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► To cite this version:

Manel Chaouachi, Takwa Marzouk, Selim Jallouli, Salem Elkahoui, Laurent Gentzbittel, et al.. Activity assessment of tomato endophytic bacteria bioactive compounds for the postharvest biocontrol of *Botrytis cinerea*. *Postharvest Biology and Technology*, 2021, 172, pp.111389 -. 10.1016/j.postharvbio.2020.111389 . hal-03493490

HAL Id: hal-03493490

<https://hal.science/hal-03493490v1>

Submitted on 7 Nov 2022

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1 **Activity assessment of tomato endophytic bacteria bioactive compounds for the**
2 **postharvest biocontrol of *Botrytis cinerea***

3

4 Manel Chaouachi^{a,b,c}, Takwa Marzouk^{a,b}, Selim Jallouli^b, Salem Elkahoui^{b,d}, Laurent
5 Gentzbittel^{c,e}, Cécile Ben^{c*}, Naceur Djébali^{b**}

6

7 ^a University of Tunis El Manar, Faculty of Mathematical, Physical and Natural Sciences of
8 Tunis, 2092, Tunis, Tunisia.

9 ^b Laboratory of Bioactive Substances, Centre of Biotechnology of Borj Cedria, BP 901,
10 Hammam-Lif 2050, Tunisia.

11 ^c Laboratoire Ecologie Fonctionnelle et Environnement, Université de Toulouse, CNRS,
12 Toulouse, France.

13 ^d Department of Biology, College of Sciences, University of Ha'il, Ha'il, PO Box 2440,
14 81451, Kingdom of Saudi Arabia.

15 ^e Digital Agriculture Laboratory, Skolkovo Institute of Science and Technology, Bolshoy
16 Boulevard 30 bld.1, Skolkovo, Moscow Region 121205, Russian Federation

17

18 Corresponding authors:

19 * cecile.ben@toulouse-inp.fr; ** dnaceur2014@gmail.com

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21

22

23 Abstract

24 *Botrytis cinerea* is one of the most important postharvest fungal pathogens causing significant
25 losses in fresh fruits, vegetables and ornamentals. Synthetic fungicides are primarily used to
26 control postharvest decay loss, but due to their hazardous use the recent trend is shifting
27 toward safer and more eco-friendly alternatives. The use of antagonistic microorganisms is
28 becoming popular throughout the world. This study investigated the antifungal, the plant
29 growth promoting activities and the identity of volatile organic compounds (VOCs) produced
30 by tomato-derived endophytic bacteria strains. The capacity of selected strains to prevent
31 postharvest *B. cinerea* infection on tomato fruit through VOCs and soluble compounds was
32 also studied. A collection of 50 bacterial strains was established from different organs of
33 tomato plants sampled from six localities in Cape Bon region (Tunisia). Despite the small
34 geographical scale, complexity and abundance of endophytic communities varied greatly
35 according to the site of origin. Healthy tomato plants harbor diverse endophytic bacteria of
36 *Bacillus* and *Enterobacter* genera colonizing mainly leaves with a significant enrichment with
37 *Bacillus* strains. The *in vitro* dual culture assays showed that 36 % of the endophytic bacterial
38 strains produce antifungal VOCs against *B. cinerea*. To our knowledge, this is the first report
39 of VOCs antifungal activity produced by *B. nakamurai*, *B. pseudomycooides*, *B. proteolyticus*,
40 *B. thuringiensis*, *E. asburiae* and *E. cloacae* against *B. cinerea*. About 14 % of bacterial
41 strains produce VOCs with *in vitro* specific promoting effects on tomato seedling length or
42 biomass production. The five selected antagonistic endophytic bacterial strains produced a
43 core set of seven VOCs along with different strain-specific and known antifungal VOCs such
44 as 3-Methylbutan-1-ol, sulfur-containing compounds, 2-Heptanone and Dodecanal. Tomato
45 fruit bio-protection assay showed that the *Enterobacter* strain TR1 produces the most
46 protective VOCs against *B. cinerea* infection with 3-Methylbutan-1-ol as a major volatile
47 compound which totally suppressed *B. cinerea* growth and infection on tomato fruit at 0.442

48 mL L⁻¹ headspace, whereas the *Bacillus* strains showed better protection against fungal
49 infection when applied as vegetative cells on tomato fruit. These results support the use of the
50 selected strains as potential biocontrol agents to reduce postharvest decay of *B. cinerea*, as
51 well as 3-Methylbutan-1-ol as promising antifungal volatile to apply during postharvest
52 commercialization of tomato fruit.

53

54 **Keywords:** Antifungal activity; Endophytic bacteria; Gray mold disease; Plant Growth
55 Promoting activity; *Solanum lycopersicum*; Volatile organic compounds; Gas
56 Chromatography–Mass Spectrometry.

57

58 **Abbreviations:** CFU: Colony Forming Unit; ITS: Internal Transcribed Spacer; GC-MS: Gas
59 Chromatography–Mass Spectrometry; LB: Luria Bertani; PDA: Potato Dextrose Agar; PGP:
60 Plant Growth Promotion; SPME-GC–MS: Solid Phase Microextraction-Gas
61 Chromatography–Mass Spectrometry; VOC: Volatile Organic Compound.

62

63 **Introduction**

64 *Botrytis cinerea* is a necrotrophic fungal pathogen that infects over 1400 plant species
65 (Williamson et al., 2007; Fillinger and Elad, 2015), leading to serious economic losses in
66 agricultural sector (Dean et al., 2012). Annual economic losses due to this disease are
67 evaluated up to 100 billion dollars worldwide (Jin and Wu, 2015). This pathogen infects all
68 aboveground parts of the plants and constitutes a threat during crop season and during storage
69 and transport of vegetables and fruit. Several management methods have been used to control
70 this disease such as cultural methods, but the most common way is the use of chemical
71 fungicides such as folpel, dichlofluanid and thiram on several crops including tomato.
72 However, due to its high genetic variability, prolific reproduction and short life cycle, *B.*
73 *cinerea* is considered a high-risk pathogen for the development of fungicide resistance
74 (Williamson et al., 2007; Haidar et al., 2017; Saito et al., 2019). In addition, the abusive use of
75 these chemicals has serious hazardous effects on human health, mainly when used to control
76 postharvest infection of *B. cinerea* on vegetables and fruit. Over the past 15 years, research
77 trends are moving toward the prospection of eco-friendly methods to control *B. cinerea*
78 including the use of biological control agents and their active compounds (Combrinck et al.,
79 2011; Abbey et al., 2019). Among the wide range of biocontrol microbial agents, endophytes
80 and especially endophytic bacteria are showing significant potential in fungal disease
81 management (Latha et al. 2019; Singh et al. 2020). Endophytic bacteria are microorganisms
82 living within plant tissues without causing any harmful effect and they are generally
83 considered as beneficial organisms for their hosts (Bolívar-Anillo et al., 2019). Endophytic
84 bacteria can promote plant growth by enhancing several mechanisms such as nutrient uptake
85 and phytohormones production (Vacheron et al., 2013; Van Kan et al., 2014). These
86 endophytes are known also to enhance plant health by modulating plant immune system
87 (Santoyo et al., 2016) and/or by direct inhibition of pathogens through the production of

88 active soluble (antibiotics and lytic enzymes) and volatile compounds (Compant et al., 2005;
89 Nair and Padmavathy, 2014; Haidar et al., 2017). Volatile organic compounds (VOCs)
90 produced by bacteria have low molecular weight and polarities and diffuse easily through the
91 porous structure of soil and over great distances in the atmosphere. As such, they have good
92 properties to be used in crop protection in the field as well as under greenhouses and storage
93 environments (Arrarte et al., 2017). Indeed, several studies showed the potential use of
94 bacterial VOCs in plant disease management (Agisha et al. 2019; Dhouib et al. 2019) and in
95 particular in postharvest disease control (Elkahoui et al. 2015; Zheng et al. 2019; Calvo et al.
96 2020). Several bacterial endophytes have shown the production of antifungal VOCs; among
97 these, *Bacillus*, *Pseudomonas* and *Enterobacter* are well known genera (Tsuda et al. 2001;
98 Agisha et al. 2019; Dhouib et al. 2019; Singh et al. 2020) producing chemical volatiles
99 belonging to different groups such as aldehydes, alcohols, terpenes, esters, ketones,
100 hydrocarbons, nitrogen-containing heterocycles, sulfur derivatives and acids (Elkahoui et al.
101 2015; Agisha et al. 2019; Dhouib et al. 2019).

102 The present study aims to prospect and select endophytic bacteria strains with VOCs
103 antagonistic activity against *B. cinerea*, combined to the ability to enhance tomato seedling
104 growth. The capacity of selected strains in reducing postharvest infection of *B. cinerea* on
105 tomato fruit was also studied along with the identification of their emitted VOCs by SPME-
106 GC/MS and characterizing the antifungal activity of interesting volatiles.

107

108 **Material and methods**

109 **1. Biological material**

110 **1.1. Bacterial strains**

111 Bacterial strains were isolated from flower, fruit, leaf, petiole, root and stem of healthy
112 tomato plants sampled in six localities in Cap Bon region in Tunisia (Fig. 1; Supplementary

113 Table 1). Tomato organs were surface-disinfected with 3 % sodium hypochlorite solution for
114 6 min and 70 % ethanol for 1 min and washed 5 times with sterile distilled water. The
115 disinfection efficiency was verified by pouring the water of the last wash on Luria Bertani
116 (LB) agar medium. Each sample was aseptically cut into small pieces and deposited on LB
117 agar medium. Petri dishes were incubated for 2 d at 30 °C in obscurity, and then growing
118 bacterial colonies were streaked onto new LB agar plates. The obtained bacterial cultures
119 were stored at -20 °C in LB broth medium containing 25 % of glycerol. Bacterial isolation
120 was balanced for each plant organ and collection site.

121

122 **1.2. Fungal strains**

123 Two fungal strains S2 and S5 of *Botrytis* sp. were isolated on Potato Dextrose Agar
124 (PDA) medium from tomato fruit collected in the locality of Teboulba in the Sahel region in
125 Tunisia. The fungal strains were molecularly characterized by sequencing the Internal
126 Transcribed Spacer (ITS) regions of the ribosomal RNA using ITS4 (5'-
127 TCCTCCGCTTATTGATATGC-3') and ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3')
128 primers (White et al., 1990). The nucleotide sequences were assembled using the CAP
129 program available on the NCBI website (<http://www.ncbi.nlm.nih.gov/blast>). Blast sequences
130 in the NCBI data bank showed that they belong to the *Botrytis cinerea* species. The sequences
131 were deposited in GenBank with accession numbers MK854558.1 and MK854559.1,
132 respectively. The 18S rDNA type material sequences of different *Botrytis* species and out
133 groups *Saccharomyces cerevisiae* culture CBS:1201, *Aspergillus niger* ATCC 16888,
134 *Beauveria bassiana* isolate CIDDB38, *Fusarium sambucinum* strain 166A, *Parastagonospora*
135 *nodorum* were aligned using the Bioedit software. Neighbor joining tree showing the
136 phylogenetic relationships among the fungal strains, based on 18S rDNA sequences and the
137 out groups, was constructed (Supplementary Fig. 1). Bootstrap values of > 50 % from a

138 sample of 1000 replicates were considered. The construction of phylogenetic tree in R was
139 done using ape (Paradis and Schliep, 2019) and phangron (Schliep, 2011; Schliep et al., 2017)
140 packages for phylogenetic study.

141

142 **1.3. Plant material**

143 Organic tomato seeds of the variety RioGrande were used for the germination tests. The
144 cherry tomato fruit used in the *in vivo* protection assay were purchased from the market.

145

146 **2. Antifungal activity tests**

147 **2.1. Test of volatile organic compounds**

148 The antifungal activity of bacterial VOCs was assessed by the double Petri dishes method
149 (Rouissi et al., 2013). A 7 mm **diameter** disc of the 7 **d** old fungal culture (in dark at 25 °C)
150 was transplanted on PDA medium 24 h before the dual confrontation with the bacterial
151 culture. A 100 µL of bacterial suspension (24 h liquid LB bacterial culture at 30 °C) and
152 adjusted to 10⁸ CFU **mL⁻¹** was poured on LB agar medium. The two plates were overlaid and
153 sealed with cellophane paper to prevent the loss of bacterial VOCs. In the control plates the
154 fungal cultures were placed in front of an LB agar plate. The fungal growth was monitored at
155 7, 14 and 21 **d** of confrontation and the antagonistic activity of bacterial strains against the
156 fungal strains was evaluated by calculating the **Area Under Growth-Progress Curve**
157 (AUGPC).

158

159 **2.2. Test of soluble compounds**

160 This test was performed for 5 selected bacteria strains against *B. cinerea* strains S2 and
161 S5. The bacterial strain was streaked at the middle of a Petri dish containing PDA medium
162 and two mycelia disks (5 mm **diameter**) of 7 **d** old *B. cinerea* cultures were placed in both

163 sides at approximately 2 cm from the bacteria culture line. Plates were incubated at 25 °C in
164 dark for 7 d and the antagonistic activity was evaluated by measuring the diameter of the
165 fungal colony. The test was repeated twice with three Petri dishes for each bacterial-fungal
166 strains combination. Control Petri dishes contained only two mycelia disks of the fungal
167 strains.

168

169 **3. Plant growth promoting (PGP) tests**

170 Tomato seeds were disinfected with 3 % sodium hypochlorite solution for 6 min and 70
171 % ethanol for 1 min, and then washed 5 times with sterile distilled water. The efficiency of
172 seed disinfection was evaluated by pouring 100 µL of the last wash water on LB agar
173 medium. The bacterial strains were cultured in LB broth for 24 h at 30 °C in the dark in a
174 shaking incubator at 150 rpm min⁻¹. Each bacterial culture was suspended in physiological
175 saline solution (0.9 % NaCl) and adjusted to 10⁸ CFU mL⁻¹ using a densimeter (Biomérieux).
176 The test was carried out in double compartment Petri dishes, in one compartment a sterile
177 Wattman paper disc (6 mm diameter) inoculated with 50 µL bacterial suspensions was placed
178 on LB agar medium and in the second compartment 20 seeds were placed on 0.9 % Agar
179 medium. Seed germination was monitored for 6 d of co-culture. At the end of the experiment,
180 the length, the fresh and the dry weights of seedlings were measured.

181

182 **4. *In vivo* protection assays**

183 **4.1. Fruit protection test by bacterial VOCs**

184 Tomato fruit free from wounds with comparable sizes and stage of maturity were used in
185 this work. The fruit were disinfected by soaking them in 3 % sodium hypochlorite for 30 min,
186 and then rinsed several times with sterile distilled water. Then, a hole of 6 mm in diameter
187 was performed in fruit skin and a fungal mycelia disc of 5 mm was placed in it. Fungal

188 inoculated fruit were placed in plastic boxes in the presence of 24 h old bacterial culture on
189 solid LB medium. Control fruit were inoculated by PDA discs. The boxes (contains two filter
190 papers imbibed with sterile distilled water to maintain high humidity) were sealed with
191 cellophane paper to prevent the release of bacterial VOCs and incubated in the dark at 24 ± 2
192 °C. The degree of *B. cinerea* infection on fruit was estimated by measuring the diameter and
193 the depth of the macerated zone due to fungal colonization of the tissues at 6 d after
194 inoculation.

195 A split plot design was used in this assay; each plastic box contains all three different
196 fungal treatments (non-infected fruit, fruit infected with either S2 or S5 *B. cinerea* strain) and
197 one Petri dish with one antagonistic bacteria strain on solid LB medium or no bacteria for the
198 control. The test was repeated three times, with two replicates per treatment.

199

200 **4.2. Fruit protection test by bacterial vegetative cells**

201 The same assay as described above was made except that tomato fruit were soaked in
202 bacterial suspension of 10^8 CFU mL⁻¹ for 30 min, then dried on autoclaved filter paper under
203 laminar flow hood and placed in the boxes. A split plot design was used in this test and the
204 experiment was repeated three times with two replicates per treatment. The degree of
205 infection of *B. cinerea* on tomato fruit was estimated as described above.

206

207 **5. Molecular characterization of bacteria strains**

208 Genomic DNA of bacteria strains was extracted according to (Andreou, 2013) method
209 with some modifications. The 16S rDNA gene sequence was amplified by PCR and
210 sequenced using the primers fD1 (5'-AGA GTT TGA TCC TGG CTC A-3') and rD1 (5'-
211 AAG GAG GTG ATC CAG C-3') (Weisburg et al., 1991). The nucleotide sequences were
212 assembled, aligned with BioEdit software, and deposited in GenBank and the accession

213 numbers obtained. The 16S rDNA sequences of the bacteria strains and the out groups
214 *Streptomyces* sp. strain N2-8 and *Paenibacillus polymyxa* strain NBRC 15309 were aligned
215 using the Bioedit software. Neighbor joining tree showing the phylogenetic relationships
216 among the bacterial strains based on 16S rDNA sequences was constructed (Supplementary
217 Fig. 2). Bootstrap values of > 50 % from a sample of 1000 replicates were considered. The
218 construction of phylogenetic tree in R was done using ape (Paradis and Schliep, 2019) and
219 phangron (Schliep, 2011; Schliep et al., 2017) packages.

220

221 **6. Biochemical characterization of bacteria strains**

222 The biochemical characterization of selected bacteria strains was performed on the basis
223 of their enzymatic activities, siderophores, hydrogen cyanide (HCN) and ammonia production
224 and solubilisation of phosphate. The cellulose (Gupta et al., 2012), pectinase (Hankin et al.,
225 1971), phytases (Kerovuo et al., 1998), xylanase (Khan et al., 1986), amylase (Vaseekaran et
226 al., 2011), protease (Durham et al., 1987) and chitinase (Hsu and Lockwood, 1975) activities
227 were tested on minimal media containing cellulose, pectine, myoinositol, xylan, strach, and
228 casein and colloidal chitin, respectively. The production of enzymes was detected by the
229 formation of a clear circular area around the bacteria colony. The production of siderophores
230 was detected by sub-culturing bacteria on blue Agar CAS medium (Schwyn and Neilands,
231 1987). When siderophores are produced they remove iron from the dye complex and the color
232 of the medium changes from blue to orange. The PVK medium was used to test the phosphate
233 solubilisation by bacteria strains (Pikovskaya, 1948). A clear circular area around the bacteria
234 colony is formed if the bacterial strain has the ability to solubilize the phosphate. Ammonia
235 was tested on peptone water by addition of the Nessler reagent (Cappuccino and Sherman,
236 2005). The change of the bacterial suspension from yellow to brown indicates the ammonia
237 production. HCN production was measured on Wattman paper (soaked in 2.5 g L⁻¹ picric acid

238 in 12.5 g L⁻¹ sodium carbonate) placed on the lid of the Petri plate. Bacterial streaks were
239 made on LB agar medium amended with glycine (4.4 g L⁻¹). The plates were then sealed with
240 cellophane and incubated at 30 °C for 4 d. A color change of the filter paper from deep yellow
241 to reddish-brown color was considered as an indication of HCN production (Bakker and
242 Schippers, 1987).

243

244 **7. Analysis of volatiles by Solid Phase Microextraction-Gas Chromatography–Mass** 245 **Spectrometer (SPME-GC–MS)**

246 Headspace vials (20 mL) were filled with 6 mL of LB agar medium and autoclaved. The
247 selected bacteria were inoculated in the prepared headspace vials and incubated at 30 °C in
248 the dark for 7 and 14 d. Vials containing LB agar medium without bacteria were used as
249 controls. The bacterial VOCs were collected using the SPME fiber (Carboxen/ DVB/ PDMS
250 (2 cm) 50/30 µm) commercially available from Agilent Technologies (USA). The fiber was
251 conditioned at 250 °C for 30 min in the hot injector of the GC and then exposed to the
252 headspace of the culture vials for 1 h. Once the adsorption/absorption was complete, the fiber
253 was desorbed in the injector in splitless mode (5 min) with a transfer line temperature of 260
254 °C. Gas chromatography analysis was carried out using an Agilent 19091S-433: 2169.66548
255 GC coupled to an HP-5MS 5 % Phenyl Methyl Silox with a DB Wax capillary column (325
256 °C: 30 m x 250 µm x 0.25 µm) and Helium as the carrier gas at a flow rate of 1 mL min⁻¹.
257 The oven temperature program was fixed at 40 °C for 5 min, followed by an increase by 20
258 °C min⁻¹ to 250 °C with holding for 15 min. Identification of the various compounds was
259 performed by matching the recorded mass spectra with those stored in the Wiley 09 NIST
260 2011 mass spectral library of the GC–MS data system. The experiment was repeated twice
261 with two replicates per strain per time of incubation.

262 **8. Antifungal activity test of pure volatile compound**

263 The activity of the pure volatile compound 3-Methylbutan-1-ol (isoamyl alcohol)
264 (Merck) was tested *in vitro* against *B. cinerea* growth and infection on tomato fruit. The *in*
265 *vitro* antifungal activity test was done as described in section 2.1 with different concentrations
266 of 3-Methylbutan-1-ol instead of the bacteria strains. The *B. cinerea* culture of S2 and S5
267 strains was facing 13 mm diameter Whatman paper disc impregnated with different
268 concentrations of 3-Methylbutan-1-ol ranging from 0.004 to 0.885 mL L⁻¹ headspace. The
269 plates were immediately sealed with cellophane and incubated for 5 d at 25 °C in the dark.
270 Plates with a non impregnated filter paper were used as control. The half maximal inhibitory
271 concentration (IC50) and the maximal inhibitory concentration (IC100) of 3-Methylbutan-1-ol
272 on *B. cinerea* mycelium growth were determined with the regression equation using the Probit
273 method. Three plates per fungal strain and compound concentration were used and the
274 experiment was repeated two times.

275 The antifungal tests of the 3-Methylbutan-1-ol against *B. cinerea* infection on tomato
276 fruit was performed as described in section 4.1 with this volatile compound instead of the
277 bacteria strains. The 3-Methylbutan-1-ol was impregnated in a 90 mm diameter filter paper in
278 the center of the box with the concentrations corresponding to IC50 and IC100. Cherry tomato
279 fruits were inoculated with 6 mm diameter of *B. cinerea* strains S2 and S5 and incubated at 24
280 ± 2 °C in the dark. The fungal infection was evaluated as described in section 4.1 at 6 d post
281 infection. A split plot design was used, with each box contains all three fungal treatments i.e.
282 non-infected fruit and fruit infected with S2 or S5 strain in presence or not (control) of 3-
283 Methylbutan-1-ol. The test was repeated two times, with 1 box for each volatile concentration
284 and 3 fruits for each treatment.

285

286 **9. Statistical analysis**

287 Mixed Linear Models were implemented for analyses of variance using lme4 and
288 lmerTest R packages. Multiple mean comparisons were performed using Dunnett
289 (comparisons to control) or Tukey (pairwise comparisons) tests at $p\text{-value} \leq 0.05$. All
290 statistical analyses were performed using R x 64 3.5.1 software.

291

292 **Results**

293 **1. Characterization of the endophytic bacteria collection**

294 Fifty endophytic bacteria strains were isolated from aerial and root organs of healthy
295 tomato plants harvested in six field sites of Cape Bon region in Tunisia (Fig. 1A).
296 Morphologically, 75 % of the strains form large confluent white colonies, with the remaining
297 strains forming small, isolated colonies. All isolated strains are characterized by a rapid
298 growth (< 24 h). The distribution of the bacterial strains according to the site of prospection
299 and organ of isolation is represented in figure 1. The collection is significantly enriched with
300 bacteria from leaves which represent 38 % of all isolated strains (*****P < 0.001**). A total of 22
301 strains were isolated from Hattouba locality showing the most abundant and diverse set of
302 strains regarding the organ of origin. The strains isolated from Tbag locality are statistically
303 enriched in bacteria recovered from roots (Fig. 1B). Endophytic bacteria collections from
304 Belyess, Chrifat and Korba-BeniKhaled road are the least abundant with only two strains
305 isolated from flowers each. This shows a broad range in the abundance of bacterial
306 endophytic communities and in their distribution within plant according to the site of
307 prospection, even at a small geographically scale.

308

309 **2. Antifungal activity of bacterial volatile organic compounds**

310 The antifungal activity tests of VOCs produced by the bacteria strains against two strains
311 of *B. cinerea* showed that the reaction varies with the bacteria and fungi strains and the time

312 of observation (Fig. 2). Indeed, the ANOVA on basis of data of the kinetics of growth (at 7,
313 14 and 21 d of co-culture with the bacteria strains) and the Area Under Growth-Progress
314 Curve (AUGPC) showed a significant effect of the bacteria strain, the fungal strain and their
315 interaction (Table 1). Eighteen bacteria strains (i.e. 36 % of the collection) showed sustainable
316 or temporary antagonistic effect against *B. cinerea* (Table 2). These antagonistic bacteria were
317 isolated from the localities of Diar Hajjaj, Hattouba and Tbag. Three antagonistic behaviors
318 were noted (i) eight strains (i.e. 62 % of the antagonistic bacterial strains) specifically
319 inhibited *B. cinerea* S2 strain, with DHT2, H1Fr2, DHF14 and H1F1 strains showing the
320 strongest antagonistic effects; (ii) three strains, namely DHF5, H2F12 and H2F1, specifically
321 reduced the mycelia growth of S5 strain of *B. cinerea*; (iii) two strains, namely TR1 and TR2,
322 significantly inhibited the mycelia growth of both fungal strains. After 21 d of co-culture,
323 mycelia growth was respectively reduced up to 76.7 % for S2 strain due to bacterial VOCs
324 produced by H1F1 strain and 64.5 % for S5 strain due to DHF5 VOCs.

325 The antagonistic bacterial strains were also selected according to the persistence of their
326 activity during 21 d of dual confrontation (Table 1). Twelve strains maintained a significant
327 antagonistic activity against *Botrytis* mycelia growth up to 21 d of co-culture, with nine and
328 three strains against S2 and S5 *Botrytis* strains respectively (Table 2). Five strains were
329 characterized by temporary antifungal activity that decline after 7 d of co-culture (e.g. TR3,
330 BF11 and H1Fr3 against S5 or H1T3 and DHF4 against both S2 and S5 strains). Some strains
331 had intermediate persistent effect. For example, the antagonistic activity of TR2 strain against
332 the two *Botrytis* strains declined after 14 d. While the TR1 strain activity against S5 *Botrytis*
333 strain declined after 14 d it was maintained against S2 *Botrytis* strain till 21 d of
334 confrontation.

335 The molecular identification using 16S rRNA gene sequences of the 18 antagonistic
336 endophytic bacteria strains (accession numbers from MN080812.1 to MN080816.1 and from

337 MN737985.1 to MN737997.1) showed a statistically enrichment in bacteria of *Bacillus* genus
338 (Supplementary Table 2, Monte Carlo simulated Khi^2 ****P <0.01**). Indeed, 16 strains (i.e. 89
339 %) belong to nine *Bacillus* species including five strains from *B. velezensis* (no statistically
340 significant enrichment). The remaining two strains belong to different species of the
341 *Enterobacter* genus. No statistical association between the endophytic bacterial species and
342 their antifungal effect on S2 or S5 *B. cinerea* strains was observed according to Khi^2
343 independence test. This suggests that no endophytic bacterial species specifically protects
344 against a specific strain of *Botrytis cinerea*. Likewise, no significant enrichment of the site or
345 organ of origin with antagonistic bacteria strains was observed.

346

347 **3. Effect of bacterial volatile organic compounds on tomato seed germination and** 348 **seedling growth**

349 The endophytic bacteria strains were screened *in vitro* towards their VOCs activity on
350 tomato seed germination and seedling growth. The studied parameters relative to seed
351 germination and seedling growth (i.e. length and fresh and dry weights) were significantly
352 affected by bacterial VOCs after 6 d of dual culture (***P <0.05**, Table 3 and 4). Three different
353 behaviors were observed depending on the bacteria strain and the measured parameter.
354 According to the measured parameter, between 66 % and 94 % of bacteria strains produced
355 VOCs with neutral effect on plant development, i.e. seed germination or seedling growth (Fig.
356 3). A promoting behavior of VOCs was noted for 2 % to 8 % of bacteria strains depending on
357 the measured parameter; indeed four strains (RK-BK F11, DHF14, H2F3 and H1F9) enhanced
358 seedling fresh weight, two strains (DHF6 and H2F13) enhanced seedling length, and one strain
359 (H2T2) enhanced seedling dry weight in comparison to control seeds in presence of the LB
360 medium (Fig. 3). However, no bacteria strain showed positive effect on seed germination rate
361 (Fig. 3). Some bacteria strains showed deleterious effect on plant development depending also

362 on the measured parameter, 22 % reduced seed germination, 26 % reduced seedling fresh
363 weight, 6 % reduced seedling length and 4 % reduced seedling dry weight (Fig. 3). The effect
364 of bacterial VOCs depends on the measured parameter. For example DHF6 and H2F13
365 increase seedling length but not seedling fresh and dry weights. Some bacterial VOCs
366 enhanced seedling growth but have no effect (e.g. DHF6, H1F9 or H2T2) or even inhibited
367 seed germination (e.g. H2F13) (Fig. 3). Finally, some bacteria strains such as H2F1 produced
368 VOCs that inhibit seed germination and seedling growth (Fig. 3).

369

370 **4. Correlation between antifungal and plant growth activities of bacterial volatile** 371 **organic compounds and clustering of strains**

372 Among the 16 possible correlations between the measured parameters related to VOCs
373 antifungal and plant growth activities, three were statistically significant (Fig. 4A). A
374 significant positive correlation was observed between fresh and dry weights of tomato
375 seedlings and between germination and seedling fresh weight. A significant and positive
376 correlation was observed between the antifungal activities against the two *Botrytis* strains S2
377 and S5. No significant correlations were observed between the parameters related to
378 antifungal and plant growth activities of bacterial VOCs.

379 Clustering of the endophytic strains based on their VOCs antifungal and plant growth
380 activities showed five bacteria clusters (Fig. 4B). The cluster I contains one bacteria strain,
381 H2F13, with VOCs activity quite similar to LB medium without bacteria; the cluster II
382 contains 4 strains with strong antifungal effect against S2 *B. cinerea* strain only whereas, the
383 cluster III contains 18 strains with strong antifungal effect against S2 *B. cinerea* strain and
384 weak (not significant) activity against S5 *B. cinerea* strain. The cluster IV contains 12 bacteria
385 strains with VOCs effects against both *B. cinerea* strains. The cluster V contains 15 strains
386 with specific antifungal activity against S5 strain of *B. cinerea*.

387

388 **5. Selection and characterization of endophytic bacteria strains with potential use for**
389 **the control of *Botrytis cinerea***

390 **5.1. Selection of endophytic bacteria strains**

391 On the basis of the clustering of endophytic bacteria, five strains belonging to the clusters
392 II, III, IV and V of bacteria producing VOCs with strong antifungal activities and, as much as
393 possible, having neutral to promoting effects on tomato seed germination and seedling growth
394 were selected for further studies (Fig. 4B, Supplementary Table 2). The 16S rRNA gene
395 sequencing of selected endophytic bacteria strains showed that they belong to the following
396 species: *Bacillus amyloliquefaciens* H1F1 (Cluster II), *Bacillus pseudomycooides* DHT2
397 (Cluster III), *Enterobacter cloacae* TR1 (Cluster IV) and *Bacillus velezensis* H2F12 and BF11
398 (Cluster V) (Table 2).

399

400 **5.2. Identification of volatile organic compounds produced by selected endophytic**
401 **bacteria strains**

402 The volatile organic compounds produced by the five endophytic bacteria strains along
403 with the control LB Agar medium were characterized and measured by SPME-GC/MS at 7
404 and 14 d (Supplementary Table 4). The overall comparison of the VOCs profiles of
405 endophytic bacteria showed a clear difference between them and according to the time of
406 culture for the same strain (Fig. 5). A total of 93 volatile organic compounds were detected
407 with 68 compounds (i.e. 73 %) of known identity (Supplementary table 4). Twenty VOCs
408 were produced by all five strains, including seven compounds which were not detected in the
409 free bacteria LB Agar medium: six identified compounds (1-Octyn-3-ol,4-ethyl ; β -Fenchol;
410 Benzene,m-di-tert-butyl; 2-Undecanone; 3,3,5-Trimethylbicyclo[3.3.0]octan-2,8-dione; 2-
411 Pentadecanone,6,10,14-trimethyl) and one unidentified compound (NI). These molecules may

412 constitute a core set of VOCs produced by all tomato bacteria endophytes, whatever the
413 bacteria species. Forty-four strain-specific VOCs (i.e. 47.31 %) were recorded, with a large
414 majority (37/46 i.e. 80.4 %) produced at a specific culture time at 7 or 14 d. The two *B.*
415 *velezensis* strains BF11 and H2F12 from the Cluster V which specifically reduced the mycelia
416 growth of S5 strain of *B. cinerea* showed the most similar patterns of VOCs (Fig. 5) with
417 Undecane,3-methyl as a common and specific volatile for both strains. BF11 produced in total
418 31 VOCs with six strain-specific compounds with Benzenamine, N-ethyl as a specific
419 compound representing 0.74 % of its total produced VOCs (Supplementary table 3). The
420 strain H2F12 produced in total 36 VOCs among which seven are strain-specific with
421 Dodecanal as the major compound representing 4.61 % and 5.54 % of all produced
422 compounds at 7 and 14 d of culture, respectively. The strain DHT2 produced in total 43
423 VOCs among which 10 are strain-specific with Dimethyl disulfide as the major compound
424 representing 37.19 % of its total produced VOCs. H1F1 strain produced in total 32 VOCs
425 with three are strain-specific compounds with an unknown major compound representing 8.35
426 % of its VOCs (Supplementary table 3). The strain TR1 showed the most distinct VOCs
427 profile in comparison to the other bacteria strains (Fig. 5). Indeed, this strain produced in total
428 53 compounds among which 18 are strain-specific (i.e. 33.96 % of the detected VOCs) with
429 Isoamyl Alcohol (3-Methylbutan-1-ol) is the major compound representing 80.91 % and
430 58.80 % of its produced VOCs at 7 and 14 d of culture, respectively (Supplementary table 3).

431

432 **5.3. Soluble antifungal compounds, enzymatic and nutrient uptake activities of selected** 433 **endophytic bacteria strains**

434 The capacity of selected bacteria strains to produce soluble antifungal compounds was
435 evaluated by dual culture of both microorganisms on the same medium (Fig. 6). Except DHT2
436 strain, the selected bacteria strains significantly inhibited at different extent *B. cinerea* growth

437 on PDA medium at 7 d of co-culture (Fig. 6, Tukey test ****P <0.01**). No significant effect of
438 interaction between the bacteria and fungi strains was observed (Table 5). The H1F1 strain
439 produced the most effective antifungal soluble compounds against *B. cinerea*, followed by
440 BF11 and H2F12 and TR1 strains (Fig. 6).

441 The antimicrobial (i.e. hydrolytic enzymes and HCN) and plant growth promoting
442 (phosphate solubilization, siderophores and ammonia production) activities of the selected
443 antagonistic bacteria were studied *in vitro* using appropriate media. Overall, the results
444 showed that, at the exception of the TR1 strain (which only produced 1 out of 7 hydrolytic
445 enzymes), the remaining four endophytic bacteria strains produced most of the studied
446 enzymes (6 out of 7 hydrolytic enzymes) and have at least 2 out of 3 PGP activities
447 (Supplementary table 4). All the selected endophytic bacteria strains did not produce HCN
448 (Supplementary table 4).

449

450 **6. *In vivo* biocontrol assay of *B. cinerea* on tomato fruit**

451 The evaluation of the bio-protection capacity of the selected endophytic bacteria strains
452 against *B. cinerea* on tomato fruit was done by two tests using VOCs and vegetative bacterial
453 cells. The results showed difference in the protection effectiveness of bacteria strains
454 depending on the protection method used. The analysis of variance of the data of the bio-
455 protection assay using bacterial VOCs, showed a sub-significant effect of the bacteria strains
456 on the diameter (***P <0.1**) but not on the depth of *B. cinerea* rotting at 6 d after infection
457 independently from the fungal strain (Table 6). Only the VOCs of TR1 strain reduced
458 significantly the diameter of *B. cinerea* rotting on tomato fruit infected by both fungal strains
459 S2 and S5 (Fig. 7).

460 The analysis of variance of the data of the bio-protection test using bacterial cells
461 revealed a significant interaction between the bacteria and the fungal strains on both infection

462 parameters (i.e. diameter and depth of rotting) at 6 d after *B. cinerea* infection (Table 7). In
463 general, the bacteria strains had the major effect on the depth in comparison to the diameter of
464 rotting, with BF11 strain showing the strongest bio-protective effect against both fungal strains
465 (Fig. 8). All the studied bacteria strains reduced the depth of rotting due to *B. cinerea* S2
466 strain infection on tomato fruit (Fig. 8C). Except TR1 strain, the used bacteria strains
467 significantly reduced the depth of rotting due to *B. cinerea* S5 strain infection on tomato fruit,
468 but at a less extent than that observed for S2 strain (Fig. 8). The endophytic bacteria strains
469 H1F1 and DHT2 also reduced the diameter of *B. cinerea* S5 strain rotting on tomato fruit in
470 comparison to mock-infected fruit without bacteria (Fig. 8B).

471

472 **7. Antifungal activity of the pure volatile compound 3-Methylbutan-1-ol**

473 The analysis of variance of *B. cinerea* mycelia growth in presence of 3-Methylbutan-1-ol
474 at 5 d of exposition depends from the fungal strain, the concentration of this compound and
475 their interaction (Table 8). The 3-Methylbutan-1-ol showed a significant antifungal activity
476 against S2 strain since 0.177 ml L⁻¹ and against S5 since 0.221 mL L⁻¹ in comparison to the
477 control (Fig. 9). The IC50 and IC100 of 3-Methylbutan-1-ol against both *B. cinerea* strains
478 were about 0.221 mL L⁻¹ and 0.442 mL L⁻¹ headspace, respectively (Fig. 9).

479 The analysis of variance of *B. cinerea* rot diameter and depth on tomato fruit in presence
480 of 3-Methylbutan-1-ol at 6 d post infection depends mainly from the concentration of this
481 compound (Table 9). The 3-Methylbutan-1-ol decreased significantly *B. cinerea* rot diameter
482 and depth for both fungal strains at IC50 and IC100 with a greater effect of the later
483 concentration which totally suppressed fungal infection, but no visible phytotoxicity
484 symptoms on tomato fruit were observed (Fig. 10A, B and C).

485

486 **Discussion**

487 *Botrytis cinerea* is considered as one of the most important plant pathogens in term of the
488 economic losses that engenders to agriculture sector worldwide (Dean et al., 2012; Jin and
489 Wu 2015). Synthetic fungicides are exclusively used to control this disease with a global cost
490 exceeding 1 billion dollar (Hua et al., 2018) and causing negative impact on the environment
491 and human health. Considering these constraints and the increasing awareness of consumers,
492 the development of safe and eco-friendly management methods is needed for developing
493 modern sustainable agriculture system. In this context, our work aims to select efficient
494 endophytic bacteria strains to control *B. cinerea* infection on tomato fruit based on their
495 VOCs antifungal activity and taking in account their effect on tomato seed germination and
496 seedling growth, along with the identification of potential antifungal VOCs.

497 A total of 50 endophytic bacterial strains was isolated from aerial and root organs of
498 healthy tomato plants in six localities in the Tunisian peninsula region of Cap Bon. Despite
499 the short average distance between prospected localities (< 53 km), an important difference in
500 term of number and species of endophytic bacteria strains was observed. Such substantial
501 differences in endophytic bacterial communities, at a small geographically scale, may be
502 explained by the difference in the soil composition and the cropping system of the farm (Vos
503 and Velicer, 2006; Vos et al., 2013). At the plant organ level, the results showed a dominance
504 of bacteria strains isolated from tomato leaves in comparison to other plant organs. Such
505 result may be related to the known faculty of endophytes to migrate from roots to the upper
506 organs of the plant to reach the leaves (Bodenhausen et al., 2013; Rekosz-Burlaga et al.,
507 2014). The abundant colonization of leaves may indicate the contribution of these
508 microorganisms to the efficiency of photosynthesis, and/or to the protection of these vital
509 organs from diseases. Indeed, several works demonstrate the beneficial use of endophytic
510 microorganisms on photosynthesis by increasing chlorophyll content, photochemical
511 efficiency, or net CO₂ assimilation rate (Rho and Kim, 2017; Mnasri et al., 2017) as well as

512 their protection properties against pathogens (Pavithra et al., 2020). Accordingly, *in vitro*
513 antagonistic tests against *B. cinerea* showed that 18 bacteria strains of the collection produce
514 VOCs with antifungal activity. As evidenced by 16S rRNA sequencing, those belong to
515 different species of *Bacillus* and *Enterobacter* genera with a significant enrichment in strains
516 of the *Bacillus* genus. *Bacillus* strains colonizing plants are involved in many physiological
517 and stress tolerance processes improving plants growth and health (Earl et al., 2008; Mnasri et
518 al. 2017). In this study, the majority of the identified antagonistic *Bacillus* strains showed
519 neutral to promoting effect on tomato seed germination and seedling growth, implying their
520 role in tomato growth and protection against at least *Botrytis* pathogen. The *Bacillus* genus is
521 already known to harbor potent antagonistic strains to plant pathogens providing effective
522 plant protection through the production of volatile organic compounds (Kefi et al., 2015;
523 Mnasri et al., 2017; Jiang et al., 2018) as well as soluble compounds such as lipopeptides and
524 hydrolytic enzymes (Ben Slimene et al., 2012; Slimene et al., 2015). To our knowledge this is
525 the first report of VOCs antifungal activity against *B. cinerea* produced by *B. nakamurai*, *B.*
526 *pseudomycooides*, *B. proteolyticus*, *B. thuringiensis*, as well as *Enterobacter asburiae* and *E.*
527 *cloacae*. Nevertheless, *Bacillus* species like *B. amyloliquefaciens* (Gotor-Vila et al., 2017), *B.*
528 *velezensis* (Gao et al., 2018; Jiang et al., 2018), *B. subtilis* (Gao et al., 2017; Zhao et al.,
529 2019), *B. vallismortis* (Liu et al., 2018) and *B. toyonensis* (Contreras-Pérez et al., 2019) are
530 known to produce antifungal VOCs against several phytopathogens including *B. cinerea*.

531 The isolated endophytic *Enterobacter* species (*E. asburiae* and *E. cloacae*) produced
532 antifungal VOCs against *B. cinerea*. Various *Enterobacter* species are known to colonize
533 internal plant tissues, improve plant growth and prevent from pathogens attacks (Tsuda et al.,
534 2001; Jha et al., 2011; Latha et al., 2019; Singh et al., 2020). Previous studies showed that *E.*
535 *asburiae* strains KDKS5-49 and Vt-7 produce antifungal VOCs against *Phytophthora capsici*
536 (Toh et al., 2016) and *Aspergillus flavus* (Gong et al., 2019), respectively. *Enterobacter*

537 *cloacae* have antifungal activity against several pathogens such as *Fusarium oxysporum* f. sp.
538 *spinaciae* (Tsuda et al. 2001), *Mycosphaerella fijiensis* (Macedo-Raygoza et al., 2019),
539 *Sclerotium rolfsii* (Mahartha and Suprpta, 2018) and *Phytophthora sojae* (Zhao et al., 2018).

540 The correlation between VOC-mediated antifungal and PGP activities did not show any
541 significant relation between both properties. Two hypotheses can be made: firstly, our results
542 may support the specialization of endophytic bacteria toward antagonism or PGP capacities;
543 secondly, the used protocol (double culture assay) for the activities testing may be in favor of
544 selecting antagonistic strains at the expense of PGP strains because of the high concentration
545 of VOCs. The first hypothesis is supported by the work of Walitang et al. (2017) showing that
546 specific type strains isolated from different rice cultivars have distinguishing characteristics,
547 which indicates a certain specificity in their activities. For example, *Flavobacterium* sp.
548 strains are phosphate solubilisers and indole-3-acetic acid producers, whereas *Pseudomonas*
549 strains are characterized as high siderophore producers while *Microbacterium* sp. and
550 *Xanthomonas* sp. strains have very high pectinase and cellulase activity (Walitang et al.,
551 2017). The second hypothesis is supported by the work of Asari et al. (2016) indicating that
552 plant promoting and antagonistic activity by VOCs are dose dependent: while PGP activity
553 needs low concentration of volatiles, the antagonistic one needs high volatiles concentration.
554 At the practical level the specification observed in biological activities of endophytic bacteria
555 constitutes a challenge for developing broad-spectrum bio-inocula for agriculture use. This
556 issue can be managed by developing inocula containing a consortium of compatible bacteria
557 strains with complementary biological activities as reported in previous works (Thakkar and
558 Saraf, 2015; Palmieri et al., 2017; Taha et al., 2019). Based on these results we selected
559 endophytic bacteria strains that have strong VOCs antifungal activities and as much as
560 possible neutral to promoting effects on tomato seed germination and seedling growth. Four
561 *Bacillus* strains (BF11, DHT2, H1F1 and H2F12) and one *Enterobacter* strain (TR1) were

562 selected and used for postharvest control of *B. cinerea* on tomato fruit using their VOCs and
563 vegetative cells. The activities of cellulase, pectinase, amylase, phytase, xylanase and
564 chitinase were also observed in almost all of the selected endophytic bacteria strains
565 indicating the importance of these characteristics for survival and colonization into the plant
566 tissues. These bacterial endophytes also showed promising PGP enzymatic activities
567 including ammonia and siderophore production and phosphate solubilization. The selected
568 *Bacillus* strains decreased significantly fruit rot caused by *B. cinerea* mainly using vegetative
569 cells probably due to their ability to produce several hydrolytic enzymes as indicated above.
570 According to Essghaier et al. (2009), the biocontrol of *B. cinerea* on strawberry fruit was
571 correlated to production of hydrolytic enzymes (chitinase, cellulase, protease, β -1,3-
572 glucanase) by *Bacillus* spp. strain 1502. In addition, Zhang et al. (2010) showed that the
573 release of glucanase, chitinase and proteases by *Aureobasidium pullulans* PL5 strain control
574 postharvest disease caused by *M. laxa* in apple and peach. In comparison to the selected
575 *Bacillus* strains, the *Enterobacter* strain TR1 showed the most protective effect of tomato fruit
576 against *B. cinerea* rotting through its VOCs. As a potential clinical specimen, this strain
577 cannot be applied directly on plants but the bioactive VOCs it emits can be used for the
578 biocontrol of gray mold and potentially tested against other postharvest fungal pathogens.

579 The GC-MS identification of bacterial VOCs showed a core set of seven compounds
580 produced by all five selected antifungal endophytic bacteria strains i.e. 1-Octyn-3-ol, 4-ethyl ;
581 β -Fenchol; Benzene, m-di-tert-butyl; 2-Undecanone; 3,3,5-Trimethylbicyclo[3.3.0]octan-2,8-
582 dione; 2-Pentadecanone, 6,10,14-trimethyl and unidentified compound. These ubiquitous
583 VOCs may be involved in vital biological processes in bacteria such as communication,
584 environmental adaptation and defense against other microbes (Chernin et al., 2013; Audrain et
585 al., 2015). Indeed, volatiles of ketone group like 2-Undecanone are synthesized by different
586 bacteria genera and known for their antibacterial activity (Melkina et al., 2017; Veselova et

587 al., 2019). Some bacteria strains such as TR1 and DHT2 emitted sulfur-containing
588 compounds, including dimethyl disulfide and dimethyl trisulfide known for their antifungal
589 activities against several pathogens including oomycete such as *Pythium myriotylum* and
590 *Phytophthora capsici* (Agisha et al. 2019) and fungi such as *Rhizoctonia solani*, *Athelia*
591 *rolfsii*, *Colletotrichum gloeosporioides*, *Magnaporthe oryzae*, *Gibberella moniliformis* and *B.*
592 *cinerea* (Elkahoui et al., 2015; Hernández-León et al., 2015; Agisha et al. 2019). The DHT2
593 and H1F1 strains produced Cyclohexanol,4-methyl and 2-Heptanone,6-methyl which were
594 related to *Bacillus* spp. antifungal activities (Fernando et al. 2005; Morita et al., 2019). The
595 TR1 and H2F12 strains produced 2-Heptanone which was reported to have insecticidal and
596 antifungal activities specially against *Pythium irregular*, *R. solani*, and *F. oxysporum* (Cole
597 and Blum, 1975) and has been also related to the antifungal activity of *B. amyloliquefaciens*
598 DA12 against *Fusarium* species (Lee et al., 2017). The H2F12 strain produced seven strain-
599 specific volatiles with the Dodecanal as a major compound; this has antifungal (*R. solani* and
600 *Bipolaris oryzae*) and antibacterial activities (*Xanthomonas oryzae*) as pure compound
601 (Chanprapai et al., 2018) and is also related to endophytic bacterial antagonism (Kai et al.,
602 2007; Li et al., 2015). Interestingly, H1F1 and BF11 *Bacillus* strains produce three and six
603 specific VOCs, respectively of unknown antimicrobial activity; suggesting that they could be
604 new bioactive VOCs for the control of *B. cinerea*. Further analysis should be done to identify
605 these volatiles and study their antifungal activities.

606 The VOCs produced by the *Enterobacter* strain TR1 had the most important *in vivo*
607 protective effect against *B. cinerea* decay on tomato fruit. In total, the TR1 strain produces 57
608 VOCs (at 7 and 14 d) with two of them (3-Methylbutan-1-ol and 2-Furanmethanol)
609 considered as bio-markers of *E. cloacae* (Lawal et al., 2018) and three compounds (3-
610 Methylbutan-1-ol, Pentadecane and 2-Octanone) previously reported for their antifungal
611 activities (Li et al., 2020). This study showed a notable antifungal activity of the major

612 volatile compound 3-Methylbutan-1-ol produced by TR1 strain which totally suppress *B.*
613 *cinerea* infection at 0.442 mL L⁻¹ headspace with no visible alteration on tomato fruit. Calvo
614 et al. (2020) showed also that 3-Methylbutan-1-ol had antifungal activity against *B. cinerea* at
615 0.1 mL L⁻¹ in table grapes. The difference between the effectiveness of 3-Methylbutan-1-ol
616 antifungal activity in this study in comparison to Calvo et al. (2020) may be explained in part
617 by the difference of the reaction of the used *B. cinerea* strains. This is supported by the fact
618 that we observed a significant difference between the sensitivity of the S2 and S5 *B. cinerea*
619 strains in reaction to 3-Methylbutan-1-ol. So, prior to the application of this potential
620 antifungal volatile, it is crucial to determine not only the active dose but also it's spectrum of
621 activity against different fungal strains, phytotoxicity on various fruit species and effects on
622 fruit quality.

623

624 **Conclusion**

625 In conclusion this study showed that healthy tomato plants harbor diverse endophytic bacteria
626 strains colonizing mainly leaves and some of them emitting VOCs with antifungal and/or
627 plant growth promotion activity. Identified antagonistic bacteria strains showed a significant
628 enrichment with *Bacillus* species with few *Enterobacter* species. The activities of several
629 hydrolytic enzymes were also observed in almost all selected endophytic bacteria, along with
630 promising plant growth promoting enzymatic activities. The characterization of bacterial-
631 released VOCs showed a core set of compounds produced by all selected bacteria, and some
632 strain-specific volatiles, with some of them already reported for their antifungal activities.
633 Finally, this work provides evidence about VOCs- and vegetative cells-mediated biocontrol
634 ability of *Enterobacter* and *Bacillus* strains, respectively to reduce postharvest infection of *B.*
635 *cinerea* on tomato fruit, as well as 3-Methylbutan-1-ol as promising antifungal volatile to
636 control grey mould during storage and trade transport. Although, no visible alteration were

637 observed on tomato fruit after the application of 3-Methylbutan-1-ol phytotoxicity tests
638 should be done prior to application.

639

640 **Funding**

641 This work was financially support by the Tunisian-French PHC-Utique collaborative project
642 (CMCU code 17G0902 / Campus France code 37119QF) and by the Tunisian Ministry of
643 Higher Education and Scientific Research.

644

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Figure 1 is provided as pdf file as including it in the ppt file will dramatically decrease the quality.

Fig. 1. Collection of endophytic bacterial strains from different organs of healthy tomato plants. (A) Localisation of collecting sites in the Cape Bon region in Tunisia. For each site, the pie chart represents the relative proportion of bacterial strains isolated from the different plant organs. The size of the pie chart is proportional to the number of isolated bacterial strains. (B) Mosaic plot showing statistical enrichment of the bacterial endophytic collection according to site of prospection and plant organ. Standardized residuals are mapped to colors and percent values to surfaces. Marginal counts of each level of site and organ variables are indicated into brackets.

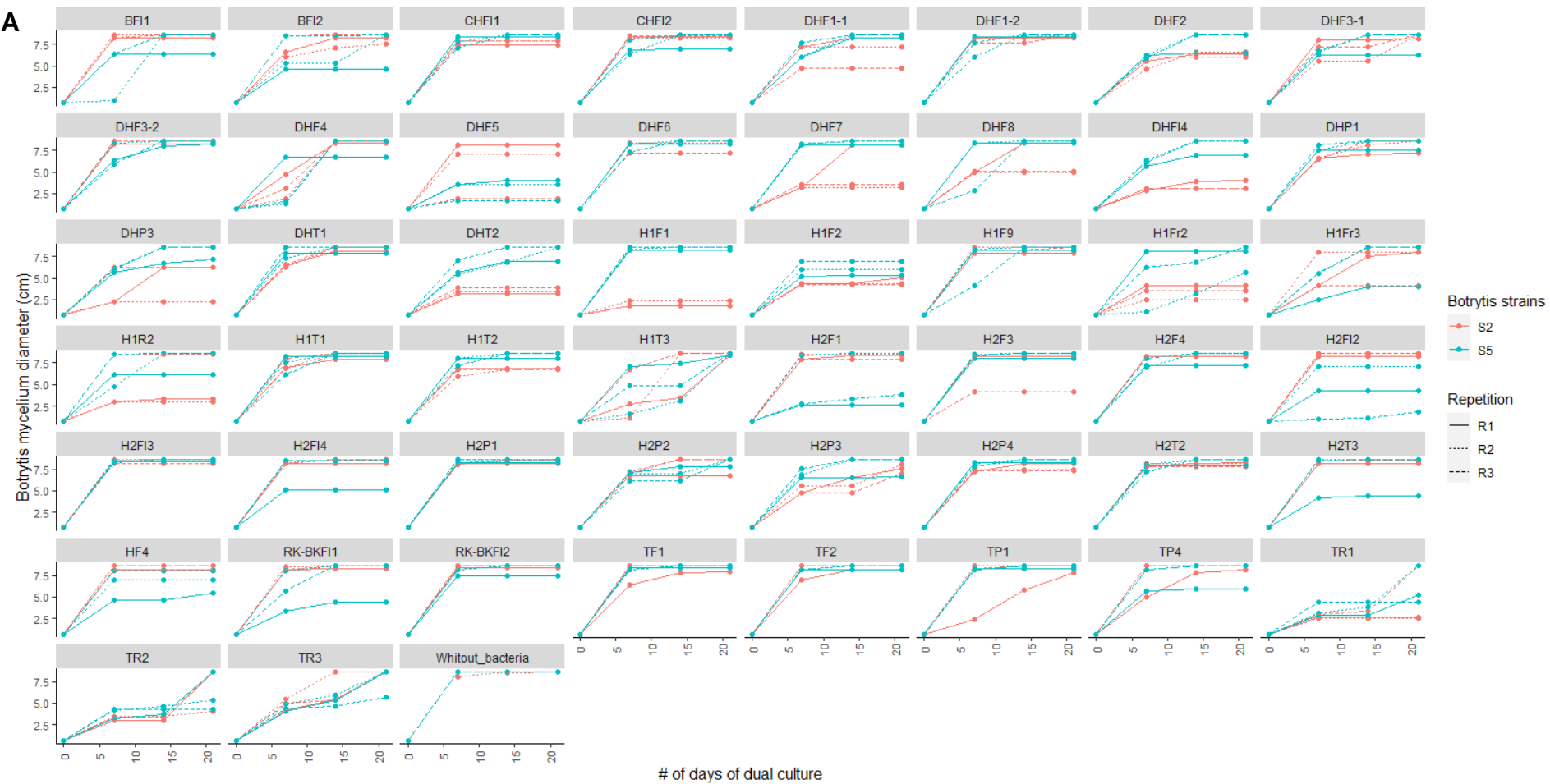
A

Fig. 2. Evaluation of the *in vitro* antifungal activity of endophytic bacterial volatile organic compounds (VOCs). (A) Radial mycelia growth kinetics, of *Botrytis cinerea* strains S2 and S5 subjected to bacterial VOCs in dual culture assay. Three independent replicates were performed for each bacterium-fungus strains combination.

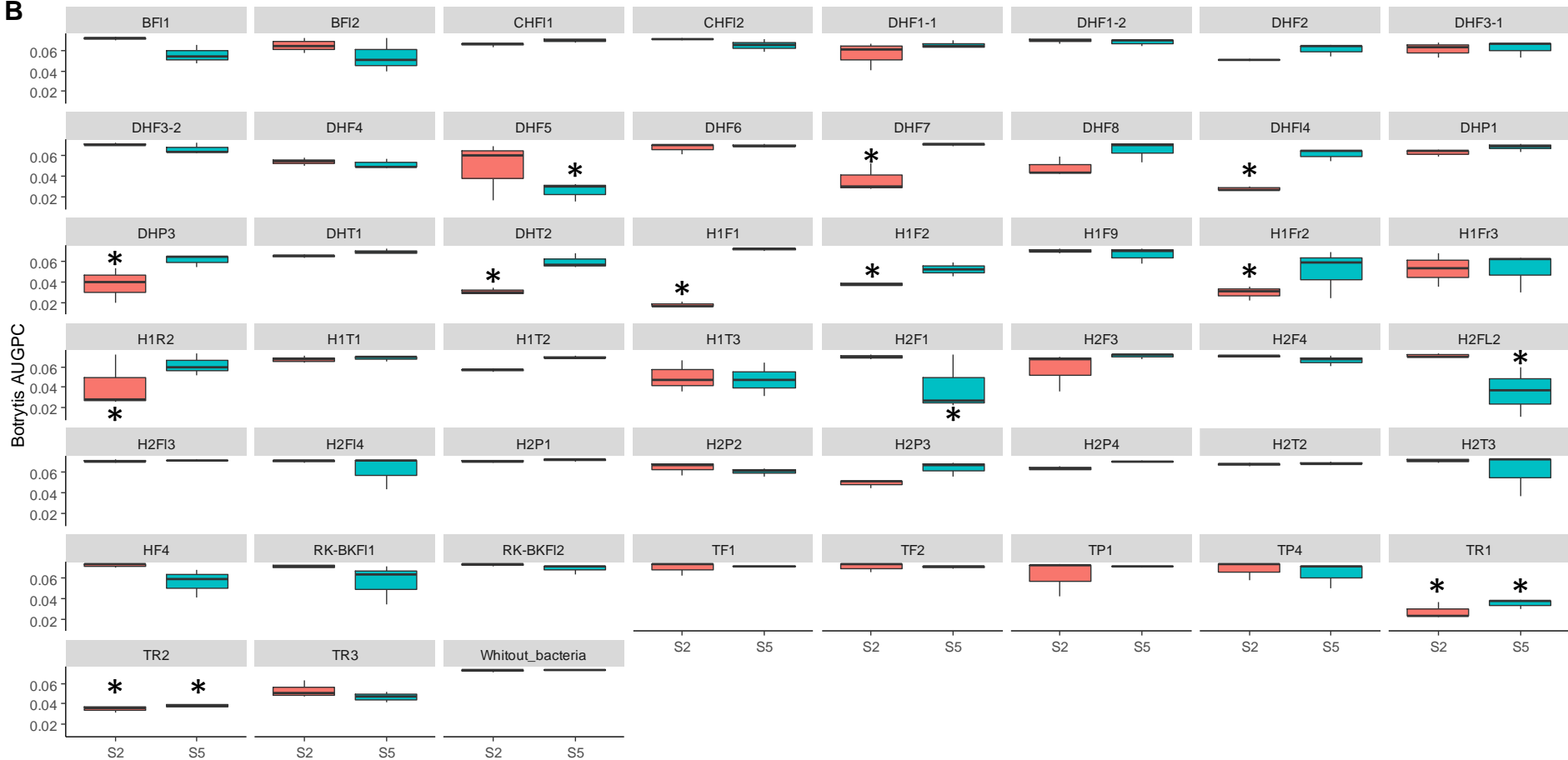
B

Fig. 2. Evaluation of the *in vitro* antifungal activity of endophytic bacterial volatile organic compounds (VOCs). (B) Area under growth progress curve (AUGPC) of *Botrytis cinerea* strains S2 and S5 subjected to bacterial VOCs in dual culture assay. For AUGPC a low value indicates a high antifungal activity of bacteria strains. Three independent replicates were performed for each bacterium-fungus strains combination. Asterisks indicate a difference in comparison to treatment “Without-Bacteria” as revealed by Dunnett’s test ($*P < 0.05$).

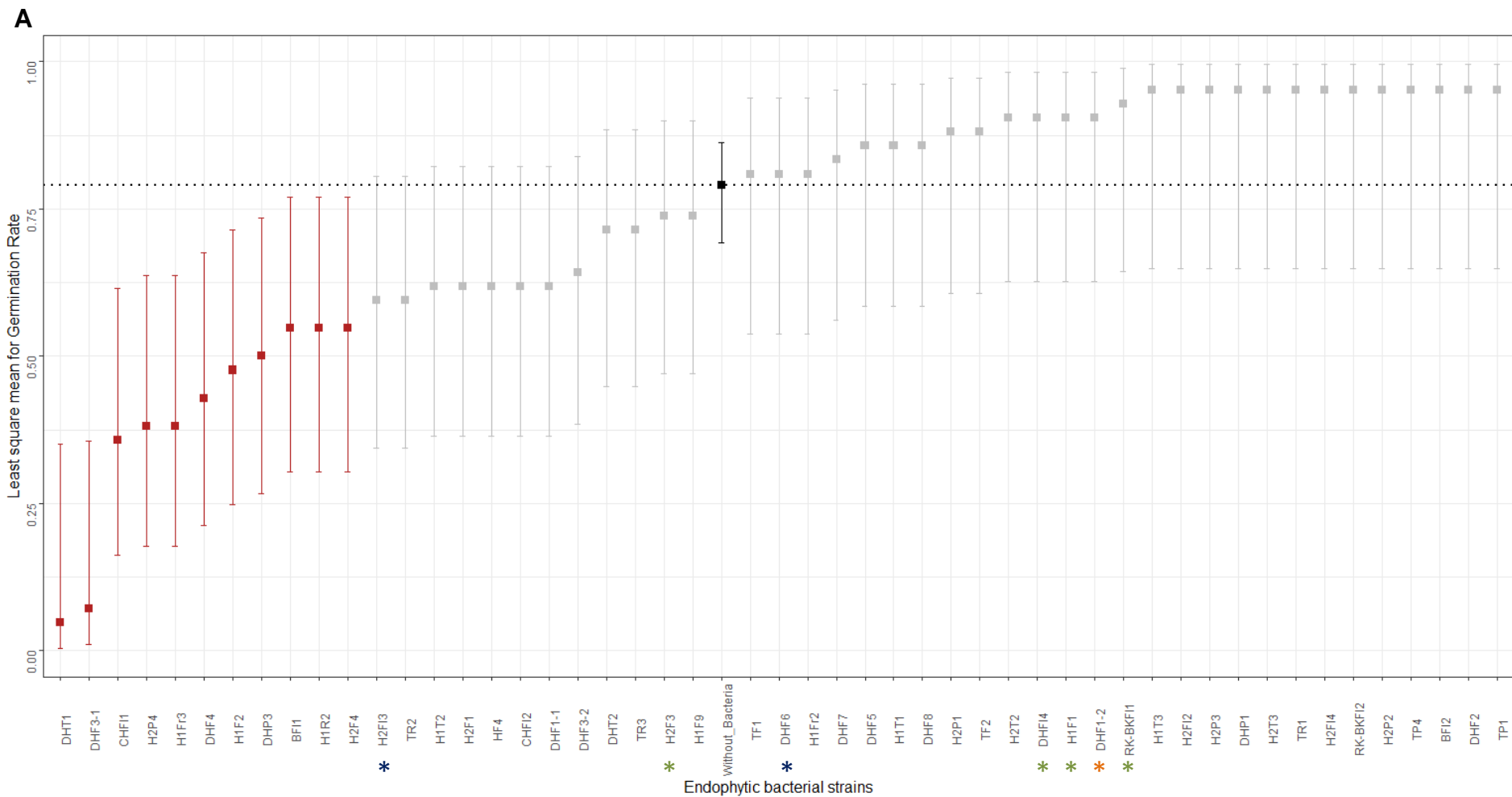


Fig. 3. Evaluation of *in vitro* effect of bacterial volatile organic compounds (VOCs) on tomato seed germination and seedlings growth at 6 d of dual culture. The values for the measured parameters represent the least square means and their standard deviations calculated from GLM for germination rate (A) and MLM for seedling length (B) and fresh (C) and dry weights (D). Red color indicates significant differences in comparison to the treatment “Without-Bacteria” as revealed by Dunnett’s test (* $P < 0.05$). Dash horizontal line represents the least square mean value of the treatment “Without-Bacteria” which is plotted in black on the graphs. Blue, green and orange asterisks represent respectively bacterial strains with significant promoting effects on seedling length and fresh and dry weights.

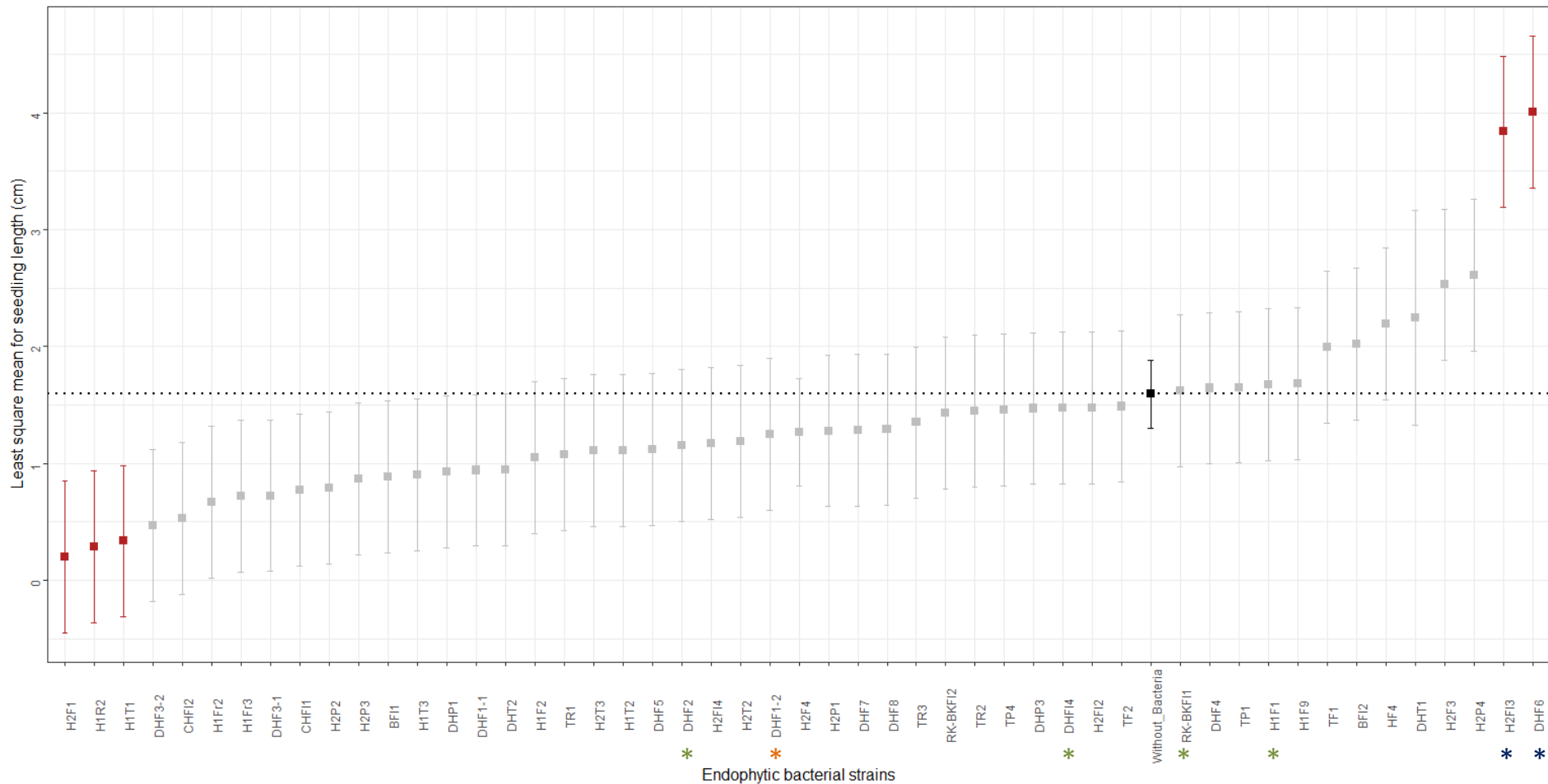
B

Fig. 3. Evaluation of *in vitro* effect of bacterial volatile organic compounds (VOCs) on tomato seed germination and seedlings growth at 6 d of dual culture. The values for the measured parameters represent the least square means and their standard deviations calculated from GLM for germination rate (A) and MLM for seedling length (B) and fresh (C) and dry weights (D). Red color indicates significant differences in comparison to the treatment “Without-Bacteria” as revealed by Dunnnett’s test (*P < 0.05). Dash horizontal line represents the least square mean value of the treatment “Without-Bacteria” which is plotted in black on the graphs. Blue, green and orange asterisks represent respectively bacterial strains with significant promoting effects on seedling length and fresh and dry weights.

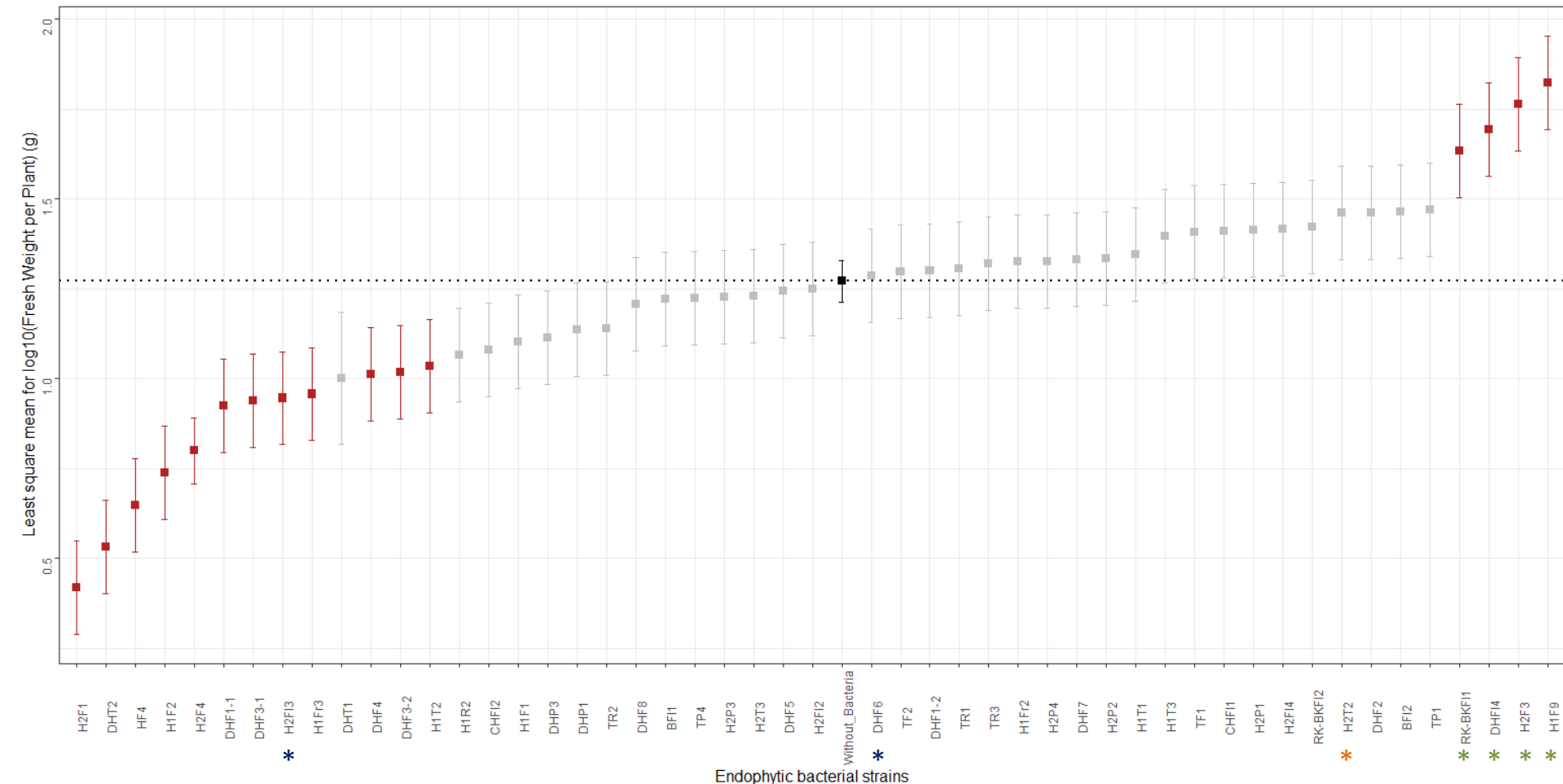
C

Fig. 3. Evaluation of *in vitro* effect of bacterial volatile organic compounds (VOCs) on tomato seed germination and seedlings growth at 6 d of dual culture. The values for the measured parameters represent the least square means and their standard deviations calculated from GLM for germination rate (A) and MLM for seedling length (B) and fresh (C) and dry weights (D). Red color indicates significant differences in comparison to the treatment “Without-Bacteria” as revealed by Dunnett’s test (* $P < 0.05$). Dash horizontal line represents the least square mean value of the treatment “Without-Bacteria” which is plotted in black on the graphs. Blue, green and orange asterisks represent respectively bacterial strains with significant promoting effects on seedling length and fresh and dry weights.

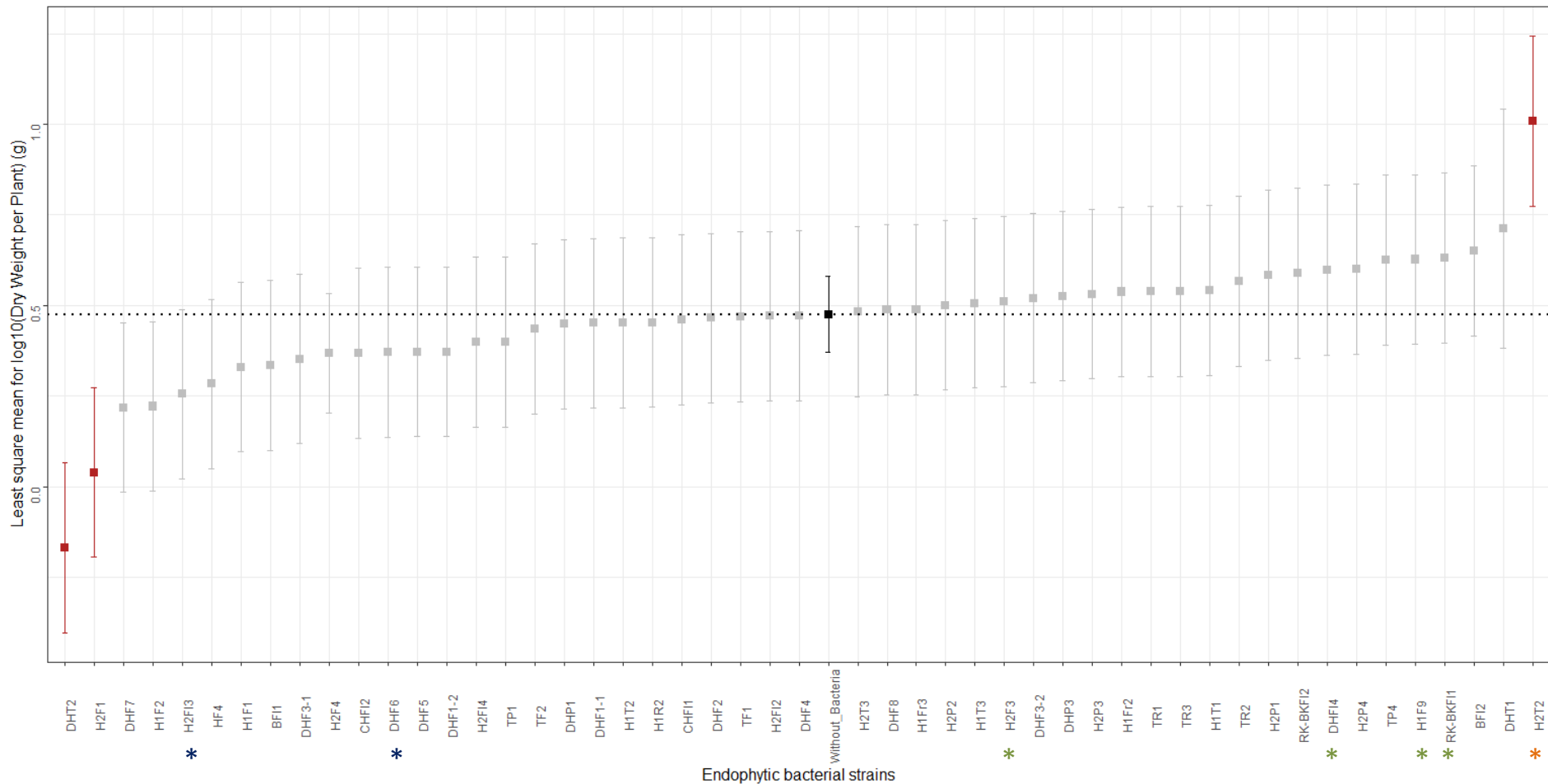
D

Fig. 3. Evaluation of *in vitro* effect of bacterial volatile organic compounds (VOCs) on tomato seed germination and seedlings growth at 6 d of dual culture. The values for the measured parameters represent the least square means and their standard deviations calculated from GLM for germination rate (A) and MLM for seedling length (B) and fresh (C) and dry weights (D). Red color indicates significant differences in comparison to the treatment “Without-Bacteria” as revealed by Dunnett’s test ($*P < 0.05$). Dash horizontal line represents the least square mean value of the treatment “Without-Bacteria” which is plotted in black on the graphs. Blue, green and orange asterisks represent respectively bacterial strains with significant promoting effects on seedling length and fresh and dry weights.

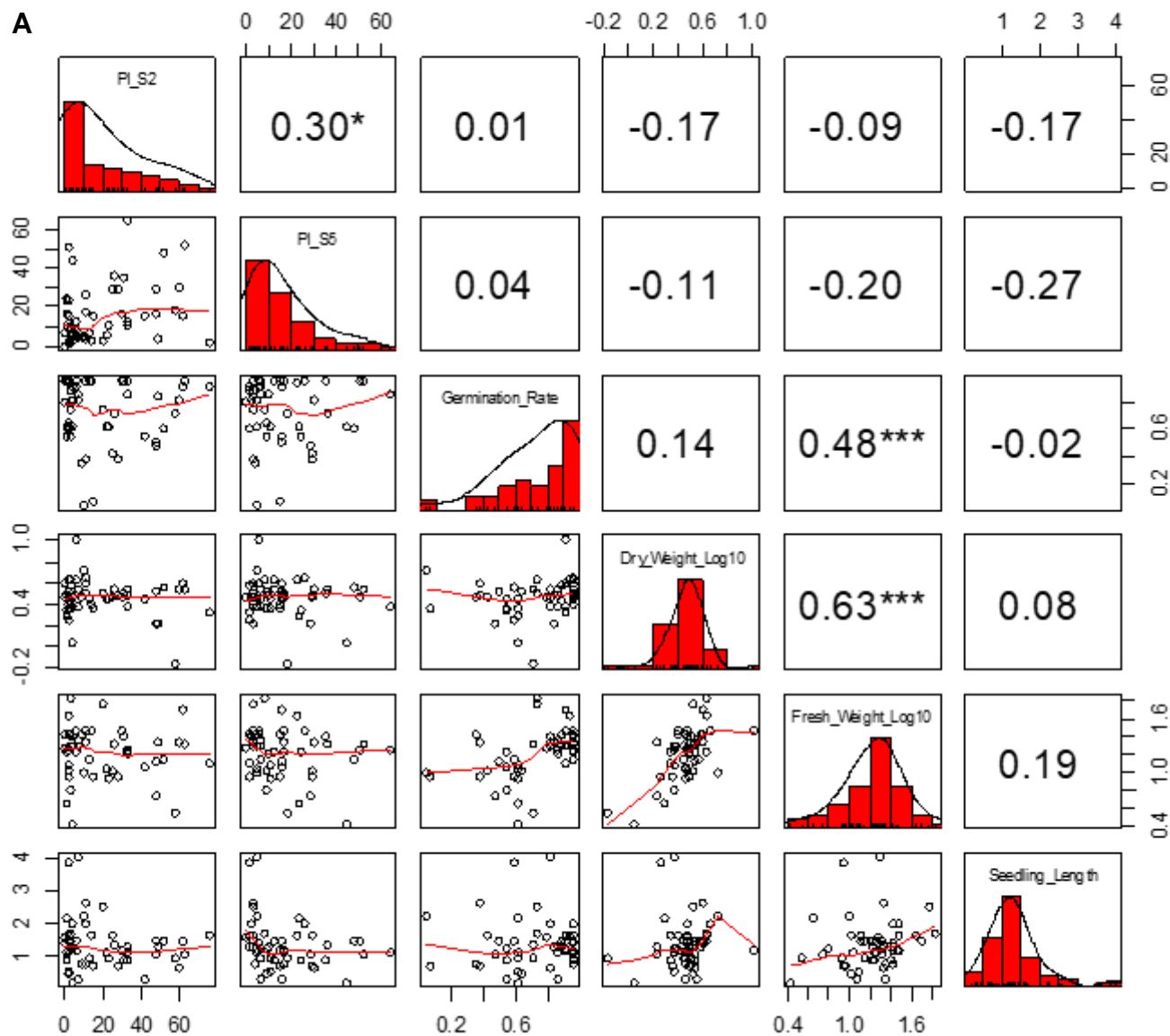


Fig. 4. (A) Correlation of the *in vitro* antifungal and plant growth activities of volatile organic compounds (VOCs) produced by 50 endophytic bacteria strains. Pairwise correlations were computed based on lsmeans from MLM and GLMM analysis. For each endophytic strain, the percentage of inhibition of *Botrytis cinerea* mycelia growth of S2 strain (PI_S2) and S5 strain (PI_S5) was calculated based on AUGPC lsmeans values reported to value of the treatment “without bacteria”. Coefficients of pairwise Pearson correlation are indicated. Asterisks represent significant correlations according to following significance code for *p*-values: 0 “***” 0.001 “**” 0.01 “*” 0.05 “.” 0.1 “.” 1. The graph was obtained using the pairs panels function of the psych package of R software.

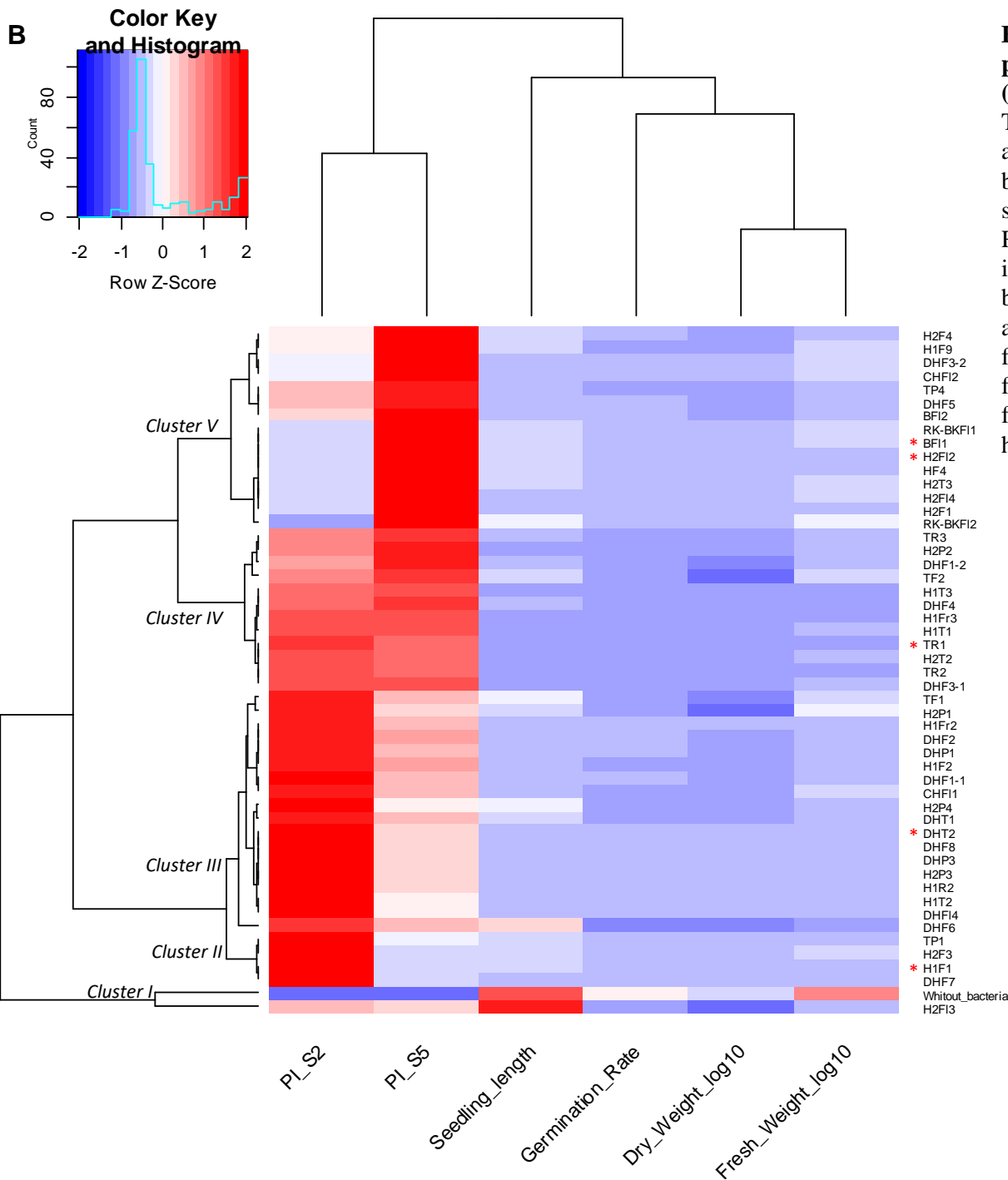


Fig. 4. (B) Heat map of the *in vitro* antifungal and plant growth activities of volatile organic compounds (VOCs) produced by 50 endophytic bacteria strains. The hierarchical clustering for bacterial strains based on antifungal and plant growth activities was computed based on Pearson correlations and complete linkage. Z-scores were calculated and used to generate heat maps. Red color indicates high activity and blue colour indicates low activity. Dendrogram on the left shows bacteria strains clustering according to both antifungal and plant growth promoting biological activities and the five bacterial clusters (I to V). Red asterisks indicate the five endophytic bacteria strains which were selected for further analysis. The graph was obtained using the heatmap.2 function of the gplot package of R software.

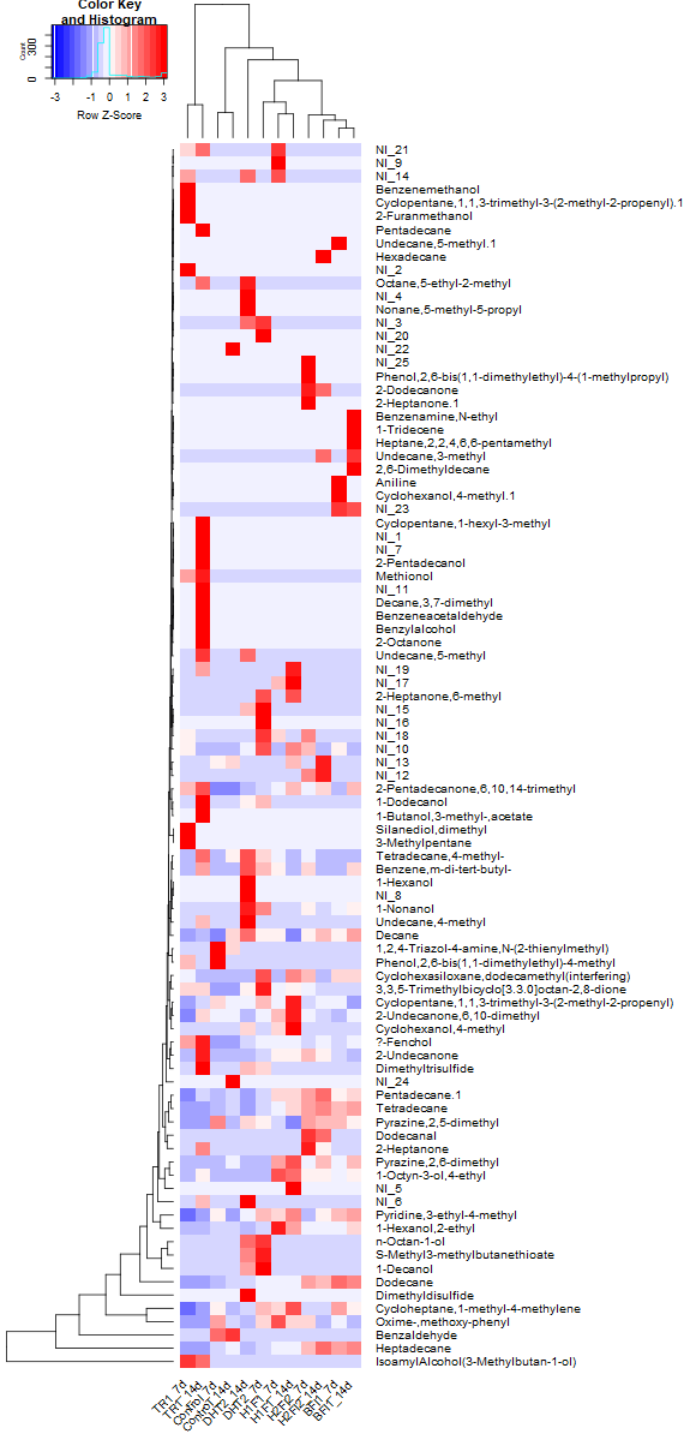


Fig. 5. Patterns of production of volatile organic compounds (VOCs) of five endophytic bacteria strains (BF11, DHT2, H1F1, H2F12 and TR1) detected by SPME-GC/MS technique. VOCs were measured at 7 and 14 d of bacterial culture on LB Agar medium (bacteria-free LB medium was used as a control condition) in two independent replicates. Means of molecules percentages for the two replicates were used to calculate Z-scores to generate heatmap. Data matrix was normalized by row to absorb the variation between VOC molecules. Dendrogram on the left shows the relationships between quantities of 92 emitted VOCs according to different samples (Bacterial strains and age of culture). Dendrogram on the upper part shows the relationships between samples according to their patterns of VOCs production. Blue colour indicates high concentration and pink color indicates low concentration of VOC. NI_#: Non identified compound.

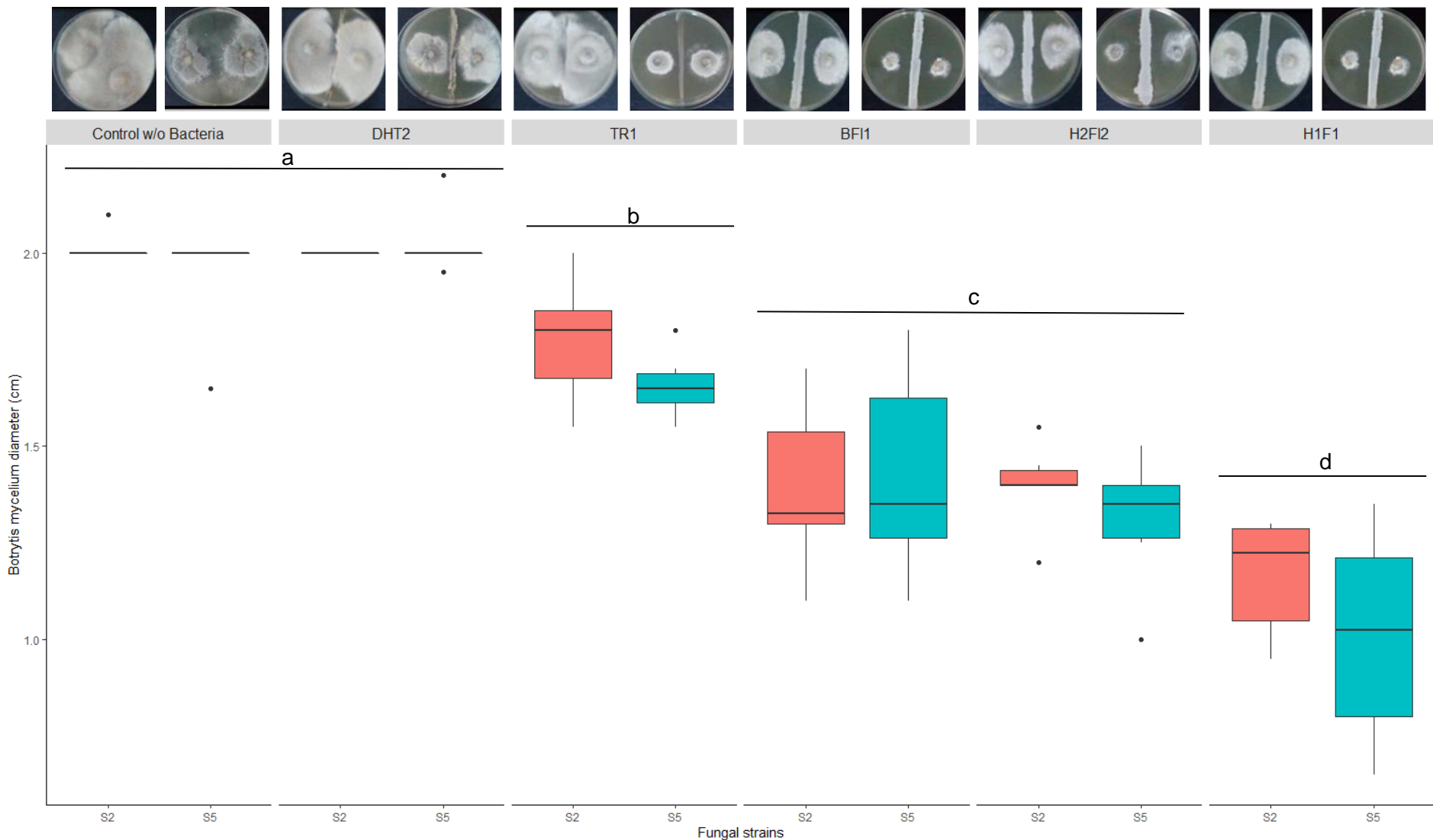


Fig. 6. *In vitro* antifungal activity through soluble compounds of five bacteria strains (BF11, DHT2, H1F1, H2F12 and TR1) against two *Botrytis cinerea* strains S2 and S5. The antifungal activity was assessed as the decrease in fungal mycelia growth compared to the control condition ‘Without bacteria’ on PDA medium at 7 d of co-culture. A representative plate at 7 d of co-culture is shown for each bacterium-fungus strains combination. Two independent replicates with three plates for each combination of bacterial and fungal strains were carried out. Letters identify significantly different groups according to Tukey test for multiple mean comparisons at $**P < 0.01$.

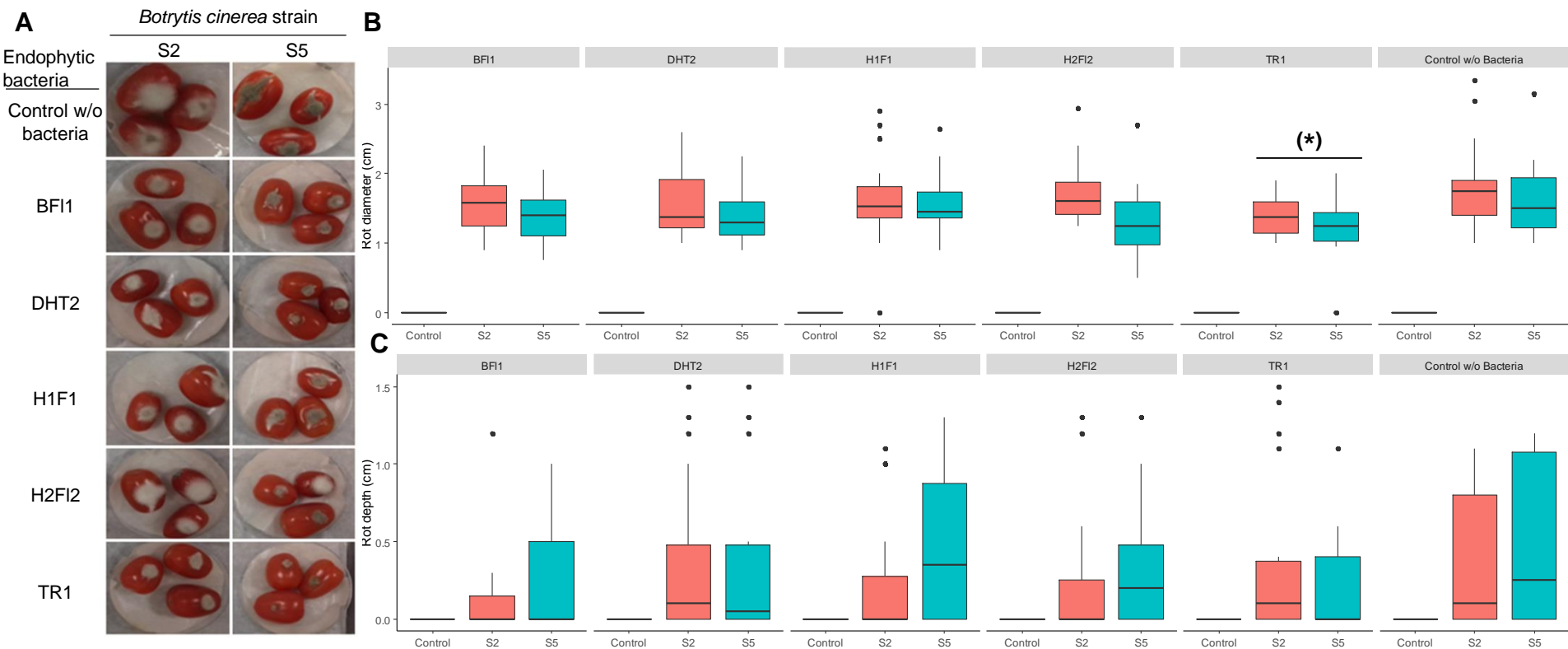


Fig. 7. Effect of volatile organic compounds (VOCs) of five endophytic bacteria strains on *Botrytis cinerea* infection on tomato fruit. (A) Photos of tomato fruit exposed to endophytic bacterial VOCs or not (control without bacteria) at 6 d after infection with S2 or S5 *B. cinerea* strains. Boxplots represent the diameter (**B**) and the depth (**C**) of fruit rotting caused by S2 and S5 *B. cinerea* strains or mock condition (Control). Data were recorded from three independent experiments. Asterisk into brackets indicates significant difference in comparison to treatment “Without bacteria” as revealed by Dunnett’s test (* $P < 0.05$).

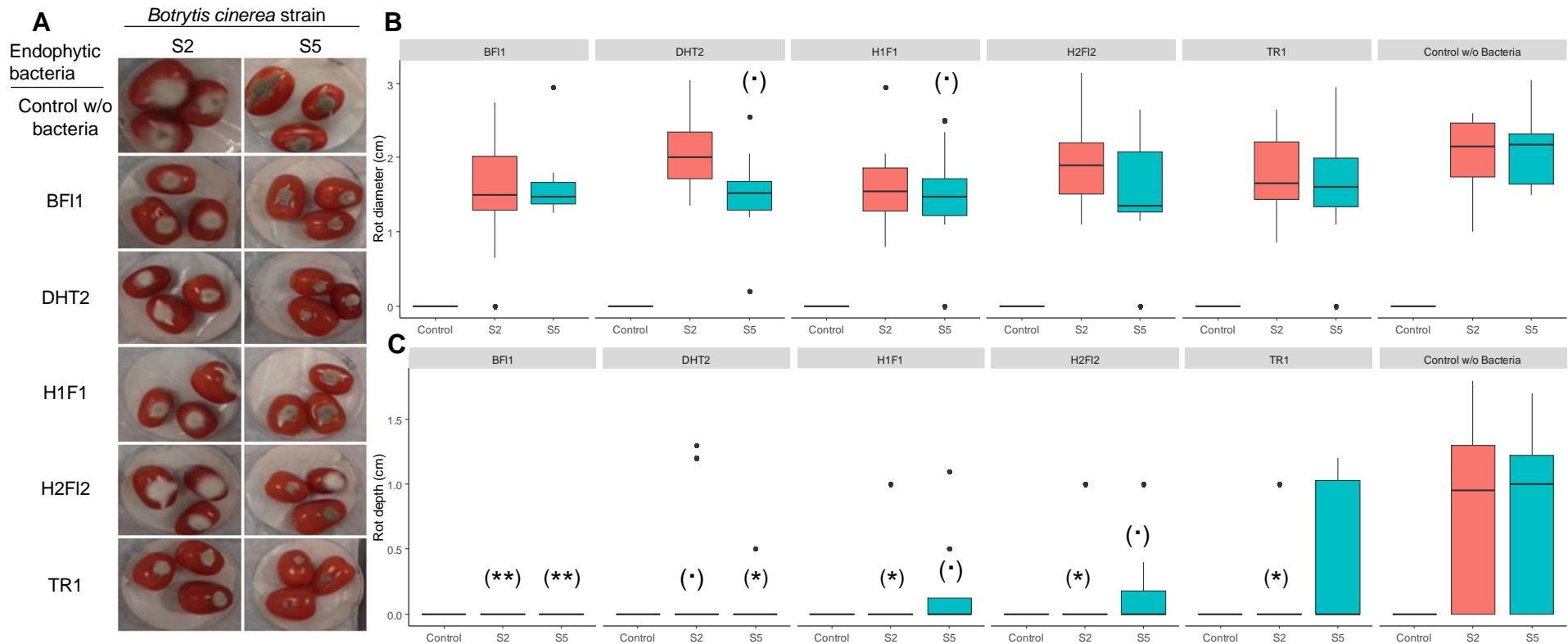


Fig. 8. Effect of treatment with vegetative cells of five endophytic bacteria strains on *Botrytis cinerea* infection on tomato fruit. (A) Photos of tomato fruits treated or not (control without bacteria) with bacterial cell suspension at six days after infection with *B. cinerea* S2 or S5 strains. Boxplots represent the diameter (B) and the depth (C) of fruit rotting caused by S2 and S5 *B. cinerea* strains or mock condition (Control) at 6 d after infection. Data are of three independent experiments. Asterisks or dots into brackets indicate significant difference compared to the treatment “Without bacteria” as revealed by Dunnett’s test. Significance code for *p*-values: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1.

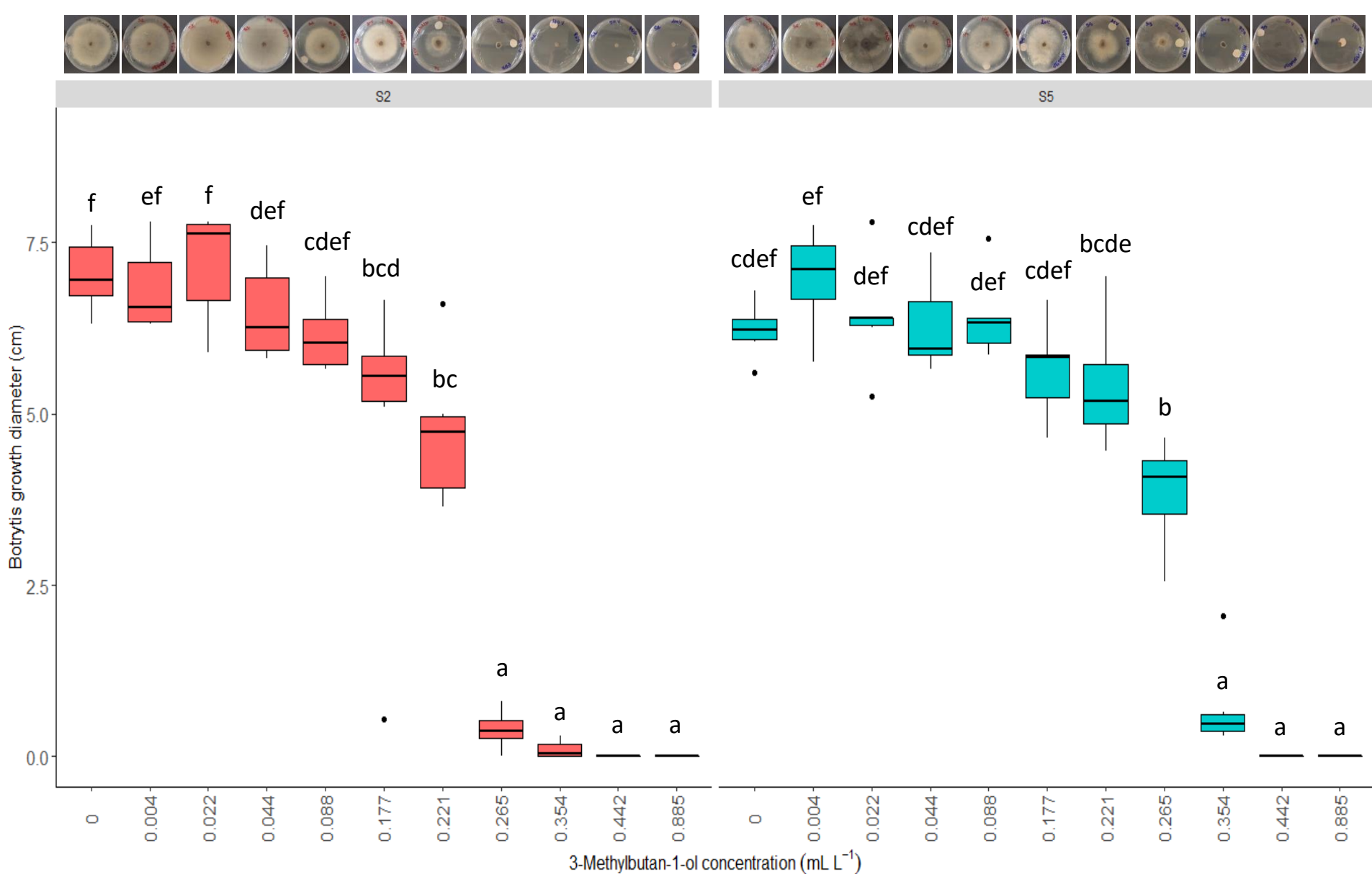
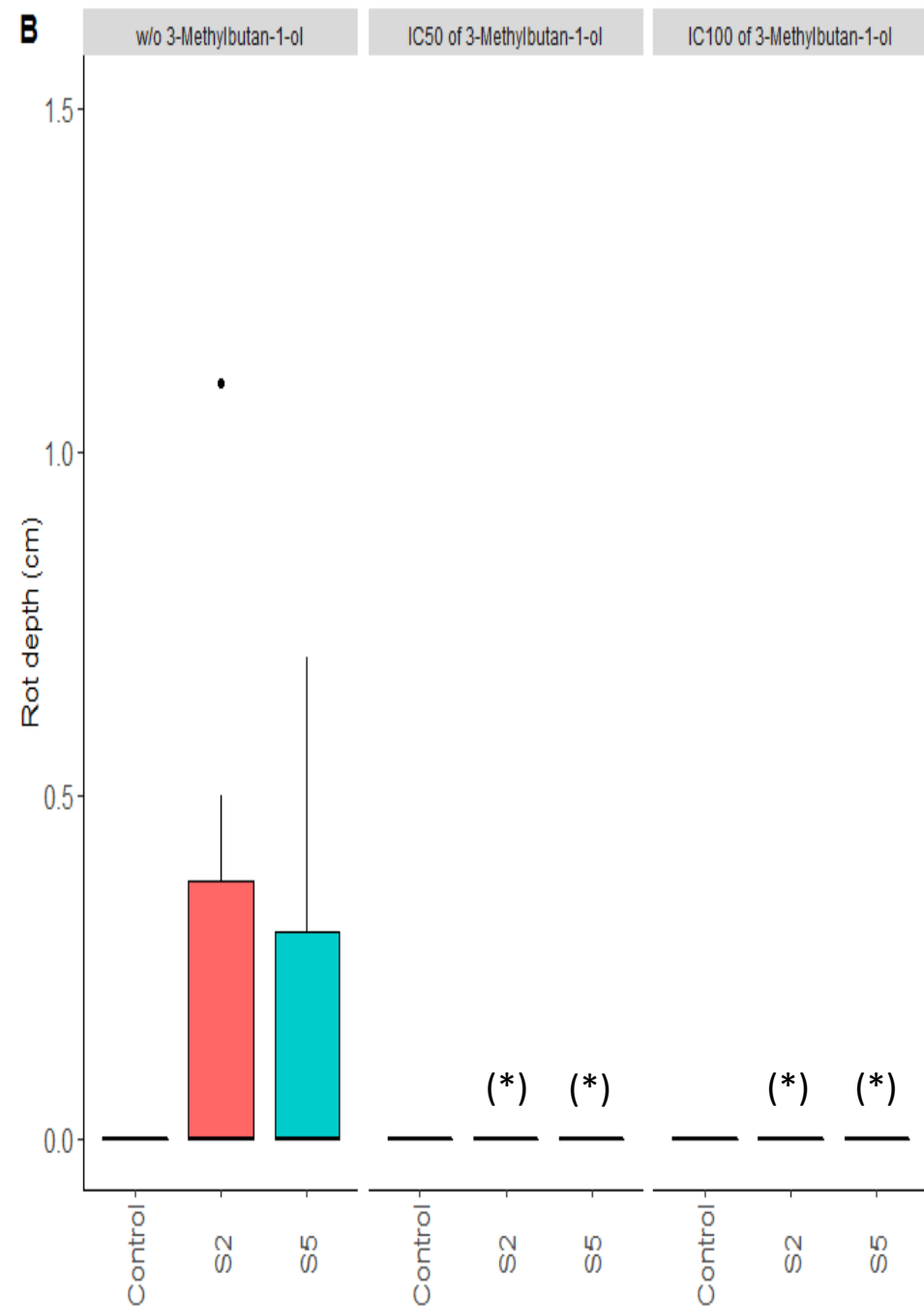
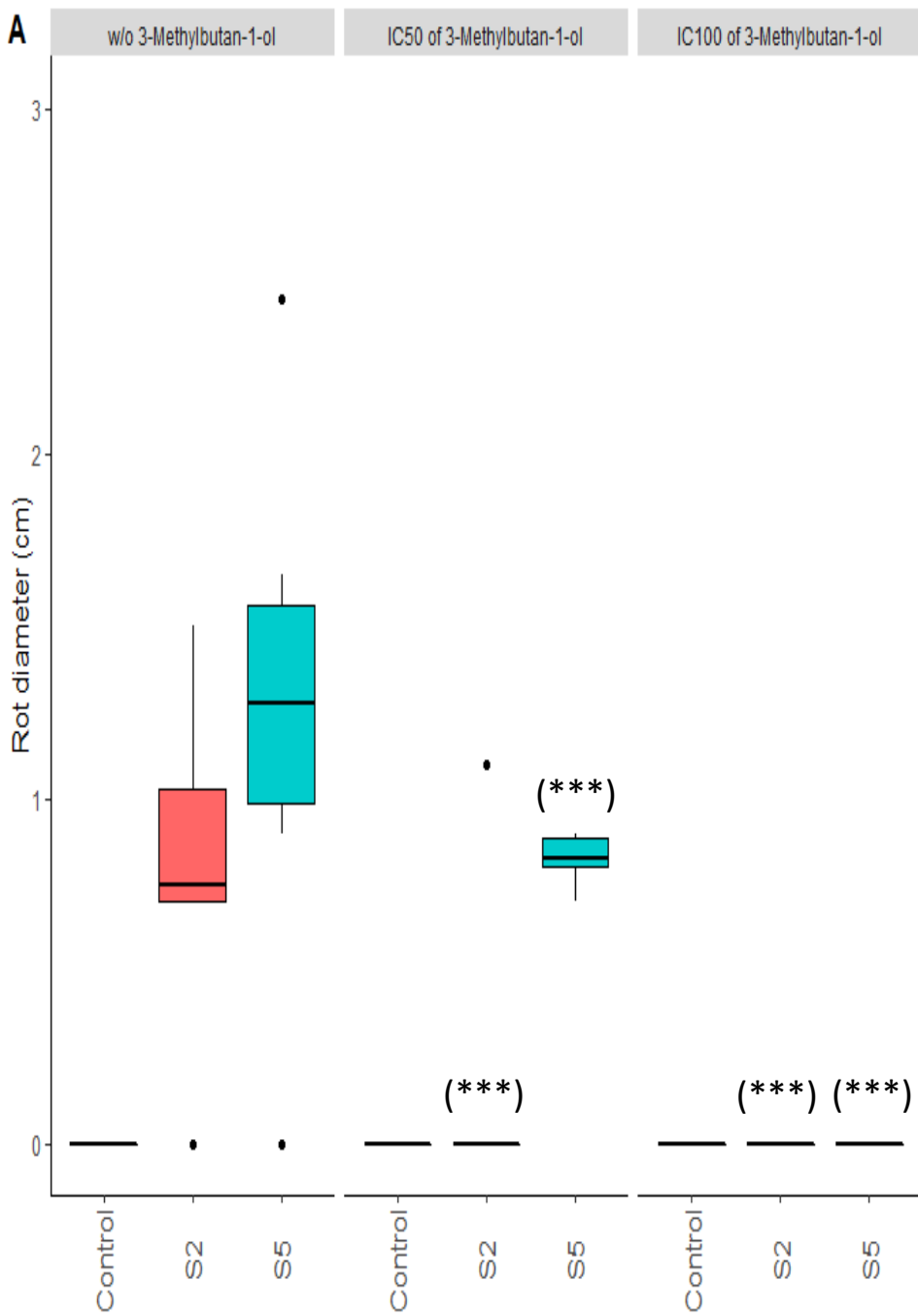


Fig. 9. *In vitro* antifungal activity of 3-Methylbutan-1-ol against two *Botrytis cinerea* strains S2 and S5. The antifungal activity was assessed as the decrease in fungal mycelia growth compared to the control condition ‘without 3-Methylbutan-1-ol’ on PDA medium at 5 d of co-culture. A representative plate at 5 d of co-culture is shown for each 3-Methylbutan-1-ol concentration-fungus strain combination. Two independent replicates with three plates for each combination of 3-Methylbutan-1-ol concentration and fungal strains were carried out. Letters identify significantly different groups according to Tukey test for multiple mean comparisons (*P < 0.05).



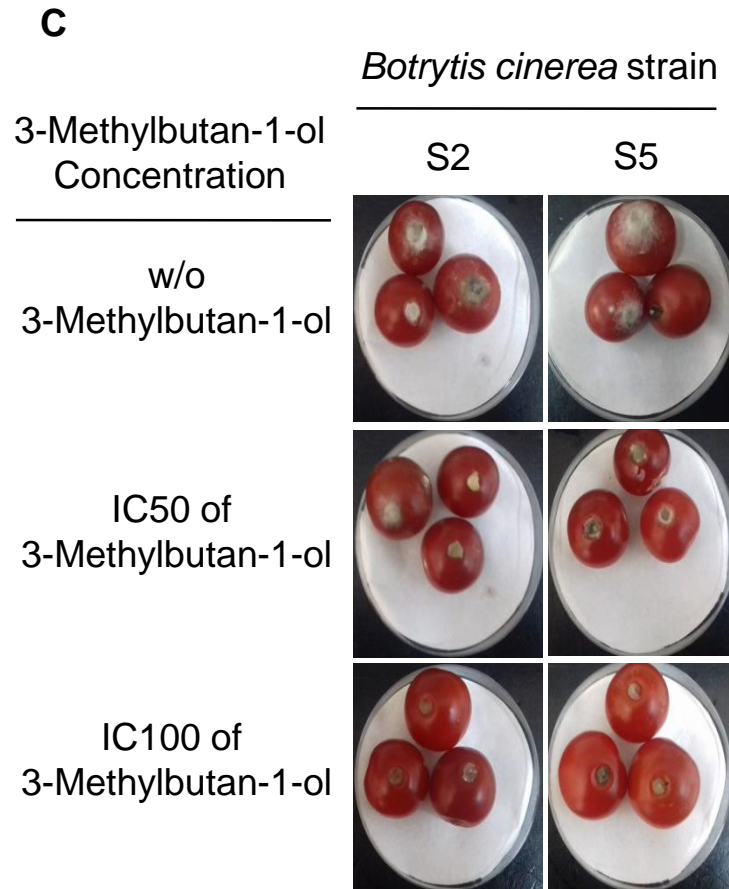


Fig. 10. Effect of 3-Methylbutan-1-ol on *Botrytis cinerea* infection on tomato fruit. Boxplots represent the diameter (A) and the depth (B) of rotting caused by S2 and S5 *Botrytis cinerea* strains or control condition (inoculated with PDA medium) at 6 d after infection on tomato fruit exposed or not to 3-Methylbutan-1-ol at the half maximal inhibitory concentration (IC50 = 0.221 mL L⁻¹) and the maximal inhibitory concentration (IC100 = 0.442 mL L⁻¹). (C) Photos of tomato fruit at 6 d after infection with *B. cinerea* strains and exposed or not to IC50 and IC100 of 3-Methylbutan-1-ol. Data were recorded from two independent experiments. Asterisk into brackets indicates significant difference between *B. cinerea* inoculated fruit in presence or not of 3-Methylbutan-1-ol as revealed by Dunnett's test (*P < 0.05). Significance codes for *p-values*: 0 '****' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1.

Table 1. Type III analysis of variance of *Botrytis cinerea* mycelia growth under the influence endophytic bacterial volatile organic compounds.

Source of variation	df	7 d		14 d		21 d		AUGPC	
		F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
Bacteria strain	50	6.8940	9.417e-15 ***	6.9942	4.022e-15 ***	4.4705	6.308e-09 ***	7.4125	5.675e-16 ***
Botrytis strain	1	1.2470	0.2655	11.6370	0.0007872 ***	11.6780	0.0007727 ***	7.8017	0.005745 **
Bacteria strain * Botrytis strain	50	3.0742	1.631e-08 ***	3.8637	8.072e-12 ***	3.7161	3.647e-11 ***	4.2633	1.909e-13 ***

The analysis was performed based on fungal growth kinetics (at 7, 14 and 21 d of co-culture with endophytic bacteria) and area under growth progress curve (AUGPC). Data were analyzed with a mixed linear model where endophytic bacteria, *Botrytis* strain and their interaction were considered as fixed effects. Independent experiment was treated as a random effect. AUGPC: Area Under Growth Progress Curve. Significance codes for *p-values*: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '.' 1.

Table 2. Kinetic and persistence of antifungal activity of endophytic bacterial volatile organic compounds (VOCs) against *Botrytis cinerea* strains S2 and S5.

Strain ID	Bacteria species	Antifungal activity against S2 strain				Antifungal activity against S5 strain			
		AUGPC	7 d	14 d	21 d	AUGPC	7 d	14 d	21 d
H1F1	<i>Bacillus amyloliquefaciens</i>	Dark Orange	Light Orange	Light Orange	Light Orange	White	White	White	White
DHF4	<i>Enterobacter asburiae</i>	Dark Orange	Light Orange	Light Orange	Light Orange	White	White	White	White
H1Fr2	<i>Bacillus vallismortis</i>	Dark Orange	Light Orange	Light Orange	Light Orange	White	Light Blue	White	White
DHT2	<i>Bacillus pseudomycooides</i>	Dark Orange	Light Orange	Light Orange	Light Orange	White	White	White	White
DHF7	<i>Bacillus subtilis subsp.spizezenii</i>	Dark Orange	Light Orange	Light Orange	Light Orange	White	White	White	White
H1F2	<i>Bacillus velezensis</i>	Dark Orange	Light Orange	Light Orange	Light Orange	White	White	White	White
DHP3	<i>Bacillus subtilis</i>	Dark Orange	Light Orange	Light Orange	Light Orange	White	White	White	White
H1R2	<i>Bacillus thuringiensis</i>	Dark Orange	Light Orange	Light Orange	Light Orange	White	White	White	White
DHF5	<i>Bacillus toyonensis</i>	White	White	White	White	Dark Blue	Dark Blue	Dark Blue	Dark Blue
H2F12	<i>Bacillus velezensis</i>	White	White	White	White	Dark Blue	Dark Blue	Dark Blue	Dark Blue
H2F1	<i>Bacillus proteolyticus</i>	White	White	White	White	Dark Blue	Dark Blue	Dark Blue	Dark Blue
TR1	<i>Enterobacter cloacae</i>	Dark Orange	Light Orange	Light Orange	Light Orange	Dark Blue	Dark Blue	Dark Blue	White
TR2	<i>Bacillus nakamurai</i>	Dark Orange	Light Orange	Light Orange	White	Dark Blue	Dark Blue	Dark Blue	White
H1T3	<i>Bacillus velezensis</i>	White	Light Orange	White	White	Light Blue	Dark Blue	Light Blue	White
TR3	<i>Bacillus amyloliquefaciens</i>	White	Light Orange	White	White	Light Blue	Dark Blue	Light Blue	White
DHF4	<i>Bacillus thuringiensis</i>	White	Light Orange	White	White	White	Dark Blue	White	White
BF11	<i>Bacillus velezensis</i>	White	White	White	White	White	Dark Blue	White	White
H1Fr3	<i>Bacillus velezensis</i>	White	White	White	White	White	Dark Blue	White	White

Only endophytic bacteria strains producing antifungal VOCs are presented here. Dark Orange and dark blue colors in AUGPC (Area Under Growth Progress Curve) and fungal mycelia growth kinetic at 7, 14 and 21 d indicate significant differences in comparison to the control ‘without bacteria’ using Dunnett test at $p\text{-value} \leq 0.05$ using adjusted means from mixed linear models. Light orange and light blue colors indicate sub-significant differences using Dunnett test at $p\text{-value} \leq 0.1$. White color indicates the absence of significant antifungal activity in comparison to the control ‘without bacteria’. Bacterial species were identified on the basis of 16S rRNA gene sequences and homology search in Genbank database. Bacteria strains in bold were selected on the basis of their high and persistent VOCs antifungal activities against one or both *B. cinerea* strains.

Table 3. Type III analysis of variance of seedling growth under the influence of endophytic bacterial volatile organic compounds.

Source of variation	df	Seedling length		Fresh weight		Dry weight	
		F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
Bacteria strain	50	5.2195	1.676e-09 ***	19.884	< 2.2e-16 ***	2.1235	0.006331 **

Data were analyzed with a mixed linear model where endophytic bacteria were considered as fixed effects and independent experiment was considered as a random effect. In order to normalize residuals of MLM, log₁₀ (Fresh weight) and log₁₀ (Dry weight) transformations were used. Significance codes for *p-values*: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

Table 4. Type III analysis of variance of tomato seed germination under the influence of endophytic bacterial volatile organic compounds.

Source of variation	df	Germination rate	
		LRT	Pr(Chi)
Bacteria strain	50	324.44	< 2.2e-16 ***

Data were analyzed with a generalized linear mixed model where endophytic bacteria were treated as fixed effects and independent experiment considered as random effect. LRT (Likelihood Ratio Test) and *p-value* comparing the model with or without fixed factor are reported. Significance codes for *p-values*: 0 '****' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

Table 5. Type III analysis of variance of *Botrytis cinerea* mycelia growth under the influence of endophytic bacterial soluble compounds at 7 d of co-culture.

Source of variation	df	Fungal growth	
		F value	Pr(>F)
Bacteria strain	5	60.2059	<2e-16 ***
Botrytis strain	1	2.6826	0.1067
Bacteria strain * Botrytis strain	5	0.6562	0.6580

Data were analyzed with a mixed linear model where endophytic bacteria and *B. cinerea* strains and their interaction were considered as fixed effects and independent experiment was treated as a random effect. Significance codes for *p-values*: 0 '****' 0.001 '***' 0.01 '**' 0.05 '*' 0.1 '.' 1.

Table 6. Type III analysis of variance of *Botrytis cinerea* infection on tomato fruit under the influence of endophytic bacterial volatile organic compounds at 6 d post-infection.

Source of variation	df	Diameter of rotting		Depth of rotting	
		F value	Pr(>F)	F value	Pr(>F)
Bacteria strain	5	3.7286	0.087486	1.4592	0.20479
Botrytis strain	1	14.5479	0.000183 ***	1.7555	0.18670
Bacteria strain * Botrytis strain	5	0.5880	0.709189	1.0594	0.38416

A split plot design was used in this assay. The experiment was repeated three times with two blocks for each replicate. Data were analyzed with a mixed linear model where endophytic bacteria, *B. cinerea* strains (S2 and S5) and their interaction were considered as fixed effects, whereas the replicate, block and whole plot (Plastic box) were treated as random effects. The data of mock-inoculated fruits i.e. fruits inoculated with PDA medium instead of the *B. cinerea* culture were not included in the statistical analysis. Significance codes for *p-values*: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '.' 1.

Table 7. Type III Analysis of variance of *Botrytis cinerea* infection on tomato fruit after treatment with vegetative cells of endophytic bacteria at 6 d post-infection.

Source of variation	df	Diameter of rotting		Depth of rotting	
		F value	Pr(>F)	F value	Pr(>F)
Bacteria strain	5	1.8734	0.25374	3.4324	0.1010336
Botrytis strain	1	370.3763	< 2e-16 ***	13.1802	4.327e-06 ***
Bacteria strain* Botrytis strain	5	1.6731	0.08952 .	3.4896	0.0003036 ***

A split plot design was used in this assay. The experiment was repeated three times with two blocks for each replicate. Data were analyzed with a mixed linear model where endophytic bacteria, *B. cinerea* strains (S2 and S5) and their interaction were treated as fixed effects, whereas replicate, Block and whole plot (Plastic box) were treated as random effects. The data of mock-inoculated fruits i.e. fruits inoculated with PDA medium instead of the *B. cinerea* culture were not included in the statistical analysis. Significance codes for *p-values*: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

Table 8. Type III analysis of variance of *Botrytis cinerea* mycelia growth in presence of the pure volatile compound 3-Methylbutan-1-ol (3-MBO) at 5 days of co-culture.

Source of variation	df	Fungal mycelia growth	
		F value	Pr(>F)
Fungal_strain	1	8.1848	0.005065 **
3-MBO concentration	10	177.8352	< 2.2e-16 ***
Fungal_strain*3-MBO concentration	10	6.9283	2.546e-08 ***

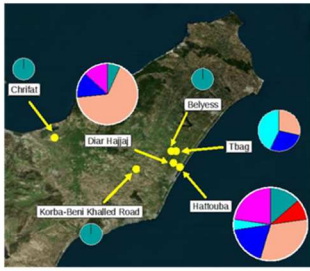
Data were analysed with a mixed linear model where 3-Methylbutan-1-ol concentration and *B. cinerea* strains and their interaction were considered as fixed effects and independent experiment was treated as a random effect. Significance codes for *p*-values: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

Table 9. Type III analysis of variance of *Botrytis cinerea* infection on tomato fruit in presence of the pure volatile compound 3-Methylbutan-1-ol (3-MBO) at 6 days post infection.

Source of variation	df	Diameter of rotting		Depth of rotting	
		F value	Pr(>F)	F value	Pr(>F)
Fungal_strain	1	6.6690	0.01513*	0.1437	0.7074
3-MBO concentration	2	17.5157	1.028e-05***	4.1905	0.0252*
Fungal_strain*3-MBO concentration	2	1.8062	0.18227	0.1437	0.8668

A split plot design was used in this assay and the experiment was repeated two times. Data were analysed with a mixed linear model where 3-Methylbutan-1-ol concentration, *B. cinerea* strain (S2 and S5) and their interaction were considered as fixed effects, whereas the replicate was treated as random effect. The data of mock-inoculated fruits i.e. fruits inoculated with PDA medium instead of the *B. cinerea* culture were not included in the statistical analysis. Significance codes for *p*-values: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

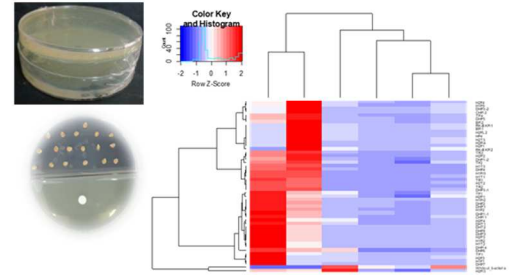
Abundance and *in planta* distribution of endophytic bacteria differ even at short distances



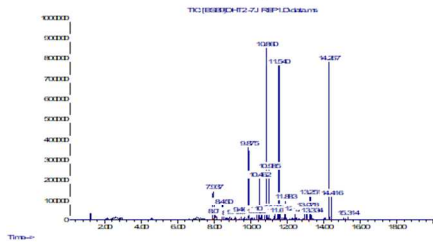
Endophytic bacteria mainly colonize leaves, with significant enrichment in *Bacillus* genus within sequenced bacteria



Selection of 5 endophytic bacteria based on antifungal and plant growth promoting activities of volatile organic compounds



Endophytic bacteria produce volatiles with antifungal activities



50 endophytic bacteria strains isolated from tomato organs



Selected endophytic bacteria produce

- hydrolytic enzymes,
- Siderophores,
- soluble compounds with antifungal activities

Bacteria volatile and soluble compounds reduce *Botrytis cinerea* infection on tomato fruits

