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Research article

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

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Abstract

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

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1 Fruit

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

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 $MgCl_2$, 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^{-1}, 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 Mikroorganisms 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^{-1};
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 (10^5
103 conidia mL^{-1}) with a hemocytometer. All the bacterial strains were maintained on nutrient agar (NA,

13 g L^{-1}, Oxoid - UK) at 4 °C until use. For the assays, bacterial cells from two-day-old plates were suspended in potassium phosphate buffer (PPB, 70 mL KH_2PO_4 0.2 M; 30 mL K_2HPO_4 0.2 M and 300 mL deionized water v/v/v pH 6·5) and adjusted to a final concentration of 106 cells mL^{-1} and 108 cells mL^{-1} according to the experiment.

2.3 *In vitro* assays

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

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

2.4 FT-IR analysis of bacterial cell filtrates

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

2.5 *In vivo* assay

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

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

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/µL
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 µL per reaction in a 96-well Bio-Rad CFX96 RealTime PCR System (Bio-Rad Inc.,
167 Hercules, CA, USA). Reaction mixtures contained 0.3 µM of each primer, 1X SsoFast™ EvaGreen
168 ® Supermix (Bio-Rad Inc., Hercules, CA, USA), molecular grade H2O; 2 µL of DNA solution
169 containing 20 ng/µL 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/µL and up to 2
177 pg/µL. 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 µL of *P. synxantha* strain 117-2b suspension (106
189 cells 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, Ø) 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 µL), 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 ± 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

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

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

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

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

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

3.3 *In vivo* assays

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

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

3.4 qPCR analysis

qPCR analysis quantified the amount of fungal pathogen DNA in each portion of the kiwifruit tissue analyzed. The standards were used to construct a standard curve, which presented an optimal 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 × 103 CFU wound^{-1}. 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 Vleesschauwer 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–

1625 cm^{-1} and 1546 cm^{-1} spectral regions (Fig. 3). These compounds could be related to the well-known production of antibiotic peptides and lipopeptides by several bacteria, such as *B. subtilis,* which produces iturin A, a heptapeptide closed in a ring with an amino fatty acid, whose antifungal activity is related to its ability to induce a massive leakage of K^+ and other cell components from the cytosol (Besson et al., 1996). The different profiles observed in the IR spectra could also explain the behavior of the antagonists in the in vivo assays. The corresponding in vivo effects of the QST713 and FZB24 strains could be related to their similar chemical composition due to the analogous positions and intensities of their peaks (Fig. 3). At the same time, the different spectral profile of the QST2808 strain may reflect the production of antifungal compounds that are less effective against the infection. However, the IR data should be interpreted carefully because it is well known that some bacteria, such as various *Pseudomonas* spp., can produce several antagonistic antifungal compounds in the form of peptides, including proteolytic enzymes (i.e., proteases, chitinases, cellulases, pectinases or amylases) or even siderophores (non-proteogenic peptides) (Karmegham et al., 2020). The similar chemical composition of these antagonistic compounds is reflected in their IR spectra, as evidenced in previous papers on non-volatile metabolites in *Aureobasidium pullulans* in which all the previous compounds were detected (Di Francesco et al., 2020, Di Francesco et al., 2021).

Nevertheless, none of the BCA cultures filtrates totally inhibited *C. luteo-olivacea* mycelial growth, even though they were active against *B. cinerea* mycelium (data not reported). This fact showed a broad spectrum of antagonism against different plant pathogens based on antimicrobial metabolites (Roselló et al., 2013).

Despite this, the curative and preventive in vivo experiments on fruits showed that *P. synxantha* strain 117-2b had higher effectiveness against *C. luteo-olivacea* (strain Cad21) than the other tested BCAs (Fig. 4). This finding was further confirmed by the IR analysis composition of culture filtrates (Fig. 3). *Pseudomonas synxantha*'s filtrates showed the most intense peaks (at 988 cm^{-1}), which can be related to the highest concentration of antifungal compounds. Also, according to the co-culturing 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 –

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

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Conflict of Interest Statement

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

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579
580 **Figures**
581 Figure 1. *In vitro* co-colture 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 (a = 0.05).
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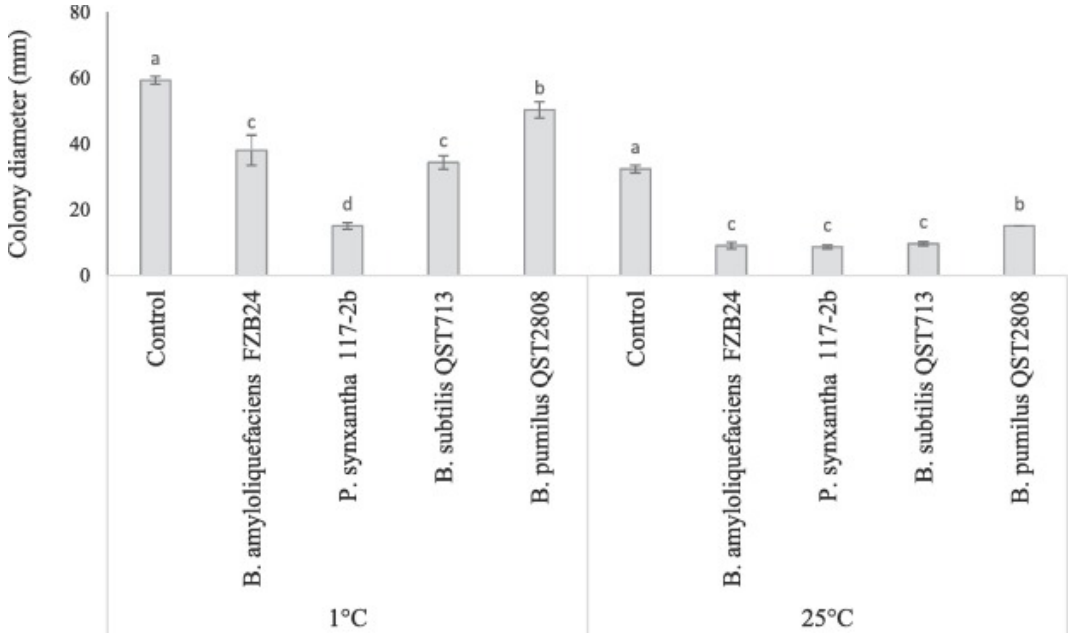

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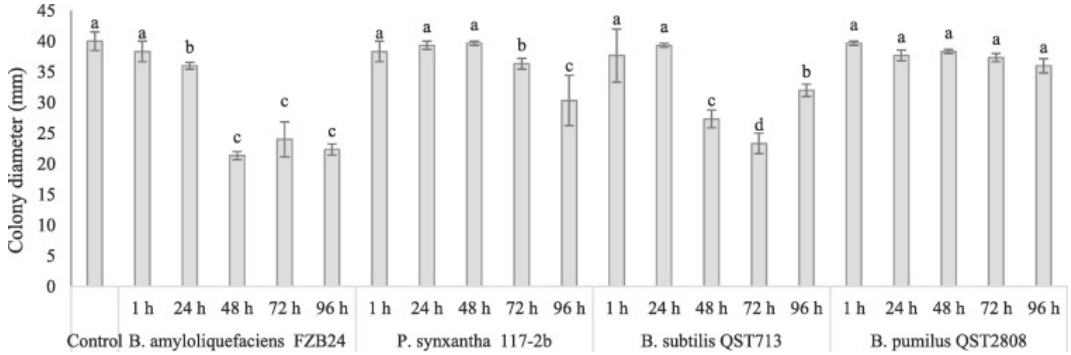
588 Figure 2. Effect of bacterial strain cell-filtrates infused onto PDA plates on the colony growth of
589 *Cadophora luteo-olivacea* (Cad21). Biocontrol agents were grown in nutrient broth at 20 °C for one
590 week and filtrates were sampled at 1 h and then every 24 h for up to 96 h. Data are the means 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 (α = 0.05).



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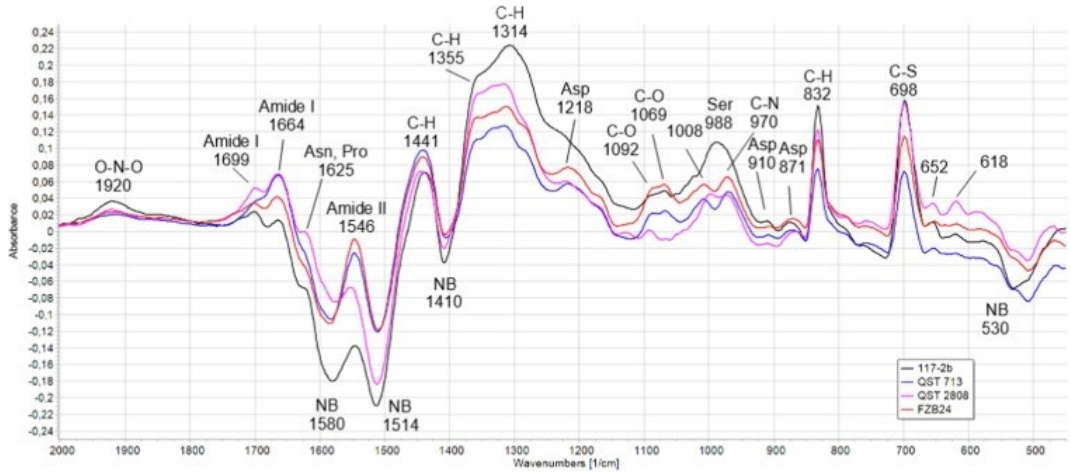
595 Figure 3. Difference FT-IR spectra of bacterial cell filtrates with the main positive bands related to
596 the production of non-volatile compounds discussed in the text. Negative peaks are related to the
597 consumption of the nutrient broth (NB).



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600 Figure 4. Curative and preventive effect of bacterial strains (108 cells mL^{-1}) and Scholar® (1.3 mL
601 L^{-1}) on disease incidence on artificially inoculated kiwifruit. Fruits were kept at 1 °C for four months.
602 For the curative effect fruit wounds were first treated with 20 µL of pathogen suspension and after air
603 drying they were inoculated with 20 µL 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 (α = 0.05).

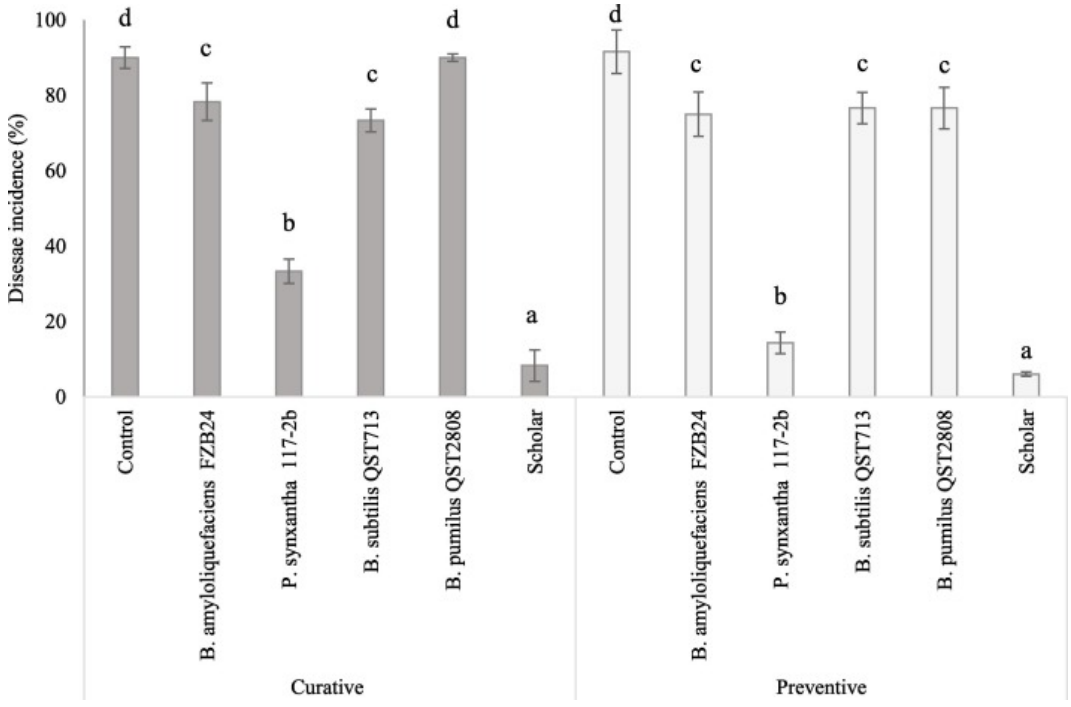

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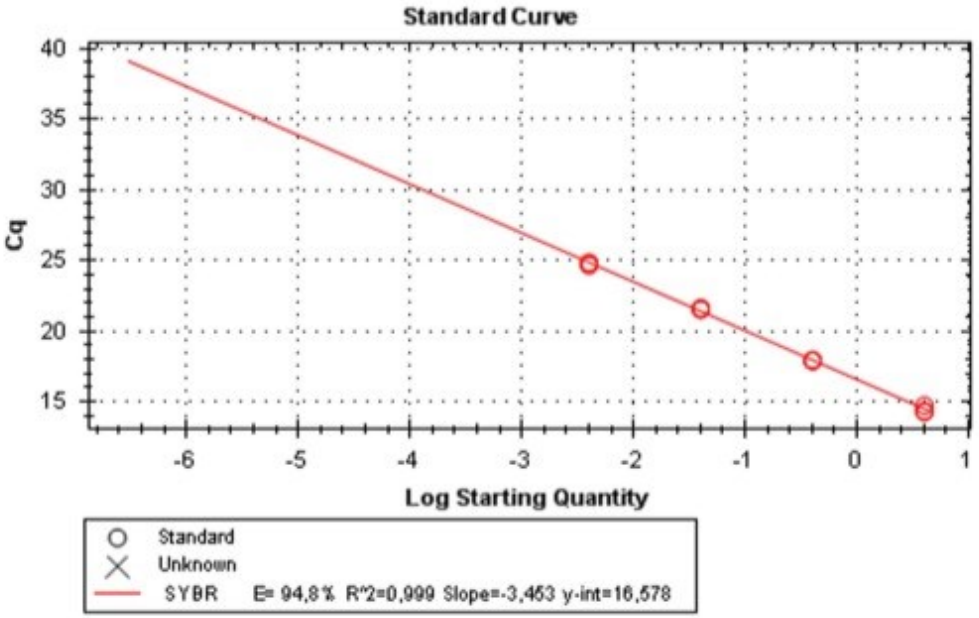
Figure 5. (a) Standard curve established for the quantification of *Cadophora luteo-olivacea* by plotting the log of the starting quantity (ng) of 1:10 serial dilutions of fungal genomic DNA versus the cycle number (Cq); (b) melting peaks of amplicons generated from 1:10 serial dilutions and genomic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

a)



b)

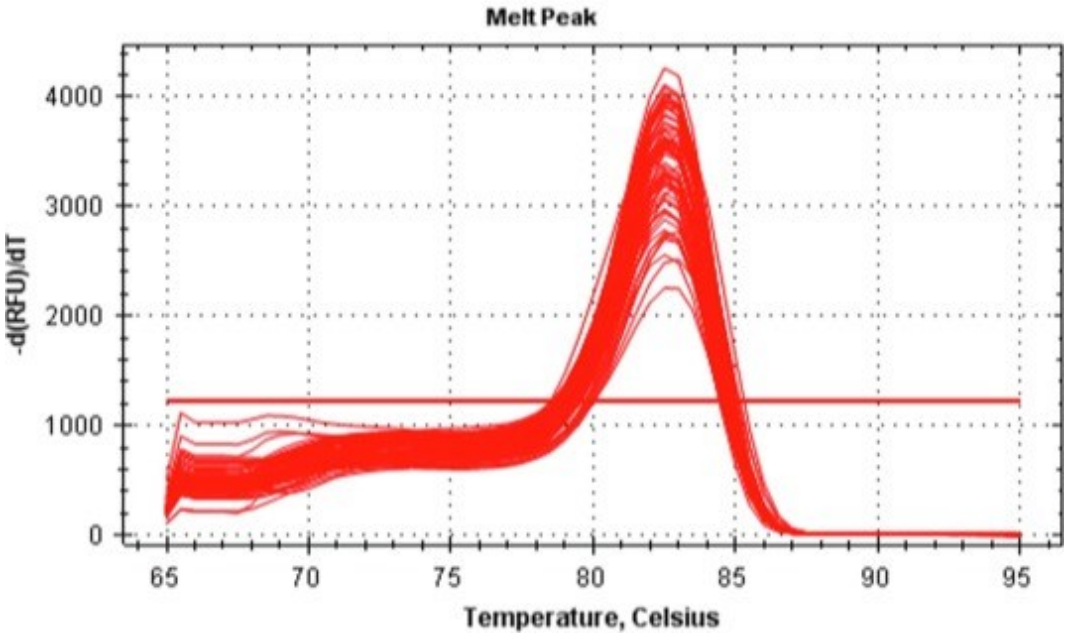
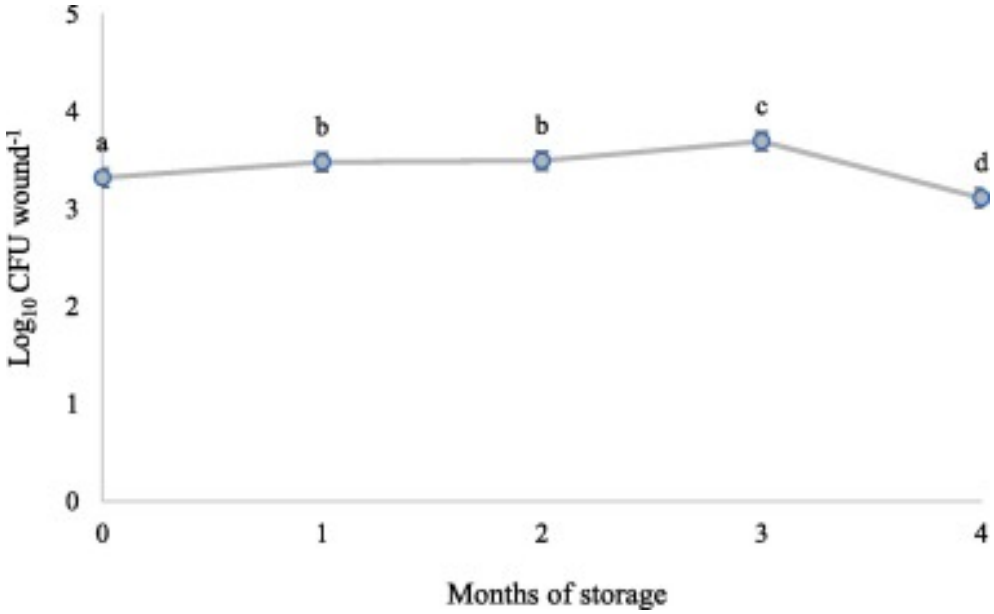


Figure 6. Population dynamics of *Pseudomonas synxantha* (118-2b) in previously wounded-inoculated kiwifruit. Fruits were stored at 0 °C in normal refrigeration for 4 months. Each point represents the mean of the number of colony forming units (CFUs) from four replicates (kiwifruit) for each sampling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



Tables

Table 1. EC_{50} values (μL mL^{-1}) of synthetic volatile organic compounds (VOCs) emitted by two strains of Aureobasidium pullulans (L1 and L8) evaluated on mycelium growth (Ø) and conidia germination (CFU) of the four Monilinia species. The evaluations were carried out after 2 days and 5 days of incubation at 25 °C respectively for CFU and mycelium growth.

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