

In vitro* and *in vivo* potential of Plant Growth-Promoting Rhizobacteria as biological control agents against *Alternaria terricola

Running title: PGPRs application to control *Alternaria terricola*

Hanene Cherif¹, Amira Oueslati¹, Bilel Bejaoui¹, Mouna Mahjoubi¹, Yosra Amara¹, Yasmine Souissi², Ameer Cherif¹, and Leila Riahi^{1*}

¹ Laboratory of Biotechnology and Bio-Geo Resources Valorization BVBGR-LR11ES31, Higher Institute of Biotechnology of Sidi Thabet, University of Manouba, ISBST, Ariana 2020, Tunisia.

² Department of Engineering, German University of Technology in Oman, P.O. Box 1816, PC 130, Muscat, Sultanate of Oman.

*Corresponding author; e-mail: leila.ba.riahi@gmail.com

ABSTRACT

In this study, the antagonistic effects of 14 Plant Growth-Promoting Rhizobacteria strains (PGPRs) against the phytopathogenic species *Alternaria terricola* Woudenb. & Crous, both *in vitro* and *in vivo* were investigated. The obtained results revealed significant inhibition effects of the 14 PGPR strains against *A. terricola* in both direct contact and indirect bioassays with significant variation. The dual *in vitro* culture tests revealed substantial inhibition rates in the growth of *A. terricola* strain, ranging from 25±5.41% (*Pseudomonas koreensis* O3RR25) to 71.87±3.12% (*Bacillus megaterium* FR1.11). Moreover, the indirect antagonism test showed that the volatile organic compounds produced by the 14 tested PGPR strains significantly inhibited the growth of *A. terricola* mycelium, with variations ranging from 36.61±0.94% (*P. brassicacearum* O3RR24) to 67.75±0.94% (*B. megaterium* FR1.11). Microscopic examination of *A. terricola* following exposure to the volatile compounds revealed significant structural damage, including inhibition of conidial germination, deformations, thin or fissured structures, irregular lengths, and the formation of empty segments. The *in vivo* application of *B. megaterium* FR1.11 resulted in the reduction of fungal development on detached leaves and tomato seedlings. This treatment engendered a significant increase in the levels of chlorophyll *a*, *b* and total, carotenoids, polyphenols, and proline in infected tomato seedlings compared to the control. Applying this PGPR strain to infected tomato plants allowed maintaining comparable level of malondialdehyde as the control. *B. megaterium* FR1.11 showed considerable *in vitro* and *in vivo* antifungal activity and could serve as a promising candidate for biological control strategies targeting phytopathogenic species of the genus *Alternaria*.

37 **Keywords:** *Alternaria* spp., Biofungicides, Environment, PGPRs, Plant protection, Tomato.

38
39 **INTRODUCTION**

40 Fungal pathogens pose a significant biotic stress that adversely affects agricultural crop
41 productivity and quality under various production systems, including fields and greenhouses
42 but also at post-harvest handling which poses a serious threat to global food security (Ferraz *et al.*,
43 2019). Fungal diseases lead to substantial additional losses during crop transportation and
44 storage (Dukare *et al.*, 2019). Among these phytopathogens, fungi of the *Alternaria* genus are
45 particularly troublesome, as they are difficult to control and have a widespread presence. They
46 cause significant yield and quality reductions in agronomic, ornamental, and medicinal crops
47 (Puvača *et al.*, 2020). Additionally, *Alternaria* species are common mycotoxigenic fungi found
48 in cereals but they cause diseases in various other plant families such as Solanaceae,
49 Cucurbitaceae, and Brassicaceae. The recorded losses attributed to *Alternaria* range from 50%
50 to 86% for tomatoes (Florea and Puia, 2020) and from 80% to 100% for potatoes (Singh *et al.*,
51 2020).

52 The modern intensification of agricultural systems, characterized by the cultivation of
53 genetically uniform crop varieties and increased international trade, combined to the drastic
54 climate changes have accelerated the spread and emergence of new fungal strains (Fisher *et al.*,
55 2018). Since the 1940s, the primary approach to controlling fungal diseases in most crops has
56 been the application of chemical fungicides (Dukare *et al.*, 2019). While the use of chemical
57 pesticides has indeed improved crop quality and yields, their effectiveness has been diminishing
58 over time, necessitating higher and more frequent doses which have led to an increase in the
59 development of fungal resistance (Gupta, 2018).

60 In recent years, there has been growing global concern regarding the harmful effects of
61 fungicides on human health, crops, fauna, flora, and the environment (Rani *et al.*, 2021). Among
62 the environmentally friendly alternatives, biological control applying beneficial
63 microorganisms such as bacteria, filamentous fungi, and yeasts, along with their metabolites
64 exhibiting antagonistic activity against phytopathogenic fungi, has gained significant attention
65 (Elnahal *et al.*, 2022). This strategy involves the application of live microorganisms to reduce
66 and/or maintain the population of plant pathogens below levels that cause economic losses
67 (Fernandez-San Millan *et al.*, 2021). Implementing this approach provides a safe, effective, and
68 environmentally friendly alternative to the use of synthetic fungicides (Karthika *et al.*, 2020).

69 Among the biological control agents against phytopathogenic fungi, several reports indicate a
70 significant potential for PGPRs bacteria (Parasuraman *et al.*, 2022). In addition to their role in

71 improving growth, PGPR bacteria act as biological control agents against fungal agent diseases
72 through various direct and indirect mechanisms which vary among the applied strains (Wang
73 *et al.*, 2021). While there is an increasing interest in the application of PGPRs to control species
74 within the genus *Alternaria* (Soliman *et al.*, 2023), only the research conducted by Cherif *et al.*
75 (2022) focused on the phytopathogenic agent *A. terricola*, and their findings were limited to *in*
76 *vitro* bioassays. Furthermore, the studies examining the impact of PGPR volatile organic
77 compounds (VOCs) and the *in vivo* effects of PGPRs against *A. terricola* are lacking.

78 This study aims to assess the *in vitro* antagonistic activity of 14 plant growth-promoting
79 rhizobacteria strains (PGPRs) against *A. terricola* Woudenb. & Crous (Woudenberg *et al.*,
80 2013) using direct contact bioassays as well as through the effects of PGPRs volatile organic
81 compounds (VOCs). The most promising bacterial strain exhibiting higher *in vitro* antagonistic
82 activity was further evaluated *in vivo* using tomato as a model plant based on detached leaf tests
83 and pot assays. The impact of the employed biological control agent on the modulation of
84 physiological and biochemical traits, including chlorophyll *a*, *b*, total chlorophyll, carotenoids,
85 proline, and malondialdehyde (MDA), was investigated.

86

87 MATERIALS AND METHODS

88 Microbial strains

89 Fourteen PGPR strains from the BVBGR-LR11ES31 laboratory collection were tested for their
90 biological control potential. PGPR strains names and accession numbers are listed in Table 1.
91 The tested PGPR strains were isolated from rhizospheric soil fractions of fig and olive trees
92 that have been irrigated for more than 20 years with treated municipal wastewater, in the region
93 of M'saken, Tunisia. The strain *A. terricola* MF480416.1 Woudenb. & Crous (Woudenberg *et*
94 *al.*, 2013) was isolated and molecularly identified from wheat leaves of the variety Karim
95 exhibiting fungal disease symptoms, collected from an agricultural field in the Beja region
96 (Cherif *et al.*, 2022). The preliminary assays conducted in our laboratory confirmed the
97 pathogenic nature of this fungal strain on tomato seedlings. Molecular identification of the
98 PGPR strains was achieved using the 16S rDNA ribosomal operon and the ITS 16S-23S
99 intergenic spaces (Cherif *et al.*, 2003).

100

101 Plant material and culture conditions

102 The tomato variety Rio Grande (*Solanum lycopersicum* L., Solanaceae family) was used in this
103 study to investigate the PGPR *in vivo* antifungal activity. The seeds were disinfected using a
104 1% sodium hypochlorite solution, rinsed three times with sterile distilled water then placed in

105 pots (10 cm x 8 cm) containing a mixture of Pindstrup commercial peat (1 V) and clay-loamy
106 agricultural soil (2 V) to germinate in a greenhouse under semi-controlled conditions
107 (photoperiod: 16/8 hours, temperature: 26.5°C, humidity: 51%).

108

109 ***In vitro* antagonism bioassays**

110 The antagonistic activity of bacterial strains against *A. terricola* strain was achieved using the
111 dual confrontation test and the *in vitro* assay for volatile metabolites following Haidar *et al.*
112 (2016). For a direct test, the percentage inhibition (PI) was calculated using the formula: $PI (\%) = [(R1 - R2) / R1] * 100$, where R1 represents the radial distance in mm of the fungus growth
113 = $[(R1 - R2) / R1] * 100$, where R1 represents the radial distance in mm of the fungus growth
114 for the control, and R2 represents the distance in mm of the fungus' growth after treatment,
115 measured from the point of inoculation towards the PGPR strain. For the indirect test, the
116 percentage of inhibition (I %) of mycelial growth was calculated using the formula $PI (\%) =$
117 $[(D1 - D2) / D1] * 100$, where D1 represents the diameter of the pathogenic fungus in the
118 absence of the antagonist agent, and D2 represents the diameter of the pathogenic fungus in the
119 presence of the antagonistic agent (Haidar *et al.*, 2016).

120

121 ***In vivo* antifungal activity**

122 The detached leaves of one-month-old tomato seedlings were disinfected with 1% sodium
123 hypochlorite and alcohol 70% for 1-2 minutes then rinsed three times with sterile physiological
124 water. The leaves were placed in compartmentalized Petri dishes on sterile filter paper soaked
125 with sterile physiological water. Aliquots of 10 μ L of the fungal spore suspension (sterile
126 distilled water for the control), adjusted to 5×10^5 conidia/mL, were applied to the adaxial
127 surface of the detached leaves. The PGPR strains were sub-cultured on Tryptic Soy Agar (TSA)
128 medium in the other compartment of the Petri dish (Bahramisharif and Rose, 2019).

129 The *in vivo* antifungal potential of the rhizobacterial strain was assessed on 45-day-old tomato
130 seedlings. The seedlings were divided into six groups, each subjected to a specific treatment:
131 (1) control, (2) infected control (10^6 conidia/mL), (3) seedlings soil-inoculated with the PGPR
132 strain 10^8 colony forming unit/mililitre (CFU/mL), (4) seedlings inoculated with PGPR then
133 infected with *A. terricola*, (5) seedlings exposed to PGPR VOCs (6) seedlings exposed to VOCs
134 then infected with *A. terricola*. All pots were covered with transparent bags to capture the
135 volatile organic compounds emitted by the PGPR strain. The experiments were conducted with
136 ten repetitions. Seedlings were exposed to the VOCs of the PGPR strain by placing the tomato
137 pots near the PGPR cultures on open Petri dishes, without lids (Attia *et al.*, 2020).

138

139 **Studied parameters**

140 The symptomatic study was conducted 10 days after the treatments. Optical microscopy was
141 used to assess the development of the fungus under various different treatments. The method
142 described by Lichtenthaler and Wellburn (1983) was employed to measure the levels of
143 Chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll (ChlT), and carotenoids (Carot) in
144 tomato leaves. The contents were expressed as milligrams per gram of fresh weight (mg/g FW).
145 The total polyphenol contents were assessed using the Folin-Ciocalteu method (Singleton and
146 Rossi, 1965). The results are reported as milligrams of gallic acid equivalent per gram of fresh
147 weight (mg GAE/g FW). Proline contents were determined following the method described by
148 Bates *et al.* (1973) and expressed as micrograms per gram of fresh weight ($\mu\text{g/g}$ FW).
149 Malondialdehyde (MDA) contents were determined based on the method outlined by Doblinski
150 *et al.* (2003) and expressed as nanomoles per gram of fresh weight (nmol/g FW).

151 **Data analysis**

152 The analysis of variance was conducted with one classification factor to evaluate the variation
153 of the studied parameters. Mean comparisons were performed using Duncan's test at a
154 significance level of 0.05. The statistical analyses were carried out using IBM SPSS Statistics
155 software, version 28.0 for Windows.

156

157 **RESULTS**

158 ***In vitro* antagonism test**

159 ***In vitro* direct bioassays**

160 The 14 tested PGPR strains induced a reduction in the growth of *A. terricola* with variable
161 degrees (Figure 1a). A noticeable change in the colour of *A. terricola* colonies from greenish
162 black (control) to whitish or greyish was recorded. The inhibition percentages obtained after 10
163 days of incubation using the direct test are presented in Figure 2a. The six PGPR strains *P.*
164 *reinekei* O3R52, *B. megaterium* FR1.11, *P. siccitolerans* O3RR17, *B. wiedmannii* FR1.35, *B.*
165 *frigotolerans* FR1.38, and *B. oceanisediminis* FR1.5 have inhibition rates over 60%. The PGPR
166 strain O3RR25 (*P. koreensis*) displayed the lowest inhibition rate ($25\pm 5.41\%$), whereas the
167 highest inhibition rate ($71.87\pm 3.12\%$) was recorded with the strain FR1.11 (*B. megaterium*).

168

169 ***In vitro* indirect bioassays**

170 The macroscopic observations obtained with the indirect antagonism test after 10-day
171 incubation showed that the growth of *A. terricola* mycelia exposed to the volatile metabolites
172 was significantly inhibited compared to the control (Figure 1b). The macroscopic observation
173 revealed a change in the appearance and the color of the colonies from greenish-black to

174 whitish, particularly at the colony's extremities. The inhibition rates obtained using the indirect
175 test reveal that all the PGPR strains produce volatile substances that inhibit significantly the
176 growth of the *A. terricola* strain, with significant variation (Figure 2b). The inhibition
177 percentages range from $36.61 \pm 0.94\%$ (*P. brassicacearum* O3RR24) to $67.75 \pm 0.94\%$ (*B.*
178 *megaterium* FR1.11). Four PGPR strains inhibited *A. terricola* by over 60%: O3R52
179 ($60.10 \pm 4.12\%$), *B. megaterium* FR1.11 ($67.75 \pm 0.94\%$), *B. zhangzhouensis* O3RR35
180 ($58.46 \pm 1.89\%$), and *B. oceanisediminis* FR1.5 ($59.56 \pm 3.41\%$).

181 A microscopic examination of the mycelium of *A. terricola* following exposure to the volatile
182 compounds produced by the applied PGPR strains was carried out. Intact cell walls with regular
183 lengths and uniform structures were observed for the hyphae of untreated *A. terricola* (Figure
184 3). However, mycelium hyphae treated with *A. terricola* VOCs displayed wrinkled surfaces,
185 deformations, and irregular lengths, often accompanied by empty segments (indicated by red
186 arrows). Thin or fissured structures (highlighted by yellow arrows), and globular swellings at
187 the ends of the mycelial strands (marked with black arrows) were noted. A significant inhibition
188 of conidial germination was observed compared to the control group (indicated by blue arrows).
189 Furthermore, certain conidia formed irregular germination tubes, notably shorter than those in
190 the control group (indicated by green arrows).

191

192 ***In vivo* antifungal activity**

193 The strain *B. megaterium* FR1.11, showing the highest *in vitro* antagonism potential, was
194 selected to conduct *in vivo* bioassays. The development and spread of disease symptoms caused
195 by *A. terricola* in detached leaves were effectively inhibited by the VOCs of the tested PGPR
196 (Figure 4). Tomato leaves exposed to VOCs exhibited discoloration and yellowing. Leaves
197 infected with *A. terricola* displayed necrotic spots (2 to 5 mm), dark brown cankers, and some
198 lesions on the tips of certain leaves. When the tomato leaves infected with *A. terricola* were
199 exposed to PGPR VOCs, no necrotic spots were shown confirming the antifungal effect of these
200 volatile substances.

201 The microscopic examination showed that *A. terricola* in infected leaves treated with PGPR
202 VOCs had lost their germination ability and/or exhibited morphological deformations (Figure
203 4). Leaves infected with *A. terricola*, in the absence of PGPR VOCs, exhibited successfully
204 germinated conidia with well developed mycelium showing regular tubes (Figure 4 a). The
205 normal hyphae displayed smooth surfaces, consistent lengths, and intact structures with
206 segmented mycelium. In the presence of PGPR VOCs, the conidia of *A. terricola* have lost their
207 capacity to germinate and developing mycelium, as shown by red arrows in Figure 4 b,

208 rendering them unable to generate appressoria or form infection structures on the leaf. Notably,
209 the volatile substances produced by the PGPR strain significantly reduced the number of
210 conidia on the detached tomato leaves.

211 For the pot seedlings bioassays, after 10 days of treatment, the treated plants displayed less
212 pronounced symptoms compared to the infected control. The symptoms were limited to pale
213 yellow spots with no signs of spreading. Seedlings infected with *A. terricola* exhibited
214 symptoms namely yellowish and brown spots and more advanced physiological decline.
215 Microscopic observations of leaves revealed variations in the developmental stages of the
216 fungus (Figure 5). The leaves infected with *A. terricola* showed full development of the fungus
217 with formed mycelia and conidia. The inhibition of *A. terricola* development was detected on
218 tomato leaves following soil-inoculation with the strain *B. megaterium* FR1.11 and exposure to
219 VOCs. Fewer conidia and morphological abnormalities were observed with these two
220 treatments, indicating their inhibitory effects on the growth of *A. terricola*.

221

222 **Variation in photosynthetic pigment contents**

223 The obtained results showed significant variations in photosynthetic pigments compared to the
224 control (Figure 6). A significant decrease in the contents of chlorophyll *a*, *b*, and total
225 compared to the control (Chla: 1.049 ± 0.008 ; Chlb: 0.321 ± 0.016 , ChlT: 1.370 ± 0.015 mg/g
226 FW) was observed for the seedlings infected with *A. terricola*. However, no significant
227 variation was detected in carotenoid content for infected seedlings compared to the control
228 (0.203 ± 0.023 mg/g FW).

229 The treatment with *B. megaterium* FR1.11 volatile compounds did not cause significant
230 changes in the contents of Chl *a*, *b*, T, and carotenoids compared to the control. PGPR treatment
231 in the absence of fungal infection resulted in the highest contents of photosynthetic pigments
232 (Chla: 1.877 ± 0.094 ; Chlb: 0.628 ± 0.023 ; ChlT: 2.505 ± 0.076 ; Carot: 0.334 ± 0.014 mg/g FW),
233 followed by the fungus + *B. megaterium* FR1.11 treatment, which exhibited a significant
234 increase compared to the control. Exposure of infected seedlings to volatile metabolites of the
235 PGPR strain resulted in a significant increase in Chl *a* and Chl T levels compared to the control,
236 with no significant variation observed for chlorophyll *b* and carotenoids.

237

238 **Variation in total polyphenol contents**

239 The contents of total phenolic compounds in the ethanolic extracts of tomato leaves were
240 determined using a standard range of gallic acid (Figure 7). The results indicate no significant
241 variation in total polyphenol content compared to untreated seedlings (0.640 ± 0.023 mg GAE

242 /g FW) for the VOCs and *A. terricola*+VOCs treatments. However, significant variations in the
243 levels of total phenolic compounds were observed in tomato seedlings with the other treatments.
244 The highest significant increase was observed in tomato seedlings inoculated with the PGPR
245 strain *B. megaterium* FR1.11 (0.828 ± 0.021 mg GAE/g FW) and those infected with *A. terricola*
246 (0.847 ± 0.026 mg GAE/g FW), followed by the *A. terricola*+PGPR treatment.

247 248 **Variation in proline levels**

249 The obtained results demonstrate a significant increase in proline levels in the treated tomato
250 leaves compared to the control conditions (40.57 ± 1.81 μ g/g FW) across all treatments (Figure
251 7). The treatment of *A. terricola* + PGPR *B. megaterium* FR1.11 exhibited the highest
252 accumulation of proline (69.78 ± 2.29 μ g/g FW), followed by the individual PGPR and *A.*
253 *terricola* treatments. A significant increase in proline levels, compared to the control, was also
254 observed with the VOCs and *A. terricola*+VOCs treatments.

255 256 **Variation in malondialdehyde (MDA) contents**

257 The contents of MDA exhibited a significant variation compared to the control (4.73 ± 0.26
258 nmol/g FW), as shown in Figure 7. The highest increase in MDA content (7.59 ± 0.41 nmol/g
259 FW) was detected following the infection of tomato seedlings by *A. terricola*. Interestingly, a
260 significant decrease in MDA levels (3.65 ± 0.24 nmol/g FW) was observed when tomato
261 seedlings were inoculated with the PGPR *B. megaterium* FR1.11 strain, compared to untreated
262 seedlings. No significant variation compared to the control was observed for this parameter in
263 the VOCs, *A. terricola*+PGPR, and *A. terricola*+VOCs treatments.

264 265 **DISCUSSION**

266 *A. terricola* is known to be a phytopathogenic agent affecting various agronomic crops,
267 including wheat (Imran *et al.*, 2011) and red pepper (Nahar *et al.*, 2004). Except the study by
268 Cherif *et al.* (2022), which focused on the effect of three PGPR strains on the species *A.*
269 *terricola* through *in vitro* direct antagonism tests, no other study has been reported for this
270 phytopathogenic agent. In this investigation, the *in vitro* antagonism assays based on both direct
271 and indirect tests, revealed that the tested 14 PGPR strains exhibited significant inhibition of
272 the growth of the *A. terricola* strain. Among the tested PGPR strains, *B. megaterium* FR1.11
273 exhibited the highest inhibition rates against the growth of *A. terricola* in both confrontation
274 and exposure to volatile compounds in the *in vitro* tests. *In vivo* investigations further supported
275 these findings, showing that the application of PGPR strain *B. megaterium* FR1.11 led to

276 reduced development of the symptoms of *A. terricola* on detached leaves and tomato seedlings,
277 whether through the effect of volatile compounds or by inoculating the strain in the soil.
278 The observed antifungal activity of the strain *B. megaterium* FR1.11 is likely attributed to its
279 volatile metabolites, as evidenced by both *in vitro* and *in vivo* studies. The effectiveness of
280 PGPR in hindering the germination and development of fungal species on detached leaves
281 confirms that volatile organic compounds are among the direct mechanisms of biological
282 control employed by PGPR strains (Bahramisharif and Rose, 2019). However, the antifungal
283 effect observed with soil inoculation suggests that PGPR strain *B. megaterium* FR1.11 may
284 employ multiple modes of action against the tested strain of *A. terricola*. *Bacillus* and
285 *Pseudomonas* species are the commonly utilized PGPRs in the biological control of plant
286 pathogens. These bacteria exhibit fast germination in soil and possess high colonization
287 capabilities (Ali *et al.*, 2020).

288 PGPR strains possess the capability to produce various secondary metabolites such as hydrogen
289 cyanide (HCN), cell wall degrading enzymes, 1-aminocyclopropane-1-carboxylate (ACC)
290 deaminase, diffusible or volatile antibiotics, and siderophores (Hassen *et al.*, 2018). These
291 metabolites play a role in limiting or eliminating fungal phytopathogens (Cherif *et al.*, 2022).
292 The biological control agents employ three primary mechanisms to combat the harmful effects
293 of plant pathogenic microorganisms, namely antibiosis, siderophore production, and parasitism
294 through the secretion of catalytic enzymes like chitinases, lipases, and proteases (Ali *et al.*,
295 2020). Additionally, PGPR bacteria can indirectly act as biological control agents by inducing
296 enhanced immunity in the target plants and by modulating endogenous phytohormones and
297 amino acid levels (Syed Nabi *et al.*, 2021).

298 The findings of this study align with previous studies that have demonstrated a significant
299 decrease in the levels of chlorophyll *a*, *b*, and total in tomato plants infected with *Alternaria*
300 species, while carotenoid contents remained relatively stable compared to the control group
301 (Attia *et al.*, 2020). A reduction in the photosynthetic pigments of tomato inoculated with
302 *Alternaria solani* was also reported by Rasool *et al.* (2021). Chlorophyll and carotenoid
303 contents are considered significant indicators of photosynthetic performance in plants (Riahi *et*
304 *al.*, 2020). The application of the PGPR bacterial strain in this study resulted in a significant
305 increase in the concentrations of chlorophyll pigments and carotenoids in tomato seedlings.
306 These results are consistent with other studies that have reported higher photosynthetic pigment
307 contents in tomato leaves treated with PGPR bacteria (Attia *et al.*, 2020).

308 The results of this study demonstrate a significant increase in proline and total polyphenol
309 content in tomato plants inoculated with the PGPR strain. Moreover, these levels were further

310 elevated in plants infected with the fungus and pre-inoculated with the PGPR strain. These
311 findings are consistent with previous investigations that have reported similar patterns of
312 variation (Kousar *et al.*, 2020). Phenolic compounds act as natural antioxidants and are
313 synthesized by plants in response to different stresses to facilitate their adaptation (Chiappero
314 *et al.*, 2019). The positive impact of PGPR inoculants on the metabolism of phenolic
315 compounds has also been observed in other plant species (Riahi *et al.*, 2020).

316 The production of proline induced by the PGPR strain highlights the ability of this inoculation
317 to enhance the plant's tolerance to osmotic stress under normal conditions. Proline serves as an
318 osmoregulator and accumulates in plants in response to a wide range of stress conditions
319 (Khanna *et al.*, 2019). The accumulation of cellular osmolytes, including proline, helps plants
320 to maintain essential cellular functions and physiological stability (Kousar *et al.*, 2020). Proline
321 and other osmolytes play a protective role by regulating water and nutrient balance, stabilizing
322 membrane structures, supporting the function of various enzymes and proteins, and providing
323 protection against reactive oxygen species (ROS) and other biochemical reactions (Khanna *et*
324 *al.*, 2019).

325 The obtained results showed a significant increase in MDA levels for tomato seedlings infected
326 with *A. terricola*. This aligns with previous findings which highlighted a significant elevation
327 in MDA, used as a stress indicator, when tomato plants were infected with *A. solani*, as
328 compared to their healthy plants (Daigham *et al.*, 2023). Furthermore, following infection with
329 *A. alternate*, the MDA contents were reported to increase in the leaves of cucumber (Wang *et*
330 *al.*, 2020) and pepper (Kazerooni *et al.*, 2021).

331 In this study, pre-treatment with the PGPR strain resulted in a significant reduction in MDA
332 content. The MDA contents decreased significantly compared to the infected plants and reached
333 levels similar to the control after treatment with the PGPR strain or exposure to its volatile
334 compounds. This indicates a reduction in the degree of membrane lipid oxidation and a decrease
335 in damage caused by *A. terricola* to tomato leaf tissue. **Indeed**, the accumulation of MDA serves
336 as an indicator of the extent of membrane peroxidation in plant cells (Gong *et al.*, 2020). These
337 findings are in line with other studies that have reported a decrease in MDA accumulation in
338 infected plants after PGPR treatments, sometimes even lower than the levels observed in control
339 conditions (Kazerooni *et al.*, 2021; Soliman *et al.*, 2023). These findings validate that one of
340 the indirect mechanisms employed by PGPR as biological control agents is their capability to
341 enhance the oxidative status of infected plants by scavenging the reactive oxygen species
342 generated during fungal infection. This was reported to occur through the upregulation of
343 antioxidative defense genes (Khanna *et al.*, 2019).

344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378

CONCLUSIONS

The obtained results showed significant *in vitro* inhibitory effects of 14 PGPR strains on the growth of *A. terricola* in both confrontation and exposure to volatile organic compounds tests. Notably, the strain *B. megaterium* FR1.11 exhibited substantial inhibition, a finding further validated through *in vivo* experiments conducted on detached leaves and potted seedlings of tomato. These promising outcomes warrant further comprehensive investigations to unravel the underlying mechanisms of action employed by these PGPR strains. Optimizing their application methods will be crucial to harness their full potential as effective biological control agents in agricultural practices. The investigation of the antifungal activity of these PGPR strains against other economically significant phytopathogens within the genus *Alternaria* will be of great interest.

ACKNOWLEDGEMENTS

The authors are grateful to the Tunisian Ministry of Higher Education and Scientific Research for financial support in the ambit of the laboratory project LR11ES31.

REFERENCES

1. Ali, S., Hameed, S., Shahid, M., Iqbal, M., Lazarovits, G., and Imran, A. 2020. **Functional Characterization of Potential PGPR Exhibiting Broad-Spectrum Antifungal Activity.** *Microbiol. Res.*, **232**: 126389.
2. Attia, M. S., El-Sayyad, G. S., Abd Elkodous, M., and El-Batal, A. I. 2020. **The Effective Antagonistic Potential of Plant Growth-Promoting Rhizobacteria Against *Alternaria solani*-causing Early Blight Disease in Tomato Plant.** *Sci. Hortic.*, **266**: 109289.
3. Bahramisharif, A., and Rose, L. E. 2019. **Efficacy of Biological Agents and Compost on Growth and Resistance of Tomatoes to Late Blight.** *Planta*, **249**: 799-813.
4. Bates, L. S., Waldren, R. P., and Teare, I. D. 1973. **Rapid Determination of Free Proline for Water Stress Studies.** *Plant Soil*, **39**: 205-207.
5. Cherif, H., Sghaier, I., Hassen, W., Amara, C., Masmoudi, A. S., Cherif, A., and Neifar, M. 2022. ***Halomonas desertis* G11, *Pseudomonas rhizophila* S211 and *Oceanobacillus iheyensis* E9 as Biological Control Agents Against Wheat Fungal Pathogens: PGPB Consorcia Optimization Through Mixture Design and Response Surface Analysis.** *Int. Clin. Pathol. J.*, **9**: 20-28.
6. Cherif, A., Brusetti, L., Borin, S., Rizzi, A., Boudabous, A., Khyami-Horani, H., and Daffonchio, D. 2003. **Genetic Relationship in the '*Bacillus cereus* group' by rep-PCR**

- 379 Fingerprinting and Sequencing of a *Bacillus anthracis*-specific rep-PCR Fragment. *J. Appl.*
380 *Microbiol.*, **94**: 1108-1119.
- 381 7. Chiappero, J., Cappellari, L. D. R., Alderete, L. G. S., Palermo, T. B., and Banchio, E. 2019.
382 Plant Growth Promoting Rhizobacteria Improve the Antioxidant Status in *Mentha piperita*
383 Grown Under Drought Stress Leading to an Enhancement of Plant Growth and Total
384 Phenolic Content. *Ind. Crops Prod.*, **139**: 111553.
- 385 8. Daigham, G. E., Mahfouz, A. Y., Abdelaziz, A. M., Nofel M. M., and Attia M. S. 2023.
386 Protective Role of Plant Growth-Promoting Fungi *Aspergillus chevalieri* OP593083 and
387 *Aspergillus egyptiacus* OP593080 as Biocontrol Approach Against *Alternaria* Leaf Spot
388 Disease of *Vicia faba* Plant. *Biomass Conv. Bioref.*, [https://doi.org/10.1007/s13399-023-](https://doi.org/10.1007/s13399-023-04510-4)
389 [04510-4](https://doi.org/10.1007/s13399-023-04510-4).
- 390 9. Doblinski, P. M. F., Ferrarese, M. L. L., Huber, D. A., Scapim, C. A., Braccini, A. L., and
391 Ferrarese-Filho, O. 2003. Peroxidase and Lipid Peroxidation of Soybean Roots in Response
392 to p-coumaric and p-hydroxybenzoic Acids. *Braz. Arch. Biol. Technol.*, **46**: 193-198.
- 393 10. Dukare, A. S., Paul, S., Nambi, V. E., Gupta, R. K., Singh, R., Sharma, K., and
394 Vishwakarma, R. K. 2019. Exploitation of Microbial Antagonists for the Control of
395 Postharvest Diseases of Fruits: A Review. *Crit. Rev. Food. Sci. Nutr.*, **59**: 1498-1513.
- 396 11. Elnahal, A. S. M., El-Saadony, M. T., Saad, A. M., Desoky, E. S. M., El-Tahan, A. M., Rady,
397 M. M., AbuQamar, S. F., and El-Tarabily, K. A. 2022. The Use of Microbial Inoculants for
398 Biological Control, Plant Growth Promotion, and Sustainable Agriculture: A Review. *Eur.*
399 *J. Plant Pathol.*, **162**: 759-792.
- 400 12. Fernandez-San Millan, A., Larraya, L., Farran, I., Ancin, M., and Veramendi, J. 2021.
401 Successful Biocontrol of Major Postharvest and Soil-Borne Plant Pathogenic Fungi by
402 Antagonistic Yeasts. *Biol. Control.*, **160**: 104683.
- 403 13. Ferraz, P., Cássio, F., and Lucas, C. 2019. Potential of Yeasts as Biocontrol Agents of the
404 Phytopathogen Causing Cacao Witches' Broom Disease: Is Microbial Warfare a Solution?
405 *Front. Microbiol.*, **10**: 1766.
- 406 14. Fisher, M. C., Hawkins, N. J., Sanglard, D., and Gurr, S. J. 2018. Worldwide Emergence of
407 Resistance to Antifungal Drugs Challenges Human Health and Food Security. *Science*, **360**:
408 739-742.
- 409 15. Florea, A., and Puia, C. 2020. *Alternaria* Genus and the Diseases Caused to Agricultural and
410 Horticultural Plants. *Bull. UASVM. Agric.*, **77**: 53-63.
- 411 16. Gong, Y., Chen, L. J., Pan, S. Y., Li, X. W., Xu, M. J., Zhang, C. M., Xing, K., and Qin, S.
412 2020. Antifungal Potential Evaluation and Alleviation of Salt Stress in Tomato Seedlings by

- 413 a Halotolerant Plant Growth-Promoting Actinomycete *Streptomyces* sp. KLBMP5084.
414 *Rhizosphere*, **16**: 100262.
- 415 17. Gupta, P. K. 2018. Chapter 45 - Toxicity of Fungicides, Editor(s): Ramesh C. Gupta,
416 Veterinary Toxicology (Third Edition), Academic Press, Pages 569-580.
- 417 18. Haidar, R., Roudet, J., Bonnard, O., Dufour, M. C., Corio-Costet, M. F., Fert, M., Gautier,
418 T., Deschamps, A., and Fermaud, M. 2016. Screening and Modes of Action of Antagonistic
419 Bacteria to Control the Fungal Pathogen *Phaeoconiella chlamydospora* Involved in
420 Grapevine Trunk Diseases. *Microbiol. Res.*, **192**: 172-184.
- 421 19. Hassen, W., Neifar, M., Cherif, H., Najjari, A., Chouchane, H., Driouich, R. C., Salah, A.,
422 Naili, F., Mosbah, A., Souissi, Y., Raddadi, N., Ouzari, H. I., Fava, F., and Cherif A. 2018.
423 *Pseudomonas rhizophila* S211, a New Plant Growth-Promoting Rhizobacterium with
424 Potential in Pesticide-Bioremediation. *Front. Microbiol.*, **9**: 34.
- 425 20. Imran, Z. K. 2011. Isolation and Identification Species of *Ulocladium Preuss* from Different
426 Regions in Iraq. *Basrah J. Agric. Sci.*, **24**: 27-47.
- 427 21. Karthika, S., Varghese, S., and Jisha, M. S. 2020. Exploring the Efficacy of Antagonistic
428 Rhizobacteria as Native Biocontrol Agents Against Tomato Plant Diseases. *3 Biotech.*, **10**:
429 320.
- 430 22. Kazerooni, E. A., Maharachchikumbura, S. S. N., Al-Sadi, A. M., Kang, S. M., Yun, B. W.,
431 Lee, I. J. 2021. Biocontrol Potential of *Bacillus amyloliquefaciens* against *Botrytis*
432 *pelargonii* and *Alternaria alternata* on *Capsicum annuum*. *J. Fungi* **7**: 472.
- 433 23. Khanna, K., Kohli, S. K., Ohri, P., Bhardwaj, R., Al-Huqail, A. A., Siddiqui, M. H.,
434 Alosaimi, G. S., and Ahmad, P. 2019. Microbial Fortification Improved Photosynthetic
435 Efficiency and Secondary Metabolism in *Lycopersicon esculentum* Plants Under Cd Stress.
436 *Biomolecules*, **9**: 581.
- 437 24. Kousar, B., Bano, A., and Khan, N. 2020. PGPR Modulation of Secondary Metabolites in
438 Tomato Infested with *Spodoptera litura*. *Agronomy*, **10**: 778.
- 439 25. Lichtenthaler, H. K., and Wellburn, A. R. 1983. Determination of Total Carotenoids and
440 Chlorophyll a and b of Leaf Extract in Different Solvents. *Biochem. Soc. Trans.*, **11**: 591-
441 592.
- 442 26. Nahar, S., Mushtaq, M., and Pathan, I. H. 2004. Seed-borne Mycoflora of *Capsicum annuum*
443 Imported from India. *Pak. J. Bot.*, **36**: 191-197.
- 444 27. Parasuraman, P., Pattnaik, S. S., Busi, S., Marraiki, N., Elgorban, A. M., and Syed, A. 2022.
445 Isolation and Characterization of Plant Growth Promoting Rhizobacteria and Their

- 446 Biocontrol Efficacy Against Phytopathogens of Tomato (*Solanum lycopersicum* L.). *Plant*
447 *Biosyst.*, **156**: 164-170.
- 448 28. Puvača, N., Bursić, V., Vuković, G., Budakov, D., Petrović, A., Merkuri, J., Avantaggiato,
449 G., and Cara., M. 2020. Ascomycete Fungi (*Alternaria* spp.) Characterization as Major Feed
450 Grains Pathogens. *J. Agron. Technol. Eng. Manag.*, **3**: 499-505.
- 451 29. Rani, L., Thapa, K., Kanojia, N., Sharma, N., Singh, S., Grewal, A. S., Srivastav, A. L., and
452 Kaushal, J. 2021. An Extensive Review on the Consequences of Chemical Pesticides on
453 Human Health and Environment. *J. Clean. Prod.*, **283**: 124657.
- 454 30. Rasool, M., Akhter, A., Soja, G., and Haider, M. S. 2021. Role of Biochar, Compost and
455 Plant Growth Promoting Rhizobacteria in the Management of Tomato Early Blight Disease.
456 *Sci. Rep.*, **11**: 6092.
- 457 31. Riahi, L., Cherif, H., Miladi, S., Neifar, M., Bejaoui, B., Chouchane, H., Masmoudi, A. S.,
458 and Cherif, A. 2020. Use of Plant Growth Promoting Bacteria as an Efficient
459 Biotechnological Tool to Enhance the Biomass and Secondary Metabolites Production of
460 the Industrial Crop *Pelargonium graveolens* L'Hér. Under Semi-Controlled Conditions. *Ind.*
461 *Crops. Prod.*, **154**: 112721.
- 462 32. Singh, A., Singh Gaurav, S., Shukla, G., Rani, P., Kumar, B., and Kumar, A. 2020.
463 Evaluation of Mycogenic Silver and Zinc Oxide Nanoparticles as Potential Control Agent
464 against Early Blight (*Alternaria solani*) of Potato (*Solanum tuberosum* L.). *J. Adv. Sci. Res.*,
465 **11**: 164-172.
- 466 33. Singleton, V. L., and Rosi, J. A. 1965. Colorimetry of Total Phenolics with
467 Phosphomolybdic–Phosphotungstic Acid Reagents. *Am. J. Oenol. Vitic.*, **16**: 144-158.
- 468 34. Soliman, S. A., Abdelhameed, R. E., and Metwally, R. A. 2023. *In vivo* and *In vitro*
469 Evaluation of the Antifungal Activity of the PGPR *Bacillus amyloliquefaciens* RaSh1
470 (MZ945930) Against *Alternaria alternate* with Growth Promotion Influences on *Capsicum*
471 *annuum* L. Plants. *Microb. Cell Fact.*, **22**: 70.
- 472 35. Syed Nabi, R. B., Shahzad, R., Tayade, R., Shahid, M., Hussain, A., Ali, M.W., and Yun,
473 B.W. 2021. Evaluation Potential of PGPR to Protect Tomato Against *Fusarium* wilt and
474 Promote Plant Growth. *PeerJ.*, **9**: e11194.
- 475 36. Wang, M., Xue, J., Ma, J., Feng, X., Ying, H., and Xu, H. 2020. *Streptomyces lydicus* M01
476 Regulates Soil Microbial Community and Alleviates Foliar Disease Caused by *Alternaria*
477 *alternate* on Cucumbers. *Front. Microbiol.*, **11**: 942.

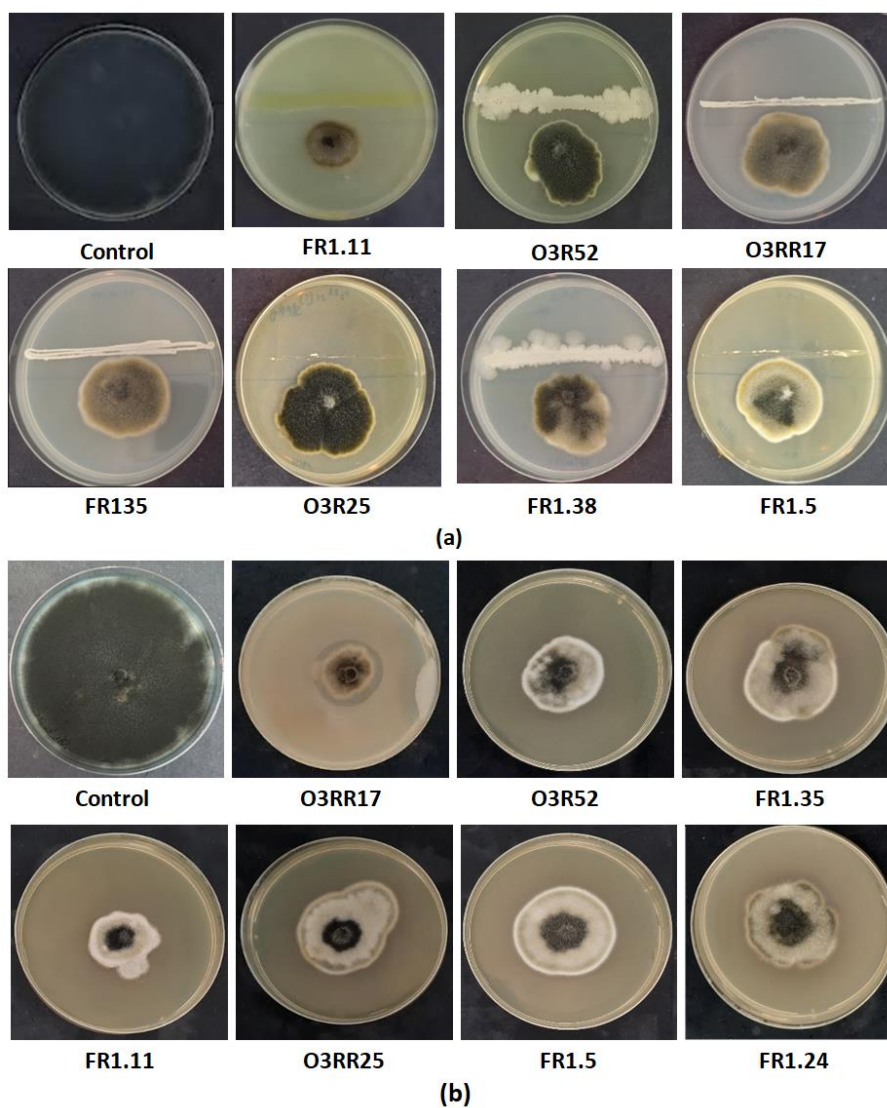
- 478 37. Wang, H., Liu, R., You, M. P., Barbetti, M. J., and Chen, Y. 2021. Pathogen Biocontrol
479 Using Plant Growth-Promoting Bacteria (PGPR): Role of Bacterial Diversity.
480 *Microorganisms*, **9**: 1988.
- 481 38. Woudenberg, J. H., Groenewald, J. Z., Binder, M., and Crous, P. W. 2013. *Alternaria*
482 Redefined. *Stud. Mycol.*, **75**: 171-212.
- 483

Table 1. List and codes of the studied PGPR strains.

Strain code	Origin	Species	Accession numbers
FR1.5	Fig tree rhizosphere	<i>Bacillus oceanisediminis</i>	NR117285
FR1.17		<i>Microbacterium azadirachtae</i>	NR116502
FR1.24		<i>Bacillus tyonensis</i>	NR121761
FR1.38		<i>Brevibacterium frigotolerans</i>	NR117474
FR1.11		<i>Bacillus megaterium</i>	NR116873
FR1.35		<i>Bacillus wiedmannii</i>	NR152692
O3R15	Olive tree rhizosphere	<i>Pseudomonas azotoformans</i>	NR113600
O3R24		<i>Bacillus muralis</i>	NR042083
O3R52		<i>Pseudomonas reinekei</i>	NR042541
O3RR17		<i>Pseudarthrobacter siccitolerans</i>	NR108849
O3RR24		<i>Pseudomonas brassicacearum</i>	NR116299
O3RR25		<i>Pseudomonas koreensis</i>	NR025228
O3RR33		<i>Arthrobacter humicola</i>	NR041546
O3RR35		<i>Bacillus zhangzhouensis</i>	NR148786

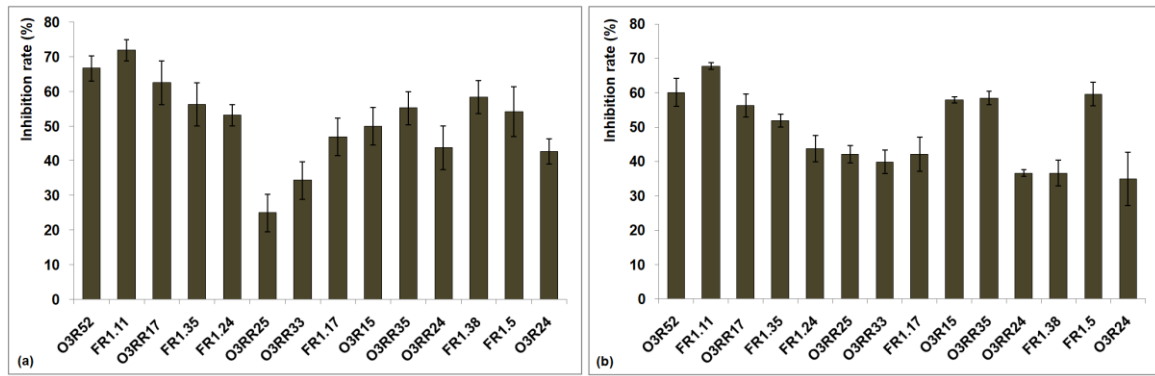
485

486

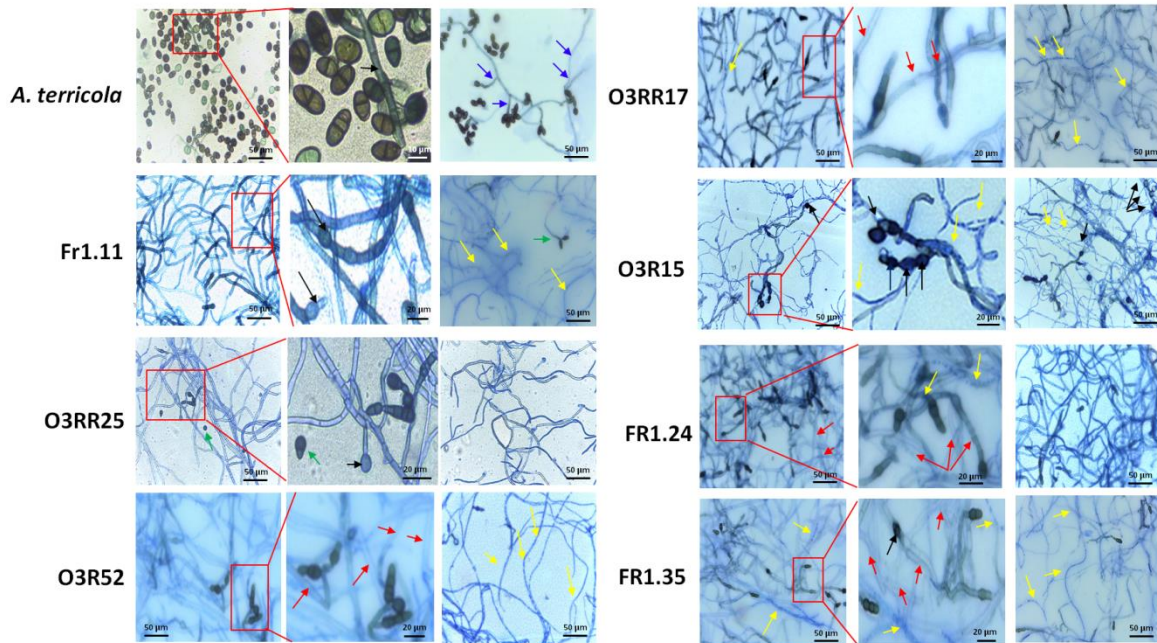


487

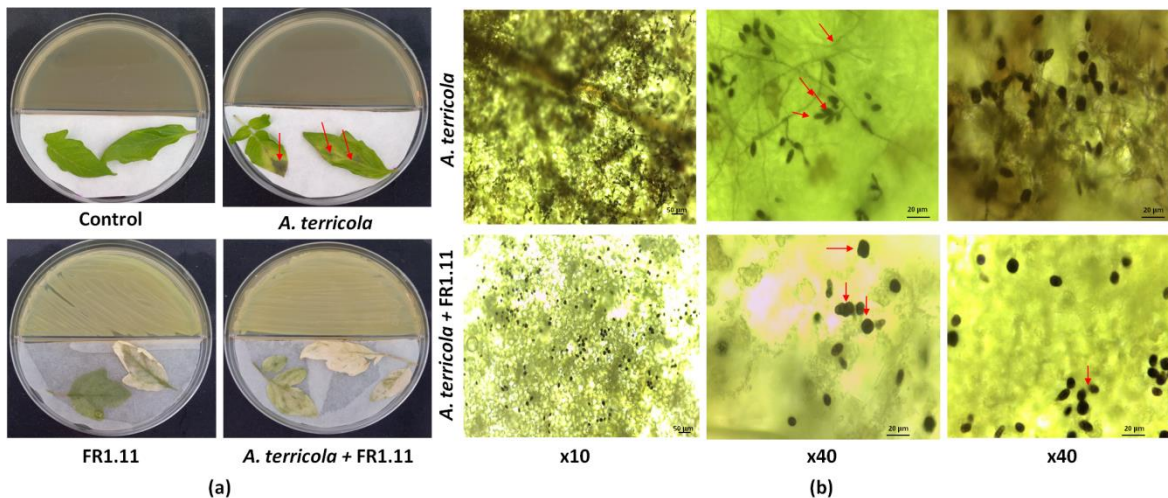
488 **Figure 1.** Macroscopic observation of the direct antagonism test (a) and indirect antagonism489 test (b) between PGPRs and *Alternaria terricola* after 10 days of incubation on PDA medium.



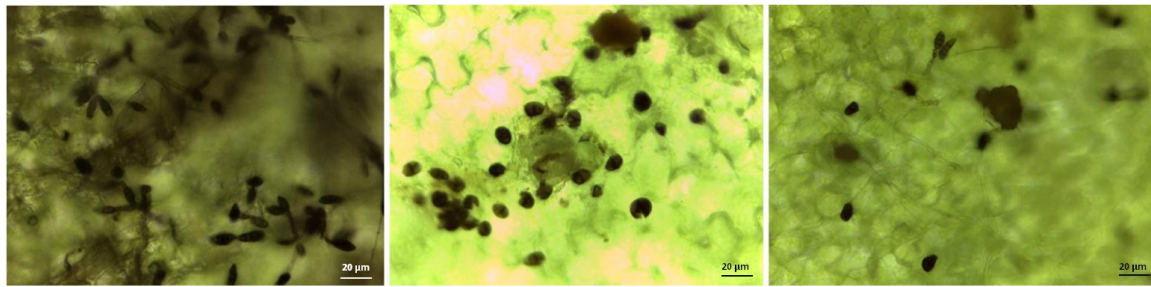
490
491 **Figure 2.** Variation in the percentage of growth inhibition of *Alternaria terricola* in direct
492 confrontation between different PGPR strains (a) and following exposure to volatile compounds
493 (b).



494
495 **Figure 3.** Microscopic observation of *Alternaria terricola* following the indirect antagonism
496 test based on the application of PGPR VOCs after 10 days of incubation. **Scale bars in µm.**
497



498
499 **Figure 4.** Macroscopic (a) and microscopic (b) observations of detached tomato leaves after 10
500 days of exposure to *Bacillus megaterium* FR1.11 volatile metabolites. **Scale bars in µm.**

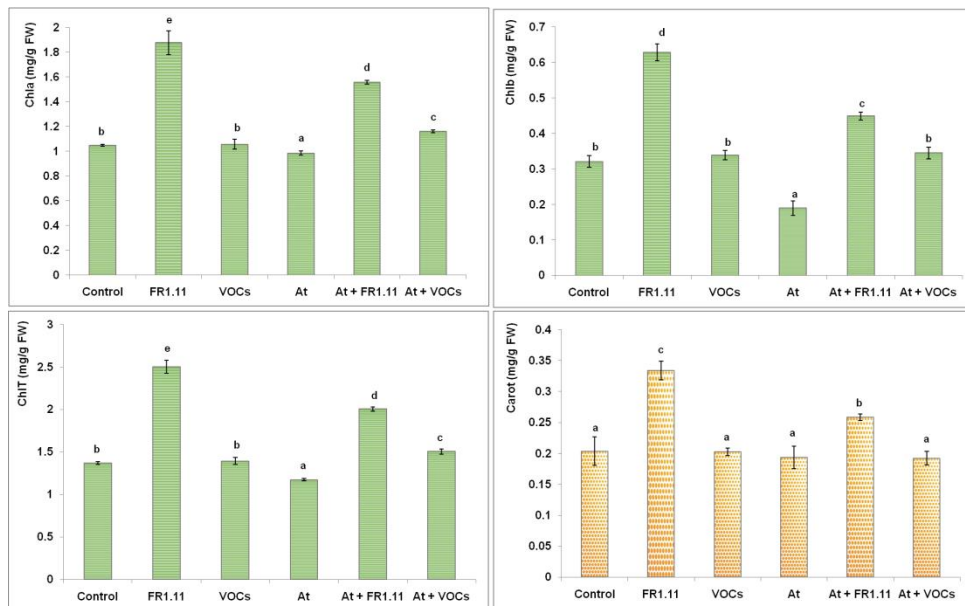


A. terricola

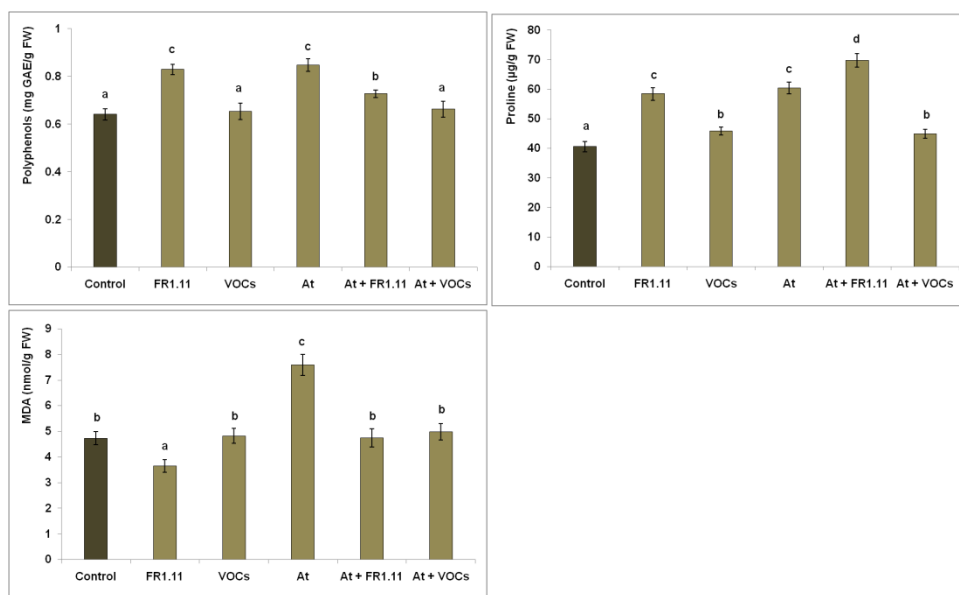
A. terricola + VOCs

A. terricola + FR1.11

501 **Figure 5.** Microscopic observation of tomato leaves infected by *Alternaria terricola* under
 502 the influence of the PGPR strain *Bacillus megaterium* FR1.11 and its **volatile organic**
 503 **compounds**. Scale bars in μm .
 504
 505



506 **Figure 6.** Variation in the content of chlorophyll *a*, *b*, total, and carotenoids (mg/g FW) in
 507 tomato seedlings according to the treatments.
 508
 509



510 **Figure 7.** Variation in the content of total polyphenols (mg GAE/g FW), proline contents ($\mu\text{g/g}$
 511 FW) and **malondialdehyde** (nmol/g FW) in tomato leaves according to the applied treatment.
 512