



Enterobacter cloacae Induces SA-Dependent Systemic Acquired Resistance of *Zea mays* Against *Fusarium oxysporum*

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Abstract

Plant Growth Promoting Bacteria have proven themselves in agricultural applications not only as biofertilizers but also as biocontrol agents against different phytopathogens. In this study, we focused on investigating the ability of *Enterobacter cloacae* to induce the immune response of *Zea mays* against *Fusarium oxysporum* infection. The bacterium was transformed with a plasmid to express Green Fluorescence Protein and used in a greenhouse experiment in combination with *Fusarium* infection in different treatments. *E. cloacae* successfully colonized the root, resulting in enhanced physical growth with great investment in leaf area, photosynthetic pigment production, and reduced anthocyanin content. *E. cloacae* left a considerable resistance to root rot caused by *Fusarium*, as the disease severity was reduced from 74.2% (in the case of *Fusarium* infection alone) to about 35.8% (in the case of *E. cloacae* addition 14 days before fungal infection). The amount of salicylic acid (SA) was markedly elevated, and Pathogen-Related Protein showed up to an eightfold increase in the expression level. From these results, we suppose that *E. cloacae* induces SA-dependent systemic acquired resistance, which allows pre-colonized plants to resist *Fusarium* infection.

Keywords *Enterobacter cloacae* · *Fusarium oxysporum* · Induced systemic resistance · Pathogen-related protein (PR1) · Systemic acquired resistance · *Zea mays*

Introduction

Plant Growth Promoting microorganisms, particularly Bacteria (PGPB), started to gain attention since the last century (Aeron et al. 2011). In that regard, various bacterial strains, including *Rhizobium*, *Pseudomonas*, *Bacillus*, and *Enterobacter*, have shown their ability to improve crop plant growth and productivity (Rodríguez and Fraga

1999). Recently, it was found that PGPB are sensed and further reprogrammed gene expression patterns in plants to help plants overcome both biotic and abiotic stresses via activation of induced systematic resistance (Jain et al. 2014). PGPB may bring many benefits to the plant; it can be relied upon as safe, environmentally compatible, and effective in both short and long terms in helping the plant to survive as well as to resist various stresses (Timmusk et al. 2017).

Among those PGPB is the *Enterobacter* genus. The genus is motile, has peritrichous flagella, is facultative anaerobic, and is Gram-negative. There are many PGP traits that *Enterobacter spp.* are known to possess, including the ability to fix nitrogen, solubilize soil phosphorus, create antibiotics, secrete siderophore products, secrete exopolysaccharides, increase soil porosity, and produce enzymes such as chitinase, ACC deaminase, and hydrolytic enzymes (Jha et al. 2011). It is well accepted that PGPB possess the ability to induce systematic resistance in plants. For PGPB to successfully inhabit roots and have an impact, they must thwart or elude plant MTI (Microbial Triggered Immunity). The localized effect of MTI and ETI (Effector Triggered Immunity) often results in SAR (Systemic

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Acquired Resistance) in distant plant tissues, particularly when subjected to pathogens. That means ISR (Induced Systemic Resistance) is generated by PGPB; however, SAR is generated by pathogens. ISR, in contrast to SAR, relies on pathways controlled by jasmonic acid (JA) and ethylene (ET) rather than the accumulation of pathogenesis-related proteins or salicylic acid.

Maize is ranked the 3rd after wheat and rice as an important cereal crop (Strable and Scanlon 2009) and the second most important crop in Egypt, according to Food and Agricultural Organization. Maize possesses many nutritional and potential health benefits, but unfortunately, the quantity and quality of maize are highly reduced by fungal diseases (ur Rehman et al. 2021). *Fusarium oxysporum* causes root rot of *Z. mays*, which is a yield limiting disease appearing as root decay with black or brown discoloration (Okello et al. 2019). *E. cloacae*, the strain of interest in this study, is regarded as an endophytic symbiont of corn (Hinton and Bacon 1995). Several researchers reported the ability of *E. cloacae* to promote millets, rice, tomato, and mung bean in both normal and abiotic stress conditions (Pattnaik et al. 2020; Sagar et al. 2020; Mayak et al. 2001). Interestingly, *E. cloacae* was also reported as a potential biocontrol agent against several pathogens, including *Ralstonia solanacearum* and *Fusarium sambucinum* (Mohamed et al. 2020; Al-Mughrabi 2010). Additionally, *E. cloacae* as a potential biocontrol agent for suppressing cucumber damping-off disease caused by *Pythium aphanidermatum*, as it reduced disease incidence significantly by 63% (Kazerooni et al. 2020). The effect was attributed to pathogen suppression and induction of systemic plant resistance that was indicated by a significant elevation of total phenols as well as salicylic acid. It was reported that several species of plant growth-promoting bacteria in general and *Enterobacter* in particular produce volatile organic compounds (VOCs) that affect plant-defense genes (Rani et al. 2023). Not only biotic stress, but also *E. cloacae* was able to alleviate cadmium and nickel toxicity

effect on tomato due to its effect on antioxidant capacity and osmoregulation (Badawy et al. 2022).

Given the above, there is an urgent need for further study to identify the mechanisms of plant immune stimulation by beneficial bacteria. This study was designed to detect the response of maize ISR upon bacterization with *E. cloacae* to evaluate the plant's readiness to deal with *Fusarium* infection.

Materials and Methods

Enterobacter DesertPSK Preparation

Enterobacter MT012825 (DesertPSK) was used to investigate maize defense response and its impact on *Fusarium* infection. *Enterobacter* DesertPSK was transformed with the plasmid *pE-BBR2-EGFP* carrying green fluorescence protein expressed under a constitutive promoter to allow tracking of the bacteria inside the plant tissues.

Plasmid Construction

Two 2.0 kb PCR fragments were amplified using the PrimeSTAR Max DNA Polymerase (Takara Bio, USA) and the vector *pE-BR42-TolC1-EGFP* (Aboulnaga et al. 2018) as a template. The first fragment was amplified using BBR1-Ori.F and BBR1-Ori.R primers (Table 1), while Kan-GFP.F and Kan-GFP.R primers were used to amplify the second fragment. The PCR reaction was set as follows: initial denaturation at 98 °C for 30 s; 30 cycles of (denaturation at 98 °C for 10 s, annealing at 55 °C for 10 s, elongation at 72 °C for 2 min); and final elongation at 72 °C for 5 min. These fragments were purified using gel extraction kit (Takara Bio, USA) and treated with *DpnI*-restriction enzyme to destroy the parent template (Aboulnaga et al. 2018). Afterward, the two fragments were ligated together after being cut with *LguI*-restriction

Table 1 List of the strains, plasmids, and primers used in PE-BBR2-EGFP construction

| | | Genotype/relevant characteristics | Source |
|---------|--|---|-------------------------|
| Strains | <i>E. coli</i> DH5 α | fhuA2 Δ (argF-lacZ)U169 phoA glnV44 Φ 80 Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | Invitrogen |
| | <i>Enterobacter</i> MT012825 (DesertPSK) | Isolated strain from Egyptian environment (Isolated from the nodules of common bean and <i>Lotus glaber</i> plants) | This study |
| Plasmid | pE-BR42-TolC1-gfp | Mob, f1ori, KanR, rrnD.T1, PTolC1, gfp, dT, TOP.tetR, RP4, pBBR1 origin | Aboulnaga et al. (2018) |
| | pEF-BRR1- TolC1-gfp | KanR, rrnD.T1, PTolC1, gfp, dT, RP4, pBBR1 origin | This study |
| Primers | BBR1-Ori.F | AAGCTCTTCTGGGTTTATTGACTACCGGAAGC | This study |
| | BBR1-Ori.R | AAGCTCTTCCCATGCCTGCCCTCCC | This study |
| | Kan-GFP.F | AAGCTCTTCGATGAATGTCAGCTACTGGG | This study |
| | Kan-GFP.R | AAGCTCTTCTCCCGTGGAGATCCGTGACGCA | This study |

enzyme and ligated with *T₄-ligase*. The ligated reaction was transformed into *E. coli* DH5 α chemically competent cells using a standard protocol (Chung et al. 1989) and plated on an LB-agar plate supplemented with 50 μ g/ml kanamycin as a selection marker. The correct colony was chosen depending on the green fluorescence (due to the deletion of the *tetR*-repressor) under the blue light. Three different correct colonies were selected and were grown overnight in LB-liquid media supplemented with 50 μ g/ml kanamycin for plasmid minprep. The plasmid was extracted using the plasmid extraction kit (Takara Bio, USA). The sequence of the plasmid was verified via Sanger sequencing.

Enterobacter DesertPSK Transformation

Enterobacter DesertPSK competent cells were prepared using calcium chloride method (Sambrook et al. 2001). Bacterial transformation was then carried out as described in (Sambrook et al. 2001). In summary, freshly prepared competent cells (200 μ l) were gently mixed with 500 ng of *pE-BBR2-EGFP* and kept in an ice bath for 20 min, then heat shocked at 42 °C for 90 s, followed by incubation in an ice bath for an additional 5 min. Afterward, 800 μ l of SOC media was added, and the culture was incubated at 37 °C for 45 min. The transformed cells were plated on LB-agar containing 50 μ g/ml kanamycin as an antibiotic selection marker. The expressed green fluorescence protein was checked by examining the bacteria by epifluorescence microscopy (Olympus BX53, Japan) using a fluorescence filter cube, a narrow band blue (FITC) excitation Filter: BP470-495, beam splitter DM505, a and barrier filter BA510-550, including plastic (Wang et al. 2017). The transformed bacterial strain was confirmed by MALDI-TOF.

Greenhouse Experiment

A mixture of peat and vermiculite soils (50:50 W) was used in this experiment. The soil mixture was analyzed before sowing, and the properties are indicated in (Table 2). The mixture was autoclaved at 121 °C for 20 min.

Two kg of the autoclaved soil mixture were packed in plastic agricultural bags. The bags were divided into five groups. Homogenous and healthy seeds were selected and subjected to surface sterilization by 20% NaClO for 3 min and washed with sterilized distilled water. The seeds were then subjected to five different treatments for 1 h as follows: Water (W/control); the recombinant *Enterobacter pE-BBR2-EGFP*, one just with the recombinant *Enterobacter* (T), and a second group which was further infected with *Fusarium oxysporum* after two weeks (T then F); recombinant *Enterobacter pE-BBR2-EGFP* along with *Fusarium oxysporum* (T + F); and *Fusarium oxysporum* (F) alone. Eight seeds were then sown in each bag, about 1 cm below

Table 2 The soil specifications in the soil mixture

| Soil properties | |
|--------------------------------|---------|
| Organic matter (%) | 94.62 |
| Organic carbon (%) | 54.88 |
| E.c (ds.m ⁻¹) | 1.24 |
| pH | 4.2 |
| Total Nitrogen (%) | 0.96 |
| Total phosphorus (%) | 0.04 |
| Total potassium (%) | 0.05 |
| Iron (ppm) | 972 |
| Zinc (ppm) | 118.6 |
| C/N ratio | 1:57.16 |
| Water-holding capacity WHC (%) | 482.17 |

the soil surface. The *F. oxysporum* spore suspension (8 \times 10⁵ spores/kg soil) was added to the irrigation water for the treatments including infection. The seeds were allowed to grow under normal day/night conditions in the greenhouse, and the samples were taken 14 days after sowing. On day 14th the *F. oxysporum* spore suspension was added to one group of the bacterially colonized seeds (T then F). Additionally, final sampling took place after 28 days of sowing for all treatments.

The physical growth parameters, photosynthetic pigments, membrane features, total phenols, flavonoids, proline, and antioxidant enzymes such as catalase, peroxidase, polyphenol oxidase, and ascorbate peroxidase were measured for both 14- and 28-day old samples.

Further analyses, such as estimation of anthocyanin, phenolics, and flavonoids profiles, salicylic acid (SA), jasmonic acid (JA), and assessment of pathogenesis-related gene (PR-1) and coronatine-insensitive 1 (COI1) expression levels, were done for the 28-day old samples only.

Physical Growth Parameters

Root length, shoot length, leaf area, root fresh and dry weight, shoot fresh and dry weight, as well as the water percentage of the shoot and root, were measured.

Estimation of Photosynthetic Pigments

The pigments were extracted by soaking 0.1 g of fresh leaves in 5 ml of dimethyl sulfoxide (DMSO) at room temperature for 24 h, followed by filtration, and the final volume was raised to 10 ml by DMSO. The absorbance was measured at wavelengths of 470 nm, 644 nm, and 662 nm. The concentration (mg/ml) was calculated for the pigment fractions by the following equations (Adhikari et al. 2020).

$$\text{Chl.a} = 12.7(\text{O.D}_{662}) - 2.69(\text{O.D}_{644}) = \text{mg/ml}$$

$$\text{Chl.b} = 22.9(\text{O.D}_{664}) - 2.69(\text{O.D}_{662}) = \text{mg/ml}$$

$$\text{Total chl.} = 20.2(\text{O.D}_{644}) + 8.02(\text{O.D}_{662}) = \text{mg/ml}$$

$$\text{Carotenoids} = [(\text{O.D}_{470}) - 1.28(\text{Chl.a}) + 5.67(\text{Chl.b})] / (256 \times 0.906) = \text{mg/ml}$$

Membrane Features

Membrane features such as electrolyte leakage, membrane injury index, and membrane stability index were measured in the fresh plant leaves.

Electrolyte Leakage (E.L.)

Electrolyte leakage was determined following the method described by (Saeed et al. (Saeed et al. 2019)). The plant leaf was cut into 1cm² pieces (away from the midrib), then placed in a tube containing 10 ml of distilled water, and the electrical conductivity was recorded using the EC meter to be (EC₁). The same tubes were then placed on a shaker for 2 h and the electrical conductivity was recorded to be (EC₂). After that, the tubes were autoclaved, left to cool down, and the electrical conductivity was recorded for the third time to be (EC₃). The electrolyte leakage was calculated by the following equation:

$$\text{Electrolyte leakage (\%)} = (\text{EC}_2) - (\text{EC}_1) / (\text{EC}_3) \times 100$$

Membrane Injury Index (MII)

Leaves (0.1 g) were weighed and sectioned into uniform small pieces and placed into tubes containing 10 ml of distilled water, the tubes were then divided into two groups: the first group was incubated at 100 °C for 15 min and the electrical conductivity was measured to be (EC₂). The second group was incubated at 40 °C for 30 min, and the electrical conductivity was measured to be (EC₁). The membrane injury index was calculated by the following equation (Deshmukh et al. 1991):

$$\text{MII} = (\text{EC}_1 / \text{EC}_2) \times 100$$

Membrane Stability Index (MSI)

Membrane stability index was calculated as follows (Mickky et al. 2019):

$$\text{MSI} = 100 - \text{MII}$$

Estimation of Total Phenols

For total phenolic content, a methanolic extract was prepared by extracting 2 g of powdered dry plant tissue in 20 ml of 50% methanol for a week at 37 °C, followed by filtration. One ml of the methanolic extract was mixed with

1 ml of diluted Folin-Ciocalteu reagent. After 3 min, 1 ml of a saturated sodium carbonate solution was added, and the mixture was completed to 10 ml by distilled water, then kept in the dark for 90 min, after which the absorbance was measured at 760 nm. The total phenolic content was expressed in microgram gallic acid equivalents (GAE) per gram dry weight (µg GAE/g) (Zeitoun et al. 2017).

Estimation of Total Flavonoids

The reaction mixture was prepared by mixing 1 ml of methanolic extract, prepared as mentioned previously, with 4 ml of distilled water and 0.3 ml of 5% NaNO₂. After 3 min, 0.3 ml of 10% AlCl₃ was added. The mixture was allowed to stand for 6 min before adding 2 ml of 1 M NaOH. The reaction mixture was completed to 10 ml using distilled water, mixed well, and the optical density was measured at 500 nm. The total flavonoid content was expressed in microgram quercetin equivalents (QE) per gram dry weight (µg QE/g) (De Souza et al. 2018).

Estimation of Proline

Plant water extract was prepared by incubating 0.1 g of dry tissue powder in 10 ml of water at 90 °C for 1 h. Then it was centrifuged and the pellet was extracted twice. The combined supernatant was raised to 10 ml. Proline was estimated by mixing 1 ml plant water extract with 1 ml glacial acetic acid and 1 ml ninhydrin reagent. The mixture was then incubated in a boiling water bath for an hour, and after cooling, the absorbance was measured at 510 nm. Serial dilutions of proline were used to construct the standard curve (Khalil et al. 2017).

Estimation of Antioxidant Enzymes

The enzymes were extracted by grinding 0.2 g of leaves in liquid nitrogen in the presence of 0.1M potassium phosphate extraction buffer (pH 6.8). The homogenate was then centrifuged at 4 °C, 10,000 rpm for 15 min. The

supernatant was obtained, fractionated into 1 ml aliquots, and kept at -20°C till use (Abd-ElGawad et al. 2020).

Detection of Peroxidase (POD) Activity (EC 1.11.1.7.)

The assay mixture contains 125 μM of potassium phosphate buffer (pH 6.8), 50 μM of pyrogallol, 50 μM of H_2O_2 , and the enzyme extract. The increase in absorbance was measured at 420 nm using spectrophotometer (Jenway 7315 UV-VIS, USA) (Kar and Mishra 1976).

Detection of Polyphenol Oxidase (PPO) Activity (EC 1.14.18.1.)

The PPO was assayed using the same mixture for peroxidase without the addition of H_2O_2 . The increase in absorbance was recorded at 420 nm (Oktay et al. 1995).

Detection of Catalase (CAT) Activity (EC 1.11.1.6.)

Catalase was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 11 mM H_2O_2 , and enzyme extract. Activity was determined by monitoring the change in absorbance at 240 nm (Mishra et al. 1993).

Detection of Ascorbate Peroxidase (APX) Activity (E.C.1.1.1.1)

APX activity was determined spectrophotometrically by observing the decrease in absorbance at 290 nm (Kochhar et al. 2003; Li et al. 2008). The enzyme extract (0.1 ml) was mixed with 1.8 ml potassium phosphate buffer (50 mM, pH 7), 0.1 ml ascorbic acid (15 mM), and 1 ml H_2O_2 (0.3 mM), then mixed thoroughly for 3 min.

Estimation of Anthocyanin

Anthocyanin was extracted by macerating 0.4 g of fresh leaves in 4 ml chilled acidified methanol (HCl 0.1%). The homogenate was then centrifuged at 6,000 rpm at 4°C for 20 min. The spectral scan was monitored from 300 to 700 nm using spectrophotometer (Jenway 7315 UV-VIS, Burlington, VT, USA). Anthocyanin content was calculated by the following equation (Abd-ElGawad et al. 2020):

$$\text{Anthocyanin content} = A_{530\text{ nm}} - A_{653\text{ nm}}$$

Phenolics and Flavonoids Profile

Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector was used. Sample extracts were accurately weighed and sonicated for 15 min, filtered

using a 0.22 μm nylon syringe filter, then 10 μl was injected into C18 Inertsil ODS 4.6×250 mm, 5 μm Column. The compounds were separated with 0.1% phosphoric acid in water and using acetonitrile as the mobile phase with a flow rate of 1 ml/min and detected under a wavelength of 280 nm. For standards, a Stock solutions of 10 different standards in methanol were prepared, filtered using a 0.22 μm syringe filter, and then 10 μl were injected (Mradu et al. 2012).

Estimation of Salicylic Acid

Salicylic acid levels were assessed as per (Zhang et al. 2002). One gram of fresh leaf was ground up in liquid nitrogen, combined with two milliliters of 90% methanol, sonicated for 15 min, and then centrifuged at 2800 rpm for 20 min. Entirely pure methanol was used to extract the pellet (2 ml). Two extractions' supernatants were mixed and then dried under a nitrogen stream. The resulting residue was resuspended in 4 ml of 5% trichloroacetic acid (TCA), followed by centrifugation at 2800 rpm for 20 min. The upper phase was collected and dried under a nitrogen stream. The supernatant was divided twice with an extraction solution (ethylacetate:cyclohexane:isopropanol 100:99:1). In 4 ml of mobile phase, the residues were resuspended, and the solution was filtered through a 0.22 μm nylon membrane. For the standard solution, 1 mg/ml salicylic acid in methanol was prepared, and serial dilutions were made to obtain a concentration of (10, 20, 30, 40, 50 $\mu\text{g/ml}$), then filtered using a 0.22 μm syringe filter, and 10 μl was injected to construct the standard curve. The used device was Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector, with C18 Inertsil ODS 4.6×250 mm, 5 μm Column, with 0.1% phosphoric acid in water and acetonitrile as the mobile phase. A gradient mode of elution was used with a Flow rate of 1 ml/min, and the SA was detected under the wavelength of 300 nm.

Estimation of Jasmonic Acid (JA)

For jasmonic acid detection, Agilent 6890N GC system equipped with an FID detector was used. The samples were prepared by dissolving an accurately weighed amount of the extract into methanol, followed by sonication for 15 min and filtration through a 0.45 μm nylon syringe filter. The first portion of the filtrate was rejected and 1 μl filtrate was injected with a split ratio of 20:1 into 0.53 mm \times 30 m fused silica, coated with a 1 μm layer of G16 stationary phase. The running conditions were: the temperature was held at 34°C for 2 min, then increased to 180°C (20°C/min), followed by an increase to 220°C (5°C/min). The carrier gas was nitrogen at a flow rate of 2 mL/min; the temperatures of the injector port and of the detector were 300°C . A standard solution of jasmonic

Table 3 The used primers nucleotide sequence, length, and the gene name

| Primers | Nucleotide sequence | Length |
|----------------|------------------------------------|--------|
| PR-1 forward | 5'-TAGCGTGCCTCCTAGCTC-3' | 18 bp |
| PR-1 reverse | 5'-GTTGTAGCTGCAGATGATGAA GAC-3' | 24 bp |
| COI1 forward | 5'-TCCAGATCAAGATGAACCTTGC-3' | 22 bp |
| COI1 reverse | 5'-CACAGATAGTGGGACCAA TGGA-3' | 22 bp |
| ZmAct1 forward | 5'-GGGATTGCCGATCGTATGAG-3' | 20 bp |
| ZmAct1 reverse | 5'-GCCACCGATCCAGACACT-3' | 18 bp |

Table 4 The temperature conditions of the thermal cycler

| Program | Temperature (°C) | Time |
|----------------------|------------------|--------|
| Initial denaturation | 95 | 10 min |
| Denaturation | 95 | 15 s |
| Annealing | 60 | 1 min |
| Extension | 72 | 30 s |

acid was prepared (100 mg/ml) and serial dilutions were prepared (0.8, 0.6, 0.4, 0.2, and 0.1 µg/ml) to construct the standard curve. (Shahzad et al. 2017; Zadra et al. 2006).

Gene Expression Levels (RT-PCR)

The expression levels of two important pathogenesis-related genes (PR1) and coronatine-insensitive 1 (COI1) were measured using RT-PCR. RNA was extracted using TRIzol™ Reagent according to the manufactures protocol (Thermo Scientific, USA), and RNA concentration and purity were measured using Nanodrop. The RNA was then converted into cDNAs using 2X Bioline SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA). For Real-Time PCR, the used primers are shown in (Table 3), which were designed using pDRAW32 software and synthesized by HVD Life Science Company, Egypt, Almazah, Heliopolis, Cairo Governate. The PCR reaction mixture was performed in a final volume of 20 µl as follows 0.5 µl cDNA template (500 ng/µl), 10 µl 2X Bioline SYBR Green/ROX qPCR Master Mix, 0.25 µl forward primer (100 µM), 0.25 µl reverse primer (100 µM), and 9 µl nuclease-free water. PCR was then performed using Step One Plus™ Real-Time PCR System. The program was adjusted as detailed in (Table 4). Maize actin ZmAct1 was used as an internal reference to standardize the RNA samples for evaluating relative expression levels (Gond et al. 2015; Huang et al. 2017). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008).

Assessment of *Fusarium oxysporum* Disease Severity

The symptoms of root rot caused by *Fusarium oxysporum* inoculation were observed on 28-day old samples, and percentages of disease incidence (DI) were calculated using the following formula:

$$DI (\%) = (\text{No. of diseased plants} / \text{Total no. of assessed plants}) \times 100$$

Symptoms were qualitatively rated as described by (Carranza et al. 2019) using a 0–4 scale: 0, healthy plant with no visible disease symptoms; 1, mild symptoms; 2, apparent symptoms with leaf yellowing, wilting, and root browning; 3, more severe symptoms with expanded lesions and root browning; and 4, massive infection resulting in plant death.

Disease severity was calculated as follows:

$$\text{Disease severity} \% = (\sum n \times r / N4) \times 100$$

n = number of diseased leaves with a rating of r . N = total number of leaves tested multiplied by the maximum numerical rate. r = rating value.

Root Colonization Assay

In order to visualize the bacterial root colonization, confocal laser scanning microscopy was performed with Leica DMI8 system as described by (Fan et al. 2011) using an excitation laser of 488 nm and collecting the emission band of 580 nm for GFP fluorescence. Transmission light was collected to visualize the root structure. Images were acquired and reconstructed by Leica Confocal Software.

Statistical Analysis

The data were statistically analyzed using COSTAT program (798 Lighthouse Ave. PMB 329, Monterey, CA, 93,940, USA). Multiple values are presented as means ± SE. Groups of values were compared by one-way ANOVA analysis of variance, and $p \leq 0.05$ was accepted as significant.

Results

Recombinant *Enterobacter DesertPSK*

The expression vector *pE-BBR2-EGFP* carrying green fluorescence protein under the constitutive *TolC*-promotor was constructed, and the final plasmid map is shown in (Fig. 1a). *Enterobacter DesertPSK* was transformed by

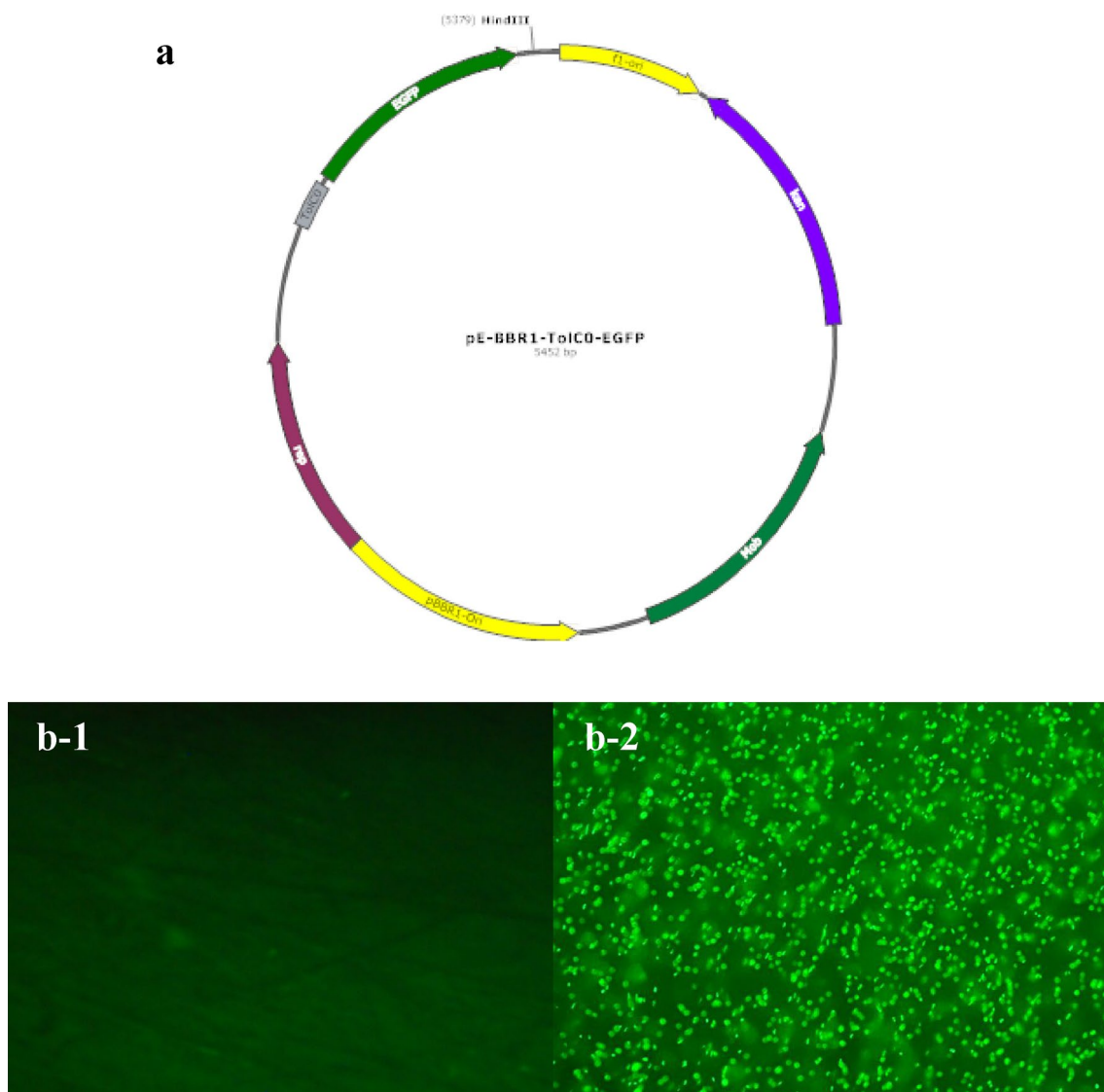


Fig. 1 a PE-BBR2-EGFP map showing key genes and selected restriction endonuclease sites. Ori, origin of DNA replication, Kan, kanamycin resistance gene, Mob, the origin of transfer, rep, replicase, EGFP, the green fluorescent protein gene, HindIII (5379)

indicate the nucleotide location of the restriction endonuclease site, **b** Florescence microscopy detection of the green florescent protein (GFP) expression; **b-1** wild type strain *Enterobacter* DesertPSK and **b-2** the recombinant *Enterobacter* harboring PE-BBR2-EGFP

this plasmid. To assure the successful transformation, a fluorescent microscopy examination was conducted, and the fluorescence was detected as clear in (Fig. 1b) indicating the functionality of the *TolC*-promotor in *E. DesertPSK*. Additionally, MALDI-TOF identified the transformed bacteria as *Enterobacter cloacae* (supplementary data).

Estimation of Growth Parameters

Root length, shoot length, root fresh weight, root dry weight, shoot fresh weight, and shoot dry weight all showed a significant increase in presence of recombinant

Enterobacter pE-BBR2-EGFP (T and T + F) treatments compared to control, while these parameters decreased with (F) treatment significantly. For root water percentage, all the treatments showed almost no difference from the control except for (F) treatment, which showed a significant decrease. Shoot water percentage was not affected by any treatment, as shown in (Figs. 2, 3). These results show the ability of recombinant *Enterobacter pE-BBR2-EGFP* to enhance *Z. mays* growth, an ability that is extending even under infection with *Fusarium*. In 28-day old samples, the additional treatment (T/F) showed enhanced growth over (T + F).



Fig. 2 a Growth response of 14 days old *Z. maize* plants to the different bacterial and fungal treatments (W=water, T=recombinant *Enterobacter* PE-BBR2-EGFP, F=*F. Oxysporum*, T+F=recombinant *Enterobacter* PE-BBR2-EGFP along with *F.*

Oxysporum), **b** Growth response of 28 days old *Z. maize* plants to the different bacterial and fungal treatments (T/F=recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition)

Changes in Photosynthetic Pigments

The results for chlorophyll a, chlorophyll b, and total chlorophyll were found to be significantly increased in presence of recombinant *Enterobacter pE-BBR2-EGFP* (T and T + F), while decreasing due to *Fusarium* infection (F). Carotenoids increased with (T) while the difference in other treatments compared to the control was not that significant, as shown in (Fig. 4). In 28-day old samples, the pigments showed a significant increase in case of bacterial colonization prior to fungal infection (T/F) over the plants with mixed bacterial colonization and fungal infection at the same time (T + F) (Fig. 4).

Changes in Membrane Features

Fusarium infection (F) disturbs membrane stability, leading to a significant increase in E.L and MII, an effect that was reduced in presence of recombinant *Enterobacter pE-BBR2-EGFP* (T + F). (Fig. 5) shows a significant decrease in E.L and MII in presence of recombinant *Enterobacter pE-BBR2-EGFP* (T and T + F). MSI values significantly increased and decreased in presence of bacteria (T and T + F) and fungi (F), respectively. When (T/F) treatment was added in the second stage, it showed enhanced membrane stability compared to (T + F) treatment.

Changes in Antioxidant Compounds

In both stages, Proline, phenols, and flavonoids increased significantly in *Fusarium* infected plant samples and

phenols showed a significant decrease in presence of recombinant *Enterobacter pE-BBR2-EGFP*. In 28-day old samples, the amounts of proline and flavonoids mostly increased considerably for recombinant *Enterobacter pE-BBR2-EGFP* treated plants (Fig. 5).

Changes in Antioxidant Enzymes

In 14-day old samples, and as shown in (Fig. 6), catalase activity increased considerably with all the treatments (T, T + F, and F), while the activity of peroxidase decreased with (T + F) treatment. Polyphenol oxidase enzyme showed maximum activity with (T + F). The treatment (T) showed almost no change and decreased in presence of *Fusarium* (F). In 28-day old samples, both POD and PPO activities significantly increased due to *Fusarium* treatment, but the presence of bacteria significantly decreased POD activity. PPO significantly increased in all recombinant *Enterobacter pE-BBR2-EGFP* treated plants.

Changes in Anthocyanin

Apparently, the leaves of *Fusarium* treated plants generated a significant amount of anthocyanins compared to all other treatments (a sixfold increase compared to control). In presence of recombinant *Enterobacter pE-BBR2-EGFP*, the amount was reduced to almost half the amount of fungal infected ones (Fig. 7).

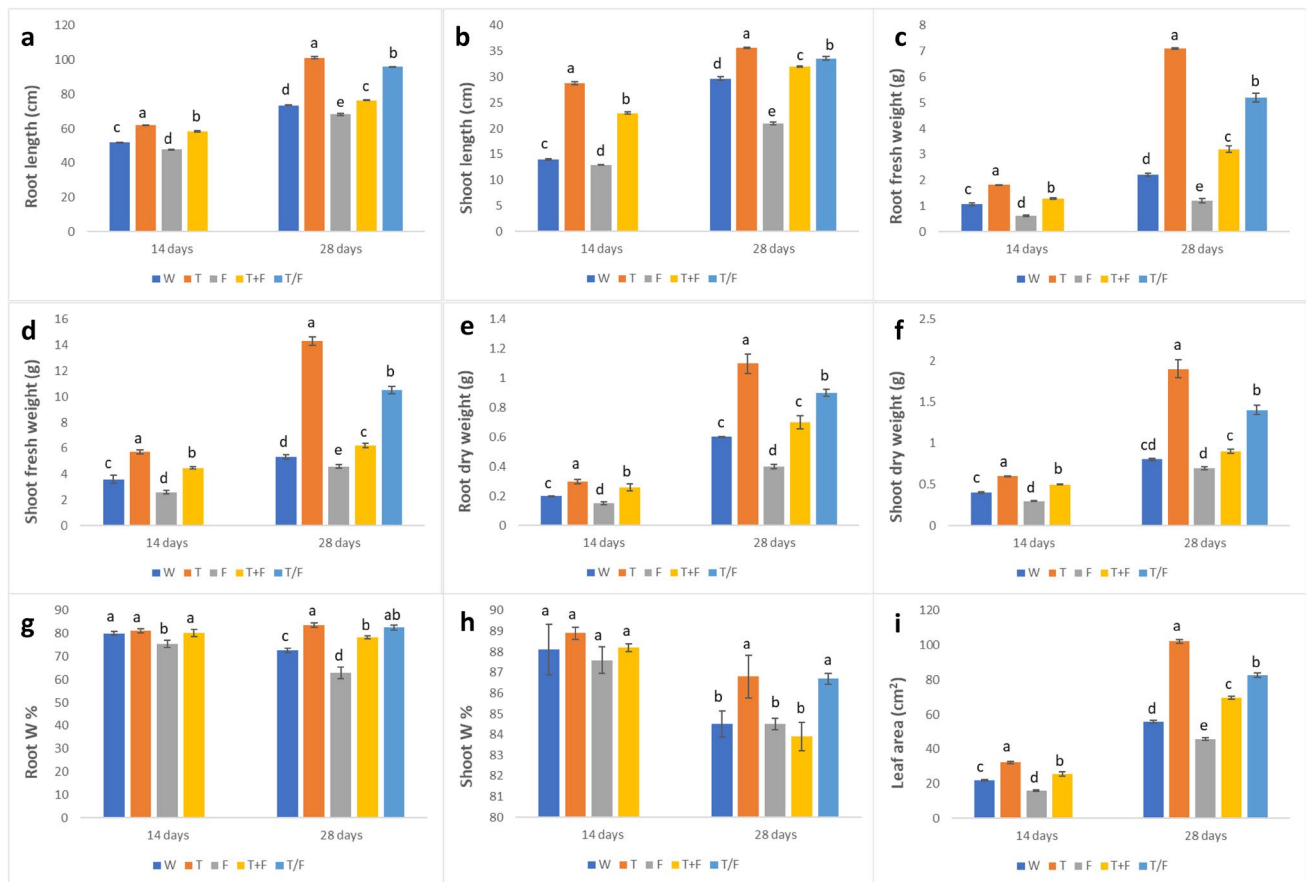


Fig. 3 Effect of bacterial and fungal treatments on the growth parameters (**a** root length, **b** shoot length, **c** root fresh weight, **d** shoot fresh weight, **e** root dry weight and **f** shoot dry weight, **g**: root water percentage, **h**: shoot water percentage, **i**: leaf area) of *Z. maize* in the three stages (7, 14 and 28 days) (W=water,

T=recombinant *Enterobacter* PE-BBR2-EGFP, F=*F. Oxysporum*, T+F=recombinant *Enterobacter* PE-BBR2-EGFP along with *F. Oxysporum* and T/F=recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition). The Duncan's test revealed that different letters differ significantly from each bar (p -value < 0.05)

Phenolics and Flavonoids Profile

Only chlorogenic acid and rutin were detected in maize plants in this study, but the amounts were varied, as shown in (Fig. 8), according to the designed treatment. Chlorogenic acid significantly increased in recombinant *Enterobacter pE-BBR2-EGFP* treated plants (1.7-fold). However, *Fusarium* infected plants accumulated the lowest amount of chlorogenic acid. The combined treatments amount of chlorogenic acid was higher than that of fungal treated ones. Rutin accumulated considerably in fungal treated plants compared to all bacterial treatments.

Estimation of Salicylic and Jasmonic Acid

All the represented treatments increased the amount of SA, but to different degrees. The highest contents of SA were

recorded for fungal treated plants. For JA, both (T/F) and (T + F) treated plants showed the highest amount of JA compared to control and other treatments (Fig. 9).

Estimation of Gene Expression Levels (RT-PCR)

Pathogen-related protein (PR1) showed an eightfold increase in the expression level in response to (T/F) treatment, and plants that received *Enterobacter* (T) showed a 2.5-fold increase. Fungal and (T + F) treatments showed almost no change and decrease in expression pattern, respectively. As to coronatine-insensitive gene (COI1), the expression pattern was increased (1.2-fold) in response to bacteria and decreased in response to (T/F) and (F). (T + F) treatment did not change the expression pattern. The results are represented in (Fig. 9).

Fig. 4 Effect of bacterial and fungal treatments on the photosynthetic pigments (**a**: chlorophyll a, **b**: chlorophyll b, **c**: total chlorophyll and **d**: carotenoids) of *Z. mays* after 14 and 28 days (W = water, T = recombinant *Enterobacter* PE-BBR2-EGFP, F = *F. Oxysporum*, T + F = recombinant *Enterobacter* PE-BBR2-EGFP along with *F. Oxysporum* and T/F = recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition). The Duncan's test revealed that different letters differ significantly from each bar (p -value < 0.05)

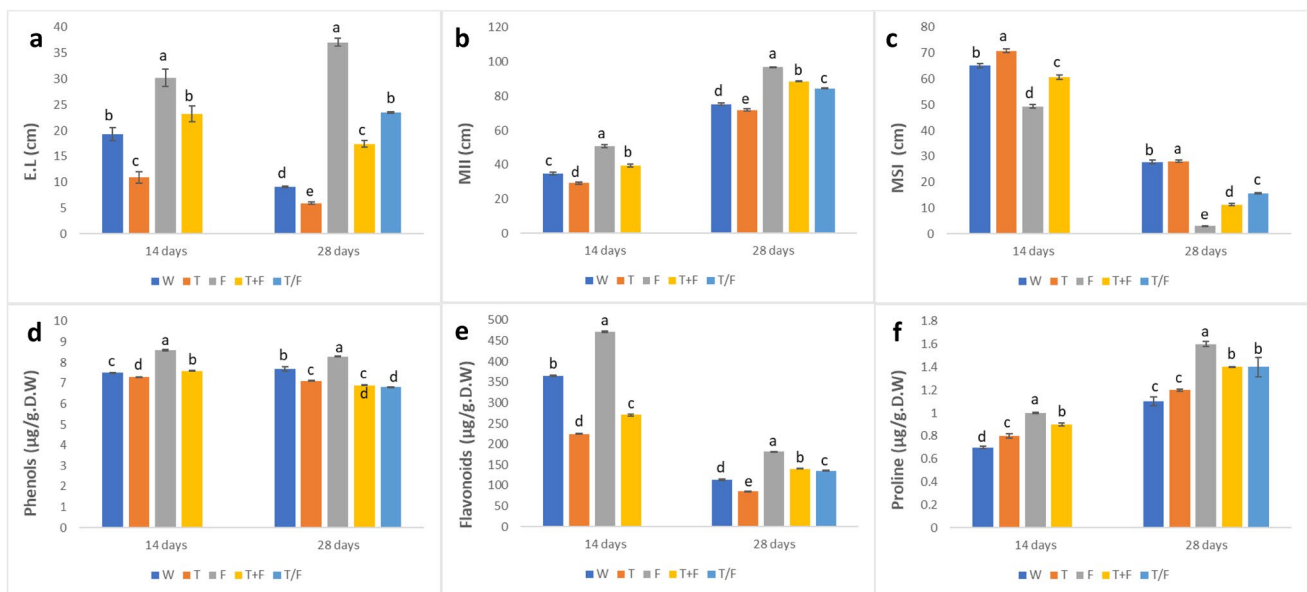
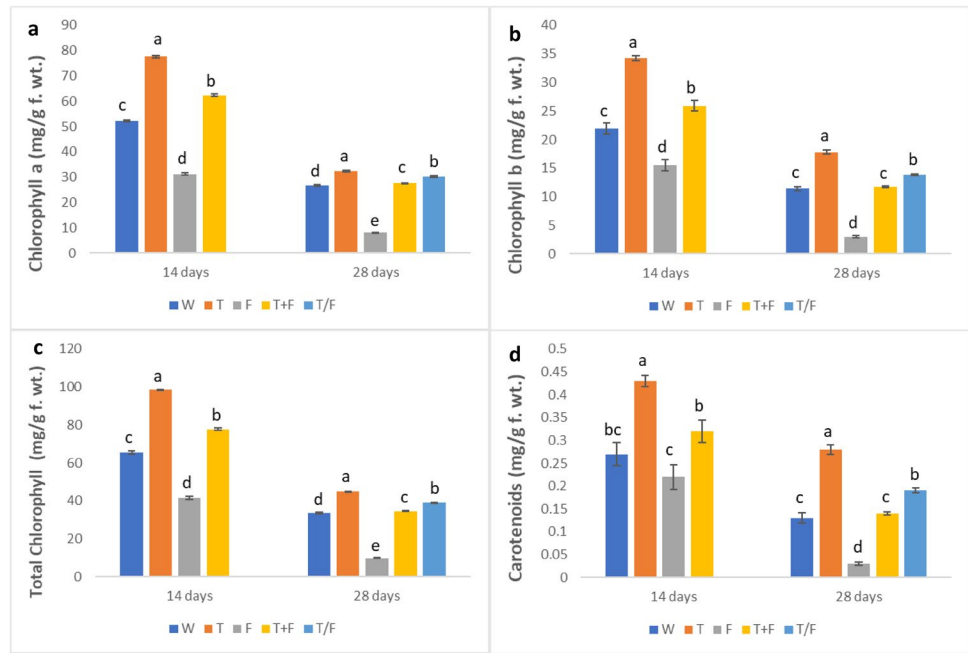


Fig. 5 Effect of bacterial and fungal treatments on (**a**: electrolyte leakage, **b**: membrane injury index, **c**: membrane stability index, **d**: phenolics, **e**: flavonoids and **f**: proline) of *Z. mays* after 14 and 28 days. (W = water, T = recombinant *Enterobacter* PE-BBR2-EGFP, F = *F. Oxysporum*, T + F = recombinant *Enterobacter* PE-BBR2-

EGFP along with *F. Oxysporum* and T/F = recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition) The Duncan's test revealed that different letters differ significantly from each bar (p -value < 0.05)

Root Colonization Assay

As shown in (Fig. 10), recombinant *Enterobacter pE-BBR2-EGFP* was detected in the root bases by the GFP fluorescence, which was significantly high in case of

treatment with bacteria only (T), while it was reduced in case of the presence of fungal including treatments (T/F) and (T + F). The water control treatment showed a minor fluorescence due to plant autofluorescence.

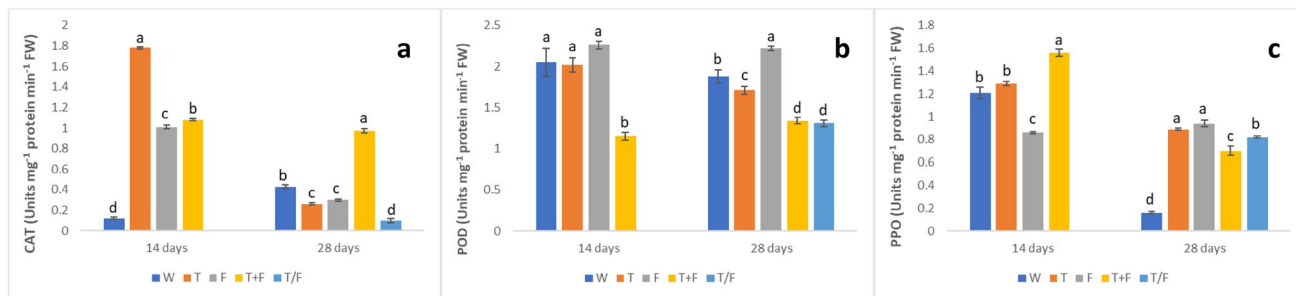


Fig. 6 Effect of bacterial and fungal treatments on the activities of antioxidant enzymes (a: catalase, b: peroxidase and c: polyphenol oxidase) of *Z. Maize* after 14 and 28 days (W=water, T=recombinant *Enterobacter* PE-BBR2-EGFP, F=*F. Oxysporum*,

T+F=recombinant *Enterobacter* PE-BBR2-EGFP along with *F. Oxysporum* and T/F=recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition). The Duncan's test revealed that different letters differ significantly from each bar (p -value < 0.05)

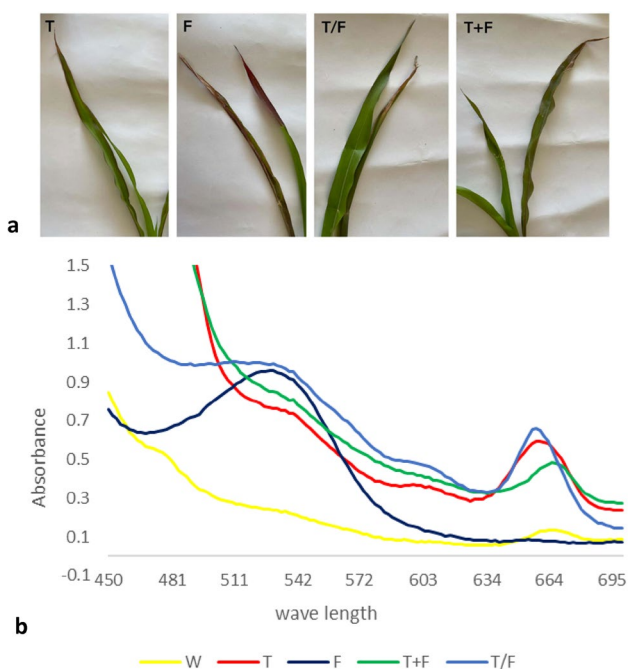


Fig. 7 Anthocyanin accumulation in the leaves of *Z. maize* plants (a), Absorbance spectra of anthocyanin (b) (W=water, T=recombinant *Enterobacter* PE-BBR2-EGFP, F=*F. Oxysporum*, T/F=recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition and T+F=recombinant *Enterobacter* PE-BBR2-EGFP along with *F. Oxysporum*)

Assessment of *Fusarium oxysporum* Disease Severity

The addition of *Enterobacter* resulted in a significant disease severity reduction, and the best results were obtained in case of *Enterobacter* addition 14 days before *Fusarium* infection (T/F), which reduced the disease severity up to 35.8% (Table 5; Fig. 11).

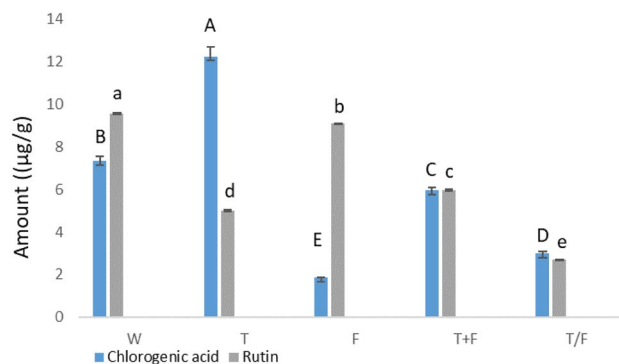


Fig. 8 The amount (µg/g) of chlorogenic acid and rutin with different bacterial and fungal treatments (W=water, T=recombinant *Enterobacter* PE-BBR2-EGFP, F=*F. Oxysporum*, T+F=recombinant *Enterobacter* PE-BBR2-EGFP along with *F. Oxysporum* and T/F=recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition). The Duncan's test revealed that different letters differ significantly from each bar (p -value < 0.05) (capital letters for chlorogenic acid and small letters for rutin)

Discussion

Plants inoculated with *Enterobacter* alone showed a significant increase in root and shoot lengths, leaf area, and root and shoot fresh and dry weights (two times increase or less) (Figs. 2, 3). The growth parameters for plants bacterized then subjected to *Fusarium* infection considerably increased compared to plants infected with *Fusarium* along with *Enterobacter* at the beginning of the experiment and *Fusarium* infected plants, which showed reduced growth. *Fusarium* causes great growth retardation and crop loss in maize and tomato (Khalil et al. 2021; Okello et al. 2019). *Enterobacter* was reported to increase maize growth and yield attributes (Mowafy et al. 2021), and to improve maize growth under drought tolerance (Maqbool et al. 2021). *Fusarium* wilt resistance and growth were promoted in *Enterobacter* inoculated plants (Bendaha et al. 2019).

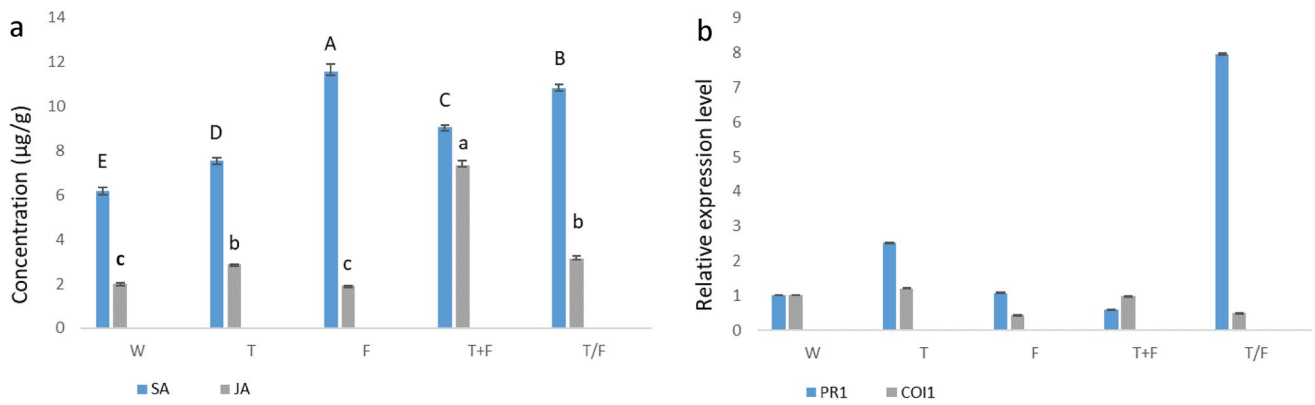


Fig. 9 Effect of bacterial and fungal treatments on the production of salicylic and jasmonic acid of *Z. maize* plants after 28 days, (capital letters for SA and small letters for JA) (**a**). Relative gene expression of PR1 and COI1 in the leaves of *Z. maize* plants (**b**) (W=water, T=recombinant *Enterobacter* PE-BBR2-EGFP, F=*F. Oxysporum*,

T+F=recombinant *Enterobacter* PE-BBR2-EGFP along with *F. Oxysporum* and T/F=recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition) after 28 days. The Duncan's test revealed that different letters differ significantly from each bar (p -value < 0.05) (capital letters for PR1 and small letters for COI1)

Fig. 10 CLSM of recombinant *Enterobacter* PE-BBR2-EGFP colonizing *Z. Maize* roots. transverse sections in the roots of different treatments (W=water, T=recombinant *Enterobacter* PE-BBR2-EGFP, T/F=recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition, and T+F=recombinant *Enterobacter* PE-BBR2-EGFP along with *F. Oxysporum*)

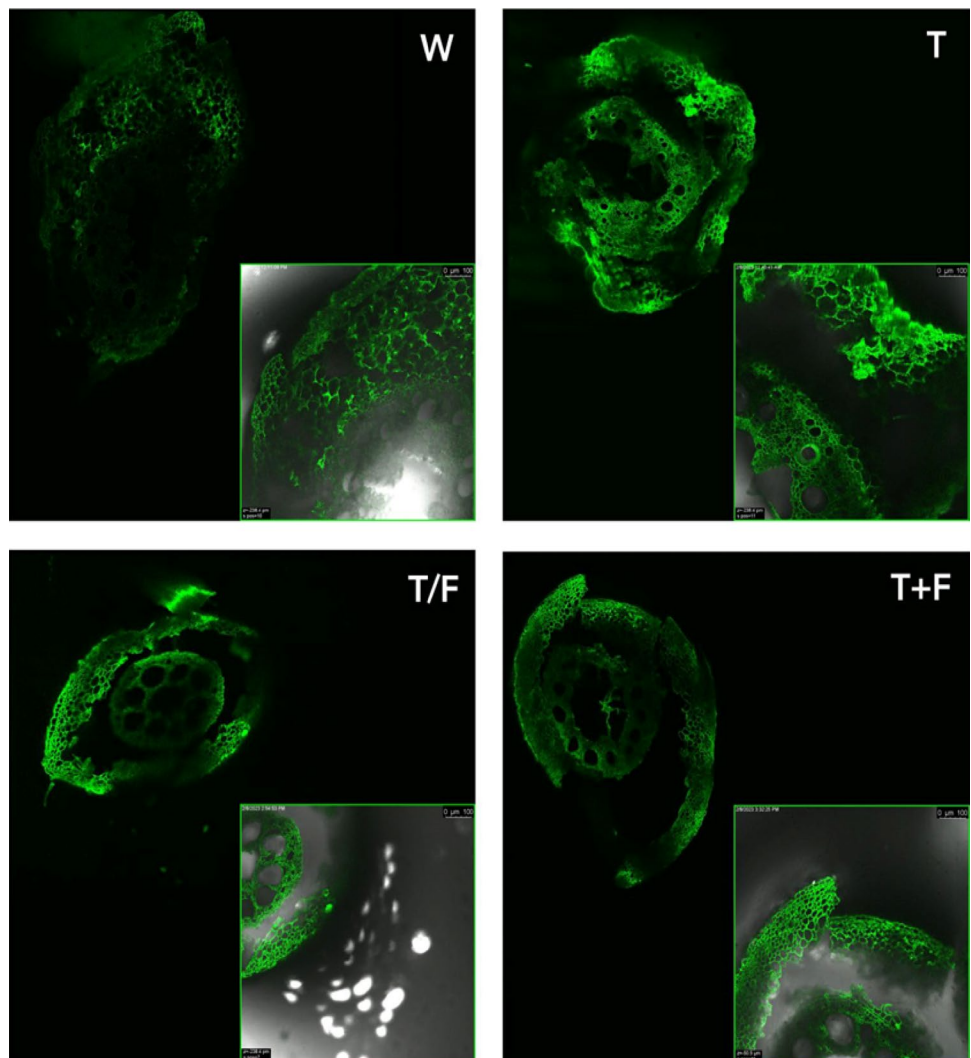
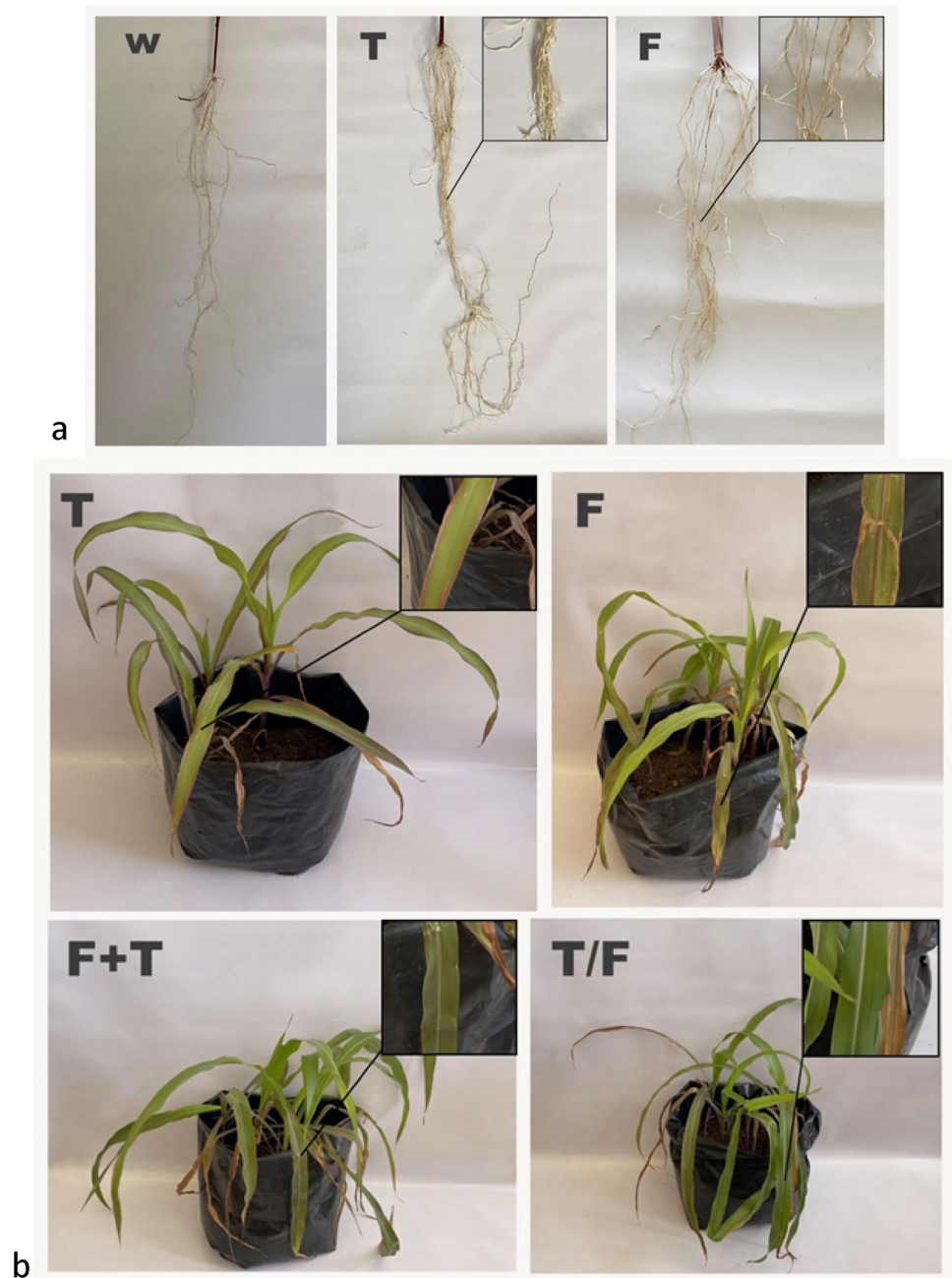


Table 5 Effect of *Enterobacter* on DI% and DSI% of maize plants infected with *Fusarium oxysporum*

| Treatment | Disease incidence (DI%) | Disease severity index (DSI%) |
|-----------|--------------------------|-------------------------------|
| W | 0 ^d ± 0 | 0 ^d ± 0 |
| T | 0 ^d ± 0 | 0 ^d ± 0 |
| F | 73.3 ^a ± 3.33 | 74.2 ^a ± 2.20 |
| T/F | 23.3 ^c ± 3.33 | 35.8 ^c ± 2.20 |
| T + F | 50 ^b ± 5.77 | 62.5 ^b ± 1.44 |

The results are recorded as Mean of triplicates ± Standard Error (S.E) Different superscript letters refer to significant variation; with the least significant difference (LSD) at $p \leq 0.05$ using COSTAT software

Fig. 11 Root rot disease symptoms of *Z. mays* roots infected with *F. Oxysporum* (W = water, T = recombinant *Enterobacter* PE-BBR2-EGFP, F = *F. Oxysporum*) (a), Fungal shoot symptoms on *Z. mays* plants caused by *F. Oxysporum* infection (T = recombinant *Enterobacter* PE-BBR2-EGFP, F = *F. Oxysporum*, T + F = recombinant *Enterobacter* PE-BBR2-EGFP along with *F. Oxysporum* and T/F = recombinant *Enterobacter* PE-BBR2-EGFP followed by *F. Oxysporum* addition) (b)



In this study, IAA (Indole Acetic Acid) and GA₃ (Gibberellic Acid) produced by *Enterobacter* may be the basis for the promoting effects observed in maize phenotype, with and without infection, as it stimulates cell elongation and division. The ability of plants to store phytohormones and the documented role of PGPB in source sink relations might explain the extended growth promotion until the end of the experiment (Rafique et al. 2021). Additionally, siderophores produced by this bacteria not only provide iron as well as other minor elements to support growth but also control *Fusarium* wilt (Bendaha et al. 2019). Phosphate solubilization by *Enterobacter cloacae* (MT012825) allows plants to gain more phosphates, which are essential for all

metabolic activities (Day and Ludeka 1993). A considerable increase in chlorophylls and carotenoids was reported in *Enterobacter* treated plants (Fig. 4) which indicates improved photosynthetic activity. This improved pigmentation might be attributed to increased iron availability by siderophores and ammonia provided by *Enterobacter* (Mowafy et al. 2023). The promoted root growth attained by *Enterobacter* leads to better nutrition absorption, which subsequently leads to an increase in chlorophyll contents (El-Ganainy et al. 2023). Maize plants bacterized before *Fusarium* infection showed better pigmentation compared to the ones that received both treatments abruptly, indicating that prior colonization with *Enterobacter* properly enhanced growth and metabolism.

The increase in photosynthetic pigments was reported for *Enterobacter* treated rice and wheat plants (Suprapta et al. 2014; Borham et al. 2017). On the other hand, *Fusarium* is known for its devastating effect on the chlorophyll contents of infected plants (Baghbani et al. 2019).

In terms of membrane indices, the presence of *Enterobacter* considerably reinforces membrane stability and decreases membrane leakage of maize plants at different stages of growth (Fig. 5). In contrast, *Fusarium* infection had the opposite effect, which was less severe in the presence of bacteria, which confirms the ability of bacteria to reduce the fungal invasion due to its ability to stimulate the production of osmo-protectants such as proline and the ability to up-regulate the activity of antioxidant enzymes (Fig. 5). In that regard, membrane stability increased in soybean plants treated with *Pseudomonas putida* (MT604992) and *Bacillus clausii* (MT604989) as biocontrol agents against charcoal rot disease (Yasmin et al. 2020). *Enterobacter* treated plants prior to *Fusarium* infection (T/F) showed higher membrane stability compared with abruptly treated plant membranes. Reduced electrolyte leakage and increased membrane stability indices are indicative signs of induced resistance against *Fusarium* due to *Enterobacter* colonization. The same conclusion was drawn in *Arabidopsis* (Dixit et al. 2019).

Proline as a stress sign increased considerably in response to *Fusarium* infections, meanwhile; it showed mostly a nonsignificant response due to *Enterobacter* colonization (Fig. 5). *Enterobacter* treated plants showed less accumulated proline compared to *Fusarium* infected ones, which is a sign of lower stress. This result agrees with the membrane stability measures that concluded the ability of *Enterobacter* to alleviate biotic stress caused by *Fusarium*. In contrast, both *Trichoderma viride* and *Pseudomonas fluorescens* as biocontrol agents led to increased proline levels in wheat and reduced *Fusarium* infection (Rajeswari 2019). *Streptomyces* sp. S-9, as a plant growth promoter, increased proline contents of *Fusarium* (Dave and Ingle 2021).

Proline, phenols, and flavonoids accumulated considerably in *Fusarium* infected plants (without *Enterobacter*).

Meanwhile, *Enterobacter* reduced the amounts of these secondary metabolites in bacterial colonized maize plants (Fig. 5). Phenols, as environmental modulating molecules are produced by plants in response to stress (Naikoo et al. 2019). In this study, total phenols increased considerably with the fungal infection, however, the presence of *Enterobacter* significantly decreased phenol accumulation (Fig. 5). The same pattern of influence was obtained when potatoes were subjected to *Enterobacter* BC-8 (Sorokan et al. 2020). On the other hand, the observed increase in phenolic compounds of pepper in response to several PGPB indicated the activation of induced systematic resistance (ISR) via phenylpropanoid pathway elicitation (Ureche et al. 2021). It has been reported that PGPB are able to modulate phenol profiles in plants according to their types, and this might be attributed to these compounds' involvement in interactions between bacteria and plants.

Total flavonoids mostly showed a considerable increase and decrease in response to *Fusarium* and *Enterobacter*, respectively. Flavonoids are potential secondary metabolites produced in response to both biotic and abiotic stresses. These compounds are species specific and could be modulated according to microbial interactions (Wu et al. 2018). Cucumber flavonoids were significantly reduced in response to *Enterobacter*, regardless of phosphate fertilization levels (Alzate Zuluaga et al. 2021). This is probably due to their consumption as a carbon source or blocking in their synthesis.

Phenolic and flavonoid profile analysis indicated the dominance of chlorogenic acid and rutin; however, other tested phenols did not show a significant amount to detect. The amount of chlorogenic acid considerably doubled and dropped in response to *Enterobacter* and *Fusarium*; respectively (Fig. 8). The observed increase due to *Enterobacter* is a sign of immune stimulation since chlorogenate is considered a storage of caffeate and an intermediate of lignin biosynthesis (Rutherford et al. 2013). Both *Azospirillum brasilense* and *Bacillus* sp.-induced chlorogenic and rutin accumulation in neotropical trees under drought stress (Tiepo et al. 2020). Chlorogenate increase in response to *Enterobacter* indicates its involvement in the activated immune response through ROS scavenging. Chlorogenate amounts produced in maize leaves are regarded as a physiological signature for larger kernel production (Cañas et al. 2017). Chlorogenic acid is considered an important metabolite working against *Fusarium* head blight and *Gibberella* ear rot (Gauthier et al. 2016). The extension of germ tubes, secondary hyphal branching, and the radial growth of *Botrytis cinerea* were significantly inhibited by rutin (Kalinova and Radova 2009).

In this study, rutin contents of maize decreased in response to *Enterobacter*. The same pattern of influence was detected in pepper in response to PGPB (Ureche et al. 2021).

Rutin might be transformed into other undetected flavonols that would protect the plants from abiotic stress or utilized as a carbon source by PGPB during germination. On the other hand, it increased in *Stevia rebaudiana* in response to *Fusarium fujikuroi* (Devi et al. 2023).

Catalase activity increased in response to *Enterobacter* in the earlier stage of growth; however, the activity was considerably decreased in 28-day old samples, particularly for fungal treated samples. The same results were observed in chickpea in response to *Fusarium* (Gayatri Devi et al. 2012). The increase in activity indicates the activation of maize immune response due to bacterial colonization. The same result was reported in wheat under salinity stress in response to PGPB (Singh and Tiwari 2021). The activity of peroxidase and polyphenol oxidase (Fig. 6) was not significantly affected in response to the used bacterium in the earlier growth stages; however, polyphenol oxidase was elevated in 28-day old plants. In response to *Fusarium*, the activity of all enzymes mostly increased in all stages, indicating a stress response. The activity of polyphenol oxidase was reported to be high in wheat resistant strains to *Fusarium* head blight (Mohammadi and Kazemi 2002).

Anthocyanin production was considerable in response to *Fusarium* infection; however, the presence of *Enterobacter* significantly reduced its amount even in presence of the fungi (Fig. 7). This result indicates that the presence of *Enterobacter* alleviated *Fusarium* stress on plants. The production of these water-soluble pigments is mostly correlated with biotic and abiotic stress. Resistance to *Erwinia* in the engineered potato tubers was found to be correlated with anthocyanin levels (Lorenc-Kukuła et al. 2005). The infection with *Fusarium circinatum* has been shown to increase anthocyanin contents (Cerqueira et al. 2017).

Plant immunity heavily relies on the phytohormones (SA) and (JA). According to numerous studies, SA- and JA-mediated signals interact with one another (SA-JA crosstalk) to coordinate plant immune responses against pathogens. Pathogens frequently take advantage of SA-JA crosstalk in order to increase their pathogenicity (Hou and Tsuda 2022). ISR is a strategy by which PGPB that are beneficial to plants develop their immunity. This immunity can promote crop growth and resistance to a variety of parasites, insects, and phytopathogens. These PGPB enhance plant performance by controlling hormone signaling, such as the SA, JA, prosystemin, PR1, and ET pathways. These pathways then induce the gene expression of ISR, the synthesis of secondary metabolites, various enzymes, and volatile compounds, which in turn trigger the plant's defense mechanism (Rabari et al. 2022). In *Brassica napus*, all the afro mentioned hormones signaling pathway relevant genes were up-regulated due to bacterization with *Pseudomonas aeruginosa*. (Cook et al. 2021). Although ISR by beneficial microbes is often regulated through SA-independent

mechanisms, several PGPB have been reported to trigger an SA-dependent type of ISR that resembles pathogen induced SAR (Zhou et al. 2022). In our study, it became evident that *Enterobacter* stimulates *Z. mays* innate immunity against *Fusarium oxysporum*. The amount of salicylic acid considerably increased in *Enterobacter* treated plants (Fig. 9), an effect that was accompanied by an increase in the expression level of pathogen-related gene (PR1). This result indicates the stimulation of *Z. mays* systemic acquired resistance (SAR), which was apparent in lowering the disease severity of T/F plants. Although SA amount increased significantly in *Fusarium* treated plants, PR1 expression levels were almost not affected, indicating disease's considerable suitability in absence of *Enterobacter*. Meanwhile, *Enterobacter* treated plants showed a considerable increase in PR1 expression levels. The same results were reported when *Z. mays* were treated with *Bacillus* lipopeptides (Gond et al. 2015) and *Arabidopsis* colonized with *Enterobacter* (Brock et al. 2013). It is evident from the present data that the accumulation of SA along with the up-regulated PR1 gene in T/F treated plants was the reason for the remarkable improvement of plant systematic acquired resistance (SAR) that could be motivated by pathogenic and non-pathogenic microbes (Choudhary et al. 2007) in a SA-dependent manner against fungal infection. Similar results were obtained in case of *Paenibacillus alvei* K165 which was able to protect *Arabidopsis thaliana* from *Verticillium dahlia* through the activation of SA-dependent ISR (Tjamos et al. 2005). Similarly, *P. fluorescens* SS101-enhanced *Arabidopsis thaliana* resistance against several bacterial pathogens based on SA-dependent resistance (van de Mortel et al. 2012). Recently, it has been found that both *Enterobacter cloacae* and *Trichoderma asperellum* stimulated potato-induced systematic resistance via the increase in SA contents, leading to *Ralstonia solanacearum* resistance (Mohamed et al. 2020). Regardless of the bacterial strain employed, the endogenous level of SA and JA in *Mentha piperita* increased 4–5 and twofold, respectively, compared to non-inoculated ones (del Rosario et al. 2019). However, soybean inoculation with *B. cereus* SA1 showed no significant difference in the endogenous ABA and SA content compared with control plants (Khan et al. 2020). The *Enterobacter* strain used in our study seems to colonize the root cortex of maize as indicated by the intensive fluorescence of this region. Probably the siderophores and/or lipopolysaccharides of this bacteria modulate SAR, leading to elevated levels of SA as well as an up-regulated PR1. The elevated chlorogenate as well as PPO activity and the decrease in anthocyanin contents are all responses to *Enterobacter* immune response effect, that led to a decrease in disease severity.

On the other hand, jasmonic acid did not show a significant change in response to *Fusarium*, but *Enterobacter* treatments showed higher levels of JA. This result indicates

the stimulation of SAR due to the bacterial treatment, although the expression level of coronatine-insensitive protein1 (COI1) was not considerably increased (Fig. 9). The COI1 gene plays a critical role in regulating plant-defense responses against herbivores and pathogens. COI1 is a key component of the JA signaling pathway, as it acts as a receptor for JA and is involved in the degradation of JAZ proteins, which are negative regulators of the JA pathway. By degrading JAZ proteins, COI1 allows for the activation of JA-responsive genes, leading to the production of defensive compounds such as protease inhibitors, phytoalexins, and volatile organic compounds (Ruan et al. 2019; Chini et al. 2009). It has been found that *Bacillus cereus* AR156 promoted the growth and immunity in *Arabidopsis thaliana* by increase in JA levels (Niu et al. 2011). It was concluded that, PGPB increase JA levels but did not result in a significant increase in the expression of JA-responsive genes, suggesting that PGPB-mediated JA signaling may involve post-transcriptional regulation (Pieterse et al. 2014). Recently, it was reported the expression of COI1 responsive gene was upregulated in pepper plants treated with *Bacillus butanolivorans* (Kim et al. 2022). *Klebsiella oxytoca* treated seedlings of potato and tobacco exhibited a significant reduction in *Potato Virus Y* (PVY) symptoms (77.3 and 59.9%, respectively) accompanied with high levels of PR1 and COI1 expression (Elsharkawy et al. 2022).

Since the beneficial microorganisms are initially perceived as plant invaders, tomato plants treated with *Pseudomonas chlororaphis* showed a 15.22-fold increase in PR-1a gene expression when compared to the control. However, when the pathogen was challenged, the pre-inoculated tomato roots showed a 38.53-fold increase, indicating the induction of systemic resistance in tomatoes and confirming our findings (Kamou et al. 2020).

Conclusion

The used *Enterobacter* strain was able to colonize root tissues of maize and induce maize growth as well as SA-dependent SAR, which allowed pre-bacterial colonized plants to resist *Fusarium* infection as the disease severity reduced to a marked level. Further studies are required to elucidate and dissect the detailed mechanism by which *Enterobacter* induced the defense against *F. oxysporum* to pave the way for using this strain as a biocontrol agent against these fungi and probably other pathogens.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00344-024-11280-4>.

Author Contributions Asmaa A. Sallam was the MSc student who did all the work, collected the data, analyzed, and represented it. Samia A.

Haroun was the main supervisor of Asmaa's thesis. She participated in planning the idea and writing the MS. Elhussiny A. Aboulnaga provides the plasmid used in this study in addition to its contribution in planning the idea, and writing the MS. Amr M. Mowafy participated in planning the idea and he was the day-by-day supervisor for this work in addition to writing the MS and representing the data.

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Declarations

Conflict of interest The authors declare that there is no interest conflict.

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