the production of antifungal compounds that are less effective against  
346 the infection. However, the IR data should be interpreted carefully because it is well known that some  
347 bacteria, such as various *Pseudomonas* spp., can produce several antagonistic antifungal compounds  
348 in the form of peptides, including proteolytic enzymes (i.e., proteases, chitinases, cellulases,  
349 pectinases or amylases) or even siderophores (non-proteogenic peptides) (Karmegham et al., 2020).  
350 The similar chemical composition of these antagonistic compounds is reflected in their IR spectra, as  
351 evidenced in previous papers on non-volatile metabolites in *Aureobasidium pullulans* in which all the  
352 previous compounds were detected (Di Francesco et al., 2020, Di Francesco et al., 2021).  
353 Nevertheless, none of the BCA cultures filtrates totally inhibited *C. luteo-olivacea* mycelial growth,  
354 even though they were active against *B. cinerea* mycelium (data not reported). This fact showed a  
355 broad spectrum of antagonism against different plant pathogens based on antimicrobial metabolites  
356 (Roselló et al., 2013).  
357 Despite this, the curative and preventive in vivo experiments on fruits showed that *P. synxantha* strain  
358 117-2b had higher effectiveness against *C. luteo-olivacea* (strain Cad21) than the other tested BCAs  
359 (Fig. 4). This finding was further confirmed by the IR analysis composition of culture filtrates (Fig.  
360 3). *Pseudomonas synxantha*'s filtrates showed the most intense peaks (at 988 cm<sup>-1</sup>), which can be  
361 related to the highest concentration of antifungal compounds. Also, according to the co-culturing  
362 assay, the strain had the best adaptation to low temperatures (1 °C), permitting long persistence during

363 the storage phase, although, as seen in population dynamics, with a significant cellular decrease.  
364 Nevertheless, different studies suggested that high spore concentrations in inoculum were not always  
365 connected to the best performance of a BCA (Verma et al., 2007). However, a higher initial cellular  
366 concentration will be required for further applications such as in the field.  
367 Indeed, this fact could be connected to the endophytic behavior of *P. synxantha* (Aiello et al., 2019),  
368 which can move, colonize, and persist inside its host's vascular tissues. This characteristic should be  
369 interesting to develop in the future for managing *C. luteo-olivacea*, whose epidemiology is not still  
370 well understood but whose long latency is known and poorly manageable.  
371 The management of kiwifruit diseases by employing BCAs has been successfully evaluated with  
372 many pathogens, but to our knowledge, *C. luteo-olivacea* had not been explored previously.  
373 According to the results obtained with qPCR analysis, *C. luteo-olivacea* showed great persistence in  
374 kiwifruit despite being inoculated with an antagonist. In fact, the pathogen DNA was detected in  
375 inoculated but asymptomatic kiwifruit, although in much smaller quantities than the control.  
376 Conversely, in the positive control (fungicide application), almost no traces of the fungal pathogen  
377 biomass were detected, confirming the different mechanisms of action between a BCA and a  
378 fungicide.  
379 Therefore, further studies such as genomic analysis are needed to deeply evaluate the mechanisms of  
380 action of *P. synxantha* strain 117-2b. It will be useful to characterize it as a good BCA and for its  
381 eventual consideration as an active ingredient in a bioformulation.

382

### 383 **CRedit authorship contribution statement**

384 A. Di Francesco: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original  
385 draft, Writing – review & editing. F. Jabeen: Investigation, Writing – original draft. M. Di Foggia:  
386 Methodology, Investigation, Writing – original draft, Writing – review & editing. C. Zanon:  
387 Investigation. R. Cignola: Methodology, Investigation. A. Sadallah: Methodology, Investigation. V.  
388 Tugnoli: Methodology, Writing – review & editing. P. Ermacora: Conceptualization, Writing –



389 original draft, Writing – review & editing, Supervision, Funding acquisition. M. Martini:  
390 Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision.

391

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396

## 397 **Conflict of Interest Statement**

398 The authors declare that they have no known competing financial interests or personal relationships  
399 that could have appeared to influence the work reported in this paper.

400

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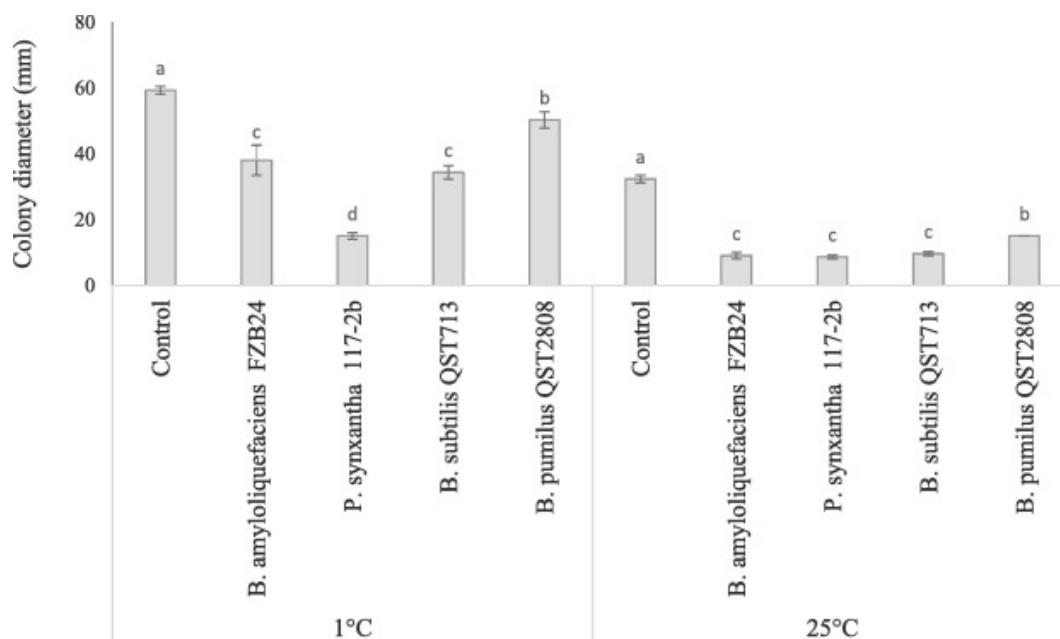
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## 580 **Figures**

581 Figure 1. *In vitro* co-culture assay. Efficacy of bacterial antagonists at two different temperatures (1  
582 °C and 25 °C) on the colony growth of *Cadophora luteo-olivacea* (Cad 21). The data were the average  
583 of two experiments. Data reporting the same letters are not statistically significant according to  
584 Tukey's test ( $\alpha = 0.05$ ).

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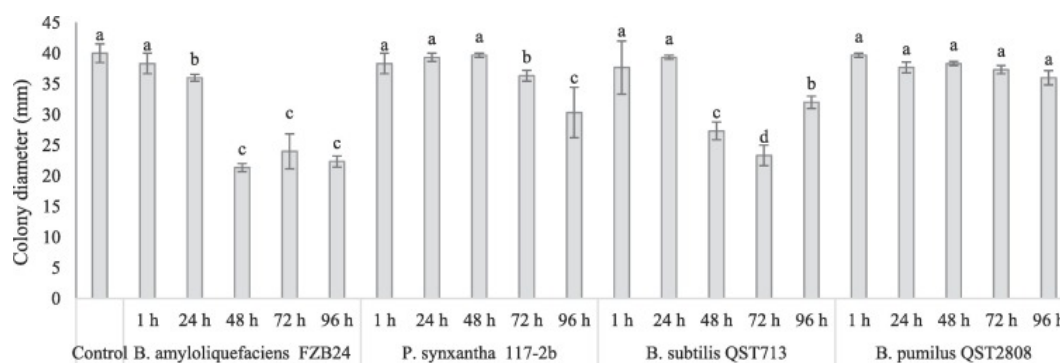




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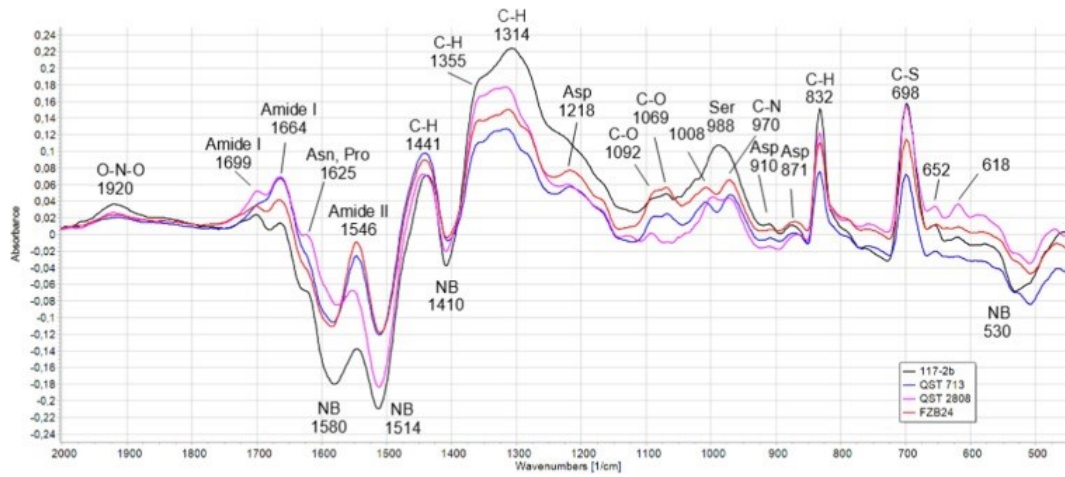
588 Figure 2. Effect of bacterial strain cell-filtrates infused onto PDA plates on the colony growth of  
 589 *Cadophora luteo-olivacea* (Cad21). Biocontrol agents were grown in nutrient broth at 20 °C for one  
 590 week and filtrates were sampled at 1 h and then every 24 h for up to 96 h. Data are the means of  
 591 *Cadophora luteo-olivacea* (Cad21) colony growth observed after 14 days at 20 °C. For each  
 592 antagonist different letters indicate significant differences according to Tukey's test ( $\alpha = 0.05$ ).



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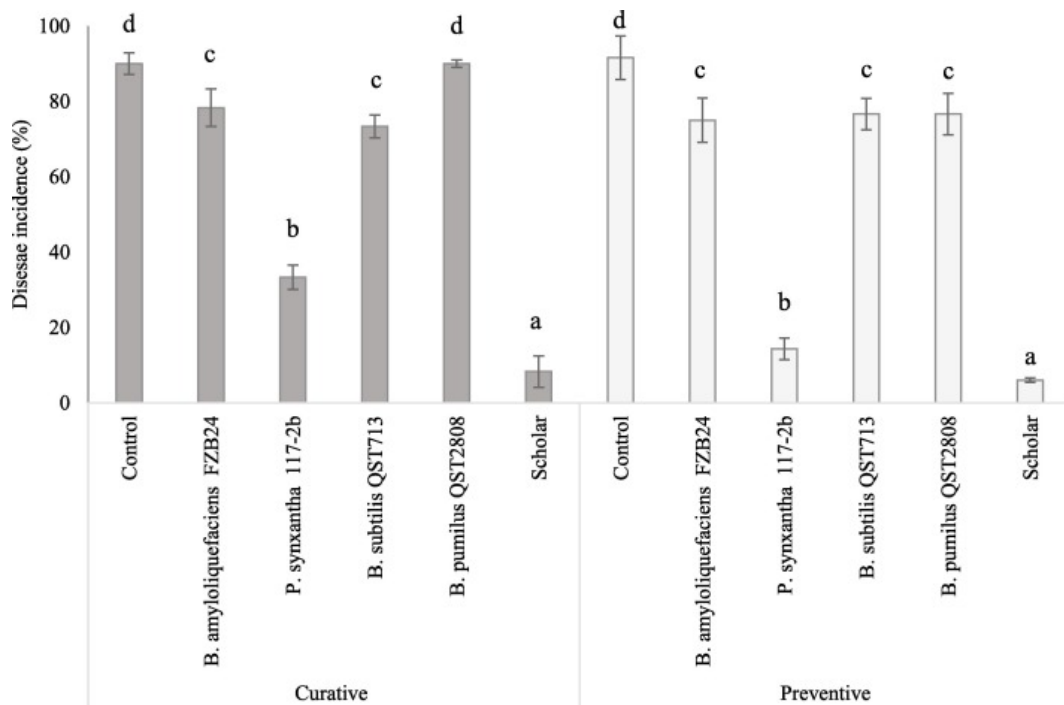
595 Figure 3. Difference FT-IR spectra of bacterial cell filtrates with the main positive bands related to  
 596 the production of non-volatile compounds discussed in the text. Negative peaks are related to the  
 597 consumption of the nutrient broth (NB).



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599

600 Figure 4. Curative and preventive effect of bacterial strains ( $10^8$  cells  $\text{mL}^{-1}$ ) and Scholar<sup>®</sup> ( $1.3 \text{ mL}$   
 601  $\text{L}^{-1}$ ) on disease incidence on artificially inoculated kiwifruit. Fruits were kept at  $1 \text{ }^\circ\text{C}$  for four months.  
 602 For the curative effect fruit wounds were first treated with  $20 \text{ }\mu\text{L}$  of pathogen suspension and after air  
 603 drying they were inoculated with  $20 \text{ }\mu\text{L}$  of each antagonist. For the preventive effect antagonists were  
 604 inoculated first. Data are the means of fifteen fruits for each antagonist and treatment effect. For each  
 605 treatment effect different letters indicate significant differences according to Tukey's test ( $\alpha = 0.05$ ).

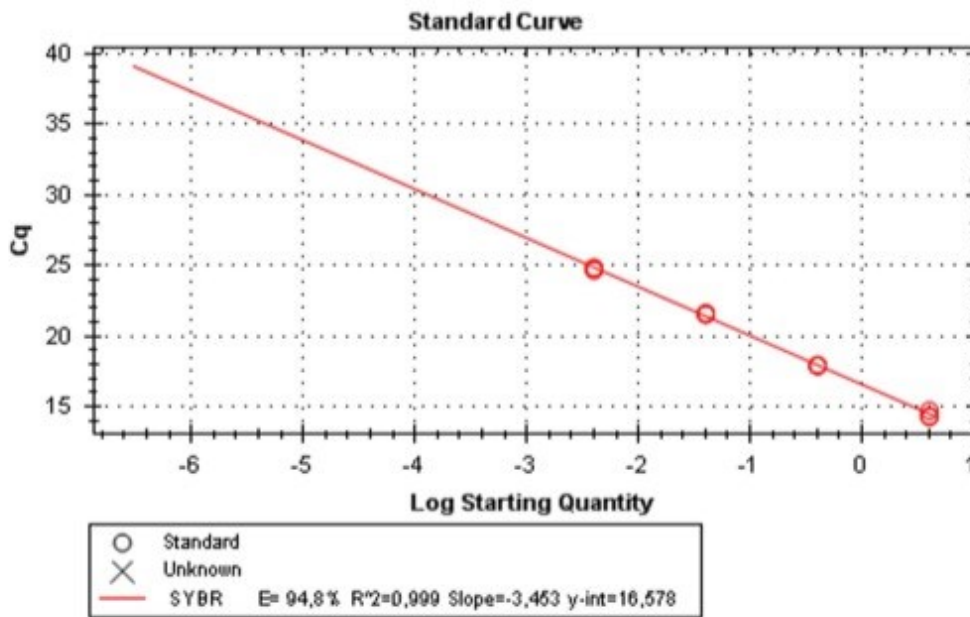


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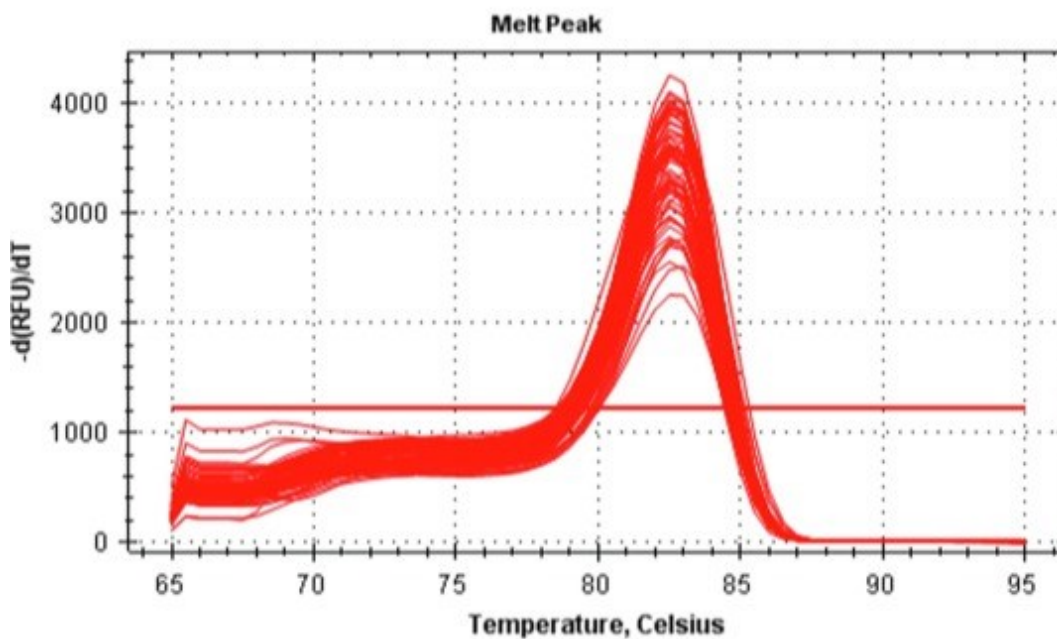
607

608 Figure 5. (a) Standard curve established for the quantification of *Cadophora luteo-olivacea* by  
609 plotting the log of the starting quantity (ng) of 1:10 serial dilutions of fungal genomic DNA versus  
610 the cycle number (Cq); (b) melting peaks of amplicons generated from 1:10 serial dilutions and  
611 genomic DNA samples extracted from artificially wounded-inoculated kiwifruit.

a)

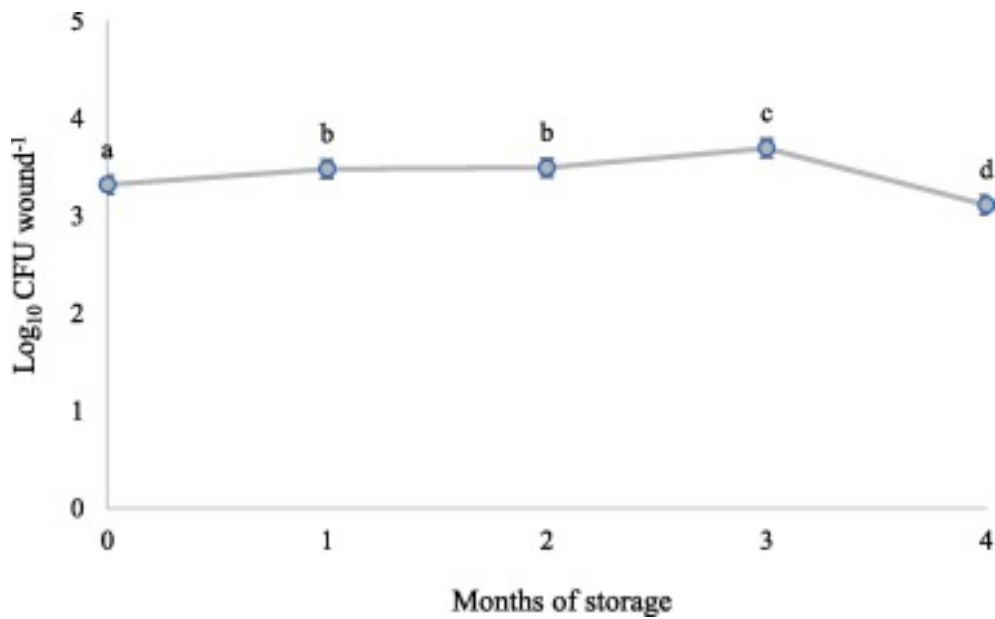


b)



612

613 Figure 6. Population dynamics of *Pseudomonas synxantha* (118-2b) in previously wounded-  
 614 inoculated kiwifruit. Fruits were stored at 0 °C in normal refrigeration for 4 months. Each point  
 615 represents the mean of the number of colony forming units (CFUs) from four replicates (kiwifruit)  
 616 for each sampling time. For each sampling time different letters indicate significant differences  
 617 according to Tukey's test ( $\alpha = 0.05$ ).



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619

620 **Tables**

621 Table 1. EC<sub>50</sub> values ( $\mu\text{L mL}^{-1}$ ) of synthetic volatile organic compounds (VOCs) emitted by two  
 622 strains of *Aureobasidium pullulans* (L1 and L8) evaluated on mycelium growth ( $\emptyset$ ) and conidia  
 623 germination (CFU) of the four *Monilinia* species. The evaluations were carried out after 2 days and  
 624 5 days of incubation at 25 °C respectively for CFU and mycelium growth.

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Treatments	Cad21 DNA pg/mg
Control	30.02 ± 9.05 a
<i>P. synxantha</i> 117-2b	0.13 ± 0.02 b
Scholar®	0.03 ± 0.00 c