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Rhizobacterial-induced defense genes and their role in tomato systemic resistance against wilt disease

Yomna Y. Ismail[1*], Nasr F. Nasr[2], Ahmed ElFatih A. ElDoliefy[3], Mohamed A. Ali[2], Manal F. Abdelall[4]

[1] *School of Biotechnology, Nile University, Giza 12588, Egypt.*
[2] *Agricultural Microbiology Department, Faculty of Agriculture, Cairo University, Giza 2613, Egypt.*
[3] *The Laboratory of Applied Plant Breeding and Microbial Biotechnology (APBMB), Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt.*
[4] *Microbial Molecular Biology Department, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt.*

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ABSTRACT

Tomato is a strategic crop grown in Egypt with unfortunate sensitivity to wilt diseases causing tons and millions of losses. Plant Growth-Promoting Rhizobacteria (PGPR) is a suitable bioagent to activate systemic resistance, improve plant health, and replace expensive chemical pesticides. Therefore, the current study investigated the potential antagonistic activity of four PGPR strains [*Pseudomonas alcaligenes* (denoted as YMB9), *Bacillus subtilis* (YMB8), *Pseudomonas indica* (YMB4), and *Bacillus licheniformis* (YMB3)] against two tomato wilt causing pathogens [*Fusarium oxysporum* and *Ralstonia solanacearum*]. Abilities to produce inhibitory metabolites [Siderophores and Hydrogen Cyanide (HCN)], plant growth hormones [Indole-3-Acetic Acid (IAA)] and solubilize soil phosphate were approved by molecular and microbiological approaches to all four strains. After 24- and 48-hours post-infection, significant changes at the level of gene expression were revealed using the semi-quantitative real-time PCR associated with the three-tomato hormonal, signaling, and defense mechanisms. Compared to the untreated plants, the YMB9 strain has differentially modulated the three genes [pathogenesis-related protein 1 (*pr.1a;* $p \leq 0.05$), protease inhibitor II (*pin2;* $p \leq 0.001$), and osmotin-like (*osm;* $p \leq 0.001$)] as indicators to the three signaling pathways of salicylic and jasmonic acids and ethylene; respectively. Supportively, the scanning electron microscopy revealed robust adherence of the rhizobacterial cells to the tomato roots causing the activation of tomato systemic resistance against the two tomato wilt pathogens. This proves the bioagent role of rhizobacteria in enhancing crop resilience and clean sustainable agriculture.

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***Corresponding author Email address*: yyoussef@nu.edu.eg (Yomna Y. Ismail)**

Introduction

Tomato is one of the most popular crops in the world, however, it is susceptible to wilt-causing pathogens that reduce productivity in many countries (Liu& Khan 2021). Ralstonia solanacearum is a bacterial causative agent to wilt disease, that significantly decreases yield and quality of tomato crop from 50 to 100% (Chen et al 2022). Another wilt-causing agent is the soil-borne pathogen Fusarium oxysporum that doubling the significance of wilting in developing countries like Egypt (Abdelaziz et al. 2022).

There is no effective management technique to control wilting disease due to the great variability, environmental survival ability, and wide host range of these pathogens. However, some field practices were found necessary in mitigating negative effects on plants including plant breeding, field sanitation, crop rotation, and bactericides (Farahat et al. 2017). This urges scientists to search outside the regularly used strategies for more effective management. Among these strategies were the alteration of soil microbiota and the application of biological control agents (Deng et al 2021). Additionally, scientists used microbial-induced plant immune system that triggers certain receptors in the primary defense system called pattern-triggered immunity (PTI). If a pathogen succeeded in avoiding the PTI, plants then use the effector-triggered immunity (ETI) as a second system of defense. The ETI recognizes pathogen effector molecules and triggers an intense immune response to stop the infection (Yu et al. 2022).

In agriculture, the conventional practices may negatively impact the soil quality and determine the potential role of Plant Growth-Promoting Rhizobacteria (PGPR) on improving plant nutrient uptake, pathogens suppressions, growth stimulation, and enhance production (Ouf et al. 2023). In sustainable agriculture, PGPR can substitute chemical pesticides. Extendedly, these beneficial bacteria induce plant systemic resistance to a variety of diseases and improve plant development in a comparable manner to the plant's own systemic acquired resistance (SAR). In plants, the phytohormone salicylic acid (SA) is triggering the SAR mechanism through the molecular signaling pathway characterized by elevated levels of pathogen-related (PR) proteins (Ismail et al. 2021). Additional triggering pathways included the jasmonic acid (JA)-dependent and the ethylene (ET)-dependent that were mediating the plant resistance in taxa with antagonistic effects against pathogens, such as Bacillus and Pseudomonas (Beneduzi et al. 2012). Through biological processes like phosphate solubilization and nitrogen fixation, PGPR boosts nutritional availability, synthesizes essential phytohormones like ET, JA, gibberellic acid, indole-3 acetic acid, and cytokinins, providing healthier and productive plants (El-Saadony et al. 2022). Furthermore, there were tremendous research efforts to understand how biocontrol agents colonize and adhere to plant roots and seeds. These research studies were successful due to the significant documentation highlighting the critical role of multitrophic interactions in many biological control organisms for soil-borne pathogens (Pliego et al. 2019).

In the current study, a dual application of local PGPR and infectious pathogens (such as R. solanacearum and F. oxysporum) on tomato was examined. Afterwards, the differential expression of some genes [glucanase A (gluA), pathogenesis-related protein 1 (pr-1a), lipoxygenase A (loxA), protease inhibitor II (pin2), osmotin-like (osm), and pr-1b)] related to SA, JA, and ET of immune response signaling pathways was assayed followed by fold-change quantification using the real-time PCR. The current four rhizobacterial isolates (YMB9, YMB8, YMB4, andYMB3) were proven to have antagonistic activity against the two soil-born and wilt-causing pathogens (R. solanacearum and F. oxysporum). In addition, the strain YMB9 has physically colonized the roots as visualized by the electron-scanning microscopy then induced the tomato systemic resistance as molecularly approved.

Materials and Methods

Isolation of the biocontrol rhizobacteria (BR)

Ten soil samples were collected from the rhizosphere of healthy tomato plants. Plants from the Agriculture Genetic Engineering Research Institute (AGERI) and the Faculty of Agriculture greenhouse at Cairo University (FACU) were harvested during the summer season (temperature ~ 41˚C). Each 2 g soil sample was placed in a stomacher bag containing 20 ml of sterile saline solution (0.85% NaCl, Sigma-Aldrich, USA) and shaken for two minutes at 100 rpm. The suspensions were left to settle for 15 minutes, then ten-fold serial dilutions (up to 10^{-5}) were prepared using the same saline solution. Of each dilution, 100 µl were spread on Luria-Bertani (LB) Agar plates, Miller, USA) (Luria & Burrous, 1957) then incubate at 30˚C for 24 h. For each soil sample, ten bacterial colonies with different morphological features were purified and reserved in 20% glycerol LB broth cultures in -20˚C (Fan et al 2018).

In vitro antagonistic activity against two soil-borne pathogens

In vitro antagonistic effect investigation of one hundred BR isolates were performed on two soil-borne pathogens using two methods. The first method was the dual culture assay against *F. oxysporum* as described by (Yang et al. 2019). As indicated in (Fig.1), a fungal growth disc (1 mm) of a 7-day culture was carefully centered on the surface of a plate of Potato dextrose agar (PDA) media that was previously streaked at three edges by the BR isolate 3 cm away from the fungal disc. After seven days of incubation at 28˚C, for both the control and the dual-cultured plates, the diameter of the fungal growth was determined using regular ruler the percentage of fungal growth inhibition was calculated using the formula given by (Yang et al. 2019).

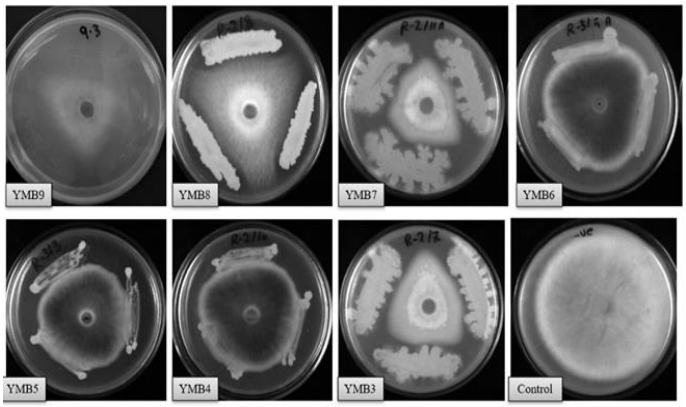

Fig 1. The antifungal activity of BR isolates against *Fusarium oxysporum*. After 7-day incubation at 28 °C, the fungal growth in the center of the plate while the BR growth streaked in the three edges of the PDA medium.

Parallelly, the second method was the plug diffusion to test the BR against the *R. solanacearum* as described by (Dogra et al 2021; Sarmiento-Vizcaino et al 2021). Briefly, each BR isolate was cultivated on LB agar media and incubated for three days at 30˚C, after which one cm-circled plugs of agar were added to LB agar plates previously inoculated with a 24-hour culture of R. solanacearum (~10^8 CFU) at a ratio of 1 ml of culture per 100 ml of melted LB agar; the inhibitory zone diameter around the plugs was then measured after 48 hours of incubation at 30˚C, as described by (Hossain 2024).

Plant Growth-Promoting (PGP) characteristics of BR isolates

The BR isolates, which showed antagonistic activity against the plant pathogens, were tested for the PGP characteristics including the ability to produce indole acetic acid (IAA), hydrogen cyanide (HCN), siderophore, and phosphate solubilization (Vaikuntapu, et al. 2014).

Molecular Characterization

DNA isolation and diversity repetitive analysis of BR isolates

The genomic DNA of the tested isolates was extracted using the QIAamp DNA mini kit (QIAGEN, Germany) from the fresh cultures of BR isolates. The diversity among the tested BR isolates was performed using the repetitive genomic PCR technique (BOX PCR) (Adiguzel et al 2010). The PCR reactions at a total volume of 25 µl have included 2.5 µl of 10x buffer, 2.5 µl of dNTPs, 0.5 µl of BoxA1R primer Table (1), 0.13 µl of DNA polymerase, 18.4 µl of H_2O, and 1 µl of DNA as a template. The PCR conditions were set using a 1st denaturation cycle at 94˚C for 7 minutes followed by 35 cycles (94˚C for 1 minute, 53˚C for 1 minute, extension at 65˚C for 8-minute), then a single final cycle at 65˚C for 16 minutes. The patterns of fragments for BR isolate were visualized using the 1.5% agarose gel electrophoresis. The pattern comparison was analyzed using the Bio-Rad Gel documentation system and pyElph 1.4 software (Pavel & Vasile 2012). The phylogenic tree was generated using the Unweighted Pair Group Method Average (UPGMA) method (Bilung et al. 2018).

Sequencing of 16s rDNA gene of BR isolates

The 16S rDNA gene was amplified and sequenced using the universal primers in Table (1) to identify the BR isolates (Wu et al 2013) using the Bio-Rad.T100.US thermal cycler and the following conditions: Denaturation cycle at 94˚C/4 min, then 35 cycles (94˚C/30 sec., 50˚C/30 sec., and 72˚C/1.30 min.), and a final extension cycle (72˚C/10 min.). The PCR product was purified using the GeneJET PCR Purification kit (Thermo Scientific, Cat. No. KO701) then sequenced through Macrogen (Seoul, Republic of Korea). Similarity hits were tested within the partial sequence of the 16S rDNA gene using the BLASTn tool published online on the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple sequence alignment (MSA) was performed using ClustalW on the highly significant hits obtained from the NCBI GenBank. Dissimilar and poorly aligned sites of obtained hits were eliminated before the phylogenetic analysis. The Jukes–Cantor (JC) model from the substitution (ML) model was used to build the Neighbor-Joining phylogenetic tree between the 16S rDNA sequences and those selected from the database. Using 1000 iterations (Idris et al. 2020), the bootstrap test and evolutionary analysis were performed using the software Molecular Evolutionary Genetics Analysis Version 11.0 (MEGA11) (Tamura et al. 2021).

Table.1: The genes name, symbol, primers, and associated purposes and citation.

Associated Purpose	Gene Name	Symbol	Primer Sequence	Reference
BOX PCR	*Box*A1	BoxA1R	5'-CTACGGCAAGGCGACGCTGACG-3'	(Adiguzel et al. 2010)
Bacterial identification	*16s rRNA* gene	27F 1492R	5-AGA GTT TGA TCM TGG CTC AG-3 5-CGG TTA CCT TGT TAC GAC TT-3	(Wu et al. 2013)
Salicylic acid-related	*Glucanase* A	*glu*A	F: TCAGCAGGGTTGCAAAATCA R:CTCTAGGTGGGTAGGTGTTGGTTAA	
	PR-1a	*pr*-1a	F:GAGGGCAGCCGTGCAA R:CACATTTTTCCACCAACACATTG	
Jasmonic acid-related	*Lipoxygenase* A	*lox*A	F:TGGTAGACCACCAACACGAA R:GACCAAAACGCTCGTCTCTC	
	Protease inhibitor II	*pin*2	F:TGATGCCAAGGCTTGTACTAGAGA R:AGCGGACTTCCTTCTGAACGT	(Baichoo &Jaufeerally-Fakim 2016)
Ethylene-related	*Osmotin-like*	*osm*	F:TGTACCACGTTTGGAGGACA R:ACCAGGGCAAGTAAATGTGC	
	PR-1b	*pr*-1b	F:TTGGTGACTGCGGGATGA R:GGCGGCGGCTAGGTTT	
Housekeeping	*GAPDH*	GAPDH	F:CTCCATCACAGCCACTCAGA R:TTCCACCTCTCCAATCCTTG	

Bacterial colonization on tomato roots

Tomato seeds (Alissa F1) were thoroughly rinsed with water followed by a sterilization and treatment process for root colonization as outlined by (Vaikuntapu et al. 2014). After 15 days, the colonization of the bacteria on the plant roots was examined using the scanning electron microscopy (Model: JEOL GM 5200) at Faculty of Agriculture, Cairo University, Egypt, as described by (Shamseldean & Platzer 1989).

Greenhouse experiment

Tomato seeds were grown in the Agricultural Genetic Engineering Research Institute (AGERI) greenhouse facility using a soil mixture of sand: clay: peat moss [1:1:1 (v/v)]. The greenhouse was set on 30°C and 16 hours of light until full germination. After about 40 days, seedlings were moved to pots of 20 cm sized diameter. After five days adaptation in the greenhouse, the experiment was started using the two soil-borne pathogens (*F. oxysporum* and *R. solanacearum*). At the level of bio-agent, four treatments were included: a) plants treated with no BR-bioagents nor pathogens (**Control**), b) plants treated with pathogens (**T1**), c) plants treated with BR-bioagents (**T2**), and d) plants treated with both BR-bioagents and pathogens (**T3**). Neither the pathogen nor the BR-bioagent was applied until after the roots were drenched, following the protocol of (Kamou et al. 2020). The BR-bioagent used was *Pseudomonas alcaligenes* (YMB9), and in the T3 treatment, the pathogen was introduced five days after the BR-bioagent application. Leaf-samples were collected for gene expression analysis after 24 and 48 hours of pathogens infection (hpi) across all treatments. Each treatment had four replicates, and the experiment followed a split-split design.

RNA extraction and cDNA synthesis

Plant leaf samples from four replicates of each treatment were harvested and combined into a single pooled sample (Kamou et al. 2020). RNA was isolated using the Gene JET Plant RNA Purification Kit (Thermo Scientific™, Lithuania, Cat. No. K0801). The Revert-Aid First Strand cDNA Synthesis Kit (Thermo Scientific™, Lithuania, Cat. No. K1622) was used to synthesis cDNA after the concentration of RNA was measured using the Thermo-Scientific Nano-Drop 2000 Spectrophotometer (Thermo Fisher Scientific – US).

Level of Gene expression of signaling pathways gene (s) and statistical analysis

The Step OnePlus Real-Time PCR System was used to assess the genes expression associated with the SA, the JA, and the ET signaling pathways in both treated and control samples according to (Baichoo & Jaufeerally-Fakim 2016). The Master Mix (2X) of Maxima SYBR Green/ROX qPCR was prepared for each primer Table (1). The cDNA was diluted (1:10 dilution) and 1µl was used from each sample as a template. The real-time PCR cycles and conditions were established following the Maxima protocol of Two-step cycling, though two technical replicates for each sample were assigned. The threshold cycles (CTs) of qPCR were used for the evaluation of the relative and quantitative expression levels. Then, the $2^{-\Delta\Delta CT}$ method was used to determine the fold-change of tested genes compared with the reference gene (GAPDH) (Livak & Schmittgen 2001). The significant differences between treatment groups and

control groups were statistically tested using Analysis of Variance (ANOVA with replications). Then, the significant treatments were further subjected to means' separation test using the Least Significant Difference (LSD $p = \leq 0.05$) and the GenStat software 17.1.(2014) (Mourtala et al. 2023).

Results

In vitro antagonistic activity of the isolated biocontrol rhizobacteria (BR)

Seven out of one hundred bacterial isolates from the tomato plant rhizosphere showed inhibitory effects against *Fusarium oxysporum* (Fig.1). The three BR isolates YMB4, YMB5, and YMB6 showed antagonistic effect by reducing the growth of *F. oxysporum* by 38% (50 mm), compared to the control (80 mm). Although YMB8 reduced fungal growth by 50% (40 mm), YMB9 reduced it by 43% (46 mm). Surprisingly, isolates YMB3 and YMB7 showed the most potent antagonistic effects, reducing fungal growth by 60% and 55%, respectively (32 mm and 36 mm). Moreover, *F. oxysporum* (46 mm) and *Ralstonia solanacearum* (10 mm) were both significantly inhibited by YMB9.

Plant Growth-Promoting (PGP) characteristics of BR isolates

The seven BR bacterial isolates that were evaluated and proved to be effective bio-control agents against investigated soil-borne diseases were also able to solubilize phosphate and produce siderophores, HCN, and IAA. The strains' IAA production levels ranged from 3 to 51 mg/ml when measured against a standard IAA curve. Every isolate exhibited favorable PGPR traits. IAA was, however, produced in varying amounts by each isolate; isolate YMB3 yielded the least (3 mg/ml), while isolate YMB4 produced a significantly greater amount (51 mg/ml). Moreover, YMB9 isolate produced 42 mg/ml of IAA, while isolates YMB8 and YMB7 produced 5 mg/ml and 8 mg/ml, respectively.

Molecular Characterization

Repetitive Genomic PCR of BR isolates

The pattern analysis of DNA Box-PCR fragments performed by the cluster analysis revealed the genetic variability among the BR bacterial isolates (Fig. 2). The total number of fragments for all isolates ranged from eight to twelve and their sizes ranged from 100 to 10,000 bp. The three isolates YMB4, YMB5, and YMB6 shared an identical pattern of DNA fragments (8-framents) and were presented as one cluster (Fig.2.A). The remaining four isolates (YMB3, YMB7, YMB8 and YMB9) each exhibited a unique pattern of DNA fragments (Fig.2. B).

Sequencing of 16S rDNA gene for BR isolates identification

Upon aligning the 16S rDNA sequences of the BR isolates that showed unique genomic profiles against the NCBI database, two isolates belonged to *Bacillaceae* family, while the other isolates belonged to the *Pseudomonadaceae* family. The four isolates (YMB3 YMB4, YMB8, and YMB9) showed high percentages of similarities (99%) with *B. licheniformis* (Accession number: OQ119577), *P. indica* (Accession number: OQ119578), *B. subtilis* (Accession number: OQ119579), and *P. alcaligenes* (Accession number: OQ119580), the phylogenetic tree is presented in (Fig.3).

BR isolates colonization on tomato roots

The ability of BR bacterial cells to colonize tomato roots was examined using the SEM. The surface of tomato roots had effectively adhered to the *B. licheniformis* (YMB3) cells, *P. indica* (YMB4), *P. alcaligenes* (YMB9) as presented in (Fig.4).

Gene expression and statistical analysis

The expression level of the genes (*glu*A, *pr*-1a, *lox*A, *pin*2, *osm*, and *pr*-1b) related to the hormonal immune response in tomato was measured. (Baichoo & Jaufeerally-Fakim 2016). Following the measurements, the fold change was calculated by comparing the expression levels of each investigated gene to those of the reference gene (GAPDH) and presented in (Fig.5).

After 24 hours of microbial treatment in comparison to the untreated group (Control) (Fig.5a), the *pr*.1a gene (*p*-value = 0.050) changed significantly by starting 3-folds increase in T1, 5-folds increase in T2, then 0.5-folds decrease in T3. The *pin2* gene (*p*-value = 0.001) displayed a different pattern. It slightly changed to 0.5-folds in T1, strongly increased to 31-folds in T2, and then slightly increased to 0.2-folds in T3. The *pr*.1b gene (*p*-value = 0.05) has changed from 0.9-folds of increase in T1 to 2-folds increase in T2, but then it rapidly has fallen to 0.05-folds in T3. In (Fig.5b), the *pr*.1a gene (*p*-value = 0.050) has changed little by 0.5-folds in T1, then increased to 5-folds in T2, then highly increased to 42-folds in T3. The *pin*2 gene (*p*-value = 0.001) was significantly greater, though, started at 1-fold in T1, then impressively rose to 31-folds in T2, and fallen back to 6-folds in T3. The *osm* gene (*p*-value = 0.001) showed a progressive increase from a mild 0.3-folds in T1 to 3-folds in T2, and a high 10-folds increase in T3.

After 48 hpi (Fig.5c), the significantly changed genes included the *pr*-1a gene (*p*-value = 0.050) that decreased to 0.7-folds in T1, 11-folds in T2, and 0.2-folds in T3. The *pin*2 gene (*p*-value = 0.001) decreased to 0.2-folds in T1,

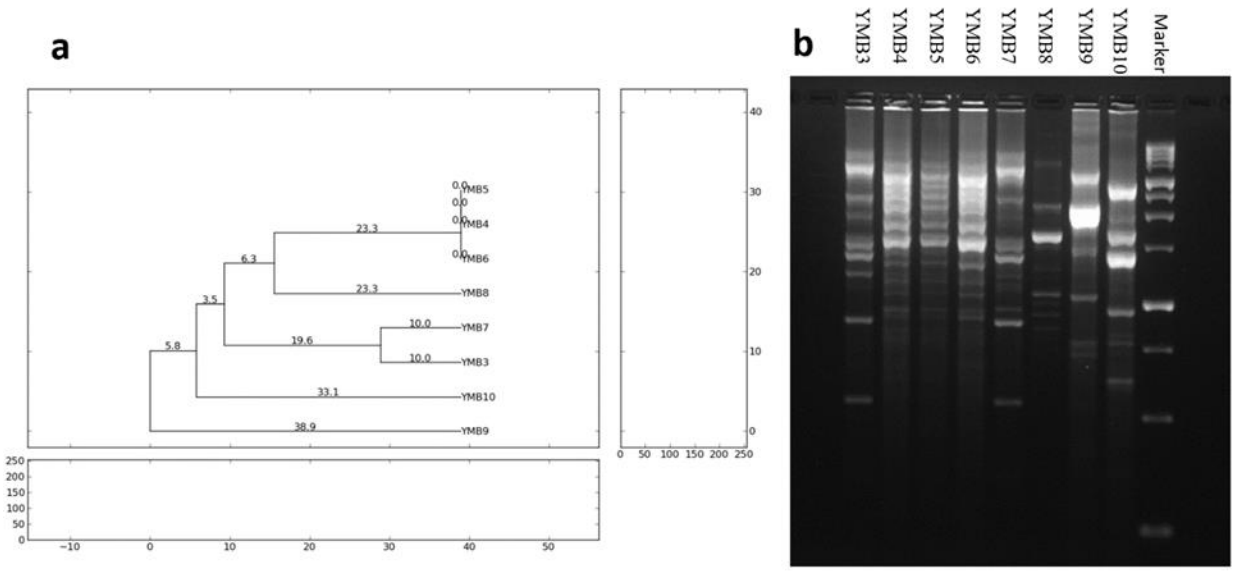

Fig. 2. Genetic diversity of BR isolates. A) Dendrogram created by pyElph 1.4 software using UPGMA clustering method obtained by BOX-PCR DNA fragments of Seven isolates study. Numbers are displayed on the branches to represent the genetic distances. B) Each isolate's DNA fragment pattern on a 1.5 % agarose gel after running at 50 volts for 4 hours, with a 1 kb DNA molecular marker in the last lane and ordered from YMB3 to YMB9.

11-folds in T2, and 0.2-folds in T3. The *pr-1b* gene (*p*-value = 0.05) was reduced to 0.03-folds in T1, 5-folds in T2, and 0.03-folds in T3. However, in (Fig.5d), the *pr*-1a gene (*p*-value = 0.050) was changed to 0.4-folds in T1, 11-folds in T2, and 2-folds in T3. The *pin*2 gene (*p*-value = 0.001) was changed to 4-folds in T1, 11-folds in T2, and 5-folds in T3. The *osm* gene was changed to 0.3-folds in T1, 8-folds in T2, and 5-folds in T3. Moreover, the treatments that caused these significant changes were identified in these genes as follows: in *pr*-1a gene (LSD= 1.16) the two treatments that caused significant differences were T1 and T2 (LSD= 72.3) and T1 and T3 (LSD= 80.5). In *pin2* gene (LSD= 0.318) the two treatments that showed significant variation were the control and T1 (LSD= 65.2), the control and T2 (LSD= 59.4), and the control and T3 (LSD= 77.6). Additionally, there were significant variations in *pr-1b* gene (LSD= 0.81) between the two treatments of control and T3 (LSD= 96), and between T2 and T3 (LSD= 95.5).

Discussion

There is an intense need to use microorganisms as natural alternatives to chemical products in agriculture as a result of their capacity to function as biofertilizers and biopesticides (Compant et al. 2019). This research focused on the beneficial application of BR strains [, *Bacillus licheniformis* (YMB3), *Pseudomonas indica* (YMB4), *Bacillus subtilis* (YMB8), and *Pseudomonas alcaligenes* (YMB9)] with proven capabilities as bioagents controlling tomato wilting pathogens (*F. oxysporum and R. solanacearum*). All isolates were collected from the rhizosphere of tomato plants. These BR
strains exhibited significant antagonistic activity against the tested soil-borne pathogens (*F. oxysporum* and *R. solanacearum*) reducing the fungal growth of *F. oxysporum* by 38% to 60%. This result is consistent with the reduced ratio against *F. oxysporum* reported by (BiBi et al 2023) that ranged from 27.66% to 49.06%.

Pseudomonas alcaligenes is known for its bioremediation capabilities, such as degrading heavy metals (such as lead) from industrial wastewater (Vélez et al. 2021). In the present study, the *Pseudomonas alcaligenes* (YMB9) strain showed antibacterial activity that inhibited the growth of *R. solanacearum* in the 10 mm-diameter zone. In addition, the antifungal activity against *F. oxysporum*. This is consistent with previous reports, (Akhtar et al. 2010) which also highlighted the antifungal activity of *P. alcaligenes* against *F. oxysporum*.

According to (Singh et al. 2022), siderophores, HCN, and IAA are essential substances for promoting plant growth and inhibiting the growth of plant pathogen. Additionally, (Vejan et al. 2016; Backer et al. 2018) suggest that isolates capable of phosphate solubilization can enhance nutrient uptake in plants. In light of these studies, the PGP characteristics of BR strains revealed that the selected isolates produced siderophores, HCN, and IAA, demonstrating their potential for use in sustainable agricultural practices. The three strains (YMB3, YMB4

and YMB9) showed strong capability to colonize and adhere to the tomato roots (Alissa F1). This finding was supported by (Wang et al. 2021; Bloemberg & Lugtenberg 2001) who stated that the effective biocontrol bacteria must first succeeded to colonize the root in the application process.

In Plants, systemic resistance (SR) response can either be induced by the secondary metabolites or the flagellin produced by the *Pseudomonas* spp. (Xing et al. 2020). Our investigation focused on activating the SR response in tomato by utilizing the Biocontrol rhizobacterial (BR) strain *P. alcaligenes* (YMB9), known for its metabolite production.

Fig 3. A phylogenetic tree based on 16S rRNA gene sequence constructed with MEGA11 software using neighbor-joining method. This tree illustrates the link between the four strains under study shown in black points and their representative species from the NCBI database.

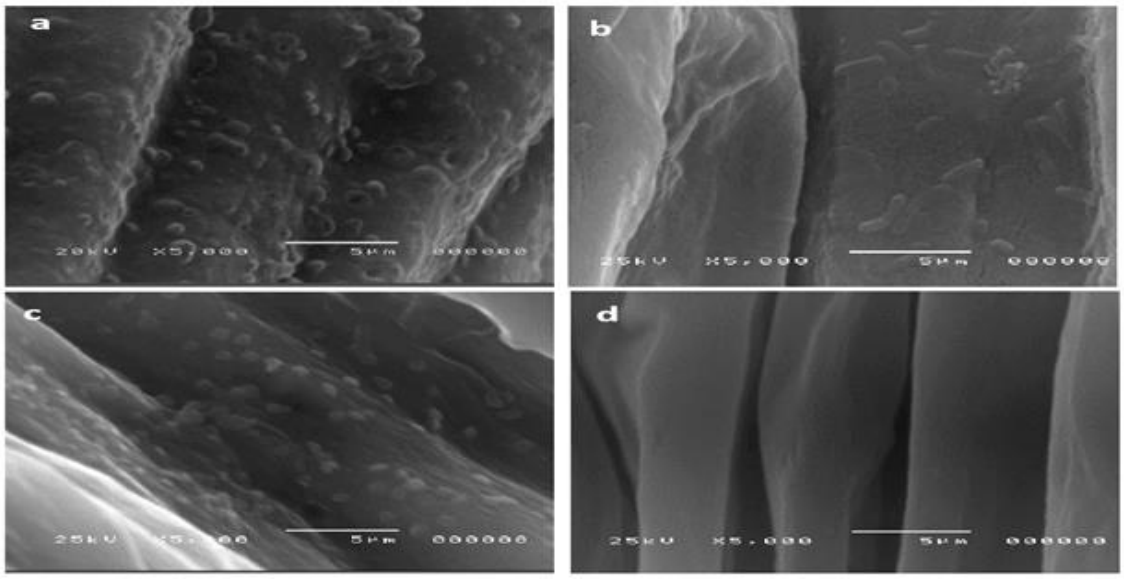

Fig 4. Scanning electron microscope images shows the surface of tomato roots colonized with BR isolates at a magnification of (X5,000). a) tomato root inoculated with *Bacillus licheniformis* (YMB3). b) tomato root inoculated with *Pseudomonas indica* (YMB4). c) tomato root inoculated with *Pseudomonas alcaligenes* (YMB9). d) Uninoculated tomato root (Control).

Through RT-PCR analysis of six genes related to the defense response and SA pathway (*pr.1a*), the JA-related gene (*pin2*), and the Eth-related gene (*osm*) were significantly upregulated in plants treated only with the YMB9 strain after 24 and 48 hpi. These results align with the highlights of (Vryzas 2016), who found that the activation of the internal molecules of SA, JA and Eth were essential for triggering the SR response in plants, which subsequently activates the expression level of PR genes. Concurrently, the high significant increase in the expression of *pin2* gene of JA to 31-folds compared to the group of untreated plants was observed after 24 hpi. The aggregation of proteinase inhibitors (*pin1* and *pin2* genes) in plants was important to activate strong defense system and decrease the pathogenicity of bacteria, fungi, and insects as described by (Turra & Lorito 2011). Consequently, this suggests that the *Pseudomonas alcaligenes* (YMB9) strain successfully activated the SR response in tomato against the tested wilting pathogens.

In comparison to the untreated tomatoes and the infected control, the tomato group infected with *F. oxysporum* showed a 42-fold increase in the expression of the *pr.1a* gene of SA after 24 hours post-infection, after five days of *Pseudomonas alcaligenes* (YMB9) colonization on roots. Furthermore, in comparison to the infected control, the expression of the *pin2* gene of JA and the *osm* gene of Eth was raised after 48 hours of propagation, however the expression of the *pr.1a* gene of SA was reduced to two times. This supports the findings of (Di et al. 2017) in plants activated the SA signalling pathway followed by the JA and Eth in response to hemibiotrophic pathogen.

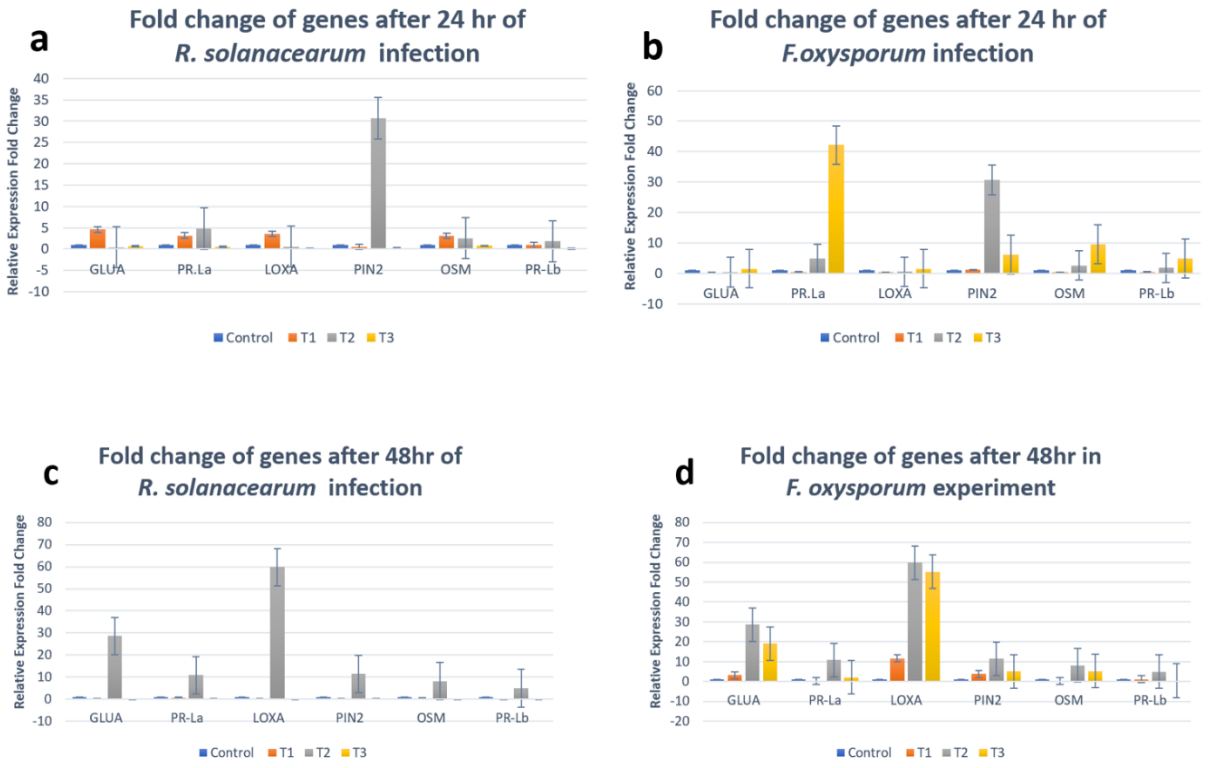

Fig 5. Gene expression relative to the control in all treatments. The fold changes in gene expression level after 24 h of a) *R. solanacearum* infection. b) *F. oxysporum* infection. The fold changes in gene expression level after 48h of c) *R. solanacearum* infection. d) *F. oxysporum* infection. The error bars were estimated at p≤0.05. Genes name is glucanase A (*glu*A), Pathogenesis-Related Protein 1 (*pr*-1a), lipoxygenase A (*lox*A), protease inhibitor II (*pin*2), osmotin-like (*osm*) and Pathogenesis-Related Protein 1 (*pr*-1b).

In the tomato group treated with the *R. solanacearum*, the expression of *pr.1a* gene of SA was upregulated to 3-folds after 24 hpi, but strongly decreased to 0.7-folds after 48 hpi. The other genes related to the hormonal immune response were strongly downregulated in all tomato groups after 48 hpi. The early activation of SA genes is likely due to SA's role in the rapid activation of defense responses, and for the induction of local defenses that contain the

growth of virulent pathogens. However, the reduction in the expression of SA genes after 48 hpi affected the induction of the genes related to JA and Eth pathways, as the SA is crucial for inducing the molecules involved in the plant immune response (Baichoo & Jaufeerally-Fakim 2016). These results suggest that the tomato (Alissa F1) was susceptible to *R. solanacearum,* even in the presence of the *Pseudomonas alcaligenes* (YMB9) strain.

Conclusion

Our investigation identified four potential biocontrol rhizobacteria that significant plant growth-promoting activities, among which *Pseudomonas alcaligenes* (YMB9) demonstrated remarkable potential in promoting tomato plants resistance to wilt-causing pathogens *Fusarium oxysporum* and *Ralstonia solanacearum*. The efficacy of YMB9 in pathogen suppression was corroborated by Scanning Electron Microscopy (SEM), which confirmed effective root colonization, and RT-PCR assays, which revealed the activation of defense-related genes of SA, JA, and Eth. Looking forward, it is crucial to scale up the cultivation of the YMB9 isolate and assess its effectiveness across a broader range of crop species. Comprehensive field trials are necessary to evaluate its long-term efficacy and economic viability in various agricultural settings. Future research should prioritize the development of stable formulations of YMB9 for field application and investigate the synergistic potential of combining YMB9 with other biological control agents. Such efforts will be essential in advancing biocontrol strategies and optimizing sustainable agricultural practices.

The significant differences between treatment groups and the control groups were statistically tested using Analysis of Variance (ANOVA with replications). Then, the significant treatments were further subjected to means' separation test using the Least Significant Difference (LSD $p = \leq 0.05$) and the GenStat software 17.1.(2014). Four replicates of each treatment were used within a split/split design.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose and have no competing interests.

Author Contributions

The research was conceptualized by **MA**, **MFA** and **YI**. **YI** wrote the original draft, with all versions reviewed, edited, and validated by **MA**, **MFA**, **YI**, and **NN**. Data collection and main analysis were conducted by **YI** and **MFA**, while software analysis was performed by **AE**. Formal analysis involved **MFA**, **AE**, **MA**, **NN** and **YI**. Data curation and statistical analysis were handled by **AE**. All authors approved the final manuscript.

Data Availability

The datasets analyzed or generated during the current study are available from the corresponding author upon reasonable request."

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