ACCEPTED ARTICLE

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 Cherif[1], Amira Oueslati[1], Bilel Bejaoui[1], Mouna Mahjoubi[1], Yosra Amara[1], Yasmine Souissi[2], Ameur Cherif[1], 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*.

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

INTRODUCTION

Fungal pathogens pose a significant biotic stress that adversely affects agricultural crop productivity and quality under various production systems, including fields and greenhouses but also at post-harvest handling which poses a serious threat to global food security (Ferraz *et al.*, 2019). Fungal diseases lead to substantial additional losses during crop transportation and storage (Dukare *et al.*, 2019). Among these phytopathogens, fungi of the *Alternaria* genus are 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 (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, Cucurbitaceae, and Brassicaceae. The recorded losses attributed to *Alternaria* range from 50% to 86% for tomatoes (Florea and Puia, 2020) and from 80% to 100% for potatoes (Singh *et al.*, 2020).

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

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 the environmentally friendly alternatives, biological control applying beneficial microorganisms such as bacteria, filamentous fungi, and yeasts, along with their metabolites exhibiting antagonistic activity against phytopathogenic fungi, has gained significant attention (Elnahal *et al.*, 2022). This strategy involves the application of live microorganisms to reduce 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 environmentally friendly alternative to the use of synthetic fungicides (Karthika *et al.*, 2020).

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 rhizobacteria strains (PGPRs) against *A. terricola* Woudenb. & Crous (Woudenberg *et al.*, 2013) using direct contact bioassays as well as through the effects of PGPRs volatile organic compounds (VOCs). The most promising bacterial strain exhibiting higher *in vitro* antagonistic activity was further evaluated *in vivo* using tomato as a model plant based on detached leaf tests 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, proline, and malondialdehyde (MDA), was investigated.

MATERIALS AND METHODS

Microbial strains

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

Plant material and culture conditions

The tomato variety Rio Grande (*Solanum lycopersicum* L., Solanaceae family) was used in this study to investigate the PGPR *in vivo* antifungal activity. The seeds were disinfected using a 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%).

In vitro antagonism bioassays

The antagonistic activity of bacterial strains against *A. terricola* strain was achieved using the 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 (%) = [(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, 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 (%) = [(D1 - D2) / D1] * 100, where D1 represents the diameter of the pathogenic fungus in the absence of the antagonist agent, and D2 represents the diameter of the pathogenic fungus in the presence of the antagonistic agent (Haidar *et al.*, 2016).

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 µL of the fungal spore suspension (sterile distilled water for the control), adjusted to 5×10^5 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 seedlings. The seedlings were divided into six groups, each subjected to a specific treatment: (1) control, (2) infected control (10^6 conidia/mL), (3) seedlings soil-inoculated with the PGPR strain 10^8 colony forming unit/mililitre (CFU/mL), (4) seedlings inoculated with PGPR then infected with *A. terricola*, (5) seedlings exposed to PGPR VOCs (6) seedlings exposed to VOCs then infected with *A. terricola*. All pots were covered with transparent bags to capture the volatile organic compounds emitted by the PGPR strain. The experiments were conducted with ten repetitions. Seedlings were exposed to the VOCs of the PGPR strain by placing the tomato pots near the PGPR cultures on open Petri dishes, without lids (Attia *et al.*, 2020).

Studied parameters

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

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.

RESULTS

In vitro antagonism test

In vitro direct bioassays

The 14 tested PGPR strains induced a reduction in the growth of *A. terricola* with variable degrees (Figure 1a). A noticeable change in the colour of *A. terricola* colonies from greenish black (control) to whitish or greyish was recorded. The inhibition percentages obtained after 10 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. frigotolerans* FR1.38, and *B. oceanisediminis* FR1.5 have inhibition rates over 60%. The PGPR strain O3RR25 (*P. koreensis*) displayed the lowest inhibition rate (25±5.41%), whereas the highest inhibition rate (71.87±3.12%) was recorded with the strain FR1.11 (*B. megaterium*).

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 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 3). However, mycelium hyphae treated with *A. terricola* VOCs displayed wrinkled surfaces, deformations, and irregular lengths, often accompanied by empty segments (indicated by red arrows). Thin or fissured structures (highlighted by yellow arrows), and globular swellings at the ends of the mycelial strands (marked with black arrows) were noted. A significant inhibition 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 the control group (indicated by green arrows).

In vivo antifungal activity

The strain *B. megaterium* FR1.11, showing the highest *in vitro* antagonism potential, was selected to conduct *in vivo* bioassays. The development and spread of disease symptoms caused 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 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 exposed to PGPR VOCs, no necrotic spots were shown confirming the antifungal effect of these volatile substances.

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 pronounced symptoms compared to the infected control. The symptoms were limited to pale yellow spots with no signs of spreading. Seedlings infected with *A. terricola* exhibited symptoms namely yellowish and brown spots and more advanced physiological decline. Microscopic observations of leaves revealed variations in the developmental stages of the 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 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 treatments, indicating their inhibitory effects on the growth of *A. terricola*.

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).

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 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), followed by the fungus + *B. megaterium* FR1.11 treatment, which exhibited a significant increase compared to the control. Exposure of infected seedlings to volatile metabolites of the 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.

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±0.023 mg GAE

/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. 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* (0.847±0.026 mg GAE/g FW), followed by the *A. terricola*+PGPR treatment.

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 ± 1.81 µg/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 µg/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.

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.

DISCUSSION

A. terricola is known to be a phytopathogenic agent affecting various agronomic crops, including wheat (Imran *et al.*, 2011) and red pepper (Nahar *et al.*, 2004). Except the study by Cherif *et al.* (2022), which focused on the effect of three PGPR strains on the species *A. terricola* through *in vitro* direct antagonism tests, no other study has been reported for this 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 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 and exposure to volatile compounds in the *in vitro* tests. *In vivo* investigations further supported these findings, showing that the application of PGPR strain *B. megaterium* FR1.11 led to

reduced development of the symptoms of *A. terricola* on detached leaves and tomato seedlings, 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 volatile metabolites, as evidenced by both *in vitro* and *in vivo* studies. The effectiveness of PGPR in hindering the germination and development of fungal species on detached leaves confirms that volatile organic compounds are among the direct mechanisms of biological control employed by PGPR strains (Bahramisharif and Rose, 2019). However, the antifungal effect observed with soil inoculation suggests that PGPR strain *B. megaterium* FR1.11 may 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 pathogens. These bacteria exhibit fast germination in soil and possess high colonization capabilities (Ali *et al.*, 2020).

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

The findings of this study align with previous studies that have demonstrated a significant decrease in the levels of chlorophyll *a*, *b*, and total in tomato plants infected with *Alternaria* species, while carotenoid contents remained relatively stable compared to the control group (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 contents are considered significant indicators of photosynthetic performance in plants (Riahi *et 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. These results are consistent with other studies that have reported higher photosynthetic pigment 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 to enhance the plant's tolerance to osmotic stress under normal conditions. Proline serves as an 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 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 membrane structures, supporting the function of various enzymes and proteins, and providing protection against reactive oxygen species (ROS) and other biochemical reactions (Khanna *et 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 content. The MDA contents decreased significantly compared to the infected plants and reached levels similar to the control after treatment with the PGPR strain or exposure to its volatile compounds. This indicates a reduction in the degree of membrane lipid oxidation and a decrease 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 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 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 enhance the oxidative status of infected plants by scavenging the reactive oxygen species generated during fungal infection. This was reported to occur through the upregulation of antioxidative defense genes (Khanna *et al.*, 2019).

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-
389 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

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

17. Gupta, P. K. 2018. Chapter 45 - Toxicity of Fungicides, Editor(s): Ramesh C. Gupta, Veterinary Toxicology (Third Edition), Academic Press, Pages 569-580.
18. Haidar, R., Roudet, J., Bonnard, O., Dufour, M. C., Corio-Costet, M. F., Fert, M., Gautier, T., Deschamps, A., and Fermaud, M. 2016. Screening and Modes of Action of Antagonistic Bacteria to Control the Fungal Pathogen *Phaeomoniella chlamydospora* Involved in Grapevine Trunk Diseases. *Microbiol. Res.*, **192**: 172-184.
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. *Pseudomonas rhizophila* S211, a New Plant Growth-Promoting Rhizobacterium with Potential in Pesticide-Bioremediation. *Front. Microbiol.*, **9**: 34.
20. Imran, Z. K. 2011. Isolation and Identification Species of *Ulocladium Preuss* from Different Regions in Iraq. *Basrah J. Agric. Sci.*, **24**: 27-47.
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: 320.
22. Kazerooni, E. A., Maharachchikumbura, S. S. N., Al-Sadi, A. M., Kang, S. M., Yun, B. W., Lee, I. J. 2021. Biocontrol Potential of *Bacillus amyloliquefaciens* against *Botrytis pelargonii* and *Alternaria alternata* on *Capsicum annuum*. *J. Fungi* **7**: 472.
23. Khanna, K., Kohli, S. K., Ohri, P., Bhardwaj, R., Al-Huqail, A. A., Siddiqui, M. H., Alosaimi, G. S., and Ahmad, P. 2019. Microbial Fortification Improved Photosynthetic Efficiency and Secondary Metabolism in *Lycopersicon esculentum* Plants Under Cd Stress. *Biomolecules*, **9**: 581.
24. Kousar, B., Bano, A., and Khan, N. 2020. PGPR Modulation of Secondary Metabolites in Tomato Infested with *Spodoptera litura*. *Agronomy*, **10**: 778.
25. Lichtenthaler, H. K., and Wellburn, A. R. 1983. Determination of Total Carotenoids and Chlorophyll a and b of Leaf Extract in Different Solvents. *Biochem. Soc. Trans.*, **11**: 591-592.
26. Nahar, S., Mushtaq, M., and Pathan, I. H. 2004. Seed-borne Mycoflora of *Capsicum annuum* Imported from India. *Pak. J. Bot.*, **36**: 191-197.
27. Parasuraman, P., Pattnaik, S. S., Busi, S., Marraiki, N., Elgorban, A. M., and Syed, A. 2022. 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.

37. Wang, H., Liu, R., You, M. P., Barbetti, M. J., and Chen, Y. 2021. Pathogen Biocontrol Using Plant Growth-Promoting Bacteria (PGPR): Role of Bacterial Diversity. *Microorganisms*, **9**: 1988.

38. Woudenberg, J. H., Groenewald, J. Z., Binder, M., and Crous, P. W. 2013. *Alternaria* Redefined. *Stud. Mycol.*, **75**: 171-212.

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

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	Olive tree rhizosphere	*Pseudomonas azotoformans*	NR113600
O3R24		*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

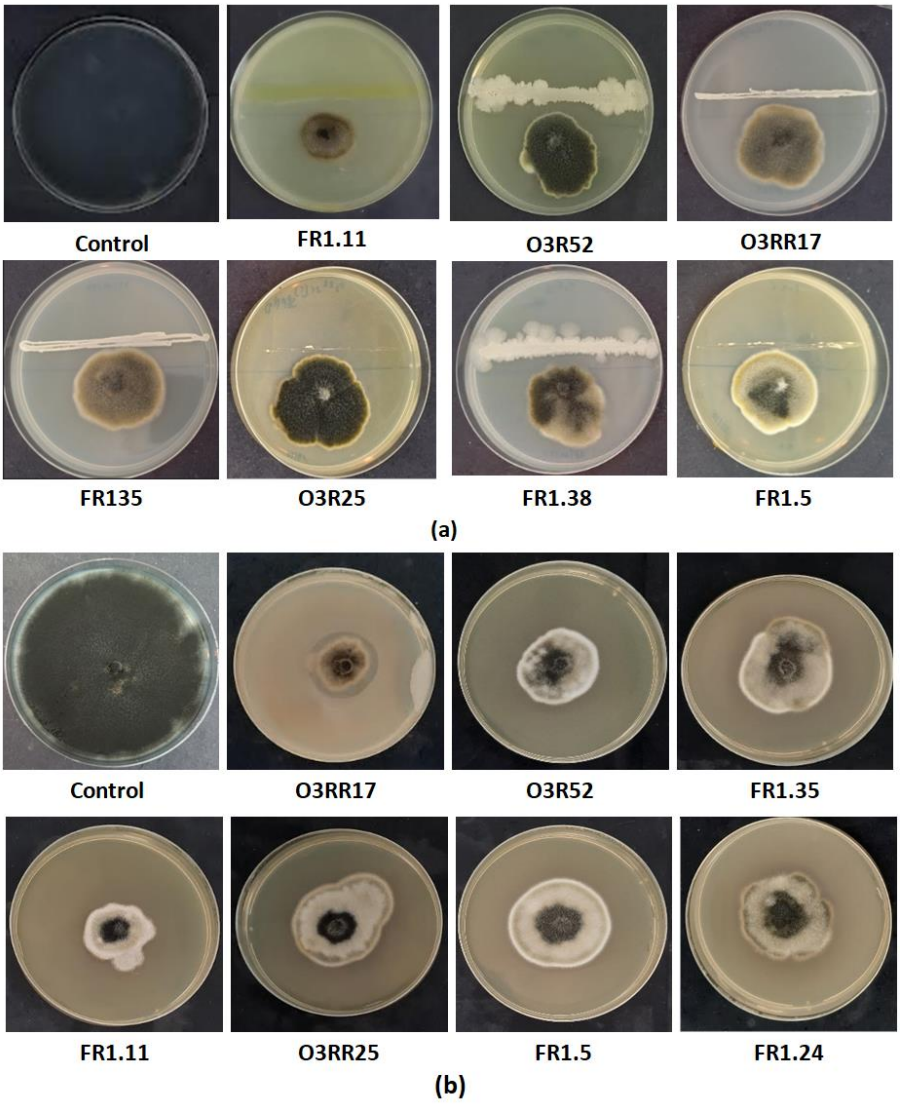


Figure 1. Macroscopic observation of the direct antagonism test (a) and indirect antagonism test (b) between PGPRs and *Alternaria terricola* after 10 days of incubation on PDA medium.

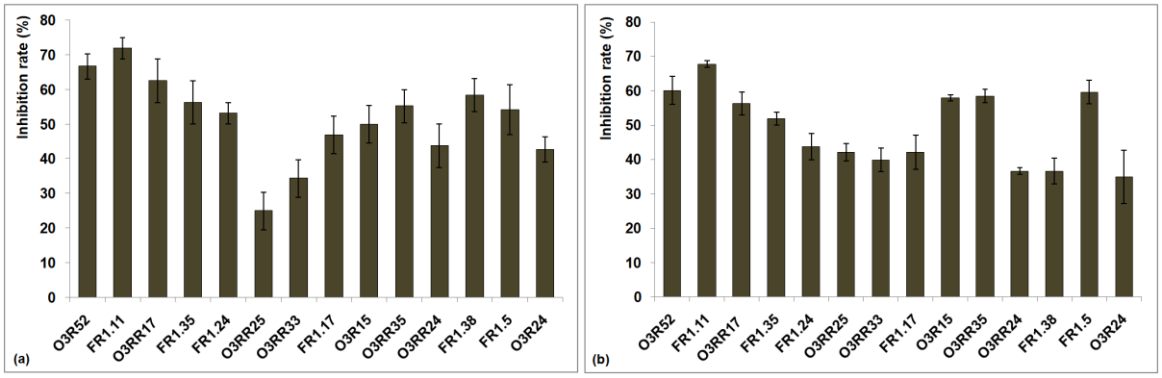


Figure 2. Variation in the percentage of growth inhibition of *Alternaria terricola* in direct confrontation between different PGPR strains (a) and following exposure to volatile compounds (b).

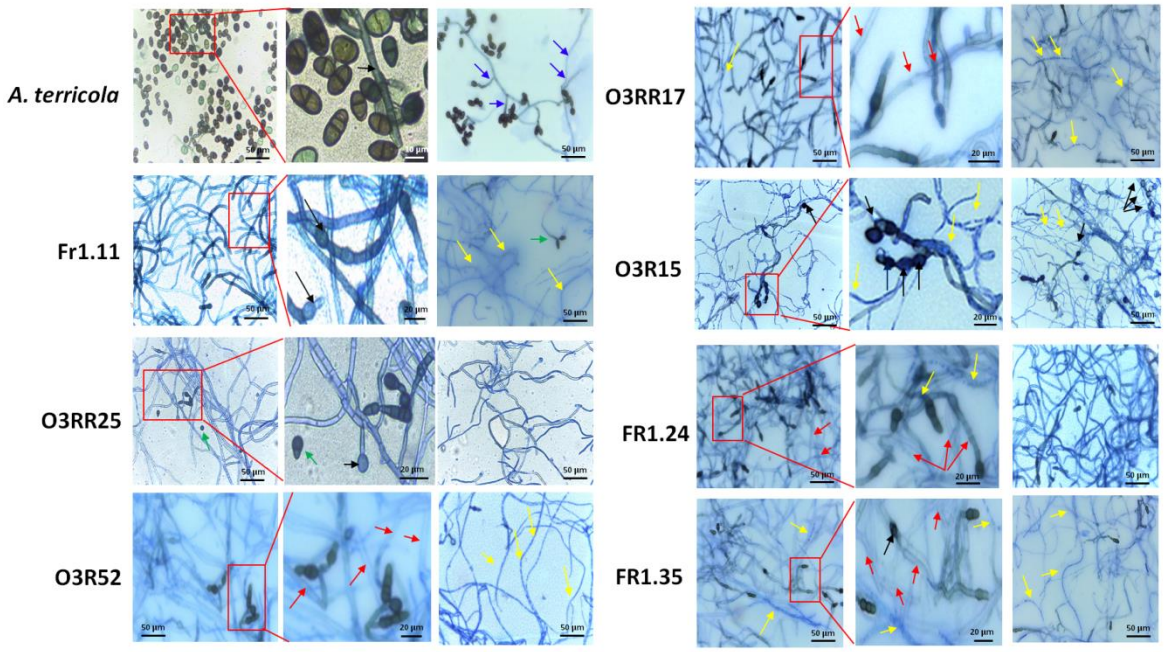


Figure 3. Microscopic observation of *Alternaria terricola* following the indirect antagonism test based on the application of PGPR VOCs after 10 days of incubation. Scale bars in µm.

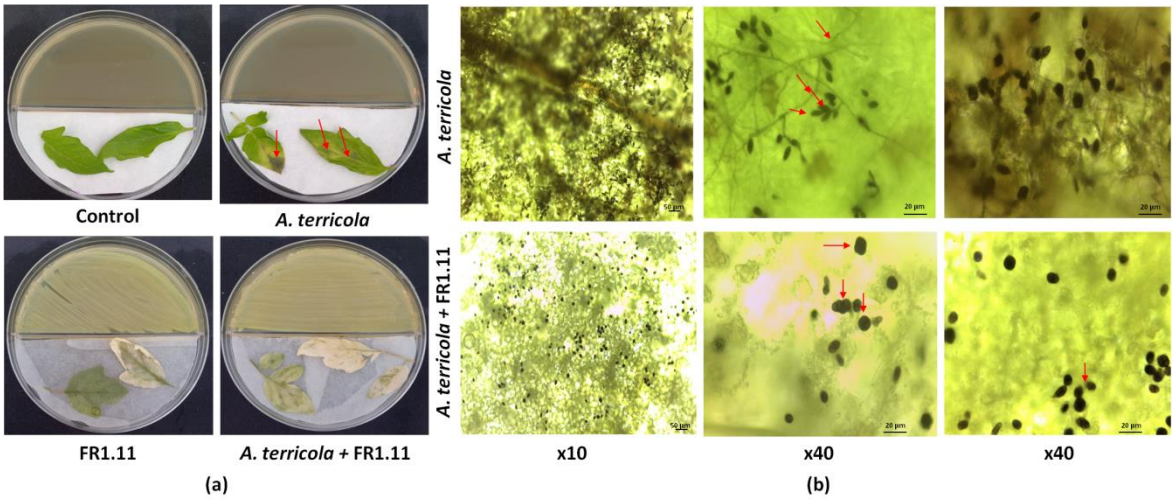
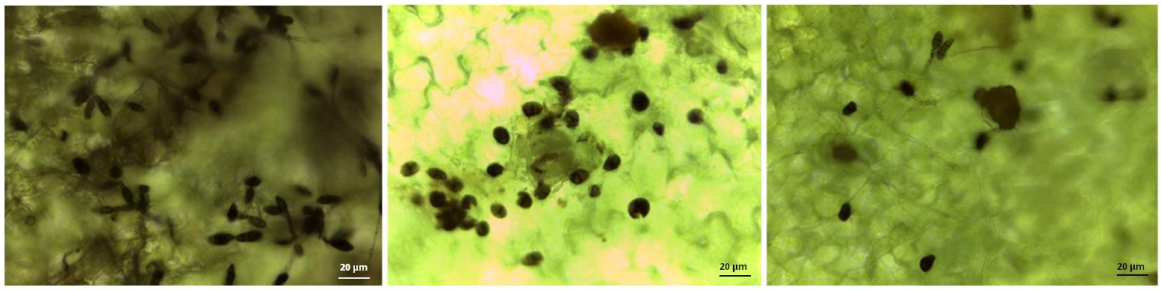


Figure 4. Macroscopic (a) and microscopic (b) observations of detached tomato leaves after 10 days of exposure to *Bacillus megaterium* FR1.11 volatile metabolites. Scale bars in µm.



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

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

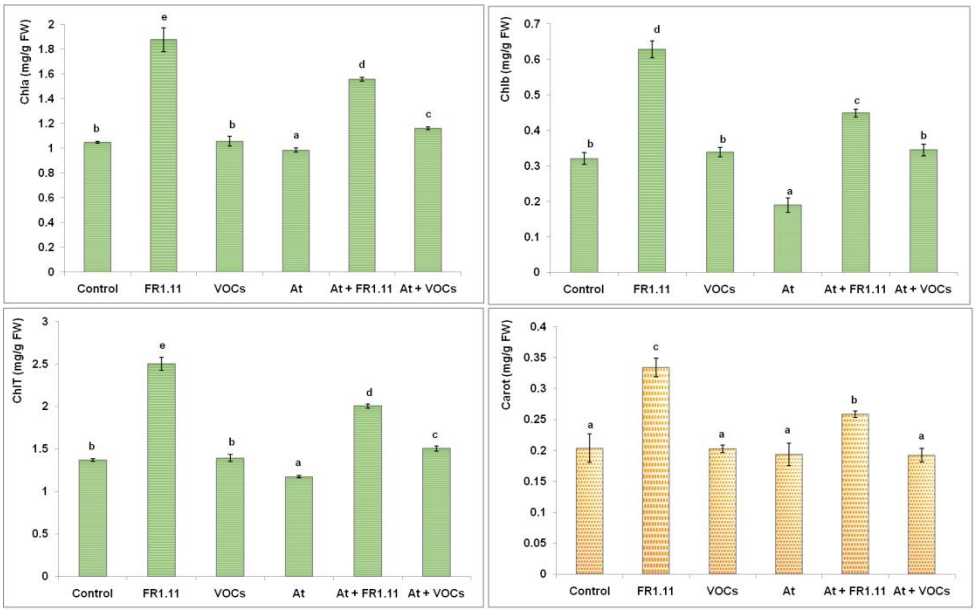


Figure 6. Variation in the content of chlorophyll *a*, *b*, total, and carotenoids (mg/g FW) in tomato seedlings according to the treatments.

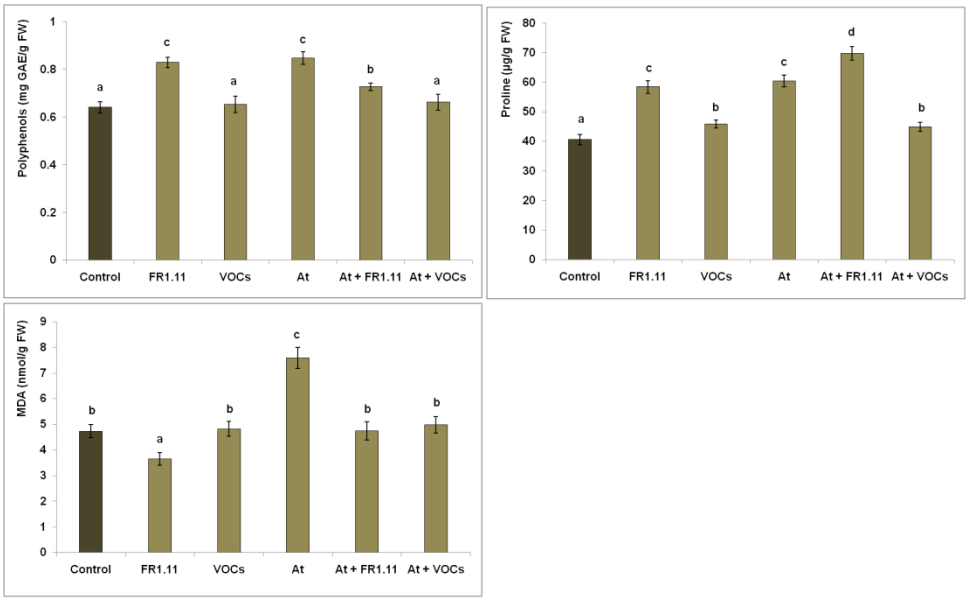


Figure 7. Variation in the content of total polyphenols (mg GAE/g FW), proline contents (µg/g FW) and malondialdehyde (nmol/g FW) in tomato leaves according to the applied treatment.