1	ACCEPTED ARTICLE
2 3 4	<i>In vitro</i> and <i>in vivo</i> potential of Plant Growth-Promoting Rhizobacteria as biological control agents against <i>Alternaria terricola</i>
6	Running title: PGPRs application to control Alternaria terricola
7 8	Hanene Cherif ¹ , Amira Oueslati ¹ , Bilel Bejaoui ¹ , Mouna Mahjoubi ¹ , Yosra Amara ¹ , Yasmine Souissi ² , Ameur Cherif ¹ , and Leila Riahi ^{1*}
9 10 11	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.
12 13	² Department of Engineering, German University of Technology in Oman, P.O. Box 1816, PC 130, Muscat, Sultanate of Oman.
14	*Corresponding author; e-mail: leila.ba.riahi@gmail.com
15 16	ABSTRACT
17	In this study, the antagonistic effects of 14 Plant Growth-Promoting Rhizobacteria strains
18	(PGPRs) against the phytopathogenic species Alternaria terricola Woudenb. & Crous, both in
19	vitro and in vivo were investigated. The obtained results revealed significant inhibition effects
20	of the 14 PGPR strains against A. terricola in both direct contact and indirect bioassays with
21	significant variation. The dual in vitro culture tests revealed substantial inhibition rates in the
22	growth of A. terricola strain, ranging from 25±5.41% (Pseudomonas koreensis O3RR25) to
23	71.87±3.12% (Bacillus megaterium FR1.11). Moreover, the indirect antagonism test showed
24	that the volatile organic compounds produced by the 14 tested PGPR strains significantly
25	inhibited the growth of A. terricola mycelium, with variations ranging from 36.61±0.94% (P.
26	brassicacearum O3RR24) to 67.75±0.94% (B. megaterium FR1.11). Microscopic examination
27	of A. terricola following exposure to the volatile compounds revealed significant structural
28	damage, including inhibition of conidial germination, deformations, thin or fissured structures,
29	irregular lengths, and the formation of empty segments. The in vivo application of B.
30	megaterium FR1.11 resulted in the reduction of fungal development on detached leaves and
31	tomato seedlings. This treatment engendered a significant increase in the levels of chlorophyll
32	a, b and total, carotenoids, polyphenols, and proline in infected tomato seedlings compared to
33	the control. Applying this PGPR strain to infected tomato plants allowed maintaining
34	comparable level of malondialdehyde as the control. B. megaterium FR1.11 showed
35	considerable in vitro and in vivo antifungal activity and could serve as a promising candidate

36 for biological control strategies targeting phytopathogenic species of the genus *Alternaria*.

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

38

39 INTRODUCTION

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

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

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

69 Among the biological control agents against phytopathogenic fungi, several reports indicate a

significant potential for PGPRs bacteria (Parasuraman *et al.*, 2022). In addition to their role in

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

- compounds (VOCs) and the *in vivo* effects of PGPRs against *A. terricola* are lacking.
- This study aims to assess the in vitro antagonistic activity of 14 plant growth-promoting 78 rhizobacteria strains (PGPRs) against A. terricola Woudenb. & Crous (Woudenberg et al., 79 2013) using direct contact bioassays as well as through the effects of PGPRs volatile organic 80 compounds (VOCs). The most promising bacterial strain exhibiting higher *in vitro* antagonistic 81 activity was further evaluated *in vivo* using tomato as a model plant based on detached leaf tests 82 83 and pot assays. The impact of the employed biological control agent on the modulation of physiological and biochemical traits, including chlorophyll a, b, total chlorophyll, carotenoids, 84 85 proline, and malondialdehyde (MDA), was investigated.
- 86

87 MATERIALS AND METHODS

88 Microbial strains

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

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

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

108

109 In vitro antagonism bioassays

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

120

121 In vivo antifungal activity

The detached leaves of one-month-old tomato seedlings were disinfected with 1% sodium hypochlorite and alcohol 70% for 1-2 minutes then rinsed three times with sterile physiological water. The leaves were placed in compartmentalized Petri dishes on sterile filter paper soaked with sterile physiological water. Aliquots of 10 μ L of the fungal spore suspension (sterile distilled water for the control), adjusted to 5×10⁵ conidia/mL, were applied to the adaxial surface of the detached leaves. The PGPR strains were sub-cultured on Tryptic Soy Agar (TSA) medium in the other compartment of the Petri dish (Bahramisharif and Rose, 2019).

The in vivo antifungal potential of the rhizobacterial strain was assessed on 45-day-old tomato 129 seedlings. The seedlings were divided into six groups, each subjected to a specific treatment: 130 (1) control, (2) infected control (10^6 conidia/mL), (3) seedlings soil-inoculated with the PGPR 131 strain 10⁸ colony forming unit/mililitre (CFU/mL), (4) seedlings inoculated with PGPR then 132 infected with A. terricola, (5) seedlings exposed to PGPR VOCs (6) seedlings exposed to VOCs 133 then infected with A. terricola. All pots were covered with transparent bags to capture the 134 volatile organic compounds emitted by the PGPR strain. The experiments were conducted with 135 ten repetitions. Seedlings were exposed to the VOCs of the PGPR strain by placing the tomato 136 pots near the PGPR cultures on open Petri dishes, without lids (Attia et al., 2020). 137

138

139 **Studied parameters**

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

151 Data analysis

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

156

157 **RESULTS**

158 In vitro antagonism test

159 In vitro direct bioassays

The 14 tested PGPR strains induced a reduction in the growth of A. terricola with variable 160 degrees (Figure 1a). A noticeable change in the colour of A. terricola colonies from greenish 161 black (control) to whitish or greyish was recorded. The inhibition percentages obtained after 10 162 163 days of incubation using the direct test are presented in Figure 2a. The six PGPR strains P. reinekei O3R52, B. megaterium FR1.11, P. siccitolerans O3RR17, B. wiedmannii FR1.35, B. 164 frigotolerans FR1.38, and B. oceanisediminis FR1.5 have inhibition rates over 60%. The PGPR 165 strain O3RR25 (*P. koreensis*) displayed the lowest inhibition rate $(25\pm5.41\%)$, whereas the 166 highest inhibition rate (71.87±3.12%) was recorded with the strain FR1.11 (*B. megaterium*). 167

168

169 *In vitro* indirect bioassays

The macroscopic observations obtained with the indirect antagonism test after 10-day incubation showed that the growth of *A. terricola* mycelia exposed to the volatile metabolites was significantly inhibited compared to the control (Figure 1b). The macroscopic observation revealed a change in the appearance and the color of the colonies from greenish-black to whitish, particularly at the colony's extremities. The inhibition rates obtained using the indirect
test reveal that all the PGPR strains produce volatile substances that inhibit significantly the
growth of the *A. terricola* strain, with significant variation (Figure 2b). The inhibition
percentages range from 36.61±0.94% (*P. brassicacearum* O3RR24) to 67.75±0.94% (*B. megaterium* FR1.11). Four PGPR strains inhibited *A. terricola* by over 60%: O3R52
(60.10±4.12%), *B. megaterium* FR1.11 (67.75±0.94%), *B. zhangzhouensis* O3RR35
(58.46±1.89%), and *B. oceanisediminis* FR1.5 (59.56±3.41%).

- A microscopic examination of the mycelium of A. terricola following exposure to the volatile 181 182 compounds produced by the applied PGPR strains was carried out. Intact cell walls with regular lengths and uniform structures were observed for the hyphae of untreated A. terricola (Figure 183 184 3). However, mycelium hyphae treated with A. terricola VOCs displayed wrinkled surfaces, deformations, and irregular lengths, often accompanied by empty segments (indicated by red 185 186 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). Furthermore, certain conidia formed irregular germination tubes, notably shorter than those in 189 190 the control group (indicated by green arrows).
- 191

192 In vivo antifungal activity

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

The microscopic examination showed that *A. terricola* in infected leaves treated with PGPR VOCs had lost their germination ability and/or exhibited morphological deformations (Figure 4). Leaves infected with *A. terricola*, in the absence of PGPR VOCs, exhibited successfully germinated conidia with well developed mycelium showing regular tubes (Figure 4 a). The normal hyphae displayed smooth surfaces, consistent lengths, and intact structures with segmented mycelium. In the presence of PGPR VOCs, the conidia of *A. terricola* have lost their capacity to germinate and developing mycelium, as shown by red arrows in Figure 4 b, rendering them unable to generate appressoria or form infection structures on the leaf. Notably,
the volatile substances produced by the PGPR strain significantly reduced the number of
conidia on the detached tomato leaves.

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

221

222 Variation in photosynthetic pigment contents

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

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

238 Variation in total polyphenol contents

The contents of total phenolic compounds in the ethanolic extracts of tomato leaves were determined using a standard range of gallic acid (Figure 7). The results indicate no significant variation in total polyphenol content compared to untreated seedlings $(0.640\pm0.023 \text{ mg GAE})$

- 242 /g FW) for the VOCs and A. *terricola*+VOCs treatments. However, significant variations in the
- 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
- strain *B. megaterium* FR1.11 (0.828±0.021 mg GAE/g FW) and those infected with *A. terricola*
- 246 $(0.847\pm0.026 \text{ mg GAE/g FW})$, followed by the *A. terricola*+PGPR treatment.
- 247

248 Variation in proline levels

- The obtained results demonstrate a significant increase in proline levels in the treated tomato leaves compared to the control conditions $(40.57 \pm 1.81 \ \mu\text{g/g} FW)$ across all treatments (Figure 7). The treatment of *A. terricola* + PGPR *B. megaterium* FR1.11 exhibited the highest accumulation of proline (69.78 ± 2.29 μ g/g FW), followed by the individual PGPR and *A. terricola* treatments. A significant increase in proline levels, compared to the control, was also observed with the VOCs and *A. terricola*+VOCs treatments.
- 255

256 Variation in malondialdehyde (MDA) contents

- The contents of MDA exhibited a significant variation compared to the control (4.73 ± 0.26) nmol/g FW), as shown in Figure 7. The highest increase in MDA content (7.59 ± 0.41) nmol/g FW) was detected following the infection of tomato seedlings by *A. terricola*. Interestingly, a significant decrease in MDA levels (3.65 ± 0.24) nmol/g FW) was observed when tomato seedlings were inoculated with the PGPR *B. megaterium* FR1.11 strain, compared to untreated seedlings. No significant variation compared to the control was observed for this parameter in the VOCs, *A. terricola*+PGPR, and *A. terricola*+VOCs treatments.
- 264

265 **DISCUSSION**

A. terricola is known to be a phytopathogenic agent affecting various agronomic crops, 266 including wheat (Imran et al., 2011) and red pepper (Nahar et al., 2004). Except the study by 267 Cherif et al. (2022), which focused on the effect of three PGPR strains on the species A. 268 terricola through in vitro direct antagonism tests, no other study has been reported for this 269 270 phytopathogenic agent. In this investigation, the *in vitro* antagonism assays based on both direct and indirect tests, revealed that the tested 14 PGPR strains exhibited significant inhibition of 271 272 the growth of the A. terricola strain. Among the tested PGPR strains, B. megaterium FR1.11 exhibited the highest inhibition rates against the growth of A. terricola in both confrontation 273 and exposure to volatile compounds in the in vitro tests. In vivo investigations further supported 274 275 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.
- The observed antifungal activity of the strain *B. megaterium* FR1.11 is likely attributed to its 278 volatile metabolites, as evidenced by both in vitro and in vivo studies. The effectiveness of 279 PGPR in hindering the germination and development of fungal species on detached leaves 280 confirms that volatile organic compounds are among the direct mechanisms of biological 281 control employed by PGPR strains (Bahramisharif and Rose, 2019). However, the antifungal 282 effect observed with soil inoculation suggests that PGPR strain *B. megaterium* FR1.11 may 283 284 employ multiple modes of action against the tested strain of A. terricola. Bacillus and Pseudomonas species are the commonly utilized PGPRs in the biological control of plant 285 286 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 cyanide (HCN), cell wall degrading enzymes, 1-aminocyclopropane-1-carboxylate (ACC) 289 290 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 of plant pathogenic microorganisms, namely antibiosis, siderophore production, and parasitism 293 through the secretion of catalytic enzymes like chitinases, lipases, and proteases (Ali et al., 294 2020). Additionally, PGPR bacteria can indirectly act as biological control agents by inducing 295 enhanced immunity in the target plants and by modulating endogenous phytohormones and 296 297 amino acid levels (Syed Nabi et al., 2021).
- The findings of this study align with previous studies that have demonstrated a significant 298 decrease in the levels of chlorophyll a, b, and total in tomato plants infected with Alternaria 299 species, while carotenoid contents remained relatively stable compared to the control group 300 301 (Attia et al., 2020). A reduction in the photosynthetic pigments of tomato inoculated with Alternaria solani was also reported by Rasool et al. (2021). Chlorophyll and carotenoid 302 303 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 increase in the concentrations of chlorophyll pigments and carotenoids in tomato seedlings. 305 These results are consistent with other studies that have reported higher photosynthetic pigment 306 307 contents in tomato leaves treated with PGPR bacteria (Attia et al., 2020).
- The results of this study demonstrate a significant increase in proline and total polyphenol content in tomato plants inoculated with the PGPR strain. Moreover, these levels were further

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

- The production of proline induced by the PGPR strain highlights the ability of this inoculation 316 to enhance the plant's tolerance to osmotic stress under normal conditions. Proline serves as an 317 318 osmoregulator and accumulates in plants in response to a wide range of stress conditions (Khanna et al., 2019). The accumulation of cellular osmolytes, including proline, helps plants 319 320 to maintain essential cellular functions and physiological stability (Kousar et al., 2020). Proline and other osmolytes play a protective role by regulating water and nutrient balance, stabilizing 321 322 membrane structures, supporting the function of various enzymes and proteins, and providing protection against reactive oxygen species (ROS) and other biochemical reactions (Khanna et 323 324 al., 2019).
- The obtained results showed a significant increase in MDA levels for tomato seedlings infected with *A. terricola*. This aligns with previous findings which highlighted a significant elevation in MDA, used as a stress indicator, when tomato plants were infected with *A. solani*, as compared to their healthy plants (Daigham *et al.*, 2023). Furthermore, following infection with *A. alternate*, the MDA contents were reported to increase in the leaves of cucumber (Wang *et al.*, 2020) and pepper (Kazerooni *et al.*, 2021).

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

345 CONCLUSIONS

The obtained results showed significant in vitro inhibitory effects of 14 PGPR strains on the 346 growth of A. terricola in both confrontation and exposure to volatile organic compounds tests. 347 Notably, the strain *B. megaterium* FR1.11 exhibited substantial inhibition, a finding further 348 validated through in vivo experiments conducted on detached leaves and potted seedlings of 349 350 tomato. These promising outcomes warrant further comprehensive investigations to unravel the underlying mechanisms of action employed by these PGPR strains. Optimizing their 351 352 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 353 354 strains against other economically significant phytopathogens within the genus Alternaria will be of great interest. 355

356

357 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.

360

361 **REFERENCES**

- 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.
- 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.
- 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.
- 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

- Fingerprinting and Sequencing of a *Bacillus anthracis*-specific rep-PCR Fragment. *J. Appl. Microbiol.*, **94**: 1108-1119.
- 7. Chiappero, J., Cappellari, L. D. R., Alderete, L. G. S., Palermo, T. B., and Banchio, E. 2019.
 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.
- Baigham, G. E., Mahfouz, A. Y., Abdelaziz, A. M, Nofel M. M., and Attia M. S. 2023.
 Protective Role of Plant Growth-Promoting Fungi *Aspergillus chevalieri* OP593083 and
- Aspergillus egyptiacus OP593080 as Biocontrol Approach Against Alternaria Leaf Spot
 Disease of Vicia faba Plant. Biomass Conv. Bioref., https://doi.org/10.1007/s13399-023-
- <u>389</u> <u>04510-4</u>.
- Doblinski, P. M. F., Ferrarese, M. L. L., Huber, D. A., Scapim, C. A., Braccini, A. L., and
 Ferrarese-Filho, O. 2003. Peroxidase and Lipid Peroxidation of Soybean Roots in Response
 to p-coumaric and p-hydroxybenzoic Acids. *Braz. Arch. Biol. Technol.*, 46: 193-198.
- 10. Dukare, A. S., Paul, S., Nambi, V. E., Gupta, R. K., Singh, R., Sharma, K., and
 Vishwakarma, R. K. 2019. Exploitation of Microbial Antagonists for the Control of
 Postharvest Diseases of Fruits: A Review. *Crit. Rev. Food. Sci. Nutr.*, **59**: 1498-1513.
- 11. Elnahal, A. S. M., El-Saadony, M. T., Saad, A. M., Desoky, E. S. M., El-Tahan, A. M., Rady,
 M. M., AbuQamar, S. F., and El-Tarabily, K. A. 2022. The Use of Microbial Inoculants for
 Biological Control, Plant Growth Promotion, and Sustainable Agriculture: A Review. *Eur. 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

- a Halotolerant Plant Growth-Promoting Actinomycete Streptomyces sp. KLBMP5084. 413
- *Rhizosphere*, **16**: 100262. 414

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

- Biocontrol Efficacy Against Phytopathogens of Tomato (*Solanum lycopersicum* L.). *Plant Biosyst.*, **156**: 164-170.
- 448 28. Puvača, N., Bursić, V., Vuković, G., Budakov, D., Petrović, A., Merkuri, J., Avantaggiato,
- G., and Cara., M. 2020. Ascomycete Fungi (*Alternaria* spp.) Characterization as Major Feed
 Grains Pathogens. *J. Agron. Technol. Eng. Manag.*, 3: 499-505.
- 29. Rani, L., Thapa, K., Kanojia, N., Sharma, N., Singh, S., Grewal, A. S., Srivastav, A. L., and
 Kaushal, J. 2021. An Extensive Review on the Consequences of Chemical Pesticides on
 Human Health and Environment. *J. Clean. Prod.*, 283: 124657.
- 30. Rasool, M., Akhter, A., Soja, G., and Haider, M. S. 2021. Role of Biochar, Compost and
 Plant Growth Promoting Rhizobacteria in the Management of Tomato Early Blight Disease.
 Sci. Rep., 11: 6092.
- 31. Riahi, L., Cherif, H., Miladi, S., Neifar, M., Bejaoui, B., Chouchane, H., Masmoudi, A. S.,
 and Cherif, A. 2020. Use of Plant Growth Promoting Bacteria as an Efficient
 Biotechnological Tool to Enhance the Biomass and Secondary Metabolites Production of
 the Industrial Crop *Pelargonium graveolens* L'Hér. Under Semi-Controlled Conditions. *Ind. Crops. Prod.*, **154**: 112721.
- 32. Singh, A., Singh Gaurav, S., Shukla, G., Rani, P., Kumar, B., and Kumar, A. 2020.
 Evaluation of Mycogenic Silver and Zinc Oxide Nanoparticles as Potential Control Agent
 against Early Blight (*Alternaria solani*) of Potato (*Solanum tuberosum* L.). *J. Adv. Sci. Res.*,
 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.
- 34. Soliman, S. A., Abdelhameed, R. E., and Metwally, R. A. 2023. *In vivo* and *In vitro*Evaluation of the Antifungal Activity of the PGPR *Bacillus amyloliquefaciens* RaSh1
 (MZ945930) Against *Alternaria alternate* with Growth Promotion Influences on *Capsicum 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.
- 36. Wang, M., Xue, J., Ma, J., Feng, X., Ying, H., and Xu, H. 2020. *Streptomyces lydicus* M01
 Regulates Soil Microbial Community and Alleviates Foliar Disease Caused by *Alternaria 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

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



FR1.11 **O3RR17** Control **O3R52** FR135 **O3R25** FR1.38 FR1.5 (a) **O3RR17** O3R52 FR1.35 Control FR1.11 **O3RR25** FR1.5 FR1.24 (b)

1

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



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



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







A. terricola + FR1.11 A. terricola A. terricola + VOCs 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



506 507

509

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





[Downloaded from jast.modares.ac.ir on 2024-05-08]