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Endophytic seed pretreatment: a strategy for boosting morphophysiological traits in tomato seedlings

Soheila Aghaei Dargiri[1] and Davood Samsampour[1*]

Abstract

This study investigated the effects of fungal (*Penicillium chrysogenum*, *Thielavia basicola*, *Curvularia hawaiiensis*) and bacterial (*Sphingomonas aquatilis*, *Bacillus licheniformis*, *Exiguobacterium aurantiacum*, *Micromonospora echinaurantiaca*, *Kocuria rhizophila*) endophytes on the growth and physiological traits of tomato plants (*Solanum lycopersicum* L.) under greenhouse conditions. Both individual and combined endophyte treatments significantly enhanced key growth parameters, including stem weight, height, and dry weight, with notable synergies observed in fungal-bacterial combinations such as *P. chrysogenum* + *E. aurantiacum* and *S. aquatilis* + *M. echinaurantiaca*. These combinations also optimised photosynthetic activity, increasing chlorophyll content, carotenoids, and photosystem II efficiency, improving plant vitality. Additionally, these endophytes stimulated a marked increase in carotenoid levels, with fungal-bacterial combinations leading to substantial improvements in antioxidant activity. Furthermore, inoculation with these endophytes promoted higher phenolic and proline content, with distinct combinations showing remarkable effects on carbohydrate accumulation. The findings underscore the synergistic potential of fungal-bacterial endophyte interactions in enhancing plant resilience, offering promising strategies for improving crop productivity and sustainability in agriculture.

Keywords Endophyte, Antioxidant, Phenol, Chlorophyll, Proline

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely consumed vegetables globally [1]. Iran, with a tomato production of 6.5 million tonnes, ranks sixth globally [1]. According to the latest report from 2018, the total area used for tomato cultivation in Iran was 158,991 hectares, with an average yield of 41.36 tonnes per hectare. Consequently, Iran produces 6,577,109 tonnes of tomatoes annually [2].

Endophytes are microorganisms that inhabit plant tissues (intracellularly or intercellularly). They do not cause symptoms and are abundant in biologically active compounds. Almost all plant species have one or more endophytes [3]. Endophytes frequently involve themselves in resistance mechanisms to alleviate the negative effects of biotic and abiotic factors in plants [4]. The interaction between endophytes and host plants seems to play a significant and definitive role in plant growth and biodiversity [5]. Endophytes stimulate plant growth and increase productivity [6] and resistance to plant biotic stresses [7], including pathogens and pests, by producing natural substances such as alkaloid compounds and antibacterial agents [8].

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Endophytes in seeds are used to accelerate germination and protect seedlings against soil-pathogenic microorganisms [9]. Due to the function of seeds as developmental organs in plant regeneration and dispersal [10], seeds inoculated with endophytes have the potential to produce high-quality seedlings [11]. In addition, utilising endophytes is a promising strategy that can be practically applied to improve seed germination [12], increase food production, and protect plants from environmental stresses [13].

The results of Irizarry et al. (2018) showed that the bacterial endophyte *Bacillus amyloliquefaciens* has positive effects on cotton seedlings [14]. Most research on seed endophytes has primarily focused on the diversity and distribution of endophytes, rather than their growth effects and practical applications [15]. It has been revealed that isolated endophytes from plants growing in saline coastal soils can increase plant yields in these environments, indicating their high commercial potential for practical application in agriculture [16]. This study aimed to investigate the impact of fungal and bacterial endophytes extracted from halophyte plants on the morpho-physiological traits of tomato seeds pretreated with these endophytes.

Materials and methods

Sample collection and surface sterilization of plants

To conduct this research, the distribution areas of halophytic plant species in Hormozgan Province, Iran, were first determined using available resources, including the Flora of Iranica, global databases (https://theplantlist.org, https://www.ipni.org), and published literature. Halophyte species of the Amaranthaceae family (Table 1) were collected from the coastal areas of Qeshm, Hormozgan Province, Iran (latitude: 26°57'59.7"N; longitude: 56°16'12.4"E). The plant leaves were surface sterilized following the method described by Szymańska et al. (2018) [17], with some modifications. The sterilization and isolation of endophytes from halophyte plant species were performed following the method of Kusari et al. [18] with minor modifications.

The tomato seeds of the 8320 variety were sourced from Pakaan Seed Company, Isfahan. The tomato seeds were washed with 70% ethanol for 30 s, followed by sterilization with 0.5% sodium hypochlorite for 90 min. Finally, they were thoroughly rinsed with autoclaved distilled water. Following this treatment, the samples were washed four times with sterile water, each wash lasting three minutes, and then subjected to a final 30-minute rinse. This procedure was repeated three times to ensure thorough sterilization and consistency across the samples. All sterilization and transfer steps were carried out in a type II laminar flow hood.

Isolation and identification of bacterial endophytes

To assess surface sterilization efficiency, 100 µL of the final rinse from each sample was plated on Nutrient Agar (NA) and monitored for microbial growth. Approximately 500 mg of leaves from each species were weighed, cut into small pieces using a sterile scalpel, and ground into a slurry with an autoclaved pestle and mortar. The slurry was transferred to sterile Petri dishes, and 30 mL of autoclaved distilled water was added. The Petri dishes were sealed and placed on a rotary shaker (150 rpm) at 25 °C for 2 h. After shaking, 100 µL of the material was inoculated in triplicate on NA plates and incubated at 28 °C for 48 h for bacterial growth. Pure bacterial colonies were cultured in nutrient broth (NB), and the resulting cell stocks were stored in 50% (v/v) glycerol at – 80 °C [19].

Genomic DNA was extracted using a modified method Lutz et al. [20]. The 16 S rRNA gene was amplified using primers 27 F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) under the following conditions: In a PCR cycle, the initial denaturation step is carried out at 95 °C for 4 min, followed by denaturation at 96 °C for 1 min. Then, during 40 cycles, the annealing step occurs at 52 °C for 30 s, followed by the extension step at 72 °C for 2 min. Finally, the last extension step is performed at 72 °C for 10 min, and the samples are stored at 4 °C [21]. The quality of the reaction was checked using 1% agarose gel electrophoresis, and

Table 1 Bacterial and fungal endophytes used in the study

Endophyte	Scientific name	Class name	Plant species	Sequence identity percentage (%)	Access number
Fungal	*Penicillium chrysogenum*	Eurotiomycetes	*Salsola imbricata*	100	MT277118
	Thielavia basicola	Sordariomycetes	*Cornulaca moncantha*	100	MT277121
	Curvularia hawaiiensis	Dothideomycetes	*Bienertia cycloptera*	100	MT277129
Bacterial	*Sphingomonas aquatilis*	Alphaproteobacteria	*Aerva javanica*	100	MW663935
	Bacillus licheniformis	Bacilli	*Halocnemum strobilaceum*	100	MW663936
	Exiguobacterium aurantiacum	Bacilli	*Suaeda vermiculata*	100	MW592843
	Micromonospora echinaurantiaca	Actinobacteria	*Suaeda agyptiaca*	100	MW663991
	Kocuria rhizophila	Actinobacteria	*Salsola imbricata*	100	MW663998

then the product was sent to Pishgam Iran for sequencing. Sequence editing was performed using CLUSTALX 2.0 and SEAVIEW software, and the sequences were compared using BLAST in the GenBank database.

Isolation and identification of fungal endophytes

Three pieces of each sample were placed on PDA agar plates for fungal growth. The plates were sealed with parafilm and incubated at 28 ± 1 °C. To ensure surface sterilization, non-sterilized tissue samples (washed with water only) were simultaneously prepared and incubated under the same conditions. The plates were checked every three days for fungal growth. After 4–6 weeks, fungal endophytes were isolated using the "tip of the hypha" method by transferring a hyphal tip from the edge of the colony to a fresh PDA plate for further growth [22].

For molecular identification, genomic DNA was extracted using the CTAB method of Baker and Mullin [23]. To identify the fungal isolates, the nuclear ribosomal ITS region was amplified and sequenced using the universal primer pair ITS1 (forward: 5′-TCCGTTGGTGAACCAGCGG-3′) and ITS4 (reverse: 5′-TCCTCCGCTTATTGATATGC-3′). The PCR conditions were as follows: Initial denaturation at 94 °C for 4 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1 min. A final extension step was performed at 72 °C for 10 min, and the samples were stored at 10 °C. PCR products were sequenced using the Sanger method, and the resulting sequences were analyzed with BioEdit software (https://bioedit.software.informer.com/). The amplified sequences were then compared to available sequences in the NCBI database using the Basic Local Alignment Search Tool (BLAST) to identify the endophytic fungi.

Preparation of fungal endophyte spores

To inoculate tomato seeds with spores of various fungi, we cultured the fungus for four weeks in Potato Dextrose Agar (PDA: Germany-Q-Lab) medium in an incubator at 24 ± 1 °C, with a photoperiod of 14:10 (L: D) hours [24]. Fungal spores were collected by placing them in Petri dishes filled with a 10 ml solution of double distilled water and 20% Tween. The number of spores in each dish was counted, and a concentration of approximately 1×10^6 (CFU) ml^{-1} was prepared using a neobar slide [24].

Preparation of bacterial endophytic suspension

To inoculate the tomato seeds with a bacterial suspension, the bacterium was cultured on Nutrient Broth (NB: Germany -Q-Lab) medium for 24 h in an incubator at 28 ± 1 °C with 130 rpm and a photoperiod of 14:10 (L: D) hours in the laboratory [25]. The combination treatments included equal amounts of fungal spores and bacterial suspensions.

Endophyte inoculation

To enhance seed-endophyte interaction, a solution of 1% carboxymethyl cellulose (1 g dissolved in 100 mL of water) was prepared. The sterilized seeds were immersed in the endophyte suspension (Table 1) and shaken for six hours. The seeds immersed in sterile distilled water served as the control treatment.

Treating tomato seeds using endophytes

The experiment was evaluated as a factorial experiment in a completely randomized design with three replications. The inoculated seeds were sown in seedling trays filled with an autoclaved soil mixture of peat moss and perlite (121 °C for 15 min). The trays were maintained in the greenhouse of Hormozgan University (27°16'09.8"N 56°18.6"E) under controlled conditions: a temperature of 20–25 °C and relative humidity of 65–70%. Irrigation was performed every other day. To initiate the experiment, tomato seeds were surface-sterilized by dipping them in 70% ethanol for 30 s, followed by immersion in 0.5% sodium hypochlorite (NaOCl) for 1.5 min. The seeds were then rinsed three times with sterile distilled water [26]. For better seed contact with endophytes, 1% carboxymethyl cellulose was used (one gram of carboxymethyl cellulose was dissolved in 100 ml of water). After that, the seeds were endophytically inoculated and shaken for six hours. Seeds immersed in sterile distilled water were used as a control. The soil mixture (peat moss and perlite) was autoclaved at 121 °C for minutes prior to sowing the treated seeds [26]. Irrigation was done every other day, and after seedling development, the desired traits were assessed.

Molecular identification and confirmation of endophytes

Molecular analyses were performed to confirm the presence of inoculated endophytes in tomato plants after harvest. Plant tissues (roots, stems, and leaves) were collected from five plants, and then the collected samples were washed first with tap water and then with sterile double-distilled water for washing. The washed leaf samples were immersed in ethanol (70%) for 1 min and then in sodium hypochlorite (1%) for 60 s. After surface sterilization, the tissue samples were cultured on selective media: NA agar for bacterial endophytes [27] and PDA for fungal endophytes [28]. Plates were incubated for 24 h at 28 ± 1 °C for bacteria and 130 rpm for fungi. Plates were also incubated for 4 weeks at 24 ± 1 °C for fungi.

Colonies suspected to be endophytes were isolated and identified by PCR using primers specific to the 16 S rRNA gene for bacterial endophytes [29] and the internal transcribed spacer (ITS) region for fungal endophytes [30]. The PCR products were sequenced and compared to sequences in the GenBank database using BLAST. In addition, control plants (non-inoculated) were

subjected to the same re-isolation procedures to confirm the absence of contamination by non-inoculated microorganisms. All PCR products were also confirmed for purity by performing amplification with universal primers.

Morphological traits

Stems and leaves were sampled at the four-leaf stage of tomato (45 days post-inoculation) to assess the studied traits. To assess the dry weight of shoots, the samples were baked for 72 h at 75 °C and then weighed to the nearest 0.0001 g on a digital scale. The stem height of leaves was determined using a 0.1 cm ruler.

Physiological traits: relative leaf water content (RWC)

To determine the relative leaf water content (RWC) of the youngest mature leaves in each plant, three leaf discs were prepared. The fresh weight (FW) of the samples was immediately recorded. Then, all leaves were placed in test tubes containing 40 ml of distilled water and kept in the dark at room temperature for 24 h, after which their saturation weight (TW) was measured. In the next step, the samples were dried in an oven at 70 °C for 72 h, and their dry weight was determined (DW). The relative water content (RWC) was calculated following Karimi et al. (2012) [31]. The obtained data were calculated using the following formulas:

$$\text{Relative lef water content} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Measurement of photosynthetic pigments: chlorophyll fluorescence

Photosystem II was evaluated using a model chlorophyll meter (Hansatech-Pocket PEA).

(Hansatech-Pocket PEA) [32].

Measurement of chlorophyll a, chlorophyll b, and carotenoids

Half a gram of fresh leaves was ground in a porcelain mortar with 5 ml of 80% acetone and then centrifuged for 15 min at 1000 rpm. A spectrophotometer (Cecil model CE2501) was used to measure the extract absorption at 663, 647, and 470 nm, respectively [33]. The results of measuring the number of photosynthetic pigments were calculated in milligrams of chlorophyll per gram of fresh weight.

Biochemical traits: measurement of antioxidant activity

This experiment was calculated according to the method suggested by Nanjo et al. [34] and based on radical inhibitory activity (DPPH). The extract absorbance was measured with a spectrophotometer at 517 nm after being left at room temperature in the dark for 30 min.

Measurement of total phenol

Total phenol content was measured using the Folin-Ciocalteu reagent and method Singleton and Rossi [35]. 0.5 g of fresh leaf tissue in 80% methanol was homogenized and then centrifuged at 5000 rpm for 15 min. To this, 1 ml of methanol solution (1.8 ml of distilled water and 0.2 ml of Folin reagent) was added. It was kept at 25 °C for 5 min, and then 1 ml of 12% sodium carbonate was added to the solution. After 30 min at room temperature, the absorption of the resulting solution was measured at a wavelength of 760 nm using a spectrophotometer.

Proline content

The proline content was calculated following Bates et al. [36]. In 0.5 mL of 3% (w/v) sulphosalicylic acid, samples (0.1 g of leaf) were homogenized. Each homogenate (0.2 mL) was mixed with 0.2 mL of glacial acetic acid, followed by 0.2 mL of ninhydrin. The mixture was cooled in a water bath at 100 °C for 30 min before being cooled in an ice bath. The toluene-containing chromophore was separated, and the absorbance of the red colour was measured at 520 nm using a UV-visible spectrophotometer.

Total carbohydrate content

The Anthrone method was used to calculate the soluble carbohydrate content [37]. The samples (0.5 g leaf) were homogenized in hot aqueous ethanol (80%). After centrifugation, 0.2 mL of the supernatant was transferred to a new test tube, and 1 mL of distilled water was added. The tubes were then filled with anthrone reagent (0.2%). The samples were heated for 8 min in a boiling water bath and then quickly cooled; the intensity of the green to dark green colour was measured using a digital spectrophotometer at 625 nm.

Statistical analysis

The data was analyzed using analysis of variance (ANOVA), and the mean values were compared with the LSD test at a significance level of ($P<0.05$) using the SAS software (version 9.1). The principal component analysis (PCA) was performed using the XLSTAT program, version 2020 (www.xlstat.com, Addinsoft SARL). Information on hierarchical cluster analysis and Pearson correlation was carried out using (www.r-project.org).

Results

Solation and identification of bacterial and fungal isolates

A total of 40 isolates, consisting of 20 bacterial isolates and 20 fungal isolates, were observed from the samples cultured on the plates. The bacterial and fungal isolates were differentiated based on their colony morphology, colour, texture, and growth patterns on selective media. Out of the total isolates, 5 bacterial isolates and 3 fungal isolates were selected for further identification based on

their distinct features from other isolates. The reisolation process was carried out to ensure the purity of the selected isolates. For the bacterial isolates, subculturing was done on fresh nutrient agar plates, and for the fungal isolates, repeated transfers to fresh potato dextrose agar plates were performed. During this process, no contamination was observed in any of the selected isolates, confirming that the strains chosen for further analysis were pure and uncontaminated. The next step in the identification process involves molecular techniques, such as 16 S rRNA sequencing for bacteria and internal transcribed spacer (ITS) region sequencing for fungi, to confirm the identity of the selected isolates.

Fresh and dry weight

The fresh weight of the stem increased by 45.94%, 45.55%, and 44.20% in the seedlings colonized by *T. basicola* + *P. chrysogenum*, *P. chrysogenum* + *E. aurantiacum*, and *S. aquatilis* + *M. echinaurantiaca*, respectively, non-treated controls (Fig. 1a). In addition, a combination of the fungal (*P. chrysogenum*) and bacterial (*M. echinaurantiaca*) endophytes increased the dry weight by 57.30% compared to non-treated controls (Fig. 1b).

The height of the stem

Different types of endophytes have had varying effects on stem height. Some endophytes, such as *E. aurantiacum* and *P. chrysogenum*, significantly increased stem height, while others, like *C. hawaiensis*, had a lesser impact. This highlights the importance of selecting specific endophyte types to enhance stem height effectively (Fig. 2). The height of the stem increased by 81.10% when seeds were inoculated with the two bacterial endophytes (*S. aquatilis* + *M. echinaurantiaca*) non-treated controls (Fig. 2).

The relative content of leaf water

The results showed that endophytes increased the relative water content of tomato seedlings (Table 2). Most endophyte treatments (except *C. hawaiiensis*) significantly increased the relative water content of non-treated controls (without endophytes) (Table 2). Different endophyte types had varying effects on relative water content; for instance, *T. basicola*, *S. aquatilis*, and *B. licheniformis* significantly enhanced relative water content, while others, like *C. hawaiiensis*, had a lesser effect (Table 2). This indicates that selecting the appropriate type of endophyte is crucial for improving the plant's water status. Accordingly, the relative content of leaf water was increased by 33.03% and 32.78% in the seedlings colonized by *P. chrysogenum* + *E. aurantiacum* and *T. basicola* + *P. chrysogenum*, respectively, compared to.

controls (Table 2). This suggests that certain endophytes may interact synergistically with one another.

Photosynthetic pigments

The different endophyte combinations had a significant effect on the total amount of photosystem II (Table 2). The combination of bacterial endophytes *S. aquatilis* + *M. echinaurantiaca* and *P. chrysogenum* + *M. echinaurantiaca* increased the total amount of photosystem II by 27.53% and 24.63%, respectively, in the colonized seedlings compared with endophyte-free seedlings (Table 2).

Endophytes increased the amount of chlorophyll a, chlorophyll b, and carotenoids in colonized seedlings (Table 2). An increase in the amount of chlorophyll up to 39.06% and 37.5% was obtained by the inoculation of *P. chrysogenum* + *M. echinaurantiaca* and *T. basicola* + *P. chrysogenum*, respectively. Similarly, the bacterial endophyte *S. aquatilis* + *M. echinaurantiaca* increased the amount of chlorophyll b up to 78.94%. Regarding carotenoid colonization by the fungal endophytes *T.*

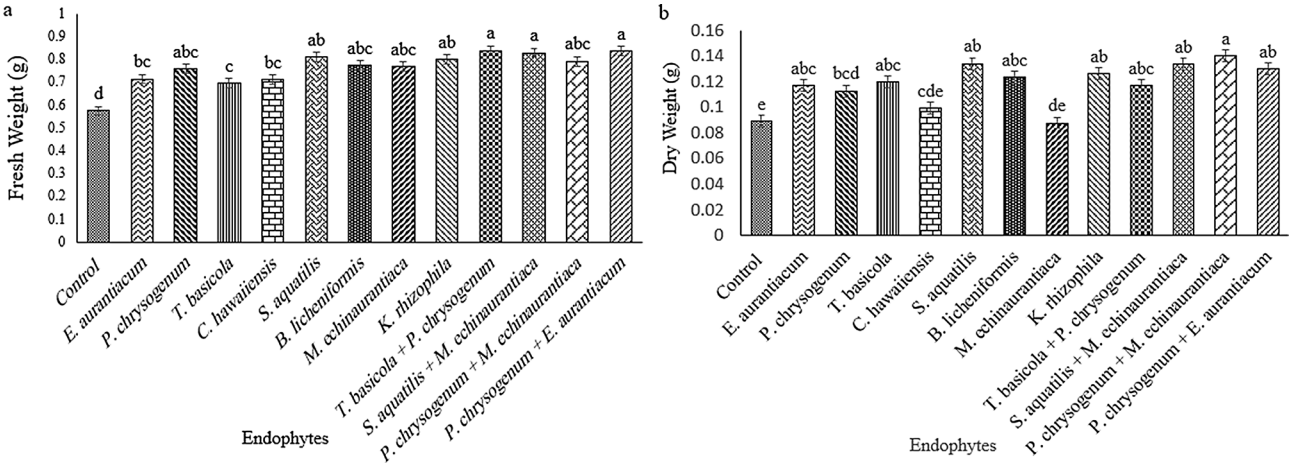

Fig. 1 The effect of endophyte on (**a**) fresh and (**b**) dry weight of tomato seedlings colonized in the seed stage. Means with the same letters are not significantly different (LSD test; $P < 0.05$)

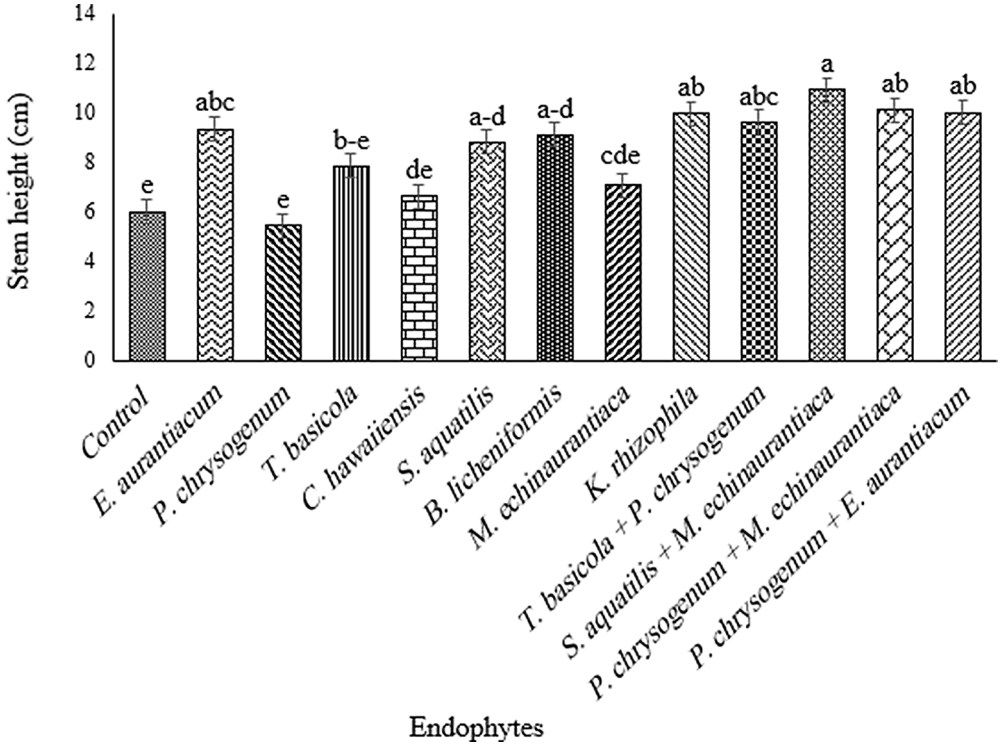

Fig. 2 The effect of endophytes inoculation on the stem height of tomato seedlings. Means with common letters did not show a significant difference statistically (LSD test; $P < 0.05$)

Table 2 The effect of endophytes inoculation on physiological parameters in the tomato seedlings

Endophytes treatment	RWC (%)	Fv/Fm	Chl a	Chl b	Carotenoids
				(mg g^{-1} FW)	
Control	65.94 ± 0.74^{d}	0.69 ± 0.002^{g}	0.64 ± 0.012^{e}	0.38 ± 0.013^{d}	0.24 ± 0.017^{f}
P. chrysogenum	68.65 ± 0.49^{cd}	0.81 ± 0.005^{bc}	0.76 ± 0.011^{cd}	0.41 ± 0.023^{d}	0.35 ± 0.040^{cd}
T. basicola	76.60 ± 0.41^{bc}	0.74 ± 0.007^{f}	0.86 ± 0.007^{abc}	0.55 ± 0.025^{bc}	0.48 ± 0.009^{a}
C. hawaiiensis	66.42 ± 0.42^{d}	0.79 ± 0.001^{cd}	$0.82 \pm 0.026^{a-d}$	0.63 ± 0.020^{ab}	0.34 ± 0.009^{cd}
S. aquatilis	75.98 ± 0.54^{bc}	0.77 ± 0.001^{def}	0.75 ± 0.045^{d}	0.45 ± 0.075^{cd}	0.25 ± 0.009^{ef}
B. licheniformis	82.66 ± 0.54^{ab}	0.79 ± 0.002^{cde}	0.77 ± 0.043^{bcd}	0.58 ± 0.028^{ab}	0.30 ± 0.021^{def}
E. aurantiacum	76.31 ± 0.58^{bc}	0.76 ± 0.001^{ef}	0.86 ± 0.005^{ab}	0.57 ± 0.032^{ab}	0.44 ± 0.009^{ab}
M. echinaurantiaca	76.18 ± 0.49^{bc}	0.79 ± 0.005^{cde}	0.77 ± 0.052^{bcd}	0.57 ± 0.025^{ab}	0.31 ± 0.033^{def}
K. rhizophila	81.97 ± 0.75^{ab}	0.78 ± 0.002^{de}	0.86 ± 0.037^{ab}	0.59 ± 0.037^{ab}	0.32 ± 0.014^{cde}
T. basicola + *P. chrysogenum*	87.56 ± 0.62^{a}	0.78 ± 0.002^{de}	0.88 ± 0.021^{a}	0.61 ± 0.034^{ab}	0.39 ± 0.030^{bc}
S. aquatilis + *M. echinaurantiaca*	83.87 ± 0.72^{ab}	0.88 ± 0.002^{a}	0.85 ± 0.028^{abc}	0.68 ± 0.002^{a}	0.47 ± 0.009^{a}
P. chrysogenum + *M. echinaurantiaca*	85.46 ± 0.41^{ab}	0.86 ± 0.002^{a}	0.89 ± 0.014^{a}	0.66 ± 0.002^{ab}	0.32 ± 0.015^{cde}
P. chrysogenum + *E. aurantiacum*	87.82 ± 0.45^{a}	0.83 ± 0.002^{b}	$0.80 \pm 0.024^{a-d}$	0.66 ± 0.002^{ab}	0.28 ± 0.023^{def}

Means with common letters based on LSD test at the level of $P < 0.05$ are not significantly different from each other

basicola and bacterial endophytes *S. aquatilis* + *M. echinaurantiaca*, an increase of 100% and 95.83% was obtained (Table 2). *E. aurantiacum* increased carotenoid levels by 83.33% in non-treated controls (Table 2).

Antioxidant activity and total phenol content

As shown in Fig. 3a, the combined inoculation of *P. chrysogenum* and *E. aurantiacum* resulted in a 16.92% increase in antioxidant activity in the colonized seedlings. In addition, the combination of *P. chrysogenum* + *E. aurantiacum* and *T. basicola* + *P. chrysogenum* caused an increase of 34.50% and 33.94% in the amount of phenol, respectively, compared to non-treated controls (Fig. 3b).

Proline and total carbohydrate content

The highest amount of proline was observed in the seedlings colonized with *S. aquatilis* + *M. echinaurantiaca* (an increase of 158.82% non-treated controls) (Fig. 4a). Additionally, *M. echinaurantiaca* and *P. chrysogenum* + *M. echinaurantiaca* increased the carbohydrate content by

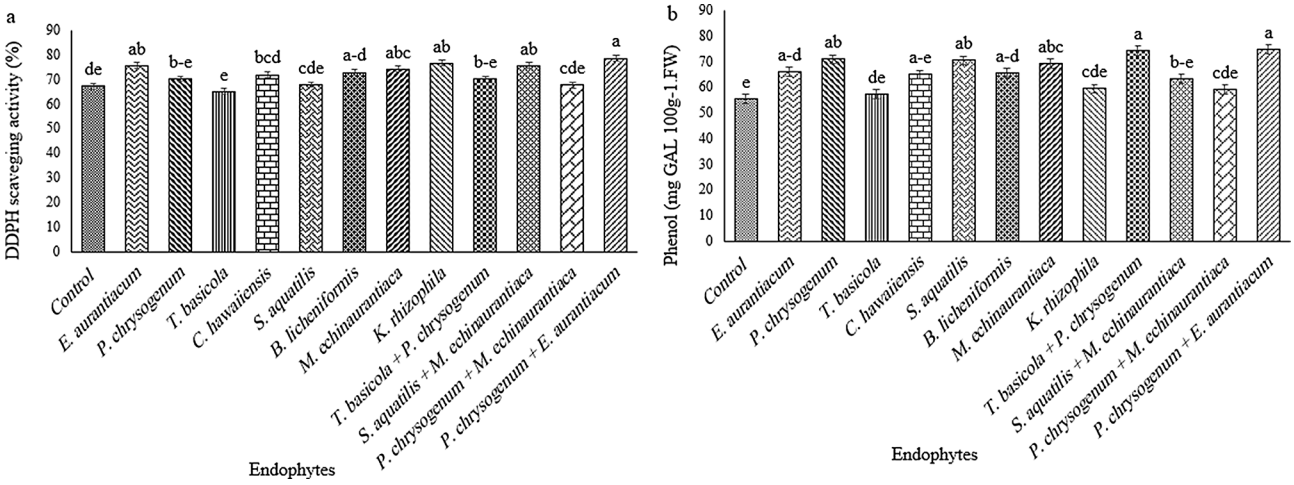

Fig. 3 The effect of endophytes colonization on (**a**) antioxidant activity and tomato seedling (**b**) phenol content. Means with the same letters are not significantly different from each other (LSD test; $P < 0.05$)

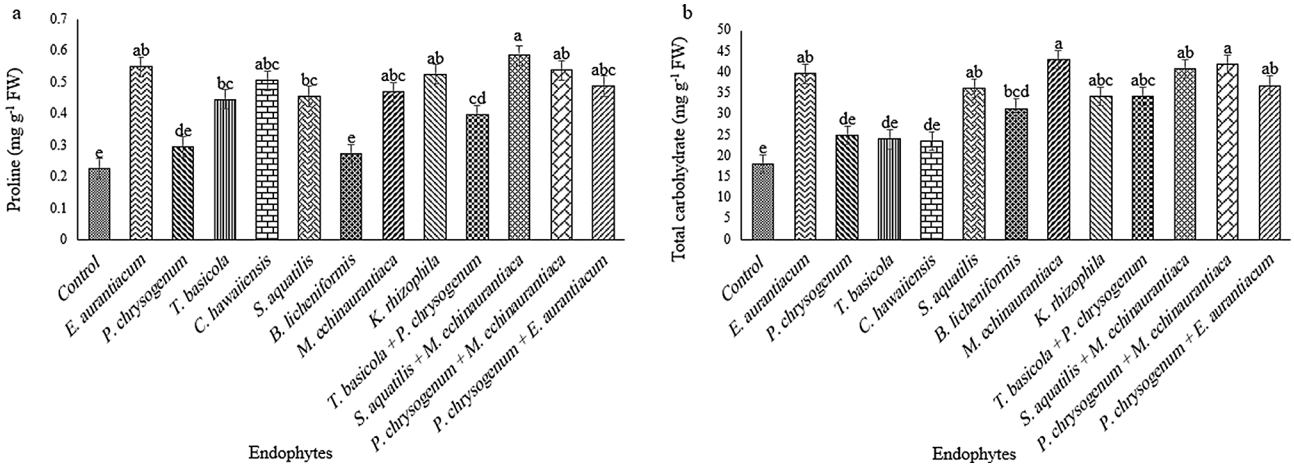

Fig. 4 The effect of endophyte-inoculation on the (**a**) proline and (**b**) total carbohydrate content in the tomato seedlings non-treated controls. Means with common letters based on are not significantly different from each other (LSD test; $P < 0.05$)

136.30% and 130.74% respectively, non-treated controls (Fig. 4b).

Correlation and similarity between traits

The heat map obtained from the hierarchical cluster analysis (HCA) showed that the different endophytes (*P. chrysogenum*, *T. basicola*, *C. hawaiiensis*, *S. aquatilis*, *B. licheniformis*, *E. aurantiacum* and *M. echinaurantiaca*) combinations and measurement parameters were divided into two groups (Fig. 5a). The first group included the dry and wet weight of shoots, fluorescence, chlorophyll a, chlorophyll b, carotenoids, proline, the relative content of leaf water, phenol, and antioxidants, and the second group embraced the carbohydrates and plant height (Fig. 5a). In the first group, it was found that the relative content of leaf water, phenol, and antioxidants had the highest correlation compared to other parameters in endophyte inoculation (Fig. 5a). In the first group, the relative content of leaf water showed the highest correlation with *P. chrysogenum* + *M. echinaurantiaca*. In addition, the clustering results showed that after the relative content of leaf water, the highest correlation was related to the phenol and antioxidant traits (Fig. 5a).

Cluster analysis also showed that according to the studied parameters, endophytes are divided into two groups. The first subgroup includes fungal endophytes (*C. hawaiiensis*, *P. chrysogenum*) and bacterial endophytes (*T. basicola*, *B. licheniformis*, and *S. aquatilis*) as well as the combination of (*T. basicola* + *P. chrysogenum*). The second group includes bacterial endophytes *K. rhizophila*, *E. aurantiacum*, and *M. echinaurantiaca* as well as the combination of *S. aquatilis* + *M. echinaurantiaca*, *P. chrysogenum* + *E. aurantiacum* which showed the highest correlation with the relative content of leaf water, phenol, and antioxidants (Fig. 5a). Hierarchical clustering analysis (HCA) showed that the highest correlation was in the

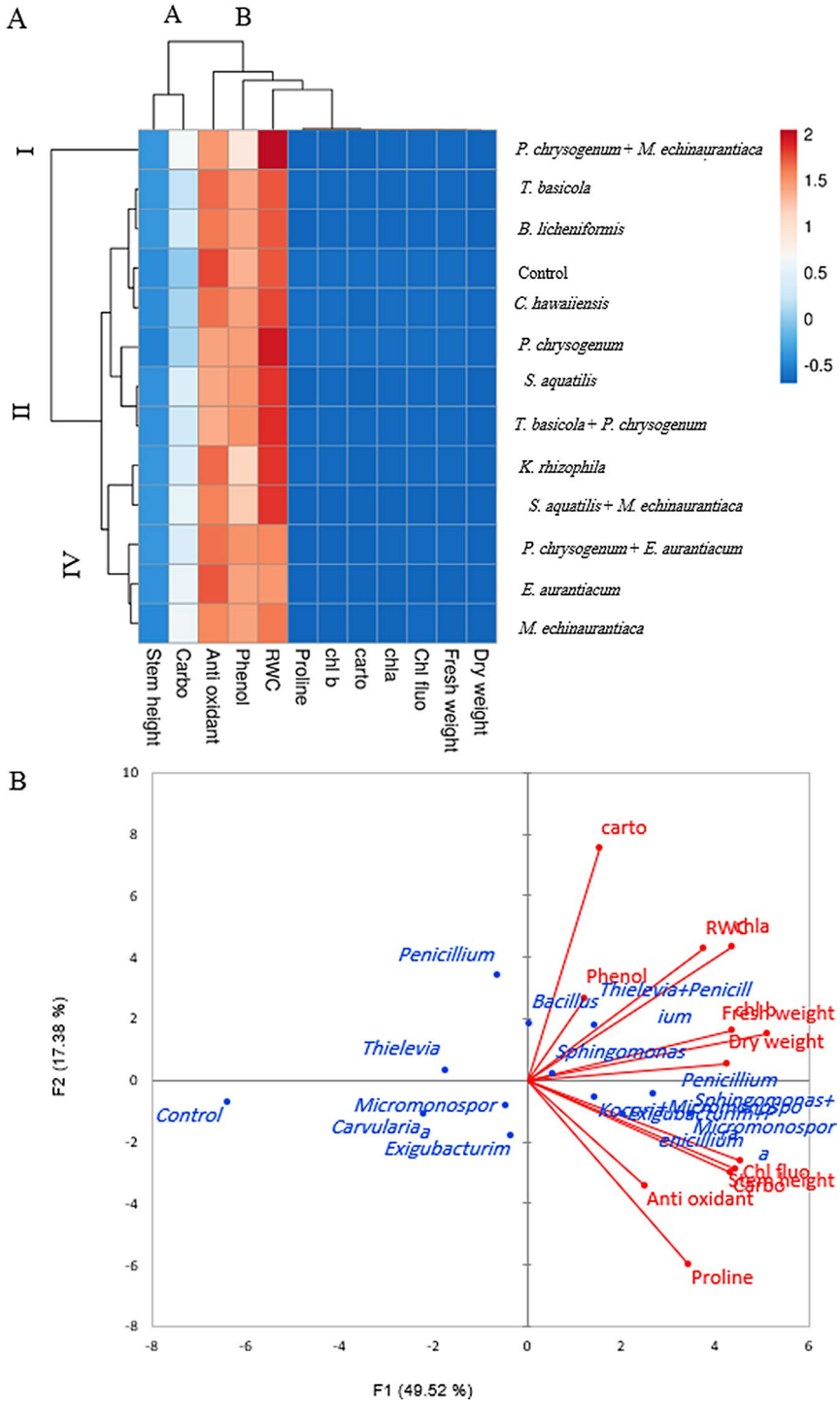

Fig. 5 (**A**) Hierarchical clustering analysis (HCA) of the endophytic treatments and variable trait relationships in tomato seedlings. Heatmap of Pearson correlation coefficient (*r*) values of variable traits, where the colored scale showing the '*r*' coefficient values (*r* = 0.5 to 2) indicates the positive (red) and negative (blue) correlations. (**B**) Dendrogram clustering of endophyte treatments in the colonized tomato seedlings and endophyte-free ones. Principal component analysis (PCA) of treatments and variable trait relationships in tomato seedlings. (**A**) PCA loading plots of the examined variable traits, and the circles indicate the most correlated variables. (**B**) PCA loading plots examined the variable traits. PCA individual plots of endophytic treatments on the inoculated tomato seedlings and non-inoculation ones. The tested variables included relative; Dry weight, Fresh weight, Chl fluo; Chlorophyll fluorance, Chl a; Chlorophyll a, Chl b; Chlorophyll b, Carto; Cartonoeid, Proline, RWC; Relative water content, Phenol, Antioxidant; Antioxidanty; Carbo; Carbohydrate; Stem height

composition of endophytes with morphological, physiological, and biochemical parameters (Fig. 5a).

All 12 biomass, physiological, and biochemical traits were loaded into two major principal components (PC1 and PC2), explaining 66.90% of the total variance (Fig. 5b). Most of the examined traits were discriminated by PC1 and explained by the higher ratio of variance (49.52%), while the lower ratio of variance (17.38%) was indicated by PC2 (Fig. 5b). According to the PCA results, the most similar parameters were carotenoid, phenol, relative leaf water content, chlorophyll a, chlorophyll b, and fresh and dry weight in the seedlings colonized by *B. licheniformis*, *S. aquatilis*, and *T. basicola* + *P. chrysogenum* (Fig. 5b). On the other hand, the results showed that the most similar parameters were plant height, proline, carbohydrate, chlorophyll fluorescence, and antioxidant in those seedlings inoculated by *K. rhizophila*, *S. aquatilis* + *M. echinaurantiaca*, *P. chrysogenum* + *E. aurantiacum*, and *P. chrysogenum* + *M. echinaurantiaca* interaction.

Discussion

Many bacterial, fungal, and mycorrhizal species have been shown to establish an intimate endophytic association with the seed of interest for priming [38], assisting in overcoming the negative effects of various stress factors [39]. Various endophytic bacterial and fungal strains have been identified and used as plant or seed inoculants to improve crop growth and yield [40].

The results of the current study revealed that specific combinations of *T. basicola* + *P. chrysogenum*, *P. chrysogenum* + *E. aurantiacum*, and *S. aquatilis* + *M. echinaurantiaca* significantly improved the fresh weight of tomato plants, indicating a synergistic effect among these endophytes in promoting stem growth. Endophytes are known to enhance the host plant's nutrient acquisition by solubilizing phosphorus, fixing atmospheric nitrogen, and increasing the availability of essential minerals [41]. Many endophytes also produce phytohormones such as auxins, gibberellins, and cytokinins, which promote cell division, elongation, and differentiation. These hormones contribute to stem and root growth, leading to greater biomass accumulation in tomato plants [42, 43]. The specific combinations of fungal and bacterial endophytes likely provide synergistic effects, where the combined action of multiple endophytes is greater than the sum of their individual effects. This synergy can more effectively enhance various plant physiological processes compared to single endophyte treatments, especially in tomato plants [44]. Egamberdieva et al. [45] demonstrated that the application of *Bacillus cereus*, *Achromobacter xylosoxidans*, *Bacillus thuringiensis*, and *Bacillus subtilis* on chickpeas (*Cicer arietinum*) increased dry biomass by 5%, 24%, 43%, and 45%, respectively, compared to untreated controls. Similarly, our results with tomato plants showed a significant increase in dry weight in endophyte-colonized plants, which can be attributed to enhanced photosynthetic capacity in symbiosis with the endophytes. As Shaik and Thomas [46] reported, inoculation of tomato seeds with endophytes led to an increase in the dry weight of both roots and leaves.

The results of Ghorbani et al. [47] indicated that the symbiosis of *Piriformospora indica* with tomato plants increased photosynthetic pigments across all treatments compared to plants without symbiosis. The enhanced chlorophyll content in symbiotic plants may suggest that greater photosynthesis is required to supply endophytic carbon to the plant symbiosis [48]. This increase in photosynthetic pigments under the symbiotic relationship may be attributed to improved phosphorus uptake from the soil, with endophytes playing a key role as energy carriers during photosynthesis [49].

Additionally, a study on *Citrus reticulata* L. colonized with a combination of three fungal endophytes (*Penicillium citrium* + *Aurobasidium pullulans* + *Dothideomycetes* sp.) showed enhanced growth when the plants were subjected to drought stress, highlighting the potential of fungal-bacterial combinations to improve stress tolerance in plants. In the case of tomato, our results align with this observation, demonstrating that co-inoculation with both fungal and bacterial endophytes improved the fresh and dry weight of the plants [50]. Wang et al. [51] also found that mycorrhizal fungi symbiosis improved plant fitness by enhancing fresh and dry weight in plant tissues. Similarly, Rai et al. [52] reported increased stem and root length, biomass, leaf area, inflorescence, and seed production in *Spilanthes calva* and *Withania somnifera* colonized by *Piriformospora indica*. This suggests that endophyte combinations can have a synergistic impact on tomato plants as well, driving growth and development. In another study, Shahab et al. [53] observed a significant increase in stem length of mung beans treated with *Bacillus thuringiensis* and *Pseudomonas aeruginosa*. These findings further support the idea that bacterial endophytes can synergistically enhance plant growth, as seen in our own study on tomato plants. Moreover, mycorrhizal fungi are known to promote root system expansion, leading to improved nutrient and water uptake, which in turn boosts photosynthesis and overall plant growth [54].

In our study, the combinations of *P. chrysogenum* + *E. aurantiacum* and *T. basicola* + P. *chrysogenum* endophytes significantly increased the relative leaf water content (RWC) in tomato plants compared to untreated controls. RWC is a key indicator of plant water status, as it is closely linked to the metabolic activity within plant tissues [55]. Previous studies have shown that plants inoculated with endophytes tend to retain higher water content than non-inoculated plants, which can enhance

both growth and stress tolerance [56]. Endophytes contribute to improving a plant's water absorption and retention capacity by altering root architecture or stimulating root growth, which increases the root surface area available for water uptake. This enhanced ability to absorb water directly contributes to the higher RWC observed in tomato plants co-inoculated with beneficial endophytes [57]. Moreover, the synergistic effects observed in our study underscore how specific combinations of *P. chrysogenum* and *E. aurantiacum*, as well as *T. basicola* and *P. chrysogenum*, can enhance the plant's ability to cope with water stress. The combined actions of these microbial endophytes likely lead to more efficient water retention mechanisms compared to individual endophyte treatments, providing a clear example of how co-inoculation can optimize plant physiological responses. This synergy is particularly valuable in tomato plants, which are known to be sensitive to water stress. Our findings are consistent with previous studies demonstrating that co-inoculation can positively influence water use efficiency and overall plant growth [58].

In this study, the combinations of bacterial endophytes *S. aquatilis* + *M. echinaurantiaca* and *P. chrysogenum* + *M. echinaurantiaca* significantly increased the total chlorophyll fluorescence in tomato plants. Chlorophyll fluorescence serves as a crucial indicator of photosynthetic efficiency and the overall health of the photosynthetic apparatus [59]. Endophytes enhance photosynthetic activity by improving the structure and function of chloroplasts, leading to more efficient light capture and energy conversion. This boost in photosynthetic efficiency can result in higher chlorophyll fluorescence values [60, 61]. Additionally, endophytes may stimulate the synthesis of chlorophyll molecules, increasing the plant's capacity to absorb light and carry out photosynthesis more effectively, thus further enhancing chlorophyll fluorescence [62]. Previous studies, including those by Abdelaziz et al. [63] and Kaboosi et al. [64], have demonstrated that symbiotic relationships with endophytes, such as *Piriformospora indica*, can increase chlorophyll content in tomato plants, particularly at the seedling stage. The increased chlorophyll content in symbiotic plants likely reflects higher photosynthetic activity, which is necessary to supply carbon for the symbiotic relationship between the plant and endophytes [62]. Moreover, the enhanced chlorophyll content observed in leaves during mycorrhizal symbiosis may be linked to improved phosphorus uptake from the soil, with endophytes acting as energy carriers during photosynthesis [65]. Supporting these findings, Sadeghi et al. [66] showed that a combination of three endophytic fungi (*Penicillium citrium* + *Aurobium* + *Dothideomycetes* sp.) significantly increased chlorophyll fluorescence in *Citrus reticulata*, with a 10.6% increase in the (Fv/Fm) ratio compared to untreated controls. In our study, the observed increase in chlorophyll fluorescence in tomato plants inoculated with *S. aquatilis* + *M. echinaurantiaca* and *P. chrysogenum* + *M. echinaurantiaca* indicates that these endophyte combinations play a synergistic role in enhancing the photosynthetic capacity of tomato plants. The Fv/Fm ratio, which is above 0.7 in healthy plants, suggests that co-inoculation contributes to improved plant health and stress resilience [67].

In this study, inoculation with the combinations of *P. chrysogenum* + *M. echinaurantiaca* and *T. basicola* + *P. chrysogenum* significantly increased chlorophyll content in tomato plants. The bacterial endophyte *S. aquatilis* + *M. echinaurantiaca* also led to a notable increase in chlorophyll b levels. Additionally, colonization by the fungal endophytes *T. basicola* and the bacterial endophytes *S. aquatilis* + *M. echinaurantiaca* resulted in higher carotenoid content. These findings suggest that co-inoculation with these specific endophyte combinations has a synergistic effect on enhancing chlorophyll and carotenoid production in tomato plants. The observed increase in chlorophyll and carotenoids aligns with previous studies. Eleiwa et al. [68] demonstrated that inoculation of wheat grains with biofertilizers such as *Bacillus polymyxa* and *Azospirillum brasilense*, which produce auxins, significantly enhanced chlorophyll a, b, and carotenoid levels compared to non-treated controls. These results are consistent with our study and highlight the potential of endophytes to promote chlorophyll synthesis and enhance photosynthetic activity. Carotenoids, in particular, are critical for stabilizing and protecting the lipid phase of the thylakoid membrane, acting as antioxidants to mitigate reactive oxygen species (ROS) and oxygen radicals [69]. The increased carotenoid content in our endophyte-inoculated tomato plants may, therefore, provide enhanced protection against oxidative stress, further supporting the synergistic role of co-inoculation in improving plant resilience.

This finding is in agreement with Dias et al. [70], who reported that inoculation with *Streptomyces* sp. endophytes significantly boosted total chlorophyll content in plants, showing that endophyte inoculation can improve photosynthetic pigments and protect plants from oxidative damage. Similarly, our study found that the increase in chlorophyll a, b, and carotenoids in endophyte-treated tomato plants may enhance their ability to cope with abiotic stresses, such as drought and extreme temperatures, by reducing oxidative damage and enhancing antioxidant defenses. Several studies have shown that inoculation with endophytes increases chlorophyll a, chlorophyll b, and carotenoids in plants compared to non-inoculated controls [71–73].These findings suggest that endophytes stimulate the host plants to produce metabolites essential for growth and stress tolerance. By promoting

antioxidant production, endophytes help plants cope with environmental stresses by scavenging ROS, enhancing the activity of antioxidant enzymes, and improving overall plant resilience [74]. Additionally, endophytes can induce the production of phenolic compounds in plants, which play a vital role in various physiological processes, such as photosynthetic pigment synthesis, cell division, and stress responses [75, 76]. Through co-inoculation, fungi and endophytic bacteria have been shown to enhance plant growth and increase phenol content, which contributes to improved plant metabolism and stress adaptation [77–79].

Our results confirmed that the endophytes significantly increased the proline and total carbohydrate content in tomato seedlings compared to the non-treated controls. These osmolytes are low-molecular-weight, highly concentrated, water-soluble organic compounds that are non-toxic and compatible with cellular functions, playing a crucial role in plant stress tolerance [80]. The increase in proline and carbohydrate levels in tomato plants inoculated with these endophytes suggests a synergistic effect of co-inoculation. Co-inoculation with multiple endophyte species likely enhances the plant's ability to accumulate these protective osmolytes, thereby improving its resilience under stress conditions. The combined action of bacterial and fungal endophytes may optimize the plant's stress response mechanisms, ensuring better survival and growth in challenging environments. Several studies have demonstrated proline accumulation in various plant species inoculated with beneficial endophytes. For example, *Zea mays* plants inoculated with *Yarrowia* showed increased proline accumulation [81], and similar responses were observed in *Hordeum vulgare* inoculated with *Epichloe bromicola*. In *Solanum lycopersicum* (tomato) plants inoculated with *Piriformospora indica*, as well as in *Medicago truncatula* (alfalfa) plants inoculated with the same fungus, proline accumulation was also significantly enhanced, helping these plants manage water and oxidative stress [82]. Additionally, inoculation with *Trichoderma harzianum* in *Brassica juncea* (Chinese mustard) resulted in a similar increase in proline levels, further supporting the role of endophytes in osmolyte accumulation under stress [83].

In this study, the bacterial endophyte *M. echinaurantiaca*, along with the combination of *P. chrysogenum* + *M. echinaurantiaca*, significantly increased the carbohydrate content in tomato seedlings compared to the non-inoculated controls. This enhancement suggests a synergistic effect of co-inoculation, where the combined actions of these endophytes lead to a greater accumulation of carbohydrates, which are essential for plant growth and stress tolerance. The role of endophytic fungi in promoting soluble sugar accumulation, especially under stress conditions such as salinity, has been widely documented [84, 85]. For instance, Yang et al. [86] showed that inoculating plants with *Phomopsis liquidambari* could stimulate the secretion and deposition of sugars, thereby enhancing growth by providing additional carbon. This mechanism is similar to the synergistic effects observed in our study, where co-inoculation with multiple endophyte species appears to boost the plant's ability to produce and store soluble sugars, supporting better growth and resilience to stress. Moreover, endophytic fungi are known to enhance fruit quality by increasing soluble sugar content, as observed in the *Malus domestica* (Honeycrisp) apple cultivar [87].

The synergistic effects of co-inoculation have been documented in several studies [88–91], but in our study, we observed that the effects were highly dependent on the specific combinations of endophytes used. Notably, the combination of *P. chrysogenum* + *E. aurantiacum* and *S. aquatilis* + *M. echinaurantiaca* demonstrated significant synergistic effects that enhanced plant growth, photosynthetic efficiency, antioxidant activity, and metabolite content. These findings suggest that the impact of co-inoculation may vary, and different microbial combinations may have positive, neutral, or even negative effects. This emphasizes the importance of carefully selecting and testing microbial consortia to optimise plant growth promotion.

A key limitation of this study is the lack of prior biocompatibility testing of the microbial strains used in combined inoculation experiments. Since microbial interactions can influence strain effectiveness, compatibility testing is crucial to ensure that strains can coexist without negatively affecting each others growth or the plants health. The absence of such testing may have impacted the outcomes of our experiments. For future research, we recommend incorporating biocompatibility testing as a standard procedure before inoculation. This will help identify suitable strain combinations, prevent potential antagonistic interactions, and improve the reliability and success of microbial inoculation experiments.

Conclusion

In conclusion, the results of this study highlight the significant role of both fungal and bacterial endophytes in enhancing the growth and physiological performance of tomato plants. The combined inoculation of specific fungal-bacterial pairs, such as *P. chrysogenum* + *E. aurantiacum* and *S. aquatilis* + *M. echinaurantiaca*, demonstrated synergistic effects that improved key plant traits, including growth parameters, photosynthetic efficiency, antioxidant activity, and metabolite content. These findings suggest that endophyte inoculation, particularly with fungal-bacterial combinations, holds great potential for boosting plant resilience, optimizing photosynthesis, and enhancing stress tolerance. This research provides

valuable insights into sustainable agricultural practices by harnessing the power of endophytes to improve crop productivity and resilience in the face of environmental challenges.

Supplementary Information

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Supplementary Material 1

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Author contributions
The experiments were planned and designed by SAD and DS. SAD performed the plant growth experiments and carried out data interpretation. SAD also drafted the manuscript, which was subsequently revised by DS. All authors read and approved the final version of the manuscript.

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Data are provided in the Supplementary Information files.

Declarations

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