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Prospects of *Bacillus amyloliquefaciens* (MZ945930) Mediated Enhancement of *Capsicum annuum* L. Plants Under Stress of *Alternaria alternata* in Terms of Physiological Traits, Thiol Content, Antioxidant Defense, and Phytohormones

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Abstract
Plants encounter many biotic entities, such as fungi, bacteria, and nematodes, which induce biotic stress that disrupts normal metabolism and limits the growth and productivity of plants. Currently, the use of plant growth-promoting bacterial endophytes instead of synthetic fungicides is intriguingly eco-friendly. An in vitro and in vivo antagonistic approach using *Bacillus amyloliquefaciens* RaSh1 was used to mimic the pathogenic effect of *Alternaria alternata*. The results showed that *B. amyloliquefaciens* significantly inhibited pathogenic fungal growth in vitro. Further, *Capsicum annuum* L. (pepper plants) were grown and subjected to inoculation with *B. amyloliquefaciens* and infected with *A. alternata,* and then the growth attributes, photosynthetic pigments, physio-biochemical parameters, and the level of endogenous phytohormones were assessed. Under the pathogen attack, the main responses, such as plant length, total fresh and dry weights, total chlorophylls, and pigments, were reduced, accompanied by increases in H_2O_2. As well, infection of pepper with *A. alternata* caused down-regulation in the plant hormonal system by significantly decreasing gibberellins, indole-3-acetic acid, abscisic acid, as well as cytokinin concentrations. Although, with *B. amyloliquefaciens* application, an enhancement in growth, photosynthetic pigments, proline, thiol content, phenylalanine ammonia-lyase, and peroxidase in pepper plant leaves appeared while the content of H_2O_2 decreased. Endogenous phytohormones were found to be upregulated in *B. amyloliquefaciens*-inoculated and diseased plants. The current study found that *B. amyloliquefaciens* RaSh1 rescued pepper plant growth by modulating antioxidant defense and regulating hormones, and could be used to control *A. alternata* in an environmentally friendly manner while maintaining sustainable agriculture and food security.

Keywords Antioxidant enzymes · Biocontrol · Nutrient contents · Phenylalanine ammonia-lyase · Phytohormones · Plant growth-promoting rhizobacteria · Total thiol

Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase
Cyts	Cytokinins
Gibs	Gibberellins
IAA	Indole-3-acetic acid
PAL	Phenylalanine ammonia-lyase
PGPR	Plant growth-promoting rhizobacteria
POD	Peroxidase
Tdwt	Total dry weight
Tfwt	Total fresh weight

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Introduction

The pepper (*Capsicum annuum* L.) plant is one of the most important vegetable crops grown worldwide, valued for its economic and nutritional value, and is a member of the large Solanaceae family (Parisi et al. 2020). Indeed, pepper is a potent source of health-promoting compounds, including significant nutraceutical and anticancer properties. The main challenges for vegetable crop improvement are linked to agricultural sustainability, food security, and

rising consumer demand for food. Despite this, several pests and diseases pose a threat to its cultivation around the world, limiting its productivity (Sarath Babu et al. 2011). *Phytophthora capsici, Rhizoctonia solani, Alternaria alternata, Verticillium dahliae,* and *Fusarium* spp. are among the phytopathogens that limit the global economic productivity of *C. annuum* (Parisi et al. 2020; Soliman et al. 2022). Leaf spot disease, triggered by *Alternaria* spp., is one of the most severe diseases, causing significant losses and lowering food quality and quantity, thereby degrading plant nutritive values. *Alternaria alternata* has successfully infected a wide range of economically important crops, including horticultural, ornamental, and weed species (Chung 2012).

Alternaria sp. is widespread in the environment and produces specific mycotoxins that have been found in various fruits and vegetables, and their derivative products such as juices, beverages, and sauces (Ostry 2008; den Hollander et al. 2022). Furthermore, toxins were found in pepper, sorghum, wheat, oats, and tomatoes infested with *A. alternata* (da Motta and Soares 2000). Toxin exposure causes genotoxic, mutagenic, and carcinogenic effects in humans and animals (Pavón et al. 2012). As a result, plants often activate a wide range of defense responses to prevent pathogen infection and induce disease resistance, such as the induction of defense-related enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO), and lipoxygenase (Wojtaszek 1997). Plants also initiate secondary metabolic responses against plant pathogens, and plant hormones play a dynamic role in plant development and resistance to biotic stresses (Katagiri and Tsuda 2010).

Pathogen diseases should be controlled in addition to plant defense mechanisms. Traditionally, commercial fungicides have been used to control plant diseases; however, their applications are highly toxic in the agro-food chain, causing serious environmental challenges and encouraging resistance in some fungi (Zouari et al. 2016; Metwally and Abdelhameed 2019; Metwally et al. 2022a; Soliman et al. 2022). To maintain sustainable agriculture and food security, environmentally friendly and nature-inspired solutions and strategies are urgently needed. Microbial enemy control strategies can be used to combat pathogenic fungi's antagonistic behavior. For pathogenic fungal attacks, biological management via plant growth-promoting rhizobacteria (PGPR) or endophytic bacteria offers an eco-friendly alternative to chemically produced fungicides (Droby et al. 2009). Plant growth-promoting rhizobacteria have also been named for their intriguing role in mitigating biotic stresses by inducing complex metabolic changes at the cellular level (Singh et al. 2013; Gupta et al. 2017). They influence physiology and phytohormonal signaling throughout pathogenic attacks by reprograming the growth of their associated host (Rosenblueth and Martnez-Romero 2006).

The most predominant PGPR isolated from different plant species and used commercially in modern farming systems are *Bacillus* spp. because of their ability to form heat and UV-resistant spores that withstand adverse environmental conditions (Zhang et al. 2014). Moreover, they have antifungal properties due to their excretions, which contain diverse plant-beneficial materials such as fengycin, surfactin, enzymes, and nutritional factors that promote plant growth (Fan et al. 2017; Zhang et al. 2018; Duan et al. 2021). *Bacillus*-based fertilizers can increase plant growth by increasing the plant-available forms of nutrients in rhizospheres, controlling disease-causing pathogenic microbial growth, inducing plant defense systems, and developing biofilms (Garcia-Fraile et al. 2015; Kang et al. 2015).

Among *Bacillus* spp., *B. amyloliquefaciens* is best known for its ability to promote plant growth while also providing health benefits to the host (Dhumal et al. 2021; Ahmed et al. 2022; Soliman et al. 2022). Co-inoculation of soybean plants with *B. amyloliquefaciens* LL2012 strain and *Bradyrhizobium japonicum* improved plant growth parameters, according to Masciarelli et al. (2014). Similarly, numerous scientists confirmed the role of *B. amyloliquefaciens* in the biocontrol of various pathogenic fungal diseases such as apple ring rot (Chen et al. 2016), charcoal rot of soybean and common bean (Torres et al. 2016), tomato damping-off (Zouari et al. 2016), white rot disease of garlic (Rashad et al. 2020), and gray mold disease of pepper (Kazerooni et al. 2021). The current study was carried out to mimic the attack of pathogenic *A. alternata* on pepper plants and to investigate the prospects of endophytic microbial application to crop disease resistance. Its goal was to determine the interaction of *B. amyloliquefaciens* RaSh1 (MZ945930) with pepper host plants in the rhizosphere via root and soil inoculation, as well as their biocontrol potential and mechanism of biocontrol of this pathogen, by examining physio-biochemical parameters and the level of endogenous phytohormones that induce defense mechanisms in pepper plants.

Materials and Methods

Microbial Inoculums Preparation

Alternaria alternata RaSh3 (OK053809.1), isolated from diseased pepper leaves with leaf spot disease symptoms, was used as a pathogen. The pure culture of *A. alternata* was grown on a PDA medium for 7 days at 25 °C, then flooded with sterile distilled water and gently agitated to obtain spore suspension, and its final concentration was adjusted (10^5 cfu/mL).

The previously isolated *B. amyloliquefaciens* RaSh1 (MZ945930) from *Brassica oleracea* leaves (Soliman et al. 2022) was cultured in Erlenmeyer flasks with 250 mL of

nutrient medium and incubated at 30 °C on an incubator shaker (100 rpm) for 48 h before being used as a biocontrol agent against *A. alternata* RaSh3.

Antifungal Activity of *B. amyloliquefaciens* RaSh1 Cell-Free Culture Filtrates Against Alternaria sp.

The poisoned food technique was used to assess the antifungal activity of cell-free culture supernatants from *B. amyloliquefaciens* RaSh1 against *A. alternata* (Kumar et al. 2008). Antagonistic *B. amyloliquefaciens* RaSh1 bacteria were cultured in nutrient broth for 48 h at 30 °C on an incubator shaker (150 rpm), and then centrifuged for 10 min at 12,000 rpm in a cooling centrifuge (Vision SCIENTIFIC CO., LTD., South Korea). The bacterial culture supernatant was filtered through a 0.2 µm sterilized syringe filter. PDA plates were prepared with different concentrations (100, 250, 500, and 1000 µL) of cell-free culture supernatants. Then, a 6-mm-diameter mycelial disc was cut from a 7-day-old *A. alternata* culture and placed in the center of the agar medium. After that, the plates were incubated at 28 °C for 2, 4, and 8 days to measure mycelial growth. Periodic observations of mycelium linear growth were made, and the average diameter of fungal growth was measured. Each treatment had three replicates.

Greenhouse Biocontrol Assays

The efficacy of a biocontrol agent (*B. amyloliquefaciens* RaSh1) against leaf spot/blight disease produced by *A. alternata* was tested in vivo under a greenhouse condition using pepper (*Capsicum annuum* L.) seedlings. The experiment was carried out in plastic bags, each filled with 2 kg of sterile clay soil. Forty-day-old seedlings that were propagated in a nursery greenhouse were used. Six treatments were conducted (Control plants, *A. alternata*-infected plants, Thiram (0.2%)-sprayed plants, *A. alternata* diseased and sprayed with Thiram (0.2%) plants, *B. amyloliquefaciens* RaSh1-applied plants, and *A. alternata* diseased and applied with *B. amyloliquefaciens* RaSh1 plants) with 10 replicates for each treatment (6 × 10). Treatments were applied to pepper seedlings as follows:

Control treatment: Seedlings were sprayed and irrigated with tap water only.
***Bacillus amyloliquefaciens* RaSh1 treatments:** Roots of pepper seedlings were initially soaked in *B. amyloliquefaciens* RaSh1 suspension for 4 h before transplanting; also, 50 mL of bacterial inoculum was used in irrigation.
Fungicide treatments: Thiram fungicide (0.2%) was sprayed on the leaves of the pepper seedlings.
Pathogen treatments: After 2 days of bacterial and fungicide treatments, healthy seedling leaves were scraped with a sterile needle to make wounds before being pipetted with individual droplets of *A. alternata* (10^5 cfu/mL).

Following pathogen inoculation, the inoculated plants were kept in polyethylene bags for 24 h to maximize pathogen ingress and were maintained at high humidity levels (80–90%). After 28 days, the plant samples were harvested at the growth stage for all analyses.

Measurements

Data Collection for Growth Parameters

After 28 days of *B. amyloliquefaciens* RaSh1 application, all pepper plants were washed with tap water and dried with paper tissues. The pepper plants' plant heights were measured. Its total fresh weight (Tfwt) was determined, and the samples were then placed in a 70 °C oven for 2 days to determine their total dry weight (Tdwt). The number of leaves was also counted.

Plant Metabolite Assays

Estimation of Total Chlorophyll and Pigments Content Fresh pepper leaves (100 mg) from each treatment were cut into small pieces and extracted with 85% cold acetone (Metzner et al. 1965). Using a spectrophotometer and a solvent (acetone) blank, the absorbance was utilized to compute the chlorophyll and carotenoid concentrations at 644, 663, and 452.5 nm. Then total chlorophylls and total pigments were further calculated using the Lichtenthaler and Wellburn (1983) formulas.

Determination of Osmolytes Content (Total Soluble Protein, Proline, and Total Carbohydrates) Using the Folin–Ciocalteu reagent at 700 nm and bovine serum albumin as a standard, the total soluble protein contents of pepper leaves from each treatment were determined (Lowry et al. 1951). In addition, the Bates et al. (1973) method was employed to determine the proline content of pepper leaves. To summarize, 0.25 g fwt of pepper leaves were extracted in 3% sulphosalicylic acid and centrifuged at 6000 rpm in a cooling centrifuge (MIKRO 200R Hettich zentrifugen, Germany) for 10 min. Two mL of filtrate was placed in new test tubes and allowed to react with 2 mL of ninhydrin reagent and 2 mL of glacial acetic acid before being placed in a boiling water bath. Four mL of toluene was added, and the mixture was thoroughly mixed until the upper colored layer appeared, which was separated from the mixture and its absorbance measured at 520 nm using pure proline as a standard. To determine total carbohydrate content, approximately 0.1 g of dried pepper leaves were heated in a water bath for 3 h at 100 °C with 10 mL of 2.5 N HCl (Dubois

et al. 1956). The pepper leaf extract (0.2 mL) was taken and incubated at room temperature with 1 mL of phenol. After 1 h, 2.5 mL of sulfuric acid was added to the mixture and thoroughly mixed, and the absorbance at 490 nm was measured using glucose as a standard.

Stress Marker (Hydrogen Peroxide [H_2O_2]) Estimation The Xylenol orange method was used to determine the amount of H_2O_2 present in tricarboxylic acid (TCA; 0.1%) extracts of pepper plant samples (Alexievia et al. 2001). In 0.1% TCA, a known fwt of pepper leaf tissue (0.5 g) was homogenized. Filtration was performed on the homogenate. To 0.5 mL of leaf extract, 0.5 mL of 100 mM K-phosphate buffer (pH 6.8), and 2 mL of reagent (1 M KI w/v) were added. The reaction was developed in the dark for 1 h, and the absorbance at 390 nm was measured.

Quantification of Total Thiol, Non-protein Thiol, and Protein Thiol Using Ellman's Reagent, the levels of total thiol, non-protein thiol, and protein thiol in fresh pepper leaves were determined in accordance with Sedlak and Lindsay's (1968) technique. The known weight of pepper leaves was homogenized in 10 mL of 0.2 M Tris–HCl (pH 7.4) and centrifuged at 8000 rpm for 15 min at 4 °C before being assayed for total thiol and non-protein thiol. To determine the total thiol, 0.5 mL of the supernatant was combined with 1.5 mL of 0.2 mM Tris–HCl (pH 8.2), 0.1 mL of 0.01 M DTNB (Ellman's Reagent) (5,5-dithio-bis-(2-nitrobenzoic acid), and 7.9 mL of absolute methanol to develop yellow color that was measured after 15 min at 415 nm against a blank containing 0.5 mL of distilled water instead of the supernatant. Total sulfhydryl groups were calculated and expressed as mg/g fwt using an extinction coefficient of 13,600. 5 mL of the supernatant was mixed with 4 mL of distilled water and 1 mL of 50% TCA to determine the non-protein thiol content. After 15 min, the mixture was centrifuged at 8000 rpm for another 15 min. Non-protein thiol concentration was determined in 2 mL of deproteinized supernatant in the same way that total thiol concentration was determined. The protein thiol content was calculated by subtracting the non-protein thiol content from the total thiol content.

Evaluation of the Antioxidant Enzymes (POD and APX) Activity Fresh pepper leaf (1 g) was homogenized in 10 mL of extraction buffer, which contained 1% (w/v) polyvinyl pyrrolidone, 0.1 mM ethylenediaminetetraacetic acid, and 100 mM phosphate buffer (K_2HPO_4/KH_2PO_4) (pH 7.0). The supernatant was used to assay enzyme activities after centrifugation at 4 °C. The Bergmeyer (1974) method was used to determine the activity of peroxidase (POD; EC 1.11.1.7) in the pepper leaf samples. The activity of ascorbate peroxidase (APX; EC 1.1.11.1) was determined using the Nakano and Asada method (1981).

Phenylalanine Ammonia-Lyase (PAL; EC 4.1.3.5) Enzyme Extraction and Assay Fresh leaf tissues (1g) were extracted with 50 mM phosphate buffer (pH 7.0) from the control and treated plant samples. The homogenate was filtered and centrifuged for 10 min at 8000 rpm.

According to McCallum and Walker (1990), PAL activity was assessed in enzyme extracts using a modification of Zucker's (1971) technique. The assay solution contained 0.06 M borate buffer and crude enzyme. The addition of L-phenylalanine started the reaction. For 30 min, the tubes were incubated at 30 °C. The denatured protein was then pelleted by centrifuging the tubes for 5 min at 5000 rpm. By using a UV spectrophotometer to measure the A290 of the supernatant in 10 mM quartz cuvettes, the yield of cinnamic acid was calculated.

Pepper Growth Hormone Extraction, Purification, and Quantification

- Extraction and purification of hormones

  It was crucial to determine how *A. alternata* pathogenic attacks and *B. amyloliquefaciens* RaSh1 management affected plant growth-regulating compounds in comparison to the control due to their considerable effects. As a result, phytohormones such as indole-3-acetic acid (IAA) and gibberellins (Gibs), cytokinins (Cyt), as well as abscisic acid (ABA) were measured in pepper leaf samples. Their concentrations were determined using a UV–Vis detector and high-performance liquid chromatography (HPLC) (Durley et al. 1982; Wurst et al. 1984). The sample was collected 4 weeks after the *A. alternata* application. Ten grams of fwt/treatment were placed in a beaker filled with 70% (v/v) methanol and stirred overnight at 4 °C. The extract was filtered, and the methanol was vacuum evaporated. The aqueous phase was adjusted to pH 8.5 with 0.1 M phosphate buffer before being partitioned three times with ethyl acetate. The pH of the aqueous phase was adjusted to 2.5 with 1 N HCl after the ethyl acetate phase was removed. The solution was partitioned three times with diethyl ether before being passed through anhydrous sodium sulfate. Following that, the diethyl ether phase was vacuum evaporated, and the dry residue containing hormones was dissolved in 2.0 mL of absolute methanol and stored in a vial at 4 °C.
- Determination and quantification of hormones using HPLC procedures

  HPLC with UV–Vis spectroscopy detection is the most commonly used analytical technique for quantifying plant hormones due to its versatility, rapidity, simplicity, and ease of optimization (Kelen et al. 2004). An Agilent Model 1260 was used for the chromatographic analysis. The mobile phases used were acetonitrile-water (26:74; 30:70%; v/v), 30 mM phosphoric acid, and sodium

hydroxide to adjust the pH. The Luna C18 column was equilibrated for each mobile phase condition for 30 mins. The column temperature was kept constant at 25 °C. The separation was accomplished through isocratic elution at a flow rate of 1.0 mL/min. For each analysis, a 10 µL injection volume was used. The retention time for each acid at wavelength: 210 nm was determined by preparing a standard solution of each acid in the mobile phase and chromatographing it separately.

Estimation of Potassium (K) and Magnesium (Mg) Contents The dried pepper leaves from all treatments were ground separately to a fine powder and then acid-digested with $H_2SO_4 + H_2O_2$. The powder (0.1 g) was first acid-digested with H_2SO_4 for 4 h at 200 °C (Lowther 1980). Following the addition of H_2O_2, the concentrations of K and Mg in the samples were measured calorimetrically using an atomic absorption spectrophotometer after proper dilution of digested materials.

Processing of Data and Statistical Analysis Data are the means ± standard error. Statistical differences between different treatments were compared using One-way ANOVA analysis and Duncan's multiple range tests (DMRTs) at a significance level of $p \leq 0.05$ (p-value less than 0.05 was considered statistically significant) using SPSS version 16. OriginPro 8.5 data analysis and graphing software were used to create the figures.

Results and Discussion

Evaluation of Antifungal Activity of *B. amyloliquefaciens* Cell-Free Culture at Different Concentration Under In Vitro Conditions

Several *Alternaria* spp. are responsible for significant yield losses in food crops (Meena and Samal 2019). *Alternaria* spp. have developed resistance to chemical fungicides, necessitating the development of novel, environmentally friendly management methods (Fairchild et al. 2013). *Alternaria alternata* growth inhibition achieved by *B. amyloliquefaciens* RaSh1 cell-free culture filtrates is presented in Fig. 1 and Table 1. Several studies agree with our findings (Raut et al. 2021; Soliman et al. 2022; Jia et al. 2023). The obtained results indicated that *B. amyloliquefaciens* RaSh1 exhibited strong antagonistic activity against *A. alternata*. *Bacillus* spp. culture filtrates contain a variety of antimicrobial compounds with distinct modes of action that target various biological processes in the pathogen (Arguelles-Arias et al. 2009; Alvarez et al. 2012). In comparison to living biological control organisms, the use of culture filtrates for plant

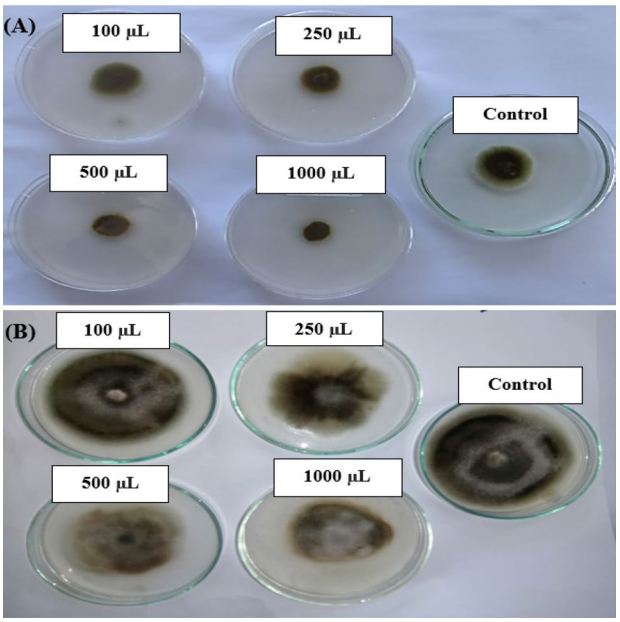

Fig. 1 Poisoned-agar technique to evaluate different concentrations of *B. amyloliquefaciens* RaSh1 cell-free culture filtrates against *A. alternata*. **A** PDA plates amended with 100, 250, 500, and 1000 µL and non-amended PDA (control) plates after 2 days of inoculation. **B** PDA plates amended with 100, 250, 500, and 1000 µL and non-amended PDA (control) plates after 8 days of inoculation

disease control has several benefits (Ali et al. 2016). This is because culture filtrates are more efficient as well as quicker to apply than biological control agents in the rhizosphere, which need time to develop and adapt to their environment. According to Calvo et al. (2017), cell-free supernatants of *B. amyloliquefaciens* BUZ-14 demonstrated strong in vitro antifungal activity against a variety of fungi.

In Vivo Biocontrol Assay

Biotic stress brought on by plant pathogens is a significant issue that costs growers a lot of money throughout the development of crops. A variety of chemical fungicides are currently used to control fungal plant pathogens. However, their use is not recommended due to public concern about hazardous residues, their selectivity, and rising plant protection expenses. The development of microbe-based control methods could result in effective crop disease management. Free-living, non-pathogenic, root-colonizing bacteria are used as bioinoculants in a variety of economically important plants in a wide range of agricultural production systems (Harish et al. 2019; Kazerooni et al. 2021). In vitro antagonistic activity of *B. amyloliquefaciens*, a plant growth-promoting bacterium, was observed against *A. alternata* in our study. The effect of this interaction on reducing *A. alternata*-caused leaf spot disease in pepper plants was studied in vivo.

Table 1 Effect of different concentrations of *B. amyloliquefaciens* RaSh1 cell-free culture filtrates against *A. alternata* radial growth

Concentrations of bacterial cell-free culture filtrates	Diameter of *A. alternata* growth (cm) after different days of incubation			
	2	4	6	8
Control	1.80 ± 0.0577a	3.50 ± 0.115a	4.50 ± 0.173a	7.83 ± 0.088a
100 µL	1.63 ± 0.0882a	3.00 ± 0.152b	4.30 ± 0.115a	6.77 ± 0.033b
250 µL	1.07 ± 0.0667b	2.27 ± 0.033c	3.10 ± 0.115b	5.70 ± 0.057c
500 µL	1.10 ± 0.011b	1.97 ± 0.145c	2.70 ± 0.21bc	5.30 ± 0.153d
1000 µL	1.00 ± 0.011b	1.53 ± 0.033d	2.40 ± 0.057c	4.30 ± 0.011e

The values are the means of 3 replicates ± SE ($n = 3$). The same letter within each column indicates no significant difference between the treatments ($p \leq 0.05$) as determined by Duncan's multiple range test (DMRT)

B. amyloliquefaciens RaSh1 Plant Growth-Promoting Traits on Pepper After *A. alternata* Infection

Biological control, which makes use of beneficial microbes, is an excellent method for limiting the negative impact of disease-causing microbes on plant health and productivity (Soliman et al. 2023). Figure 2a–d depicts the effects of *B. amyloliquefaciens* RaSh1 application on pepper plant growth traits under control and biotic stress conditions. Generally, when pepper plants infected with *A. alternata* were compared to healthy control plants, growth parameters, namely Tfwt, Tdwt of shoot and roots, and leaf number were significantly reduced. Likewise, Meena et al. (2016) and Kazerooni et al. (2021) detected growth reductions in tomato and pepper plants infected by *A. alternata*. This growth reduction may be attributed to cell damage induced by the pathogen, which could manifest as specific symptoms such as wilting, growth suppression, chlorosis, and necrosis. Furthermore, *A. alternata* pathogen exposure disrupts normal physiological processes such as photosynthesis, respiration, translocation, and transpiration, resulting in decreased growth and development (Meena et al. 2016).

Whether or not the plants were biotically challenged by *A. alternata*, *B. amyloliquefaciens* RaSh1 treatment significantly boosted growth parameters in the plants. *B. amyloliquefaciens* inoculation of plants increased plant height (7.69%), Tfwt (8.15%), and Tdwt (6.71%) in comparison to their respective controls in non-stressed plants (Fig. 2). A similar trend of improved growth characteristics was observed in diseased plants, where in *A. alternata*-infected pepper, application of *B. amyloliquefaciens* significantly improved plant height, Tfwt and, Tdwt as compared with *A. alternata*-infected plants (Fig. 2).

Both Zouari et al. (2016) and Rashad et al. (2020) previously confirmed that *B. amyloliquefaciens* RaSh1 has a growth-promoting impact on tomato and garlic plants. Similarly, Jamal et al. (2018) showed that pepper plant fwt and dwt were found to increase with *B. amyloliquefaciens* application compared to the control. This may be attributed to *B. amyloliquefaciens'* role in producing a diverse range of secondary metabolites, which are thought to be important

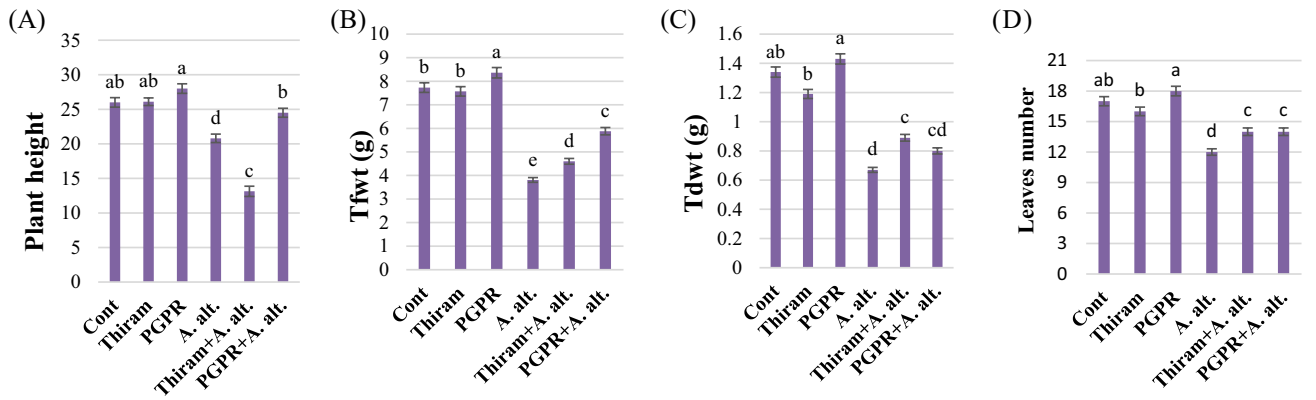

Fig. 2 **a** Plant height (cm), **b** total fresh weight (Tfwt, g), (**c**) total dry weight (Tdwt, g), and (**d**) leaves number of peppers plants grown under normal and *A. alternata* stress conditions and treated with plant growth-promoting rhizobacteria (PGPR). Treatments: Cont (control), PGPR (*B. amyloliquefaciens* RaSh1), Thiram (0.2% Thiram fungicide), Thiram + *A. alt* (0.2% Thiram fungicide + *A. alternata*), *A. alt* (*A. alternata*), PGPR + *A. alt* (*B. amyloliquefaciens* RaSh1 + *A. alternata*). The results are expressed as means of 10 replicates ± standard error (SE). Different Duncan's letters denote significant difference at $p < 0.05$

for improving plant growth and mitigating various biotic and abiotic stresses (Luo et al. 2022). In this study, it was discovered that *B. amyloliquefaciens*'s ability to produce phytohormones (IAA, Gibs, and Cyto) and defense-related antioxidant enzymes enhanced the *C. annuum* growth. Furthermore, increased plant growth has been shown to induce resistance to *A. alternata* pathogen infection. Another *Bacillus* growth promotion mechanism is the enhancement of water absorption (Khan et al. 2020) and nutrient uptake from the rhizosphere through processes such as phosphate solubilization and N-production (Pii et al. 2015).

Total Chlorophylls and Photosynthetic Pigments of Pepper Plants in Response to *A. alternata* and *B. amyloliquefaciens* RaSh1 Inoculation

The photosynthetic pigment is a vital indicator of plant physiological status (Selem et al. 2018; Metwally and Al-Amri 2019; Abdelhameed et al. 2021a; Abdelhameed and Metwally 2022). Pepper plants' total pigment and chlorophyll content were measured in both unstressed and stressed environments (Fig. 3a, b). Indeed, infection of pepper plants with *A. alternata* adversely influenced the total chlorophyll and pigments content compared to untreated plants. In comparison to control plants, *A. alternata*-stressed plants had lower total chlorophyll and pigment contents by 58% and 56%, respectively. Our results agree with Sharma et al. (2011) who showed that total chlorophyll content in *A. alternata*-diseased plants was drastically reduced in diseased tissue, with about a 16-fold reduction compared to healthy tissues.

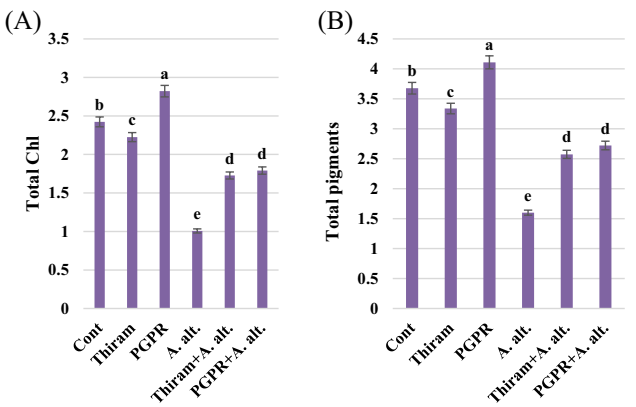

Fig. 3 **a** Total chlorophylls and **b** total pigments content (mg g^{-1} fwt) in the leaves of peppers grown under normal and *A. alternata* stress conditions and treated with plant growth-promoting rhizobacteria (PGPR). Treatments: Cont (control), PGPR (*B. amyloliquefaciens* RaSh1), Thiram (0.2% Thiram fungicide), Thiram + *A. alt* (0.2% Thiram fungicide + *A. alternata*), *A. alt* (*A. alternata*), PGPR + *A. alt* (*B. amyloliquefaciens* RaSh1 + *A. alternata*). Values show the means ± SE ($n = 3$) and significant differences are indicated at $p < 0.05$ in accordance with Duncan's multiple range test (DMRT). Bars with different letters are significantly different from each other

Similarly, comparing pepper plants under *Botrytis* and *Alternaria* stress to healthy plants, Kazerooni et al. (2021) found declines in total chlorophyll content of 9.96% and 40.34%, respectively. This drop in total chlorophylls and pigments may be attributed to the disorganization of the plastid membrane upon infection as reported by Alwadi and Baka (2001).

However, under *A. alternata* stress conditions, *B. amyloliquefaciens* RaSh1 inoculation was successful at $p \leq 0.05$ and led to about 77.8% (total chlorophylls) and 70% (total pigments) increases in comparison to those of infected plants (Fig. 3). A similar tendency of improved chlorophylls and pigments was noted by Kazerooni et al. (2021), where *B. amyloliquefaciens* caused 31.07% and 57.88% increases in total chlorophyll in pepper plants under *Botrytis* and *Alternaria* stress conditions, respectively, compared to those of infected plants. Additionally, our findings are consistent with those of Yildirim et al. (2008), who demonstrated that *Pseudomonas* and *Bacillus* caused an increase in chlorophyll content in *Raphanus sativus*. This increase in chlorophyll content may be attributed to the bacteria's function as a biofertilizer or attributable to the rise of 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase enzymes in PGPR-treated plants, which postpone the breakdown of chlorophyll. As well, the more effective absorption of nutrients by PGPR increased the chlorophyll content. In a similar manner, Srivastava et al. (2016) discovered that *B. amyloliquefaciens* SN13 improved carbon assimilation in rice plants with or without *R. solani*, which is well correlated with increased dry mass, chlorophyll content, and starch accumulation.

B. amyloliquefaciens RaSh1 Optimized the Osmolytes Production in *A. alternata*-Infected Pepper Plants

The osmolyte contents in pepper plants changed as a result of the pathogen (*A. alternata*) and biocontrol agent (*B. amyloliquefaciens* RaSh1) treatments (Fig. 4). When compared to the control, the *B. amyloliquefaciens* RaSh1 application improved the total carbohydrates and proline content. Similarly, *A. alternata*-infected pepper plants showed an increase in soluble proteins, proline, and total carbohydrates, respectively. Moreover, an additional increase in the osmolyte contents was recorded with the dual inoculation. The osmolytes such as carbohydrates, proline, and protein accumulate as antioxidants, neutralizing harmful ROS under stressful circumstances. Furthermore, during stressful circumstances, these osmolytes defend against cell membrane destruction and protein denaturation and also cause enzymatic proteins to become structurally stable, maintaining their functionality (Ghanbary et al. 2018; Abdelhameed and Metwally 2022; Metwally et al. 2022a). Besides, plant survival is extended by the osmotic components of cells, which

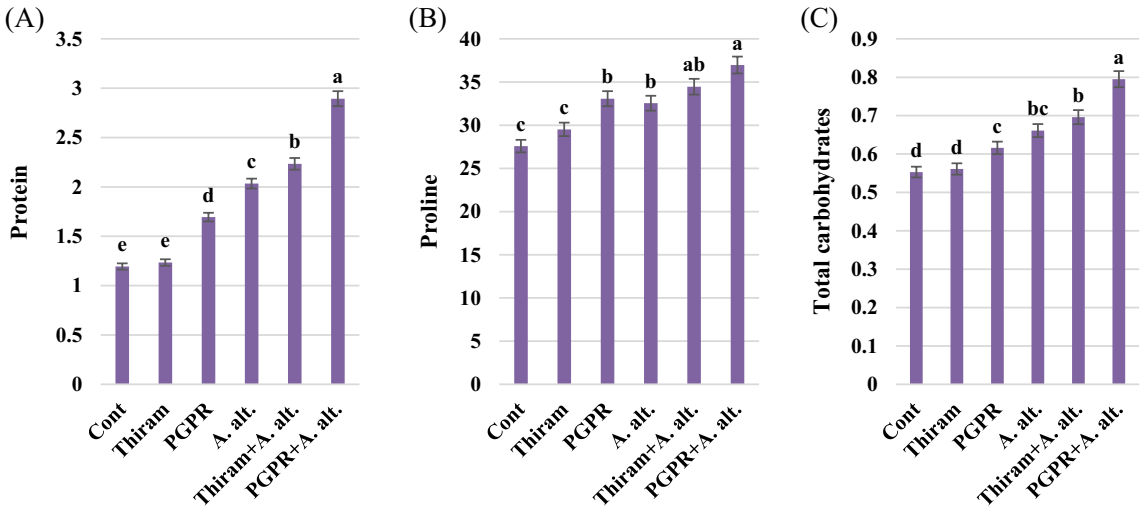

Fig. 4 **a** Protein (mg/g fwt), **b** proline (µmols/g fwt), and **c** total carbohydrates (mg/g dwt) content in the leaves of peppers grown under normal and *A. alternata* stress conditions and treated with plant growth-promoting rhizobacteria (PGPR). Treatments: Cont (control), PGPR (*B. amyloliquefaciens* RaSh1), Thiram (0.2% Thiram fungicide), Thiram + *A. alt* (0.2% Thiram fungicide + *A. alternata*), *A. alt* (*A. alternata*), PGPR + *A. alt* (*B. amyloliquefaciens* RaSh1 + *A. alternata*). Values show the means ± SE ($n = 3$) and significant differences are indicated at $p < 0.05$ in accordance with DMRT. Bars with different letters are significantly different from each other

lower the water requirement for active metabolism (Hashem et al. 2017).

Our results are consistent with that of Hashem et al. (2017) where *B. subtilis* application accelerated soluble sugars and proline synthesis in mung bean plants, and a further increase was reported with *Macrophomina phaseolina* infection. Additionally, the inoculation of *Mesorhizobium ciceri* IC53 and *B. subtilis* NUU4 together considerably raised the proline and protein levels in chickpea leaves (Egamberdieva et al. 2017). Furthermore, according to Ghanbary et al. (2018), the inoculation of *Biscogniauxia mediterranea* or *Obolarina persica* (agents of charcoal disease) increased the proline, sugar, and protein contents in the seedling foliage of *Quercus brantii* Lindl. Increased levels of proline, carbohydrates, and proteins are vital means by which plant cells maintain cellular integrity (Ahanger and Agarwal 2017; Abdelhameed and Metwally 2018; Metwally and Abdelhameed 2018; Metwally et al. 2021, 2022b). Thus, the buildup of proline, sugars, and proteins in pepper plants that received a treatment of *B. amyloliquefaciens* RaSh1 offers immunity to the leaf/blight spot disease (Upadhyay et al. 2012).

Figure 4 indicated that the use of Thiram (0.2%) fungicide did not enhance total protein, proline, and carbohydrate contents in the leaves of both the control and *A. alternata* infested pepper plants. Our findings are consistent with Metwally and Abdelhameed (2019) finding that Ridomil fungicide has no effect on total protein content in cucumber roots. This is opposite to what was observed by Siddiqui and Ahmed (2002) who conveyed that fungicides reduced total protein content in *Triticum aestivum*. Like our findings, Kengar et al. (2014) found that as hexaconazole concentration increased in spinach and guar, the amount of carbohydrates increased. Whereas Thiram altered the ratios of NAD and NADP, interfered with the electron transport system, and raised ATP levels, which led to a rise in the amount of carbohydrates.

Effect of *B. amyloliquefaciens* RaSh1 on Total Thiol, Non-protein Thiol, and Protein Thiol in *A. alternata*-Infected Pepper Plants

Several studies have been done on how thiol and non-protein thiol compounds affect plants under different stressors, including herbicides, salt, heavy metals, fungal and viral infections, and herbivores (Jain et al. 2010; Garg and Kaur 2013; Sytykiewicz 2014, 2016). As shown in Fig. 5a–c, results of total thiol, non-protein thiol, and protein thiol significantly increased in *A. alternata*-infected pepper plants, showing an increase of 31.5, 30.7, and 31.6%, respectively, as compared with control. Interestingly, the application of *B. amyloliquefaciens* RaSh1 in control or diseased pepper plants had a further increase and a significant effect on total thiol, non-protein thiol, and protein thiol compared with their corresponding controls. The previous results indicated the role of these compounds in plants under normal or diseased conditions, where glutathione, as a non-protein thiol compound, has been repeatedly reported as playing an important role in plant responses during biotic stresses (Dubreuil-Maurizi and Poinssot 2012). The low glutathione content increases vulnerability to several fungi infections (*Botrytis cinerea, A. brassicicola, Pseudomonas syringae,*

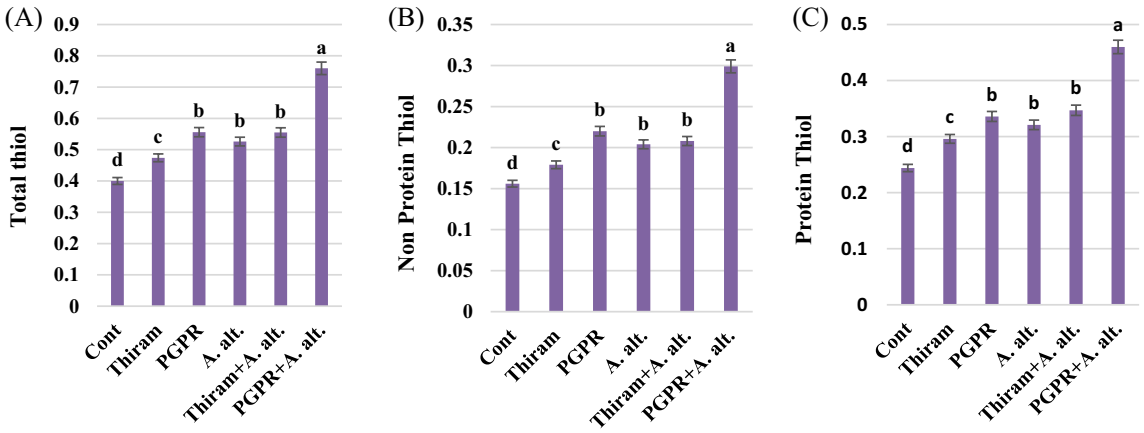

Fig. 5 **a** Total thiol, **b** non-protein thiol, and **c** protein thiol content (mg/g fwt) in the leaves of peppers grown under normal and *A. alternata* stress conditions and treated with plant growth-promoting rhizobacteria (PGPR). Treatments: Cont (control), PGPR (*B. amyloliquefaciens* RaSh1), Thiram (0.2% Thiram fungicide), Thiram + *A. alt* (0.2% Thiram fungicide + *A. alternata*), *A. alt* (*A. alternata*), PGPR + *A. alt* (*B. amyloliquefaciens* RaSh1 + *A. alternata*). Values show the means ± SE ($n = 3$) and significant differences are indicated at $p < 0.05$ in accordance with DMRT. Bars with different letters are significantly different from each other

and *Phytophthora brassicae*) (Roetschi et al. 2001; van Wees et al. 2003; Parisy et al. 2007).

Effect of *B. amyloliquefaciens* RaSh1 on H_2O_2 Concentration in *A. alternata*-Infected Pepper Plants

H_2O_2 serves as a stress-related signaling molecule in plants because biotic and abiotic stresses can increase H_2O_2 synthesis (Egamberdieva et al. 2017; Abdalla et al. 2022). The increased H_2O_2 content in cells directly correlates with oxidative alterations (Cui-Juan et al. 2020). In our result, infection by *A. alternata* resulted in a rapid rise in the amount of H_2O_2 in pepper plant leaves, followed by pepper plants infected with *A. alternata* and treated with Thiram fungicide and *B. amyloliquefaciens* RaSh1-treated samples over control plants (Fig. 6a). Even though inoculation of pepper plants with *B. amyloliquefaciens* RaSh1 decreased H_2O_2 content in their leaves by 32.4% as compared to *A. alternata*-infected ones, this reflects the positive interaction of *B. amyloliquefaciens* RaSh1 with pepper plants. Thiram (2%) fungicide causes an increase in H_2O_2 concentration in pepper plant leaves relative to the control. Our findings are in line with those of Egamberdieva et al. (2017) who found that, compared to control plants, a single inoculation of chickpea plants with *M. ciceri* IC53 reduced H_2O_2 by 18%, and a dual inoculation with *M. ciceri* IC53 and *B. subtilis* NUU4 reduced H_2O_2 by 29%. Cui-Juan et al. (2020) also stated that the pre-treatment of sweet potatoes with

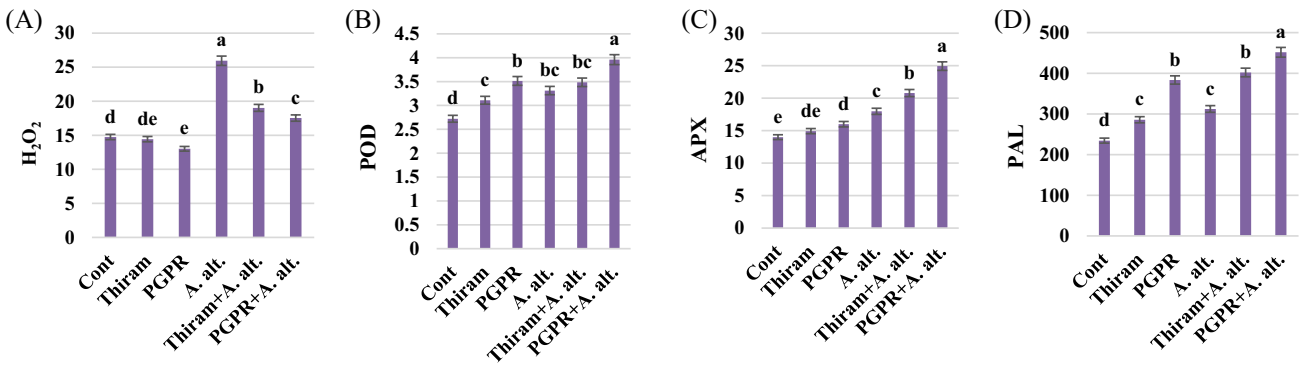

Fig. 6 **a** H_2O_2 (mg/g fwt), **b** peroxidase (POD, U/g fwt), **c** ascorbate peroxidase (APX, U/g fwt), and **d** phenylalanine ammonia-lyase (PAL, U/μg) content in the leaves of peppers grown under normal and *A. alternata* stress conditions and treated with plant growth-promoting rhizobacteria (PGPR). Treatments: Cont (control), PGPR (*B. amyloliquefaciens* RaSh1), Thiram (0.2% Thiram fungicide), Thiram + *A. alt* (0.2% Thiram fungicide + *A. alternata*), *A. alt* (*A. alternata*), PGPR + *A. alt* (*B. amyloliquefaciens* RaSh1 + *A. alternata*). Values show the means ± SE ($n = 3$) and significant differences are indicated at $p < 0.05$ in accordance with DMRT. Bars with different letters are significantly different from each other

B. amyloliquefaciens YTB1407 reduced H_2O_2 to enhance resistance against root rot and black rot diseases, caused by *F. solani* and *Ceratocystis fimbriata,* respectively. As previously indicated by Zhou et al. (2016), inoculation with PGPR has shown promise in regulating ROS levels, aiding plants in carrying out their regular activities under both favorable and unfavorable situations.

Modulation in the Activity of Antioxidant Enzymes in *A. alternata*-Infected Pepper Plants and Inoculated with *B. amyloliquefaciens*

A potential mechanism for the biological control of fungal diseases is to maintain the stability of the host plant's defensive mechanisms (Jamali et al. 2020). Scavenging ROS and preventing the oxidative stress that causes harmful effects on many sensitive molecules is a crucial function of antioxidant enzymes (Abdelhameed et al. 2019; Abdelhameed and Metwally 2019; Nasrallah et al. 2020; Metwally and Soliman 2023). The effects of the applications of *B. amyloliquefaciens* RaSh1 on the activities of POD and APX enzyme activities of pepper plants infected with *A. alternata* are summarized in Fig. 6b, c. The activity of POD and APX was significantly increased in *A. alternata*-infected pepper plants compared to the control. Our results agree with the previous observations by Kazerooni et al. (2021) on *A. alternata*- and *Botrytis pelargonii*-diseased pepper plants. Peroxidase and APX are responsible for removing excessive H_2O_2 or reducing H_2O_2 to water. It has been well documented that the level of APX transcript and enzymatic activity increases during the plant–pathogen interaction (Agrawal et al. 2000).

In addition, further stimulation of POD and APX was observed in pepper plant leaves due to *B. amyloliquefaciens* RaSh1 inoculation. Thiram (2%) application increased POD and APX enzyme activity in the diseased pepper plant leaves more than the control. Hashem et al. (2017) stated that *B. subtilis* significantly increased CAT, POD, APX, and SOD enzyme activities in *M. phaseolina*-infected mung bean plants.

Phenylalanine Ammonia-Lyase Activity

The consequences of the application of *B. amyloliquefaciens* RaSh1 and Thiram fungicide on PAL of pepper plants infected with *A. alternata* leaf spot disease are depicted in Fig. 6d. Inoculation with *A. alternata* and *B. amyloliquefaciens* RaSh1 and application of Thiram fungicide led to a significant increase in PAL activity compared with untreated control plants. The highest increase was recorded for the combined treatment of *B. amyloliquefaciens* and *A. alternata* (451.62 U/µg) followed by Thiram and *A. alternata* (401.97 U/µg) compared with the corresponding pathogen-treated plants (312.21 U/µg) and control (234.37 U/µg). The increase in PAL activity due to *A. alternata* infection is a response mechanism of pepper plants to this pathogen. This finding is consistent with those of Wang et al. (2004) and Geetha et al. (2005), who found that the blast pathogens *Pyricularia oryzae* and *Sclerospora graminicola*, respectively, increased PAL activity in rice and pearl millet. Moreover, Rashad et al. (2020) showed an increase in PAL enzyme in garlic plants as a result of *S. cepivorum* infection, and an extra increase was recorded with the application of *B. amyloliquefaciens* GGA.

From our findings, PAL played an important role in the protection of pepper plants and increased its defense against *A. alternate* as it is a physiological marker for measuring the plant's resistance (Whetten and Sederoff 1995; Dempsey et al. 1999; Melo et al. 2006). It also produces a variety of defense-related secondary metabolites like phenols, lignin, suberin, phytoalexins, and flavonoids (Hemm et al. 2004).

Endogenous Phytohormonal Regulation in *A. alternata* Diseased Pepper Plants Inoculated with *B. amyloliquefaciens* RaSh1

Phytohormones produced by endophytes influence a plant's morphology and structure and encourage plant growth. The mechanism used by rhizobacteria to promote plant development through the secretion of gibberellins (Gib), indole-3-acetic acid (IAA), abscisic acid (ABA), and cytokinins (Cyts) is similar to that used by endophytes to produce phytohormones in the host plant (Patel and Patel 2014; Khan et al. 2014; Kudoyarova et al. 2019; Fadiji and Babalola 2020). Although, infections of plants with fungal pathogens often cause an imbalance in the plant's hormonal system and bring about growth responses incompatible with the healthy development of the plant. According to Waqas et al. (2015), endophyte inoculation of sunflower plants increased their levels of endogenous hormones in comparison to control plants with or without *S. rolfsii* infection. Therefore, the increased growth of pepper plants colonized by *B. amyloliquefaciens* RaSh1 could be attributed to the optimization of the endogenous concentration of plant growth-promoting hormones. To investigate whether the regulation of pepper responses to the pathogen is linked with the coordinated activity of plant hormones, the levels of IAA, Cyt, Gib, and ABA were measured in leaves of control, *A. alternata* infected, and *A. alternata* infected and inoculated with *B. amyloliquefaciens* RaSh1, and the data are presented in Fig. 7a–d.

Cytokinins Content in *B. amyloliquefaciens* RaSh1-Inoculated Diseased Plants

Cytokinins are produced in plants as well as PGPR and are known to increase a plant's resilience to pathogen infections

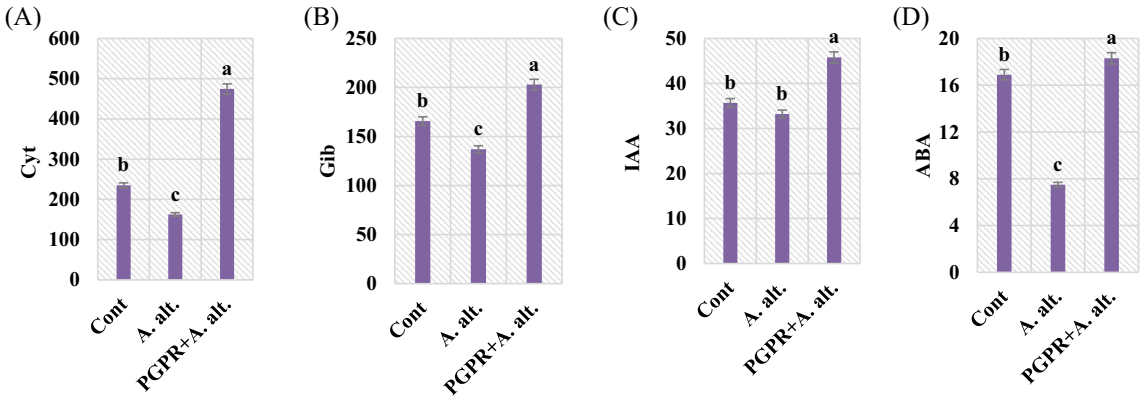

Fig. 7 **a** IAA, **b** Gib, **c** Cyt, and **d** ABA content (µg/g fwt) in the leaves of peppers grown under normal and *A. alternata* stress conditions and treated with plant growth-promoting rhizobacteria (PGPR). Treatments: Cont (control), *A. alt* (*A. alternata*), PGPR + *A. alt* (*B. amyloliquefaciens* RaSh1 + *A. alternata*). Values show the means ± SE ($n = 3$) and significant differences are indicated at $p < 0.05$ in accordance with DMRT. Bars with different letters are significantly different from each other

by enhancing Cyt content (Li et al. 2021). This is in harmony with our results (Fig. 7a) that the inoculation of pepper plants with *B. amyloliquefaciens* RaSh1 altered the levels of endogenous Cyt under the effect of *A. alternata* attack, compared to those with or without *A. alternata* pathogenic infection. Arkhipova et al. (2005) and Kudoyarova et al. (2019) reported that endophytic bacteria can influence plant growth by producing phytohormones, such as Cyts, or through the regulation of hormone internal levels in plants. As a result of our findings, Cyt generated by *B. amyloliquefaciens* RaSh1 can be employed as a biocontrol agent against *A. alternata* disease in addition to being a biostimulant for pepper growth. Cytokinins contribute to plant cell proliferation, long-lasting leaves, shoot differentiation, and nutrient mobilization (Choi and Hwang 2007). However, Spallek et al. (2018) discovered that Cyt had a different impact on plant development and may compromise plant defenses while also boosting disease virulence.

Gibberellins Content in *B. amyloliquefaciens* RaSh1-Inoculated Diseased Plants

Our research shows that pepper interactions *with B. amyloliquefaciens* RaSh1 endophytes during an *A. alternata* pathogenic infection conferred pathogen disease tolerance and enhanced the Gibs content in their leaves. Our results showed (Fig. 7b) that the pathogen-infected plants exhibited lower Gibs content (137.0 ± 3.62c) as compared with the control (165.7 ± 4.38b µg/g). Conversely, the dual inoculation with *B. amyloliquefaciens* RaSh1 and the pathogen showed the maximum Gibs content with an increase of 48.17%, which indicates the active role of the *B. amyloliquefaciens* RaSh1 endophyte to tolerate *A. alternata* infection. These findings concur with earlier findings by Kudoyarova et al. (2019). Gibberellins are mainly involved in cell division, cell elongation, and internode elongation. Moreover, Fulchieri et al. (1993) reported that Gibs increase root hair density in root zones that are involved in water and nutrient uptake and enhance the growth of plants to tolerate pathogen attack. Shahzad et al. (2017) also confirmed that the Gibs-producing ability of *B. amyloliquefaciens* RWL-1 offers additional assistance to tomato plants, and the resulting improvement in tomato growth can induce resistance to *F. oxysporum* disease in tomato plants, suggesting interference with early infection processes that further resulted in limiting disease development (Mei and Flinn 2010). Therefore, it was assumed in this study that the *B. amyloliquefaciens* RaSh1 inoculation lessened the negative effects of *A. alternata* infection on pepper plants.

Indole-3-acetic Acid Contents of *B. amyloliquefaciens* RaSh1-Inoculated Diseased Plants

Our results (Fig. 7c) revealed that IAA concentration ranges from 33.2 µg/g fwt in *A. alternata*-infected pepper plant leaves to 45.8 µg/g fwt registered in pepper leaves inoculated with *B. amyloliquefaciens* RaSh1 endophyte and infected with *A. alternata* pathogen compared to controls (35.7 µg/g fwt). *Alternaria alternata* pathogen attack lowered the concentration of IAA, suggesting the ability of this bacterial endophyte to stimulate plant growth and stimulate its resistance to pathogens. A similar observation was shown by Hashem et al. (2017) on mung bean plants infected by *M. phaseolina* and controlled by *B. subtilis*. Even in adverse environmental conditions, IAA generated by bacterial endophytes stimulates plant development, increases root area, and ultimately improves nutrient uptake from the soil (Overvoorde et al. 2010). The activation of IAA in *B. amyloliquefaciens* RaSh1-inoculated and *A. alternata*-infected pepper plants may be responsible for the roots' growing length and

biomass, thus better-facilitating nutrient access (Fig. 8) and enhancing overall growth, assisting plants in mitigating the negative impacts of pathogens and inducing defense systems (Dimopoulou et al. 2019). Moreover, auxin activates plant cell division, differentiation, and extension and plays a role as a microbe signaling molecule, directly influencing the biology of several pathogens (Overvoorde et al. 2010).

Abscisic Acid Contents of *B. amyloliquefaciens* RaSh1-Inoculated Diseased Plants

Another hormone detected in plants is ABA, which is thought to be a stress signaling molecule, as well is involved in regulating stomatal functions, plant defense, and adaptability to harsh environmental conditions (Mauch-Mani and Mauch 2005). Endogenous ABA contents were found to have significantly higher levels in *B. amyloliquefaciens* RaSh1-treated pepper plant leaves infected with *A. alternata* (18.3 µg/g fwt). Also, the pathogenic attack with *A. alternata* caused a considerable restriction in the endogenous ABA contents (7.5 µg/g fwt) in comparison to the control plants (16.9 µg/g fwt) (Fig. 7d). This suggests that *B. amyloliquefaciens* RaSh1 may counteract the pathogen attack by stimulating ABA production in pepper plants infected with *A. alternata.* Our findings are consistent with those of Siciliano et al. (2015), who stated that high ABA levels could reduce rice plant responses to *Fusarium*. On the other hand, Kang et al. (2015) demonstrated that *B. amyloliquefaciens* inoculation decreased *R. solani*-induced accumulation of ABA in plants. The optimum endogenous concentration of ABA is crucial for the stimulation of plant growth because it controls physiological processes like Ca^{2+} signaling and plasmodesmata control, which inhibit pathogen infection (Rezzonico et al. 1998).

We found that pathogenic infection resistance may be produced based on changes in endogenous hormonal contents (Cyt, Gib, ABA, and IAA) of the pepper plant, demonstrating the significant involvement of endophytes intolerance to a variety of pathogens, as previously indicated by Pieterse et al. (2012).

B. amyloliquefaciens RaSh1 and *A. alternata* Induced Changes in Nutrient (K^+ and Mg^{2+}) Acquisition in Pepper Plants

While considerable researchers have examined the impacts of endophytes on plant growth, few have simultaneously and comprehensively looked into their effects on nutrient content. Tissue mineral content is important because it greatly impacts other plant characteristics. Higher levels of tissue macronutrients are linked to increased plant growth and chlorophyll content (Ai et al. 2017; Abdelhameed et al. 2021b; Macuphe et al. 2021). The nutrients uptake in the pepper leaves was significantly influenced by *B. amyloliquefaciens* RaSh1 inoculation and *A. alternata* infection (Fig. 8). *Bacillus amyloliquefaciens* RaSh1 association with pepper plants promoted macronutrients (K^+ and Mg^{2+}) uptake by pepper roots compared with the untreated control plants. However, *A. alternata* reduced the absorption of those nutrients.

The accumulation of K^+ and Mg^{2+} was shown to decrease by 20% and 11%, respectively, in leaf spot-affected plants compared to the control plants, indicating that *A. alternata* infection inhibits nutrient uptake by plant roots. Our findings coincide with Hashem et al. (2017) results that *M. phaseolina* caused macro- and micronutrient reductions in mung bean plants. Plant pathogenic fungi damage the root system and prevent nutrients from being absorbed, assimilated, and transported through the roots and other parts of the diseased plant (Dordas 2008). In addition, pathogens use nutrients for growth and development, leaving the plant with a nutritional deficiency that makes it more susceptible to disease (Spann and Schumann 2009). In addition, mycotoxins, which are secondary metabolites of pathogenic fungi, stimulate the H^+-pump in the plasma membrane, resulting in an electrochemical gradient that increases K^+ influx into guard cells and promotes stomatal opening (Dong et al. 2012; Dehgahi et al. 2014).

Conversely, *B. amyloliquefaciens* RaSh1 significantly ameliorated the detrimental effect of *A. alternata* infection by improving K^+ (30.5%) and Mg^{2+} (9.1%) uptake, which led to an increase in plant growth under disease conditions and the regulation of different metabolic pathways, including the antioxidant system and chlorophyll synthesis

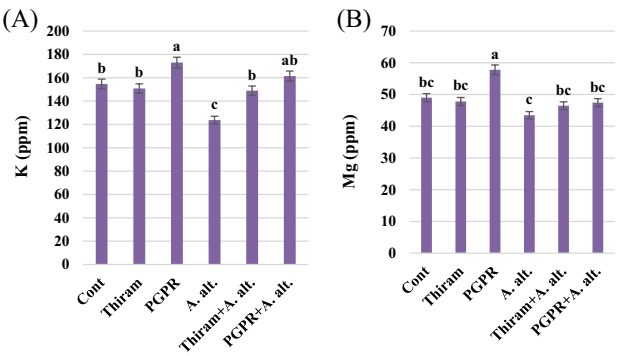

Fig. 8 **a** Potassium (K, ppm) and **b** magnesium (Mg, ppm) content in the shoot of peppers grown under normal and *A. alternata* stress conditions and treated with plant growth-promoting rhizobacteria (PGPR). Treatments: Cont (control), PGPR (*B. amyloliquefaciens* RaSh1), Thiram (0.2% Thiram fungicide), Thiram + *A. alt* (0.2% Thiram fungicide + *A. alternata*), *A. alt* (*A. alternata*), PGPR + *A. alt* (*B. amyloliquefaciens* RaSh1 + *A. alternata*). Values show the means ± SE ($n = 3$) and significant differences are indicated at $p < 0.05$ in accordance with DMRT. Bars with different letters are significantly different from each other

(Hashem et al. 2017). A few researchers have looked into the processes by which nutrients mediate the effects of bacterial endophytes on plant characteristics. One of the potential ways by which endophytic fungus *Epichloë festucae* increases the survival of *Lolium perenne* in less fertile soil is through the increase in the concentration of numerous nutritional components in leaves and roots, as Chen et al. (2020) recently demonstrated. According to Radhakrishnan and Lee (2016), *B. methylotrophicus* boosted nutrient uptake of NPK, and chlorophyll production. Additionally, according to Egamberdieva et al. (2017), inoculating chickpea plants with *M. ciceri* IC53 and NUU4 enhanced N contents compared to control plants. PGPR strains may have a direct beneficial impact on plant metabolism by enhancing the uptake of water, minerals, and enzyme activity in their host plants (Pérez-Montano et al. 2014). The greater mineral content in plants infected with *B. amyloliquefaciens* RaSh1 may improve the production of metabolites, proteins, and the expression of defense genes against Alternaria leaf spot disease (Luo et al. 2022). Moreover, results in Fig. 8a, b obviously showed that K and Mg nutrient contents were reduced with Thiram (2%) application in healthy and *A. alternata*-infected pepper plant leaves. This is in agreement with Al-Garni (2005) and Metwally and Abdelhameed (2019) who reported that the NPK, and Ca levels in watermelon and cucumber plants were decreased by the use of fungicides including Rizolex, Furadan, and Bavistin.

Conclusions

Overall, the findings of this study strongly indicate that the endophyte, *B. amyloliquefaciens* RaSh1 (MZ945930), has growth-inhibitory properties against the fungal pathogen *A. alternata* in vitro and protection against leaf spot/blight disease in pepper plants in vivo. In pepper plants, during pathogenic infection, endophytic associations boost the growth and total pigments of pepper plants, reduce disease, and elicit a series of responses to the microbial attacks; this may be attributed to the prevention of pathogenic infection and high nutrient uptake, enhancing thiol content and defense-related enzymes (POD, APX, and PAL), adjustment of the osmolyte synthesis, and promotion of plant growth hormones (IAA, Gib, Cyts, and ABA). Employing endophytic bacteria like *B. amyloliquefaciens* offers a non-chemical and economic alternative for future food security and sustainable agriculture.

Author Contributions RAM, REA, and SAS sharing in Conceptualization, Methodology, Data curation, Writing—Reviewing and Editing. All authors read and approved the final manuscript.

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Declarations

Conflict of interest Authors have no conflict of interest to declare.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Ethical Approval Not applicable.

Informed Consent Not applicable.

Research Involving Human and Animal Participants Not applicable.

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