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Alcaligenes faecalis—a maize endophytic bacterium to reduce Stewart wilt disease

Syahri[a,*,†], Giyanto[b,†], Kikin Hamzah Mutaqin[b,†]

[a] *Research Center for Food Crops, National Research and Innovation Agency (BRIN), Cibinong Science Center, Kabupaten Bogor, Jawa Barat 16911, Indonesia*

[b] *Department of Plant Protection, Faculty of Agriculture, IPB University, Dramaga, Bogor, Jawa Barat 16680, Indonesia*

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Abstract

Importance of the work: *Alcaligenes faecalis* can effectively reduce Stewart wilt disease, one of the most important maize diseases.

Objectives: To evaluate the effect of *A. faecalis* on the inhibition of Stewart wilt disease and growth enhancement of maize.

Materials and Methods: Three potential isolates of *A. faecalis* (JCB-1, JCJ-7 and MS-X) were used to decrease the disease. The antagonistic activity, volatile organic compounds (VOCs) and growth-promoting activities were evaluated *in vitro*. The effectiveness of endophytes for stimulating maize growth was assessed using a seed germination test, while their ability to reduce the disease was tested on seedlings. The responsiveness of plant defense was detected by containing phenylalanine ammonia-lyase and polyphenol oxidase (PPO) in seedlings. In addition, the chemical compounds of the bacterial endophytes were identified using gas chromatography-mass spectrometry.

Results: All the tested endophytic isolates could fix nitrogen, solubilize phosphate and produce indole. The *A. faecalis* MS-X isolate had the highest VOC activity (78.94%) and efficacy in reducing disease severity (52.4%), while the most significant antibiosis activity was produced by the isolate JCJ-7 (100%). In addition, the isolate MS-X had the best capacity to induce PPO, along with excellent ability to reduce pathogen infection and growth promoters, while JCB-1 effectively enhanced the germination of maize.

Main finding: *A. faecalis* had the potential to reduce Stewart wilt diseases by both induction resistance and enhancing maize growth.

† Equal contribution.
* Corresponding author.
E-mail address: syah026@brin.go.id

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Introduction

The bacteria *Pantoea stewartii* subsp. *stewartii* Smith causes Stewart's wilt disease (Rahma et al., 2013; Harahap, 2016) that can infect various stages and types of maize (Lipps et al., 2003) and cause yield losses of up to 0.8% for every 1% of disease incidence in seedlings (Freeman and Pataky, 2001). The disease has spread widely and has been reported in Europe (except Austria, Greece, Poland, Romania, Russia), Asia, North America (Canada, Mexico, USA), Central America (Costa Rica, Puerto Rico) and South America (Brazil, Guyana, Peru) (European and Mediterranean Plant Protection Organization, 2016; CABI Compendium, 2020). Nalis et al. (2015) combined 100 parts per million (ppm) streptomycin sulfate before dry heat treatment at 55°C for 24 hr to eliminate bacteria in seeds. However, physical and chemical seed treatment is ineffective because pathogens exist in the chalazal tissue, between the chalazal tissue and the endosperm and in the endosperm (European and Mediterranean Plant Protection Organization, 2016). Furthermore, some countries, including Indonesia, have prohibited using pesticides containing antibiotics in agriculture (Regulation of the Minister of Agriculture of the Republic of Indonesia, 2019). Increasing plant resistance through endophytic bacteria is an alternative to decrease disease development (van Loon et al., 1998). Rahma et al. (2014) reported the capacity of endophytes to reduce Stewart's wilt by 49 -56% reductions in disease severity.

Endophytic bacteria are known to induce resistance in various plants, such as rice (Leiwakabessy et al., 2018; Mao et al., 2019), maize (Thanh and Cao, 2014; Santos et al., 2018; Abedinzadeh et al., 2019), eggplant (Saridewi et al., 2020), tomato (Purwanti et al., 2019) and patchouli (Harni et al., 2012). Endophytic bacteria can colonize internal tissues, intercellular spaces and plant vascular tissue without causing negative impacts on plants (Thanh and Cao, 2014; Santos et al., 2018; Abedinzadeh et al., 2019). Induction of resistance occurs through increases in salicylic acid, peroxidases, phytoalexins, pathogenesis-related proteins (PRs) and plant phenolic compounds (Harni et al., 2012; Dalal and Kulkarni, 2015; Bodhankar et al., 2017; Abedinzadeh et al., 2019; Mao et al., 2019).

Alcaligenes faecalis, an endophytic bacterium species, is generally found in water, soil, and plant environments. This bacterium has been reported as an endophyte in rice roots (You et al., 1991), *Mimosa calodendron* (Felestrino et al., 2017), *Coleus forskohlii* (Mastan et al., 2020), *Ricinus communis* (Trivedi et al., 2020) and *Abelmoschus esculentus* (Ray et al., 2020). *A. faecalis* has been reported to induce plant resistance. For example, the interaction of *A. faecalis* as a maize root endophytic bacterium has been reported by Rahma (2013); however, there has been no comprehensive information regarding its ability. It has been used to control damping-off disease by *Sclerotium rolfsii* on okra (Ray et al., 2020), brown rot disease by *Monilinia fructigena* (Lyousfi et al., 2021), *Agrobacterium tumefaciens* on tomatoes (Sanchez-Jimenez et al., 2022), clubroot disease in cabbage (Jia et al., 2022) and *Fusarium oxysporum* and *Macrophomina phaseolina* (Chowhan et al., 2023). The degradation of phenolic compounds (Rashid et al., 2022), the production of enzymes that degrade organic pollutants and the production of R-(-)-mandelic acid are also essential precursor for various drugs (Ju et al., 2016). In addition, this bacterium has been reported to increase seed germination and growth (Felestrino et al., 2017; Latef et al., 2021; Jia et al., 2022). Therefore, the current study investigated the enzymatic activity of *A. faecalis* with maize roots, its ability to control disease and enhance growth and its capacity to produce important secondary metabolites.

Materials and Methods

Bacterial pathogen preparation

The pathogenic bacteria used in this study were prepared by Syahri et al. (2023). *Pantoea stewartii* (accession number NR_044800.1) was isolated from an infected plant in the West Java region, Indonesia and it was prepared according to the procedures of Desi and Novia (2017). Colony morphological characterization followed the procedures of Desi et al. (2014), while the physiological or biochemical followed the protocol of Schaad et al. (2001). The bacterial inoculum was cultured in a nutrient broth (NB) medium (Rahma et al., 2013). Hypersensitivity and pathogenicity assays were performed to confirm the pathogen isolate.

Preparation of endophytic bacteria Alcaligenes faecalis

A. faecalis isolates were prepared according to Syahri et al. (2023) and the protocol of Thanh and Cao, (2014). The samples were isolated from healthy maize roots. Around 1 g of roots were cleaned with running water, then sterilized progressively with 70% ethanol for 3 min, 2% sodium hypochlorite for

5 min, 70% ethanol for 30 s and finally five times with d istilled water. The roots were dried on sterile filter paper. Root pieces (0.5 cm in length) were macerated in a mortar containing 9 mL of sterilized water. The suspension was serially diluted until 100 µL of each 1 × 10^{-4} dilution was spread on tryptic soy agar (TSA) medium. After incubation for 5 d, the colonies were purified to obtain pure culture. Each isolate was assessed for hypersensitivity test on tobacco leaves, and a hemolysis test was conducted on blood agar medium. Isolates that did not necrosis on leaves or hemolysis in agar were used.

Molecular identification

DNA extraction was performed according to the PrestoTM Mini gDNA Bacteria Kit protocol from Geneaid Biotech Ltd. (Taiwan) The polymerase chain reaction (PCR) process for pathogenic bacteria used the specific primer (ES16: 5'-GCG AAC TTG GCA GAG AT-3' and ESIG2c: 5'-GCG CTT G TGT TAT GAG-3'), which have a 920 bp amplicon. The endophyte PCR process only uses the universal primer 16S rRNA (27F/1492R). The electrophoresis was performed on a 1% agarose gel and visualized using an ultraviolet-transilluminator. Each DNA sequence was sequenced at First Base Malaysia and the NCBI database (https://blast.ncbi.nlm.nih.gov) were used to analyze similarities.

Antagonistic activities of Alcaligenes faecalis

The antagonistic activity of endophytes used the cross-streak method (Velho-Pereira and Kamat, 2011) modified by Dewi et al. (2022). The *in vitro* test was used to measure the non-volatile activities of endophytic isolate, namely by streaking the endophytic thoroughly on one-third of the side of the Petri dish. A day after incubation, a single line streak (4 cm in length and 0.2 cm in width) of the pathogen was streaked 0.5 cm from the opposite side of the endophyte. The endophyte streak was repeated four times on each Petri dish. The percentage of inhibition against pathogenic bacteria (PPb) was calculated using Equation 1:

$$\text{PPb (\%)} = \frac{\text{AWG}}{\text{TSA}}\, x\ 100\% \tag{1}$$

where AWG is the length of the streak area where there is no bacterial growth and TSA is the total length of the streak (Velho-Pereira and Kamat 2011)

Volatile organic compound (VOC) activities were calculated *in vitro* using the method of Vespermann et al. (2007). Endophytes were streaked on TSA at the bottom of the Petri dish, with 10 µL of the pathogen dropped onto filter paper (6 mm diameter) at the NA surface and placed on the dish's cover. Then, the dishes were held in opposite positions using cling wrap. Pathogenic bacteria inhibition was determined after 5 d by comparing the pathogen diameters between the control and treatments. All endophytic isolates (the treatments) were repeated in three replications.

Identification of secondary metabolites

The endophytic bacterial filtrate was prepared by modifying the method of Basumatary et al. (2021). Endophytic bacteria were cultured in TSB media for 48 hr and then centrifuged at 6000 rpm for 15 min. Next, the supernatant was passed through a 0.22 µm sterile filter syringe to collect the culture filtrate. The filtrate culture was tested using a paper disc diffusion test (Ruslan et al., 2022), by dripping 15 µL of endophyte filtrate onto a paper disc on the surface of the NB media containing pathogens. The effect of the crude extract was measured based on the halo zone around the disc paper. The secondary metabolite in the isolate with the highest activity in pathogen inhibition was examined using gas chromatography-mass spectrometry (GC-MS), with the extraction of metabolites was performed using the methods of Ruslan et al. (2022) and Islam et al. (2012). The culture filtrates were dissolved in ethyl acetate solvent (1:1 ratio volume per volume). The liquid phase of the extract was isolated using a funnel and evaporated using an evaporator at 40 °C to collect the extract. Finally, the extract was analyzed using GC-MS to identify metabolic compounds.

Determination of plant growth-promoting activity

Indole production

The ability of endophytes to produce indole was calculated according to the procedures of Anugrah et al. (2021). Endophytic bacteria were tested for qualitative ability using Salkowski reagent (7.5 mL $FeCl_3.6H_2O$, 150 mL H_2SO_4 in 250 mL distilled water). The endophytic bacteria were cultured in 4 mL of TSB medium supplemented with 100 mg/L of tryptophan. The culture was cultivated in a shaking incubator at 120 rpm, at room temperature, for 3 d. The suspension was centrifuged at 12,000 rpm at 4 °C for 10 min. After centrifugation, 1 mL of the liquid was mixed with 2 mL of Salkowski reagent and incubated in a dark room for 30 min. A positive indole activity was indicated by a change in the suspension color to pink.

The quantification of indole activity was determined using a spectrophotometric method at 530 nm absorbance wavelength. The crude indole level was estimated using the indole standard curve (Oattern and Glick, 2002).

Nitrogen fixation activity

The capability of endophytic bacteria to fix nitrogen (N) was tested by streaking the bacteria in Burk's nitrogen-free culture medium. The growth of bacteria after being incubated for 7 d at room temperature indicated positive activity (Mashiane et al., 2018).

Phosphate solubilization capability

The test was carried out by growing the isolates on Pikovskaya agar medium in three replications (Rahma et al., 2014). A halo zone surrounding the endophyte colony indicated phosphorus (P) solubilization capacity. The phosphate solubilization index (PSI) was determined by comparing the halo zone and colony diameter (Teymouri et al., 2016).

Effectiveness of endophytic isolates for maize resistance induction

Testing was carried out using a randomized complete block design, with endophytic bacterial isolates as the treatment and five replications, each consisting of five plants. Following Rahma et al. (2014), testing was done by soaking seeds in endophytic bacteria suspension (1×10^8 colony forming units (cfu)/mL) for 24 hr, while seeds were only soaked in sterile water as an untreated control. Two seeds were planted in each seed tray containing a soil-to-manure ratio of 3:1 (weight per weight). A maize pseudostem (aged 1 wk) was injected with 1×10^8 cfu/mL bacterial suspension for pathogen inoculation. Untreated plants were injected with sterile water only. The incidence and severity of the disease were observed at 1 wk after application. Disease incidence was calculated based on the percentage of infected seedlings, while disease severity was calculated based on Equation 2:

$$DS = \frac{\Sigma\,(n_i \text{ x } v_i)}{Z\,x\,N} \text{ x } 100\% \quad (2)$$

where DS is the disease severity as a percentage, n_i is the number of the infected plant at each scale, v_i is the scale level of disease symptoms (0 = healthy; 1 = water soaking 0-20% on inoculation spot; 2 = water soaking 21 -40% on inoculation spot, chlorotic and necrotic; 3 = water soaking 41 -60% on inoculation spot, chlorotic and necrotic; 4 = water soaking 61 -100% on inoculation spot, plant wilted and stunted), *Z* is the highest scale level and *N* is the total number of plants observed.

Disease severity and incidence data were utilized to determine the percentage of disease reduction.

Plant growth enhancement by endophytic bacteria

The ability of endophytic bacteria to promote plant growth was characterized by the method of Rajendran et al (2015), based on a completely randomized design, consisting of four treatment and three replications. Each replication was applied with 50 seeds. The seeds were sterilized with 95% ethanol for 10 s, soaked in 5% H_2O_2 for 5 min, rinsed seven times with sterile distilled water and then air-dried in a room (Putrie, 2013). The seeds were soaked in a suspension of 1×10^8 cfu/mL of bacteria isolates for 24 hr as the treatment while only soaked in sterilized water as a control. The seeds were germinated on wet tissue paper in a plastic box for 7 d (International Seed Testing Association, 1999). The seed germination, stem length, root length, number of roots and fresh weight of seedlings were calculated after germination.

Enzymatic responsibility in plant tissue

Enzymatic activity was detected based on the phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) activities in maize. The activities were conducted on composite leaves from each treatment. PAL activity in leaves was identified twice, first when the seedlings were aged 1 wk or before inoculation with the pathogen and then 1 wk after inoculation. PPO analysis was carried out on plant roots aged at 1 wk after pathogen inoculation. Initially, the total protein was analyzed to measure PAL and PPO (Lowry et al. 1951). This procedure was started by mixing 1 mL of the sample with 5 mL of reagent (2% Na_2CO_3 in 0.1 N NaOH and 0.5% $CuSO_4$ in 1% NaK tartrate) and incubating at room temperature for 10 min. Then, 0.5 mL of Follin-Ciocalteau reagent was added and incubated at room temperature for 30 min. The absorbance was determined at 500 nm wavelength and subsequently compared to the Bovine Serum Albumin standard curve.

PAL analysis was carried out according to Cahill and McComb (1992). A sample (300 mg) of leaves was crushed on ice with 4 mL of 0.1 M Tris buffer (pH 8.9) containing 30 mg of polyvinylpyrrolidone and 10 mM mercaptoethanol. The extract was centrifuged at 10,000 rpm at 4°C for 30 min. A 500 µL sample was mixed with 100 µL of 80 mM borate buffer (pH 8.9) and 30 mM phenylalanine. The mixture was incubated at 30°C for 1 hr before adding 2 M HCl. The trans-cinnamic acid concentration was measured at 290 nm wavelength (A_{290}) using a spectrophotometer (UV-1280 UV-Vis Spectrophotometer; Shimadzu). The enzyme activity was expressed as A_{290} absorbance per milligram protein weight.

The PPO analysis followed the methodology outlined by Mayer et al. (1965). A total of 1.5 mL of a phosphate buffer solution with a concentration of 0.1 M (pH of 6.5) was combined with 0.5 mL of enzyme preparation extract and 0.5 mL of catechol solution with a concentration of 0.01 N. The spectrophotometer measured the absorbance at the 495 nm wavelength at regular intervals of 30 s. PPO activity was determined by measuring the rate of change in absorbance per minute per milligram protein weight.

Statistical analysis

Differences among groups in plant growth-promoting activity and disease reduction were determined using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) at the 5% significance level. Statistical analyses were performed using Minitab version 20.3 (Minitab, LLC, State College, PA, USA). Data visualization was carried out using Microsoft Excel 2016 (Microsoft Corporation; Redmond, WA, USA).

Results and Discussion

Molecular identification of endophytic species

The gene sequence analysis for 16S rRNA successfully identified three endophyte isolates, with similarities to *Alcaligenes faecalis* species (Table 1). The endophytic bacterial isolates from West Java, Indonesia, namely JCJ-7 and JCB-1 and isolate MS-X from South Sumatra were identical to the *A. faecalis* strain Japan (97 -98%). The isolate was also recorded as an endophytic bacteria associated with plant roots. The isolate JCJ-7 had a similarity of 98.43% with the *A. faecalis* strain NBRC 13111 (accession number NR_113606.1), while the similarities of isolate JBC-1 (accession number NR_113606.1) and MS-X (accession number NR_113606.1) were 97.05% and 97.58%, respectively. These findings further validated other investigations that reported a relationship between *A. faecalis* and the roots of maize (Naseem and Bano, 2014; Akintokun et al., 2019; Zafar-Ul-hye et al., 2020). However, these results were insufficient to determine whether the bacteria obtained belong to the facultative or obligatory endophytic bacteria group.

Effect of endophytic bacterial inhibition of pathogen in vitro

Based on the results of the *in vitro* assays, the endophytic bacteria impeded pathogenesis and enhanced plant growth (Table 2). Specifically, isolate MS-X produced 11.65 ppm of indole and fixing nitrogen ability and phosphate solubilization activity of PSI 1.85. indole acetic acid (IAA) is the major auxin hormone in plants that regulates growth (Herlina et al., 2017). Bacterial-produced IAA can enhance root growth and promote the development of lateral roots or root hairs, facilitating the absorption of water and nutrients by the plant, as well as enhancing or diminishing the levels of naturally occurring plant auxin (Etesami and Glick, 2024). In contrast, the best antibiosis activity was shown by the isolate JCJ-7. However, no volatile organic compound activity was detected.

Table 1 Similarity of isolates based on 16S rRNA gene analysis.

Isolate	Species	Query cover (%)	Similarity (%)	Accession number	Origin
JCJ-7	*Alcaligenes faecalis* strain NBRC 13111	100	98.43	NR_113606.1	Japan
MS-X	*Alcaligenes faecalis* strain NBRC 13111	100	97.58	NR_113606.1	Japan
JCB-1	*Alcaligenes faecalis* strain NBRC 13111	98	97.05	NR_113606.1	Japan

Table 2 Effects of endophytes and their metabolite extract on pathogen growth.

Treatment	Growth promoter activity			Pathogen inhibition activity		
	IAA production (ppm)	PSI	N-fix	Antibiosis (%)	VOC (%)	IZD (mm)
JCB-1	-	-	+	82.50	18.60	14.71
MS-X	11.65	1.85	+	71.88	78.94	8.14
JCJ-7	3.40	-	-	100.0	0	7.98

ppm = parts per million; IAA production = concentration of indole acetic acid produced by endophytic bacteria; PSI = phosphate solubilization index; N-fix = the ability of endophytes to fix nitrogen; VOC = volatile organic compound; IZD = inhibition diameter zone produced by endophytic metabolite.

Based on Table 2, all endophytic metabolites inhibited pathogen growth. Crude extracts of endophytic bacterial cultures have been reported to contain secondary metabolic compounds that can inhibit pathogen growth (Ruslan et al., 2022). Metabolites produced by endophytes can help them survive or cause damage to pathogens (Alam et al., 2021). The inhibition zone indicated inhibition of pathogenic growth around the colony. However, the percentage of pathogen suppression differed between isolates and there was relatively low inhibition activity. The highest inhibition through the antibiosis mechanism and metabolite extract occurred in the isolate JCB-1, which produced a maximum clear zone diameter of 14.71 mm. Similar results were reported by Widiantini et al. 92018), who tested the secondary metabolites of endophytic bacteria to reduce the growth of *Ganoderma boninense*. Their results indicated that the production of secondary metabolite compounds by bacteria was greatly influenced by the nutritional content of the compounds and the ability for communication among the bacteria (known as quorum sensing). On the other hand, the bioactive compounds produced by endophytes also depend on the species, the plant part and environmental factors (Mishra et al., 2022) in addition to the concentration of the bioactive compounds. This result was supported by Mishra et al. (2022), who stated that most bioactive compounds were produced after axenic culture for 2 -4 wk. In addition, Islam et al. (2012) noted that the presence of an acetyl-butanediol compound from *Bacillus subtilis* decreased the growth of *R. solani*.

The GC-MS analysis of the metabolic extract produced by the endophytic bacteria is shown in Table 3. It was explicitly conducted on isolate JCB-1 due to its more notable inhibition of pathogen growth. The compounds produced by the isolate JCB-1 were detected at retention times (RTs) in the range 30–35 min. The compound bis (2-ethylhexyl) phthalate was synthesized by the isolate JCB-1 at RT 34.98%, while the other compounds detected for the two isolates were relatively the same, namely the pyrrolo [1,2-a] pyrazine-1, 4-dione and hexahydro groups of compounds. Based on these result, the pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro ($C_7H_{10}N_2O_2$) is known to have antimicrobial properties, with the same compound being produced by *Bacillus tequilensis* MSI45 and reducing the growth of *Staphylococcus aureus* (Kiran et al., 2018). This same compound from *Staphylococcus xylosus* was reported to play a role as an antibacterial and anticancer agent (Mangrolia and Osborne, 2020). Another metabolite, 2-piperidinone (C_5H_9NO), has been reported to have antibiotic properties against pathogenic bacteria such as *Pseudomonas aeruginosa*, *P. mirabilis* and *Candida albicans* (Al-Bahadily et al., 2019). The compound bis (2-ethylhexyl) phthalate ($C_{24}H_{38}O_4$), at 34.98% of the extract, was reported to play an essential role as an antimicrobial, antiviral, larvicide and antioxidant compound (Fahmy and Abdel-Tawab, 2021; Javed et al., 2022). It is produced by several bacterial species, including *Lactiplantibacillus plantarum* (Javed et al., 2022), *Streptomyces* sp. (Fahmy and Abdel-Tawab, 2021) and *Aspergillus awamori* (Lotfy et al., 2018). Furthermore, the existence of antibiotic metabolites should help endophytic bacteria to reduce pathogen growth directly.

Disease reduction on maize seedlings by endophytic bacteria

Based on the results endophytic bacteria significantly reduced both the incidence and severity of Stewart wilt diseases in maize seedlings (Fig. 1). The highest disease incidence was recorded in the control, reaching 84.0%, while treatment with *Alacaligenes faecalis* isolate MS-X reduced the incidence to 44.0%, representing a 47.6% reduction. In contrast, the lowest suppression of disease incidence occurred after applying the isolates JCJ-7, with only a 4.8% reduction.

Table 3 Composition of antimicrobial compounds produced by metabolic extracts from endophytic bacteria isolate JCB-1

Retention time (min)	Chemical composition	Percentage (%)	Activity
30.268	Phenol, 3,5-dimethoxy-	6.26	Antimicrobe (Adebiyi, 2020)
30.951	Phenanthrene, 1-methyl-	1.91	Antimicrobe and antioxidant (Magnibou et al., 2022)
31.206	Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)-	3.64	Antimicrobe (Chen et al., 2018; Kiran et al., 2018)
31.468	3-Isobutylhexadydro pyrrolo [1,2-a]pyrazine-1,4-dione	9.82	Antimicrobe (Chen et al., 2018; Kiran et al., 2018)
31.585	Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)-	4.96	Antimicrobe (Chen et al., 2018; Kiran et al., 2018)
34.019	3-Benzylhexahydro pyrrolo [1,2-A] pyrazine-1,4-dione	7.00	Antimicrobe (Chen et al., 2018; Kiran et al., 2018)
34.585	Bis (2-ethylhexyl) phthalate	34.98	Antibacterial and larvicide (Javed *et al*. 2022)
35.798	Pyridine, 2-methoxy-5-nitro-	5.52	Antimicrobe and antiviral (Marinescu and Popa, 2022)

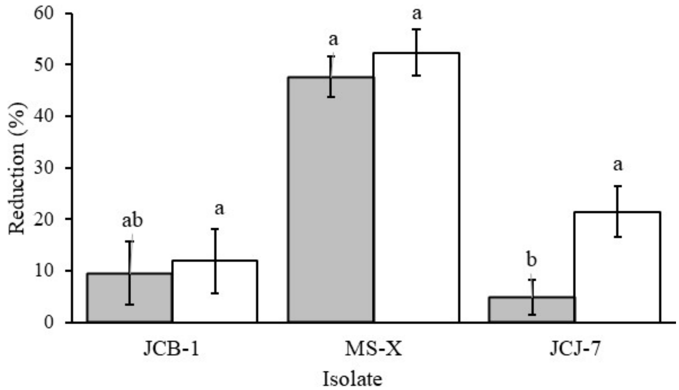

Fig. 1 Relative suppression (compared to the control) of Stewart's wilt disease incidence and severity in maize treated with endophytic bacteria (■ disease incidence, □ disease severity). Different lowercase letters above the columns indicate significant differences ($p < 0.05$) among means of each parameter. Error bar = ± SE.

At 1 wk after pathogen inoculation, the suppression of disease severity varied in the range 11.90–52.38%, indicating the pathogen could infect the plant, but the severity of the disease did not kill seedlings. The disease development was limited to initial symptoms, namely narrow chlorosis at the inoculation site or on a few leaves. The disease triangle principle could explain the low disease severity, where various factors, such as variety resistance, unsuitable environment and lower pathogenicity of pathogens, could cause fluctuations in pathogenesis. Based on the results, treating the seeds with endophytic bacteria significantly reduced the incidence and severity of disease, with the highest reduction in disease severity in isolate MS-X (52.38%), while the lowest suppression occurred in the isolate JCB-1 (11.9%).

Guo et al. (2019) demonstrated that the management of soil-borne pathogens was closely related to the structure of the microbial community in the rhizosphere. In contrast, disease inhibition can be caused by indirect mechanisms, including plant growth-promotion or through plant resistance induction (Figueredo et al., 2017). Resistance can be induced when the plants are inoculated with endophytic bacteria, leading to an increase in the activity of defense enzyme synthesis. PAL, POD and PPO are among the enzymes responsible for plant resistance activity (Guo et al., 2019; Wang et al., 2020), which increased in plants that were inoculated with *B. altitudinis* (Sahu et al., 2020) and *B. amyloliquefaciens* (Gowtham et al., 2018). In the current research, the PAL activity increased after pathogen inoculation, especially in isolates JCJ-7 and JCB-1 (Fig. 2).

In contrast, at seedling age 7 d, or 1 d before pathogen inoculation, the isolate MS-X had high PAL activity, which decreased at 1 wk after pathogen inoculation. The PAL activity greatly increased in isolate JCB-1 and isolate JCJ-7. The same activity was evident in the untreated plants, which experienced increased PAL activity after inoculation with the pathogen. The fluctuation in activity is a normal response showing the existence of a defense mechanism provided by the plant to suppress pathogens. Based on thesystemic acquired resistance (SAR) mechanism, plants respond by activating their defense system when a pathogen appears. Pattern-recognition receptors will be established, recognizing compounds produced by the pathogen as pathogen-associated molecular patterns (PAMPs). The plant response to the damage caused by pathogens is the formation of damage-associated molecular pattern (DAMP) compounds. Then, the plant translates this signaling pattern to form PAMP-triggered immunity (PTI).

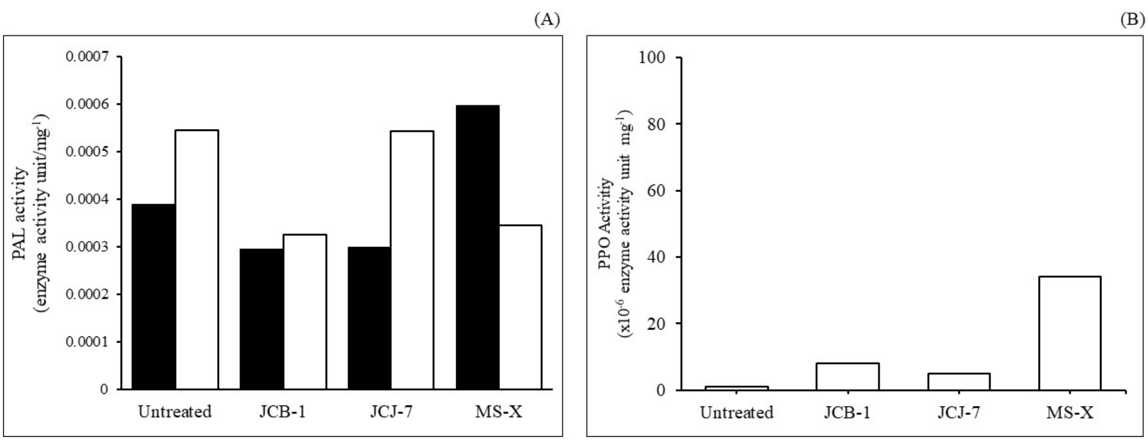

Fig. 2 (A) Phenylalanine ammonia-lyase (PAL) activity levels in maize seedlings before and after pathogen inoculation: PAL activity measured before (black bars) and after (white bars) inoculation; (B) Polyphenol oxidase (PPO) activity after inoculation.

Pathogens utilize virulence effector molecules to block the PTI system and prevent detection by the host (Pieterse et al., 2014). Therefore, as a precautionary measure, plants produce a second defense system called effector-triggered immunity (ETI) to recognize specific pathogen effectors, that increase salicylic acid in plants, ultimately triggering PR-protein expression (Pieterse et al., 2014).

The current study confirmed that disease infection induced the plant defense system through the SAR mechanism. Shobha and Mahadeva (2018) reported an increase in the activity of peroxidase enzymes and PR-protein in pepper when inoculated with *Phytophthora capsici*; however, it was lower than when applied with plant growth promoting rhizobacteria. An increase in PAL activity was reported when chili plants were inoculated with *Ph. capsici*-22 (Koç and Sülü Üstün, 2012). Apart from responding to pathogens, plants also respond to the presence of applied endophytes by forming different resistance or ISR mechanisms (Khare et al., 2018). In contrast to SAR, ISR is generally related to forming some plant defense compounds characterized by increased activity of jasmonic acid and ethylene in plants; in this mechanism, no PR-protein expression occurs due to the absence of the PTI signaling molecule (Andersen et al., 2018). Some plants only form ETI effectors, which will ultimately be closely related to the induction of many plant defense enzymes (Andersen et al., 2018).

The reduction in PAL activity after pathogen inoculation also indicated the utilization of PAL to suppress pathogen activity and inhibit disease progression. This was confirmed by the highest efficacy of reducing disease severity in the isolate MS-X (52.4%). The PAL enzyme in plants plays a crucial role in facilitating the conversion of L-phenylalanine to trans-cinnamate, which serves as the first step in phenolic metabolism in plants and strengthens and regenerates plant cell walls by synthesizing lignin, suberin and phenolic compounds in plant tissues ((Koç and Sülü Üstün, 2012). It also promotes antimicrobial activity by producing furanocoumarin, pterocarpan and isoflavonoid phytoalexins compounds ((Koç and Sülü Üstün, 2012). Furthermore, it is closely associated with the production of signaling salicylic acid compounds in plants (Koç and Sülü Üstün, 2012; Kim and Hwang, 2014).

Endophytic bacteria produced different enzymes in plant defense, including PPO activity. PPO activity in plant tissue is an indicator for evaluating the activation of the plant's defense mechanism triggered by endophytic bacteria (Rajendran et al., 2006). The current findings revealed that the endophytic bacteria treatment produced significantly increased PPO activity compared to the control (Fig. 2). Fig. 2 illustrates the established correlation between the activity of endophytic bacteria in roots and the activity of the PPO enzyme in plants inoculated with pathogens. The isolate MS-X produced the greatest (and significant) PPO activity. The notably elevated PPO activity observed in the isolate MS-X also considerably affected the isolate's capacity to decrease the severity of Stewart's wilt by 52.4%. The induction of defense in plants results from the enzyme's function facilitating the production of substances involved in the cell wall lignification and suberization processes (Purwantisari et al., 2019). In addition, this enzyme helps to counteract the effects of reactive oxygen species in plants. Sahu et al. (2020) introduced *Bacillus* sp. into rice plants infected with *R. solani* which increased PPO activity. Comparable findings were documented when chilies were exposed to *Bacillus amyloliquefaciens* to inhibit anthracnose, resulting in a two-fold increase in PPO activity (Gowtham et al., 2018).

There is strong scientific agreement PPO is an enzyme that catalyzes the hydroxylation and dehydrogenation of phenolic compounds, producing reactive o-quinones (Zhang, 2023). Subsequently, these o-quinones chemically react with nucleophilic groups and undergo the process of polymerization, resulting in the formation of melanin polymers (Fuerst et al., 2014; Zhang, 2023). The activity of PPO has been reported to be strongly associated with plant defense mechanisms triggered by enhanced levels of systemin, salicylic acid and jasmonate (Fuerst et al., 2014). Therefore, the presence of PPO in plants indicates the initiation of defensive processes, either through SAR or ISR. PPO can oxidize o-phenolic molecules, forming catechol, a crucial chemical in plant defense mechanisms (Zhang 2023). O-quinone and melanin, produced through PPO activity, are essential plant antibacterial chemicals (Fuerst et al., 2014; Fitria and Khotimah, 2017).

Plant growth enhancement by endophytic bacteria

Fig. 3 demonstrates the capability of the endophytic bacteria to enhance maize growth. According to Fig. 3, the seeds treated with endophytic bacteria had higher values of germination ability and vigor index than the untreated control, although there was no significant difference between treated and untreated seeds. The seed germination capability in the endophyte treatments varied in the range 81.0 – 86.0% on average, with the vigor index in the range 78.81 – 84.00%.

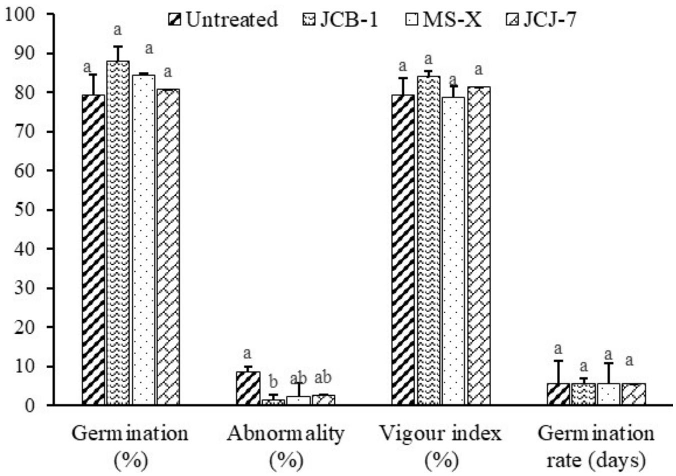

Fig. 3 Effect of endophytic bacterial activities on germination, seedling abnormality, vigor index and germination rate of maize. Different lowercase letter above bars indicates significant differences ($p < 0.05$) among mean values of each parameter. Error bar = ± SE.

In contrast, the average vigor index in the control was 79.33%. The JCB-1 isolate had the maximum germination capability, reaching 86.0%. Based on the these results, there was a trend for seed germination to increase during the last observation period, which aligned with the study conducted by (Rana et al., 2021), who demonstrated that the application of endophytes to maize resulted in enhanced germination and crop growth, with the enhanced development and germination attributed to the endophyte's capacity to generate IAA and siderophores and to solubilize phosphate and synthesize ammonia. The current research findings demonstrated that the application of endophytic bacteria via seed soaking enhanced germination in seeds, resulting in an estimated optimization rate of 1.67 -13.6%. It is crucial to minimize the overutilization of seeds resulting from the embroidery process during field-level planting.

Utilizing endophytic bacteria on seeds also decreased the proportion of abnormal seedling development, being 1.33 -2.67% lower than the control, which had an emergence rate of 8.67%. There was a suppression of 69.2 -84.7% compared to the control. The higher number of abnormal seedlings in the control could have been due to the pathogenic bacteria in the spermosphere that can survive but cannot be cultivated. Surface sterilization does not entirely ensure the total removal of organisms with such properties. Furthermore, the expected growth following germination might enhance plant tolerance in the face of the biotic and abiotic challenges encountered during the growth phase.

Fig. 4 demonstrates the impact of endophytic bacteria on plant growth. At 7 d after planting (7 DAP), the application of endophytic bacteria without further pathogen inoculation resulted in a considerable increase in stem length and

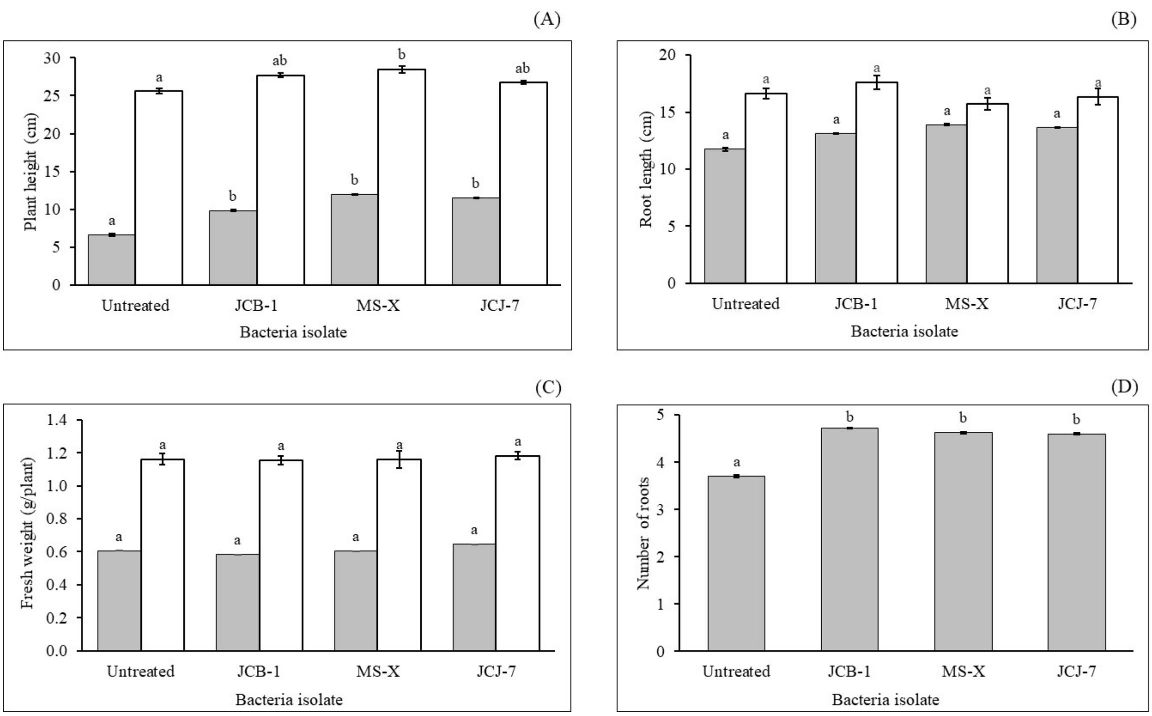

Fig. 4 Effect of endophytic bacterial inoculation on: (A) Plant height; (B) Root length; (C) Fresh weight; (D) Number of roots. Different lowercase letters above bars indicate significance differences ($p < 0.05$) among means of each parameter (■ non-inoculated seedlings at day 7; □ inoculated seedlings at day 14). Error bar = ± SE.

the number of roots compared to the control. However, no significant differences were observed in root length and plant wet weight among all treatments. The findings indicated that the isolate JCJ-2 had superior development characteristics regarding stem height, root length and fresh weight of seedlings compared to the other treatments. These results were consistent with its capacity to fix nitrogen, produce the highest concentration of IAA (32.78 ppm) and exhibit a PSI index of 1.66. The isolate JCB-1 exhibited the highest ability to stimulate lateral root production.

The application of endophytic bacteria *Lysinibacillus* sp. and *Paenibacillus dendritiformis* to maize seeds was reported to enhance germination, root quantity, average root length and fresh and dry weight of sprouts in seeds affected by pathogenic fungus (Pal et al., 2022). In the current study, there were substantial variations for all isolates in stem length and number of lateral roots compared to the control group. The seedlings injected with endophytic bacteria had more robust growth and a vibrant green leaf color compared to the untreated maize. Endophyte treatment with isolate MS-X resulted in superior growth compared to other isolates without pathogen inoculation. Subsequently, upon introducing pathogens, the activity of endophytic bacteria appeared consistently across all isolates. As well as the isolate JCB-1 having the highest capacity to induce lateral root development, it also stimulated the formation of the most lateral roots. These results correlated with the ability of isolates to promote plant growth associated with phosphate solubilization. Phosphate-soluble bacteria can release organic acids that bind phosphate-bound cations such as Ca (Mugiastuti et al., 2020), providing elemental P to plants and protecting plant roots from pathogens (Teymouri et al., 2016). In addition, the higher the concentration of IAA produced by endophytic bacteria, the better the growth of stem length, root length and growth rate of maize. However, the level of IAA will stimulate an increase in ethylene, which can impact reducing root length (Naveed et al., 2013).

Disease infections in maize seedlings impact their growth. It can be seen from the growth of the roots, stems and plants' fresh weight at day 14 (around day 7 after pathogen inoculation), with the same effect for all treatments (Fig. 4). The highest root length was for isolate JCB-1 (17.60 cm). Only stem length was affected by endophytic bacteria after pathogen inoculation, as the induction of plant defense compounds by endophytic bacteria could inhibit growth. The same research results were shown when chilies were applied with endophytic bacteria and the chemical inducer benzothiazole (Yi et al., 2013), resulting in lower root and shoot fresh weight than the control, perhaps because endophytic bacteria boost growth in specific ways such as due to differences in host plant cultivars from which the endophytes were obtained (Sahu et al., 2020). Another factor is that when endophytic bacteria induce defense compounds in plants, the assimilate produced by the plant will focus on the production of plant defense compounds, thereby reducing the synthesis of compounds that play a role in influencing growth. Resistance induction activity will stimulate an increase in plant metabolism to produce proteins and metabolites as a defense system so that there is an increase and diversion of energy for the production of defensive compounds (Shoresh and Harman, 2008). This negatively influences plant adaptation when defense expression occurs in growing conditions associated with beneficial or harmful organisms (Messa, 2021). However, the ability of plants to prevent more severe diseases is of particular value due to the activity of endophytic bacteria.

Conclusion

All of the isolates of *A. faecalis* reduced Stewart wilt disease and increased maize growth. The induced resistance mechanisms increased PPO and PAL enzyme synthesis, reducing disease. Plant growth was promoted by the ability of the endophytes to fix N, solubilize P and generate IAA.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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