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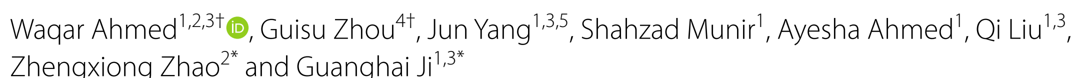

RESEARCH **Open Access**

Bacillus amyloliquefaciens WS-10 as a potential plant growth-promoter and biocontrol agent for bacterial wilt disease of flue-cured tobacco

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Abstract

Background: Bacterial wilt disease caused by the soilborne bacterium *Ralstonia solanacearum* is a serious threat to flue-cured tobacco production. In this study, an indigenous disease suppressive *Bacillus* strain was isolated from the rhizosphere soil of healthy tobacco plants, and its biocontrol and plant growth promoting (PGP) potential were evaluated in *in-vivo* and *in-vitro* assays.

Results: Through isolation and screening of 250 isolates, WS-10 was found to be the best candidate antagonistic strain against *R. solanacearum* (WS-001). *In-vitro* assays revealed that the isolated strain WS-10 (*Bacillus amyloliquefaciens*) showed an effective antagonistic activity against *R. solanacearum* WS-001 and several plant-pathogenic fungi. As promising PGP rhizobacteria, WS-10 had the ability of nitrogen fixation, solubilization of inorganic potassium and phosphate, and biosynthesis of indole-3-acetic. In a co-culture assay, it significantly inhibits the growth of WS-001. Our greenhouse experiments showed that the soil physicochemical properties and accumulation of dry matter contents in different plant parts (roots, stems, and leaves) were significantly increased in the presence of *B. amyloliquefaciens* WS-10. The soil treated with *B. amyloliquefaciens* WS-10 displayed significantly higher values of the average well color development index, the utilization ability of 6 types of carbon sources by rhizosphere microorganisms, and the diversity indices of the rhizosphere microbial communities. *In planta* assay, *B. amyloliquefaciens* WS-10 significantly reduced tobacco bacterial wilt disease incidence by up to 73.36, 43.82, and 86.82% under three different treatments by improving the functional diversity and biological activity of the soil microbial community.

Conclusions: Obtained findings suggested that *B. amyloliquefaciens* WS-10 had an excellent potential as a growth-promoting and biocontrol agent of tobacco bacterial wilt disease due to its multiple beneficial traits of nutrient solubilization and disease suppression. Thus, we conclude that *B. amyloliquefaciens* WS-10 was a high potential PGP and biocontrol strain for healthy production of tobacco crop.

Keywords: *Bacillus amyloliquefaciens*, Flue-cured tobacco, Biological control, *Ralstonia solanacearum*, Plant growth-promoter

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Background

Ralstonia solanacearum is a soilborne phytopathogenic bacterium that causes bacterial wilt disease in many economic field crops, including tomato, potato, tobacco, eggplant, ginger, and banana (Wu et al. 2014). The bacterium

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has a broad host range and is widely distributed in the temperate, tropical, and subtropical regions of the world (Paret et al. 2008). It infects more than 250 plant species of 54 different families and is ranked as the world's second most important phytopathogenic bacterium (Paudel et al. 2020). Due to broad host range and wide geographical distribution, *R. solanacearum* forms a highly diverse species complex that is classified into 4 phylotypes, 5 races, and 6 biovars (García et al. 2019). The worldwide yield losses caused by *R. solanacearum* range from 15 to 55% (Kim et al. 2016).

Ralstonia solanacearum survives in the soil and infected plant tissues, wild host, and freshwater for a long time (up to 40 years), even without a host plant (Genin and Denny 2012). Infected soil acts as a primary source of inoculum, and the bacterium invades the plant through spots of primary and secondary roots development, and wounds formed on roots due to mechanical operation (Singh et al. 2018). After entering a susceptible host, it multiplied systematically to disrupt xylem tissue. Infected plants generally show symptoms of yellowing and unilateral wilting of leaves leading to marginal necrosis. The color of parenchyma cells changes from light yellow to dark brown, and dark water-soaked lesions develop on the stem surface (García et al. 2019).

Tobacco (*Nicotiana tabacum* L.) is an economically important industrial crop worldwide, including China (Ma et al. 2018). Tobacco production is adversely affected by bacterial wilt disease (Wu et al. 2020). The incidence of tobacco bacterial wilt is recorded at around 15–35% but can extend up to 75% when it prevails with other soilborne root rot diseases (Black Shank) caused by *Phytophtora nicotianae*. In the regions of mono-cropping and high humidity, yield losses range between 50 and 60%, and in the case of a severe outbreak, they can reach 100% (Cai et al. 2021). Long-term continuous mono-cropping has resulted in an epidemic form of tobacco bacterial wilt disease, and makes it difficult to control its incidence (Chen et al. 2020).

In recent years, many integrated disease management practices in the form of cultural control, crop rotation, resistant cultivars, bioorganic fertilizers, and chemical control have been adopted to control bacterial wilt disease (Qi et al. 2020). Crop rotation, the use of cover crops, soil amendments with biochar and organic matter were found to be practical approaches to successfully manage many soilborne diseases through breaking the pathogen life cycle (Fan et al. 2020). The amendment of biochar in the soil improves soil health, functional diversity of rhizosphere microorganisms, mitigates the pathogen load, and reduces the incidence of tobacco bacterial wilt disease (Li et al. 2021).

Nowadays, biocontrol via disease suppressive potential strains, i.e., *Bacillus* sp., *Pseudomonas* sp., and *Lysobacter* sp., are considered to be an efficient management strategies against soilborne diseases. These biocontrol agents produce special antimicrobial compounds (Wei et al. 2021) and are used as microbial consortia to alter the soil microbial diversity (Zhang et al. 2020). Previous studies suggested that rhizobacteria and endophytic fungi such as *Bacillus* sp. (Wu et al. 2020), *Pseudomonas* sp. (Zhuo et al. 2019), and *Trichoderma harzianum* (Jogaiah et al. 2013) have strong plant growth promotion and biocontrol activity against many phytopathogens. Similarly, *P. aeruginosa* NXHG29 efficiently control the incidence of tobacco bacterial wilt and black shank disease through mechanisms of direct antagonism and niche exclusion (Ma et al. 2018). The present study aimed to develop safe and potential microbial biocontrol strategies by screening *Bacillus* sp. strains from the rhizosphere soil of healthy tobacco plants to manage pathogen causes flue-cured tobacco bacterial wilt disease. Additionally, the plant-beneficial traits of candidate strains related to disease suppression and plant growth promotion were characterized in *in-vitro* and *in-vivo* assays.

Methods

Bacterial strains, culture media, and growth conditions

In this study, *Ralstonia solanacearum* WS-001 (Accession No. MW730714) was isolated from the tissues of tobacco plants infected with bacterial wilt disease and *Bacillus* strains were isolated from the rhizosphere soil samples of healthy tobacco plants collected from Wenshan (23° 36′ N, 104° 24′ E), Yunnan Province, China. A colony showing the typical morphology (Additional file 1: Fig. S1) of *R. solanacearum* on Kelman's tetrazolium chloride (TTC) agar medium was picked and grown on Casamino acid-Peptone-Glucose (CPG) medium (Casein hydrolysate 1 g/L; Peptone 10 g/L; Glucose 5 g/L; and pH 7.0) and incubated at 28 °C for 48 h (Kelman 1954). Antagonistic *Bacillus* strains were isolated on Luria–Bertani (LB) medium (Bacto tryptone 10 g/L; Yeast extract 5 g/L; NaCl 10 g/L; Agar 18 g/L and pH 7.0) based on the colony morphology as described by De Vos et al. (2009). Pure cultures of bacterial strains were stored at − 80 °C in 50% glycerol (v/v) for future use.

Pathogenicity test

A pathogenicity assay was performed to confirm the virulence of isolated strain WS-001 and to fulfill Koch's postulates by adopting the methodology of Yuan et al. (2014). For the pathogenicity assay, 30 days old seedlings of flue-cured tobacco cultivar Hongda (highly susceptible) and K326 (resistant) were uprooted and washed with sterilized distilled water to remove the

soil and slightly injured with a needle. The seedlings were placed in a 100 mL suspension of WS-001 (1×10^7 CFU/mL) incubated at 160 rpm and 28 °C for 30 min and then transplanted into pots containing a mixture of double sterilized peat and soil (1:3). In comparison, the control plants were treated with the same volume of CPG broth, and symptoms were observed as described by Yuan et al. (2014).

In-vitro screening test for antimicrobial activity

The antibacterial activity of isolated strains against *R. solanacearum* WS-001 was determined on LB medium plates using the disc-diffusion technique (Li et al. 2014). For the screening test, *R. solanacearum* WS-001 and isolated bacterial strains were cultured overnight at 28 °C and 160 rpm in CPG broth and LB broth, respectively; and adjusted to an optical density of $OD_{600\ nm} \approx 0.5$ (1×10^7 CFU/mL) using a spectrophotometer (GE Uitrospec 2100 pro) (Zhang et al. 2020). Briefly, ≈150 µL aliquot culture of the pathogenic bacterium was spread on LB medium plates and dried for 2–3 min. A sterilized filter paper disc (0.5 cm) was taken and placed in the middle of the LB medium plates. Subsequently, 10 µL aliquot culture from each isolated strain (1×10^7 CFU/mL) and sterilized distilled water (control) were punched on the filter paper disc and incubated at 28 °C for 48 h to observe the growth inhibition. The isolated strains with an inhibition zone diameter (≥ 1.5 cm) were selected as biocontrol strains for further study. The antibacterial activity was expressed as a growth inhibition ratio (GIR) using the following formula (Li et al. 2014): GIR (%) = [(Inhibition zone diameter − Colony diameter)/Inhibition zone diameter] × 100. Antifungal activity of isolated strain WS-10 was determined by a dual culture technique on potato dextrose agar medium plates as previously described by Cui et al. (2019).

Raising of nursery

Seeds of flue-cured tobacco (*Nicotiana tabacum* L.) cultivar Yun87 were provided by the College of Tobacco Sciences, Yunnan Agricultural University, Kunming, China. The nursery was grown in floating foam polystyrene trays (162 wells) in the greenhouse 45–60 days before use according to the method of Dai et al. (2009). The seeds were sown in a mixed nursery medium composed of perlite, vermiculite, and turf at a ratio of 3:3:4. One to two seeds of the flue-cured tobacco cultivar Yun87 were placed in each well and slightly covered with medium.

In-vivo assay for biocontrol activity of candidate *Bacillus* strains

A pot experiment was conducted in the greenhouse of Yunnan Agricultural University, Kunming, China, to evaluate the biocontrol effect of three selected *Bacillus* strains against tobacco bacterial wilt disease as described by Yuan et al. (2014). Seedlings (35 days old) of tobacco cultivar Yun87 were transplanted into pots (40 × 35 cm) containing 10 kg of disease-free red soil. In addition, fertilizer was applied to overcome the nutrient deficiency (Additional file 1: Table S1). Tobacco seedlings were inoculated with pathogenic strain WS-001 (1×10^7 CFU/mL) and biocontrol *Bacillus* strains (1×10^7 CFU/mL) 50 mL/pot respectively, through the root drenching method by slightly injuring the roots one day after transplantation. The experiment was carried out in a completely randomized design with ten plants in each treatment and each treatment was repeated thrice. The greenhouse conditions were maintained as a day/night temperature (28/20 °C) with a 14 h light and 10 h dark photoperiod. The incidence of tobacco bacterial wilt disease was graded according to GB/T 23222-2008 (National Standardization Management Committee, China. 2009). Bacterial wilt symptoms were observed with the interval of 5 days after post-inoculation and evaluated using a disease rating scale as described by Cai et al. (2021). The disease index (DI) and protective value (PV) were calculated using the following formula: DI (%) = [Σ (Disease index × Number of diseased plants in this index)/(Highest disease index × Total number of plants investigated)] × 100 and $PV = [(DI_{ck} - DI_t)/DI_{ck}] \times 100$. Here: DI_{ck}; disease index of the control group, DI_t; disease index of treatment.

Molecular characterization of potential biocontrol and pathogen strains

Total genomic DNA of isolated strains WS-001 and WS-10 were extracted using a TIANamp Bacteria DNA isolation kit (TIANGEN®) according to the manufacturer's instructions. Molecular identification of isolated strains was made by the PCR amplification of the 16S *rRNA* gene (Zhang et al. 2020). The PCR amplification conditions for the 16S *rRNA* gene were as follow: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s, and a final extension at 72 °C for 7 min.

Multilocus sequence analysis (MLSA) was performed for the high-resolution phylogenetic relationship of species within a genus (Huang et al. 2017). For sequence alignment, MLSA for 2 housekeeping genes *rpoB* and *gyrB* of *Bacillus* strain WS-10; *egl* and *mutS* of WS-001

were performed using ClustalW. The primers used for 16S *rRNA*, *rpoB*, *gryB*, *egl*, and *mutS* genes are shown in Table 1. The PCR amplification conditions for *rpoB*, *gryB*, *egl*, and *mutS* genes were similar with 16S *rRNA* except for annealing (Table 1). The PCR amplification products were then sent to the company (TSINGKE® Co. Ltd Beijing, China) for sequencing, and the obtained sequences were analyzed online using the BlastN program (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed with the neighbor-joining method using MEGAX software (Kumar et al. 2016).

In-vitro assay for plant growth-promoting traits of the biocontrol agent

Phosphate solubilization

Pikovskaya (PVK) medium (Glucose 10 g/L, $Ca_3(PO_4)_2$ 5 g/L, NaCl 0.2 g/L, $(NH_4)_2SO_4$ 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.1 g/L, KCl 0.2 g/L, Yeast extract 0.5 g/L, $MnSO_4$ 2 mg/L, $FeSO_4{\cdot}7H_2O$ 2 mg/L, Bromphenol blue 25 mg/L, Agar 20 g/L, and pH 7.0) was used to determine the phosphate solubilizing activity of *Bacillus* strain WS-10 (Jeon et al. 2003). Briefly, *Bacillus* strain WS-10 was cultured on PVK medium plates and incubated at 28 °C for 7 days. The growth was associated with using inorganic phosphate in $Ca_3\,(PO_4)_2$ as a sole phosphate source which was determined as a clear zone around the bacterial colony.

Potassium solubilization

Potassium feldspar (PF) solid medium (Sucrose 10 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $(NH_4)_2SO_4$ 0.2 g/L, NaCl 0.1 g/L, $CaCO_3$ 0.1 g/L, Potassium feldspar 5.0 g/L, Bromophenol blue 25 mg/L, Agar 20 g/L, pH 7.2) was used to assess the potassium-solubilizing capability of *Bacillus* strain WS-10 (Zhang and Kong 2014). *Bacillus* strain WS-10 was cultured on PF medium plates, incubated at 28 °C for 7 days, and a clear zone around the bacterial colonies was observed.

Table 1 Primers used in this study

Gene	Primers	Sequence (5′—3′)	Annealing temperature (°C)
Bacillus			
rpoB	rpoB-F	AACCAGTTCCGCGTTGGCCTGG	
	rpoB-R	CCTGAACAACACGCTCGGA	59
gyrB	GyrB-F	GAAGTCATCATGACCGTTCTGCAY	
	GyrB-R	AGCAGGGTACGGATGTGCGAG CCR	
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	
	1492 R	TACGGYTACCTTGTTACGACTT	55
Ralstonia solanacearum			
egl	Endo-F	ATGCATGCCGCTGGTCGCCGC	63
	Endo-R	GCGTTGCCCGGCACGAACACC	
mutS	mutS-F	ACAGCGCCTTGAGCCGGTACA	59
	mutS-R	GCTGATCACCGGCCCGAACAT	
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	55
	1492R	GGYTACCTTGTTACGACTT	

Nitrogen fixation

To determine the nitrogen fixation ability, *Bacillus* strain WS-10 was grown on nitrogen-free (NF) Ashby medium (Glucose 5 g/L, Mannitol 5 g/L, $CaCl_2{\cdot}2H_2O$ 0.1 g/L, $MgSO_4{\cdot}7H_2O$ 0.1 g/L, $Na_2MoO_4{\cdot}2H_2O$ 5 mg/L, K_2HPO_4 0.9 g/L, KH_2PO_4 0.1 g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L, $CaCO_3$ 5 g/L, Bromphenol blue 25 mg/L, Agar 20 g/L, and pH 7.3) (Wang et al. 2018), incubated at 28 °C for 7 days and a circle was observed around the bacterial colonies.

Indole-3-acetic acid production

Salkowski reagent was used to determine the indole-3-acetic acid (IAA) production ability of *Bacillus* strain WS-10 (Abdallah et al. 2018). Briefly, the isolated strain was grown in a 200 mL Landy medium without L-tryptophan, incubated at 28 °C and 160 rpm for 60 h. Cell-free supernatants were collected by centrifugation at 10,000 rpm for 10 min, and 1.5 mL supernatants were added with 1.5 mL of Salkowski reagent and incubated for 30 min in the dark at room temperature to observe color variation. In contrast, the same volume of Landy medium was added in the Salkowski reagent as a control.

In-vitro co-culture assay

In-vitro co-culture assay was performed according to the method of Cui et al. (2019) to confirm the antagonistic activity of *Bacillus* strain WS-10 against the pathogenic bacterium *R. solanacearum* WS-001. For the co-culture assay, overnight cultures of *Bacillus* strain WS-10 and *R. solanacearum* WS-001 were prepared in LB (broth) and CPG (broth), respectively, and adjusted to an optical density of $OD_{600\ nm} \approx 0.5$ (1×10^7 CFU/mL). In brief, WS-10 (25 mL) culture was used to initiate the co-culture with WS-001 (25 mL) in CPG liquid medium, incubated at 160 rpm and 28 °C for 12 h, while the same volume (50 mL) of WS-001 cultured in CPG broth was used as a control. Serial dilutions (up to 10 folds) of co-culture were made, and CFU/mL of co-cultured strains were recorded separately based on their distinct colony morphologies on the LB and TTC medium.

Greenhouse assay

A greenhouse experiment was conducted from during the growing season in 2020 Bofeng County, Jinning

(24° 40′ N, 102° 35′ E), Yunnan Province, China, to understand the biocontrol effect of candidate *Bacillus* strain WS-10 on tobacco bacterial wilt disease. Infected free red soil was collected from the mountains, and tobacco was grown for the first time in the soil (no other crop had been grown before). Tobacco seedlings of cultivar Yun87 (45 days old) were transplanted in the pot (40 × 35 cm) containing 13 kg of soil and 1 kg peat. In addition, fertilizer was also applied in each pot as a base (before plantation) and top fertilizer (20 days after transplantation) to overcome the nutrient deficiency (Additional file 1: Table S1), and greenhouse conditions were maintained as mentioned above.

One month after transplantation, when tobacco plants reached 5–6 leaves stage, *R. solanacearum* WS-001 (1×10^7 CFU/mL) and biocontrol agent WS-10 (1×10^7 CFU/mL) culture were prepared and inoculated via root drenching method. The experiment was performed under 4 conditions: CK; [control; application of *R. solanacearum* WS-001 (100 mL/pot)], T1; [combined application of *R. solanacearum* WS-001 (100 mL/pot) and biocontrol strain WS-10 (100 mL/pot)], T2; [first application of WS-001 (100 mL/pot), then 3 days later application of WS-10 (100 mL/pot)], T3; [first application of WS-10 (100 mL/pot), then 3 days later application of WS-001 (100 mL/pot)]. Bacterial wilt symptoms were observed once a week after post-inoculation to the end of the experiment in each treatment using a disease rating scale to evaluate the disease index and protective value as described above in "*In-vivo* assay for biocontrol activity of candidate *Bacillus* strains" section (Cai et al. 2021). The experiment was conducted under a completely randomized design with 15 plants in each treatment and each treatment was repeated thrice.

Analysis of soil physicochemical properties

Soil samples were collected in replicates from each treatment at the end of the experiment using the root shaking method as described by Cai et al. (2021). Bulk soil samples collected for physicochemical analysis were stored at room temperature, while rhizosphere soil samples collected for functional diversity and diversity indices analysis of rhizosphere microorganisms were stored at 4 °C. The alkali-hydrolysable nitrogen method was used to measure the available nitrogen (mg/kg). Soil organic matter (g/kg), available phosphorus (mg/kg), and available potassium (mg/kg) were determined by acidified potassium dichromate ($K_2Cr_2O_7$–H_2SO_4) heating method, 0.5 mol/L $NaHCO_3$ solutions (pH 8.5), and CH_3COONH_4 extraction method as described by Cai et al. (2021), and pH was determined using pH meter.

Determination of functional diversity and diversity indices of soil microbial community

The functional diversity of a rhizospheric microbial community was analyzed using the Biolog EcoPlate™ method following the manufactures instructions. The metabolic activity of the rhizospheric microbial community on Biolog EcoPlate™ was recorded in the form of average well color development (AWCD) and the utilization ability of 6 types of carbon sources (carbohydrates, amino acids, polymers, amines, carboxylic acid, and phenolic acids) as described by Cai et al. (2021). The following formulas were used to calculate the AWCD and diversity indices [Shannon index (*H*) and McIntosh index (*U*)]:

$$\mathrm{AWCD} = \sum (\mathrm{C_i} - \mathrm{R})/31;$$
$$\mathrm{H} = -\sum \mathrm{P_i} \ln \mathrm{P_i};\ \ \mathrm{McIntosh;}$$
$$\mathrm{U} = \sqrt{\sum \mathrm{n_i^2}}$$

Here *Ci* represents the absorbance value of the *i*-th carbon source, *R* represents the absorbance value with water holes, *Pi* represents the comparison between *ni* and the sum of the relative absorbance values of the entire plate, and ni represents the relative absorbance value of the *i*-th hole.

Assessment of tobacco plant dry matter

The dry matter content of tobacco plant parts (roots, stem, and leaves) was determined in each treatment at the end of the experiment. Briefly, 5 tobacco plants per treatment were uprooted, washed to remove extra soil, and air-dried naturally in the shade. The roots, stems, and leaves were separated and incubated at 105 °C for 30 min and then dried to constant weight at 80 °C for 48 h to measure the contents of the dry matter.

Statistical analysis

Data were statistically analyzed using analysis of variances in Microsoft ExcelTM (2013) and SPSS version 22.0 (SPSS Inc., Chicago, IL, USA); the means were subjected to Duncan's multiple range tests at $P \leq 0.05$. All figures were processed and analyzed using Adobe Illustrator CS5 (Adobe Systems Inc., San Francisco, CA, USA) and GraphPad_Prism (8.0.2).

Results

Isolation of pathogenic bacterial strain

The pathogen was successfully isolated from the diseased tobacco plant parts. A differentiation test was performed on the TTC medium to identify virulent and avirulent strains of *R. solanacearum*. Virulent strains produce fluid-like white color colonies with red or pink center,

while colonies produced by avirulent type were smaller, dry, and non-fluidal after 48 h of incubation (Additional file 1: Fig. S1).

Pathogenicity analysis

Based on the molecular identification of the isolated strain WS-001, a pathogenicity test was performed according to Koch's postulates using the flue-cured tobacco cultivar Hongda and K326 (Additional file 1: Fig. S2). Plants of flue-cured tobacco cultivar (Hongda) treated with *R. solanacearum* WS-001 generally showed symptoms of yellowing and unilateral wilting of leaves with marginal necrosis, dark water-soaked lesions on the stem (Additional file 1: Fig. S2-i), and death of the whole plant, while control plants remained healthy. Whereas, no visible symptoms were observed on the plants of flue-cured tobacco cultivar (K326), except stunting plant growth (Additional file 1: Fig. S2-ii). The stem streaming test proved that *R. solanacearum* WS-001 was successfully colonized in the vascular tissue and produced high cell densities. A large number of milky-white bacteria oozed out from the infected stem when placed in the water, while no bacteria oozed from the healthy stem (Additional file 1: Fig. S3).

Isolation, identification, and functional traits of antagonistic bacteria

A total of 250 bacterial strains were isolated from soil collected from the rhizosphere of healthy tobacco plants. They produced rounded white-colored mucus colonies on LB medium with a rough to dry appearance (Additional file 1: Fig. S4). Among these isolated strains, 23 isolates (9.2%) showed their antibacterial activity against the *R. solanacearum* WS-001 bacterial strain at 1×10^7 CFU/mL on LB agar plates (data not shown). In the second round, the antibacterial activity of these 23 isolates was screened against *R. solanacearum* WS-001 at a higher concentration of $OD_{600\ nm} \approx 0.6$ (3×10^8 CFU/mL). Fourteen (60.86%) of these 23 isolates showed strong antibacterial activity with an inhibition zone diameter of about 1.5 to 2.86 cm (Table 2). From the 14 isolates, a subset of 3 *Bacillus* strains WS-05, WS-10, and WS-25 were found to be the most superior with effective reduction of pathogen growth in the *in-vitro* antibacterial assay and were retained for further study (Fig. 1).

Table 2 Antagonistic effect of selected *Bacillus* sp. isolates against tobacco bacterial wilt pathogen *Ralstonia solanacearum* WS-001

Isolated strains	Inhibition zone diameter (cm)	Colony diameter (cm)	Growth inhibition ratio (%)[A]
WS-10	2.86 ± 0.04	0.7 ± 0.057	75.57 ± 0.644a
WS-25	2.26 ± 0.03	0.9 ± 0.068	57.54 ± 1.164b
WS-05	2.89 ± 0.05	1.33 ± 0.044	51.30 ± 1.380cd
WS-18	2.54 ± 0.06	1.23 ± 0.057	55.20 ± 0.557bc
WS-61	2.82 ± 0.03	1.42 ± 0.068	49.63 ± 1.170cde
WS-115	2.16 ± 0.04	1.14 ± 0.093	47.10 ± 2.364def
WS-47	2.56 ± 0.04	1.28 ± 0.068	50.00 ± 0.771cde
WS-133	1.84 ± 0.05	1.00 ± 0.100	45.64 ± 1.883def
WS-96	2.68 ± 0.11	1.44 ± 0.093	45.62 ± 3.102def
WS-117	2.32 ± 0.09	1.28 ± 0.106	44.77 ± 0.542efg
WS-87	1.8 ± 0.06	1.06 ± 0.073	40.81 ± 2.151fgh
WS-151	2.32 ± 0.08	1.44 ± 0.093	37.67 ± 1.996h
WS-196	1.6 ± 0.02	0.98 ± 0.089	38.82 ± 2.262gh
06	2.56 ± 0.07	1.64 ± 0.093	35.36 ± 2.435h

Significance difference ($P < 0.05$) between growth inhibitions ratios (%) of different isolated strains are indicated by different small letters within a column according to Duncan's multiple range test at $P < 0.05$ of five replicates (± SEM)

[A] Growth inhibition ratio (%) = [(Inhibition zone diameter − Colony diameter)/Inhibition zone diameter] × 100

Assessment of biocontrol effect of candidate *Bacillus* strains on tobacco bacterial wilt

A pot experiment was conducted to evaluate the efficacy of biocontrol agents (WS-05, WS-10, and WS-25) against *R. solanacearum* WS-001 (Fig. 2). Bacterial wilt symptoms were observed 3 days after the inoculation of pathogenic strain WS-001, and the whole plant died after 4 weeks of inoculation (Fig. 2-i; CK). However, a significant difference was observed in the biocontrol efficacy of each isolated strain WS-05, WS-10, and WS-25 compared with control (Fig. 2-i; A–C). Furthermore, plants inoculated with WS-10 showed minimum DI and maximum PV compared with plants inoculated with WS-05 and WS-25 (Fig. 2-ii). Therefore, WS-10 was selected as a potential biocontrol agent against tobacco bacterial wilt disease for further study.

Antifungal activity

Results of the in-vivo assay revealed WS-10 as a potential biocontrol agent compared with other isolated strains WS-05 and WS-25 ("Assessment of biocontrol effect of candidate *Bacillus* strains on tobacco bacterial wilt" section). Then the antifungal activity of selected strain WS-10 was checked against many pathogenic fungi such as; *Fusarium oxysporum* caused root rot of *Panax notoginseng*, *F. graminearum* caused *Fusarium* head blight in wheat and barley, *F. oxysporum* caused *Fusarium* wilt of *Dendrobium chrysotoxum*, and *Colletotrichum capsici* caused pepper anthracnose (Fig. 3).

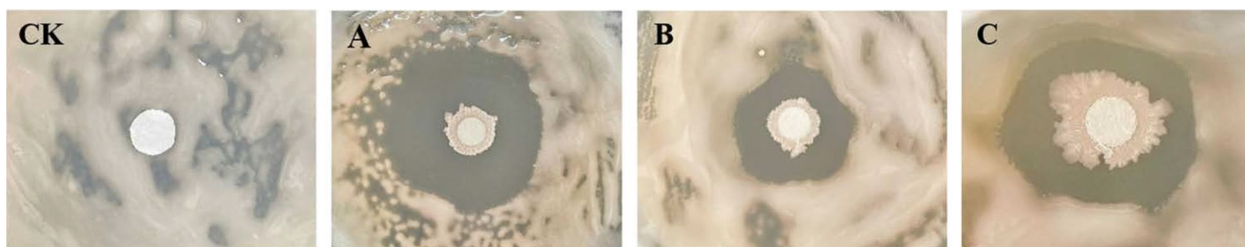
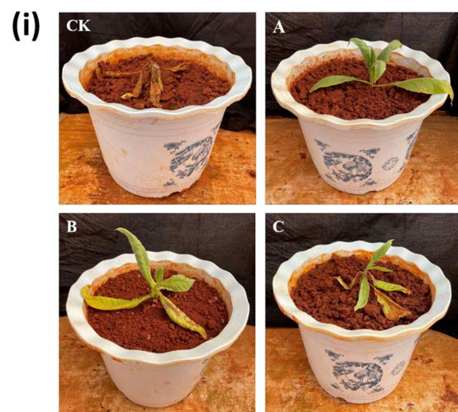


Fig. 1 Antibacterial activity of three *Bacillus* isolates against bacterial wilt pathogen *Ralstonia solanacearum* WS-001 isolated from rhizosphere soil of healthy tobacco plants. Here: **(CK)** Distilled water as control. **A** *Bacillus* strain WS-10. **B** *Bacillus* strain WS-25. **C** *Bacillus* strain WS-05



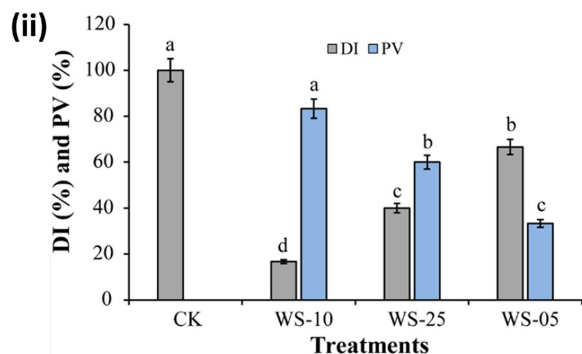


Fig. 2 Biocontrol efficacy of candidate *Bacillus* strains on tobacco bacterial wilt disease. (**i**); Symptoms produced on tobacco plants inoculated with bacterial wilt pathogen *Ralstonia solanacearum* WS-001 alone (CK) and in combination with each isolated strain WS-10 (**A**), WS-25 (**B**), and WS-05 (**C**). (**ii**); Disease incidence (%) and protective value (%). According to Duncan's multiple range test, the significant difference among the treatments is shown by different small letters on the error bars, at $P < 0.05$

Molecular characterization and identification of pathogenic and biocontrol bacterial strains

Multilocus sequence analysis was performed for 3 housekeeping genes 16S *rRNA* (1465 bp), *egl* (850 bp), and *mutS* (750 bp) of pathogenic strain WS-001 and 16S *rRNA* (1453 bp), *rpoB* (1089 bp), and *gyrB* (1173 bp) of biocontrol strain WS-10 for the high-resolution phylogenetic relationship of species within a genus. MLSA for 3 housekeeping genes revealed that isolated strains WS-001 and WS-10 were identified as *R. solanacearum* (Accession No. MW730714) and *B. amyloliquefaciens* (Accession No. MW730713) with 99% similarity (Fig. 4 and Additional file 1: Fig. S5).

Characterization of plant-beneficial traits of WS-10 *in-vitro* assay

Several traits of *B. amyloliquefaciens* WS-10, as plant growth promoters, were tested in the *in-vitro* assay (Fig. 5). After 7 days of incubation at 28 °C, visible dissolution and clear halos were formed around WS-10 colonies grown on NF (Fig. 5A), PVK (Fig. 5B), and PF (Fig. 5C) solid medium, which indicated that WS-10 had the ability to utilize air-nitrogen and decompose rock phosphate and potassium. Salkowski reagent produced the orange-red color, which indicated the biosynthesizes of Indole-3-acetic acid (Additional file 1: Fig. S6).

In-vitro co-culture assay

In-vitro co-culture test was performed for isolated strain WS-10 to evaluate the growth suppression ability against *R. solanacearum* WS-001 in LB and TTC medium, respectively. The colony count method was adopted based on their distinct colony morphologies WS-10 (rounded white-colored mucus) and *R. solanacearum* WS-001 (white-colored colonies with a red center) to quantify the growth of each isolate. This study revealed that the population dynamic of strain WS-10 was recorded 10^8 CFU/mL in co-culture assay compared with *R. solanacearum* WS-001 (10^3 CFU/mL). In contrast, strain WS-001 showed 10^{10} CFU/mL when cultured alone (Additional file 1: Fig. S7).

Soil physicochemical characteristics

This study revealed that compared with control (application of WS-001), the application of biocontrol *Bacillus*

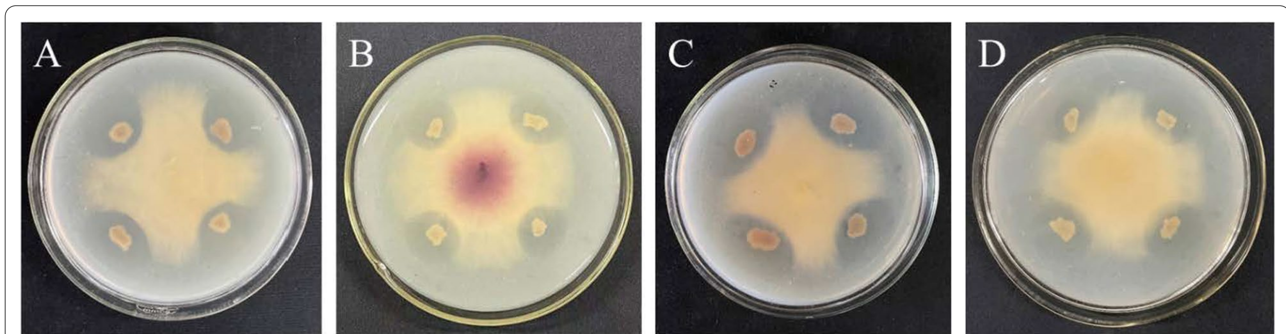


Fig. 3 Antifungal activity of *Bacillus amyloliquefaciens* WS-10 against different pathogenic fungi. *Fusarium oxysporum* (**A**), *F.* graminearum (**B**), *F. oxysporum* (**C**), and *Colletotrichum capsica* (**D**)

A

42
38
97
56
28
55
98
48
30
26

Ralstonia solanacearum strain YS-Y (MN508408)
Ralstonia solanacearum strain black heart (KX785160)
Ralstonia solanacearum strain Dong Thap 9 (MK041548)
WS-001
Ralstonia pseudosolanacearum strain RS 476 (CP021762)
Ralstonia syzygii R24 (FR854086)
Ralstonia syzygii subsp. celebesensis strain UGMSS Db01 (CP068285)
Ralstonia pickettii (AB767276)
Ralstonia pickettii DTP0602 (CP006668)
Ralstonia mannitolilytica strain Guangzhou-RMAB10 (CP049133)
Ralstonia mannitolilytica strain SN83A39 (CP011258)
Erwinia carotovora subsp. atroseptica SCRI1043 (BX950851)
Plasmodiophora brassicae (AB037917)

B

42
56
92
92
93
100
88
100
93

Bacillus amyloliquefaciens strain B.amy SS1 (MT226660)
WS-10
Bacillus amyloliquefaciens strain B81 (MW776614)
Bacillus amyloliquefaciens FZB42 (NC009725)
Bacillus megaterium strain BF245 (MK491077)
Bacillus megaterium strain SR81 (MT158522)
Bacillus pumilus strain FSL h8519 (EU147230)
Bacillus pumilus strain FSL h8467 (EU147211)
Bacillus subtilis strain ATCC 19217 (CP009749)
Bacillus velezensis strain UCMB5007 (CP041143)
Dickeya dadantii strain BAC5003-4 (MH045065)
Burkholderia glumae strain R95 (MT765058)

Fig. 4 Neighbor-Joining phylogenetic tree based on 16S *rRNA* **A** and **B** genes sequences of WS-001 and WS-10 showing the phylogenetic relationships between *Ralstonia solanacearum* and *Bacillus amyloliquefaciens*, respectively. Bootstrap values of 1000 replications are shown next to the branches. The accession numbers of the NCBI sequences are indicated in parentheses

strain WS-10 significantly affects the soil physicochemical properties associated with tobacco plants (Table 3). A significant difference was observed in the availability of soil pH, organic matter, total nitrogen, and available

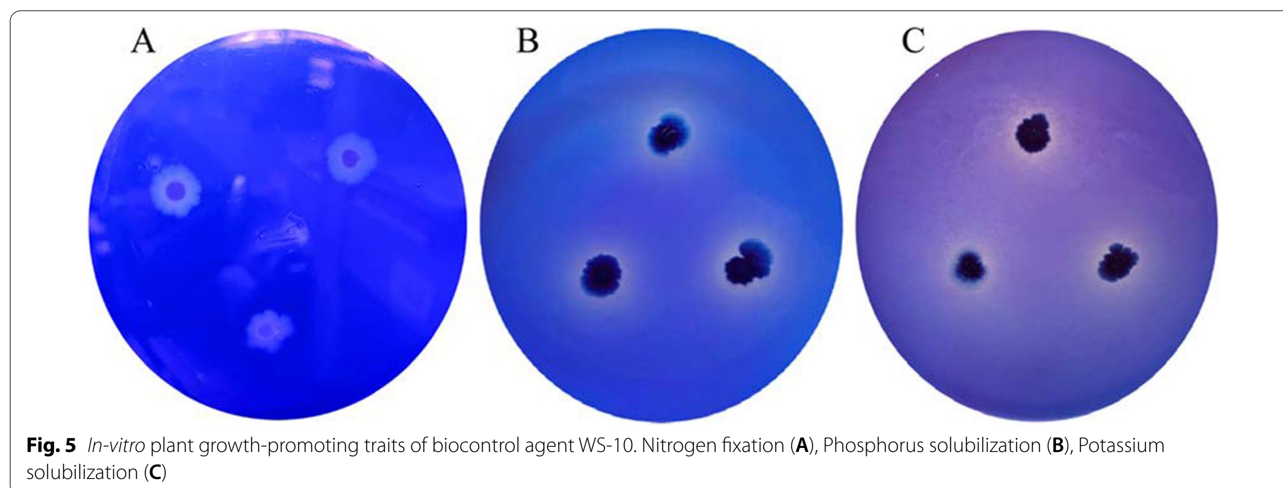


Fig. 5 *In-vitro* plant growth-promoting traits of biocontrol agent WS-10. Nitrogen fixation (**A**), Phosphorus solubilization (**B**), Potassium solubilization (**C**)

Table 3 Analysis of soil physicochemical properties

Items	pH	OM (g/kg)	T.N (mg/kg)	A.P (mg/kg)	A.K (mg/kg)
CK	6.63 ± 0.06c	15.3 ± 0.26c	2.47 ± 0.30c	16.3 ± 0.88d	115.35 ± 1.24c
T1	6.89 ± 0.02b	17.2 ± 0.07b	3.14 ± 0.15a	26.9 ± 1.90a	128.15 ± 4.65a
T2	6.82 ± 0.06bc	16.9 ± 0.41bc	2.95 ± 0.27b	19.7 ± 0.43c	121.86 ± 3.84b
T3	7.31 ± 0.12a	19.9 ± 0.14a	3.21 ± 0.19a	24.1 ± 1.75b	129.18 ± 4.21a

Here *OM* organic matter, *TN* total nitrogen, *AP* available phosphorus, *AK* available potassium

Different letters in the same column meant significant differences among treatments according to Duncan's multiple range test at $P < 0.05$

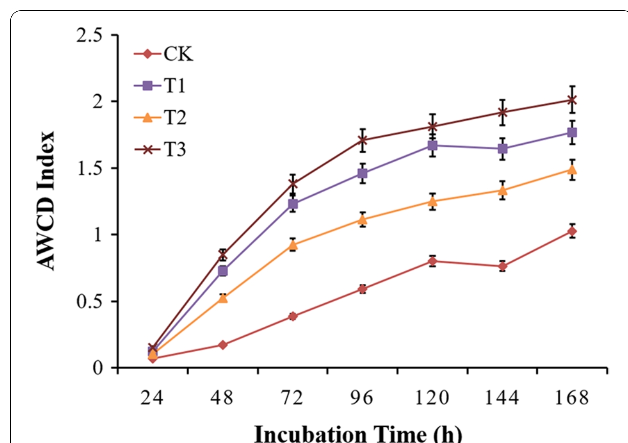


Fig. 6 Average well color development (index) of soil microbial diversity under different treatments after particular hours of incubation. Significant difference among the treatments is shown by Duncan's multiple range test at $P < 0.05$

phosphorus and potassium contents in the soil treated with WS-10 compared with control.

Average well color development of rhizospheric microbial community

This study showed that the AWCD of the rhizospheric microbial community increased with the application of *Bacillus* strain WS-10. A significant difference was observed in the AWCD of the rhizospheric microbial community treated with WS-10 compared with control (Fig. 6). The AWCD of T3 was found to be the highest than T1, T2, and CK; however, a non-significant difference was observed among the AWCD values of T1 and T3.

Utilization ability of six types of carbon sources by rhizospheric microbial community

The metabolic activity of the rhizospheric microbial community was determined according to differences in the utilization ability of 6 types of carbon sources in Biolog EcoPlate™. This study showed that the utilization ability of six types of carbon sources (Carbohydrates, Amino acids, Polymers, Amines, Carboxylic acid, and Phenolic acids) increased in all treatments treated with biocontrol *Bacillus* strain WS-10 than control (Table 4). However, the utilization ability of T1 and T2 remained almost the same, and a non-significant difference was observed between them.

Table 4 Utilization of six types of carbon sources under different treatments

Items	CH	AA	PM	AM	CA	PA
CK	1.21 ± 0.01c	1.45 ± 0.02c	0.89 ± 0.04c	1.15 ± 0.05c	1.26 ± 0.01c	1.33 ± 0.01c
T1	1.71 ± 0.02a	2.75 ± 0.16a	1.36 ± 0.03a	1.44 ± 0.14a	1.52 ± 0.10a	1.85 ± 0.09a
T2	1.56 ± 0.02b	2.45 ± 0.02b	1.23 ± 0.05b	1.27 ± 0.15b	1.39 ± 0.07bc	1.69 ± 0.04b
T3	1.80 ± 0.18a	2.78 ± 0.15a	1.39 ± 0.01a	1.48 ± 0.23a	1.42 ± 0.12b	1.81 ± 0.09a

Here *CH* carbohydrates, *AA* amino acids, *PM* polymers, *AM* amines, *CA* carboxylic acid, *PA* phenolic acids

According to Duncan's multiple range test, different small letters in the same column show significant differences among treatments at $P < 0.05$

Table 5 Diversity index of soil microbial communities under different treatments

Items	Shannon index (*H*)	McIntoch index (*U*)
CK	2.86 ± 0.01c	6.89 ± 0.01c
T1	3.24 ± 0.03b	7.51 ± 0.39b
T2	3.19 ± 0.02b	7.45 ± 0.45b
T3	3.39 ± 0.02a	8.87 ± 0.42a

Different small letters in the same column meant significant differences among treatments according to Duncan's multiple range test at $P < 0.05$

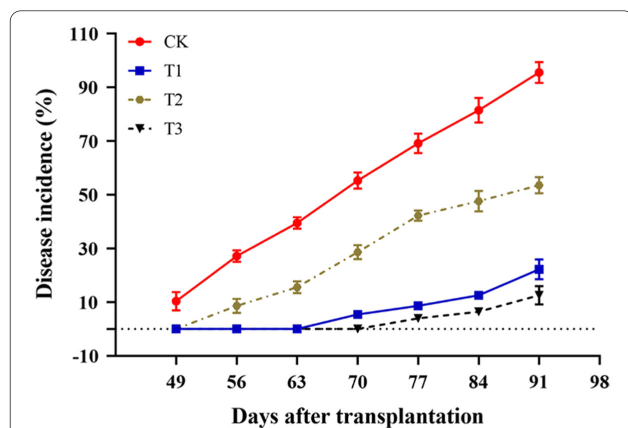


Fig. 7 Effect of biocontrol agent WS-10 on the disease incidence in a greenhouse experiment. Significance differences among the treatments are shown according to Duncan's multiple range test ($P < 0.05$) after particular days of transplantation

Functional diversity of the rhizospheric microbial community

The functional diversity of the rhizospheric microbial community significantly increased in all treatments treated with biocontrol *Bacillus* strain WS-10 compared with control (Table 5). The species evenness index (McIntosh index) and species richness index (Shannon index) were increased in all treatments after the application of WS-10 than control. However, a non-significant difference was observed between T1 and T2.

Biocontrol efficacy of *Bacillus* strain WS-10 against tobacco bacterial wilt disease

The result revealed that *Bacillus* strain WS-10 effectively reduce the tobacco bacterial wilt disease (Fig. 7). Tobacco bacterial wilt symptoms were first observed after 49 days (at the vigorous growing stage) of transplantation in control, whereas they emerged in treatments T1, T2, and T3 after 70 days, 56 days, and 77 days of transplantation, respectively. These results indicated that the biocontrol strain WS-10 delayed the development of tobacco bacterial wilt disease by 7 to 28 days. Significant differences ($P < 0.05$) in the occurrence of tobacco bacterial wilt disease between the treatments treated with *Bacillus* strain WS-10 and control were observed after 49, 56, 63, 70, 77, 84, and 91 days of transplantation. At the end of the experiment, it was observed that the application of antibacterial strain WS-10 successfully reduced the incidence

Table 6 Control effect of bacillus strain WS-10 on the occurrence of tobacco bacterial wilt disease

Items	Time (days)						
	49	56	63	70	77	84	91
CK	0.00 ± 0.00b	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d
T1	100 ± 0.00a	100 ± 0.00a	100 ± 0.00a	88.3 ± 0.92b	87.49 ± 0.76b	84.55 ± 0.56b	73.36 ± 0.47b
T2	100 ± 0.00a	68.45 ± 3.62b	60.72 ± 1.85b	54.54 ± 3.47c	38.91 ± 0.46c	41.30 ± 3.23c	43.82 ± 2.19c
T3	100 ± 0.00a	100 ± 0.00a	100 ± 0.00a	100 ± 0.00a	94.25 ± 0.84a	92.12 ± 0.61a	86.82 ± 1.68a

Different small letters in the same column meant significant difference among the treatments at $P < 0.05$ according to Duncan's Multiple Range test after the particulate days of transplantation. Protective value = $[(DI_{ck} - D_{it})/DIck] \times 100$. Here; DI_{ck}; disease index of control group, D_{it}; disease index of treatment. Data is extended from a subset of Fig. 7

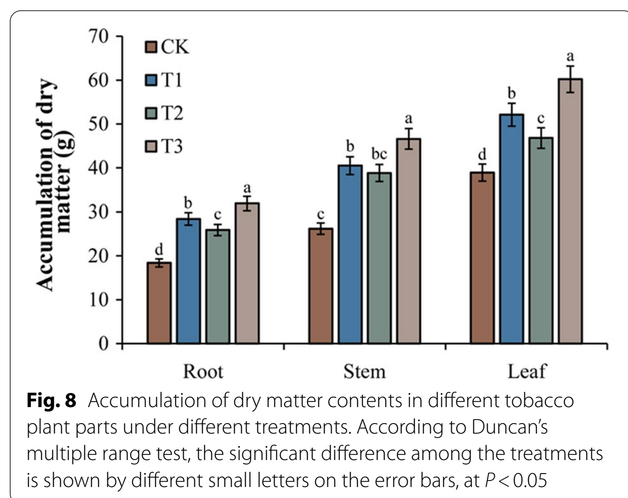


Fig. 8 Accumulation of dry matter contents in different tobacco plant parts under different treatments. According to Duncan's multiple range test, the significant difference among the treatments is shown by different small letters on the error bars, at $P < 0.05$

of tobacco bacterial wilt disease by 73.36, 43.82, and 86.82% in T1, T2, and T3, respectively (Table 6).

Accumulation of dry matter contents

This study revealed that the accumulation of dry matter contents in different parts (roots, stems, and leaves) of tobacco plants increased in all treatments after the application of *Bacillus* strain WS-10 than the control (Fig. 8). These results indicated that WS-10 had a strong plant growth promoter ability and increased the dry matter contents of tobacco plants.

Discussion

Tobacco bacterial wilt caused by *R. solanacearum* is one of the most devastating tobacco diseases resulting in unprecedented loss worldwide. The notoriously damaging pathogen persists in soil for a longer time, making disease management nearly an unachievable goal (Yuan et al. 2016). Currently, antimicrobial compounds derived from bacteria and chemical pesticides (e.g., zinc thiazole, bismerthiazol, and saisentong) are the only measures used to control the disease, but they are ineffective (Chen et al. 2020). Because the improper use of agrochemicals posed a significant risk to the environment, public health, and evolved pathogen resistance. Thus, it is necessary to shift from chemical-based treatments to nature-based cures, where biocontrol offers an environmentally friendly alternative for more promising disease management (Ahmed et al. 2022).

Plant growth-promoting rhizobacteria improve plant health and represent a great source of biocontrol against various phytopathogens. *Pseudomonas* sp., *Bacillus* sp., *Streptomyces* sp., and other bacterial species have shown efficient biocontrol against bacterial wilt (Zhuo et al. 2019). In the present study, a total of 250 strains were isolated from the rhizosphere of healthy tobacco plants, out of which 14 isolates showed significant antibacterial activity against *R. solanacearum* (WS-001) in a dual culture assay. Among the 14 isolates, 3 isolates WS-05, WS-10, and WS-25 were found to be superior strains as they significantly inhibited the growth of pathogenic strain WS-001 in the *in-vitro* antibacterial assay and were selected as candidate strains for initial evaluation of biocontrol efficiency. The percentage representing 1.2% of the 250 isolated strains is consistent with the reported percentage (0.1–1%) of antibiotic-producing *Bacillus* spp. in nature (He et al. 2021). Among the 3 isolated strains, only WS-10 was selected and retained as a potential biocontrol strain because of its efficient protective value (>80%) against tobacco bacterial wilt disease in the pot experiment. Further, antifungal activity results revealed that WS-10 inhibited the growth of different pathogenic fungi in *in-vitro* conditions, which makes it a strong antagonistic agent with dual antimicrobial characteristics. The molecular identification based on 16S *rRNA*, *rpoB*, and *gyrB* revealed that WS-10 belongs to *Bacillus amyloliquefaciens.*

Many previous studies have reported that the application of biocontrol agents (PGPR) in the soil could reduce the incidence of soilborne diseases, improve yields, and biomass in many plants (Liu et al. 2013). Obtained results are similar to the above findings; biocontrol agents WS-10 promote the tobacco plant's growth and dry matter contents in different parts (roots, stem, and leaves) of tobacco plants. Growth promotion in tobacco plants relates to the *in-vitro* production of IAA (ug/mL) (one of the pivotal phytohormones involved in cell expansion, division, and differentiation), nitrogen fixation, and potassium and phosphate solubilization. Similarly, the application of a biocontrol agent (WS-10) improved the soil physicochemical properties and functional diversity of rhizosphere microorganisms, which directly promotes plant growth and enhances plant resistance to disease; and obtained results are similar to the findings of Tang et al. (2020).

The biocontrol aspect of plant growth-promoting rhizobacteria (PGPR) has recently gained attention for sustainable agriculture. In the present study, WS-10 performed dual roles as a PGPR and a biocontrol agent against the bacterial wilt pathogen. It is evident from the experiment conducted under greenhouse conditions that WS-10 remarkably reduced the disease incidence of WS-001 in tobacco plants. Corroborating the *in-vitro* antagonism between WS-10 and WS-001 as shown in co-culture assays, WS-10 could directly antagonize pathogens by producing antimicrobial compounds. *Bacillus* sp.

is known to produce a plethora of bioactive compounds that directly inhibit plant pathogens. Especially, a large part of their genome encodes several bioactive compounds such as antibiotics, enzymes, and volatile compounds (Mülner et al. 2020). It is reported that, like other phytopathogenic bacteria, *R. solanacearum* also required an entry site such as wounds to develop a systemic infection (Denny 2007). Obtained results of 2 different greenhouse assays demonstrated that when *R. solanacearum* was inoculated with root injury technique, the development of systemic infection was quick compared with the root drenching method (without causing an injury).

In addition to directly inhibiting pathogens, rhizobacteria also activate the plant's immune system, a process termed induced systemic resistance or priming (Vannier et al. 2019). In the present study, when WS-10 treatment was applied to a plant before pathogen inoculation, the disease incidence was lowest (86.82%) than when both were applied at the same time or when the pathogen was applied before WS-10. It can be speculated that WS-10 induced resistance in tobacco when applied before the pathogen and activated plant defense responses so that the plant could limit the disease infection; thus, the disease incidence was lowest. On the other hand, when both WS-10 and pathogen were applied simultaneously (T1), the disease incidence was lower than when the pathogen was applied before WS-10 (T2). The disease incidence was higher in T2 because the pathogen had probably already developed an infection in tobacco plants. However still, WS-10 was able to reduce the disease incidence by up to 43.82%. Thus, it was concluded that the application of WS-10 before pathogen attack proved more beneficial for plant growth and developed resistance in plants against the pathogen. The present results are in accordance with previous studies, that advanced application *B. amyloliquefaciens* YN201732 exhibited a better protective effect than therapeutic effect against tobacco powdery mildew (Jiao et al. 2020).

Soil microbial diversity plays an important role in the long-term sustainability of the soil ecosystem. Rich soil biodiversity can stabilize the soil ecosystem and enhance the function and biological activity of the soil (Chaer et al. 2009). In this study, analysis of diversity indices, AWCD, and utilization ability of carbon sources proved that the application of biocontrol agent WS-10 significantly improved the functional diversity and biological activity of the rhizosphere microbial community. Obtained results are similar to the findings of Tang et al. (2020), who proved that the application of YH-07 effectively suppresses the incidence of tomato *Fusarium* wilt by improving the soil functional of soil microorganisms.

Conclusions

Bacillus amyloliquefaciens WS-10 isolated from the rhizosphere of healthy tobacco plants showed remarkable PGP and biocontrol properties. Improve the soil physicochemical properties and functional diversity of the soil microbial community, makes it a valuable tool for flue-cured tobacco cultivation. Furthermore, an in-*planta* study validated the biofertilizer and biocontrol potential of WS-10, whereas *in-vitro* antagonism showed a positive correlation with *in-vivo* disease suppression. Also, WS-10 performed more efficiently when applied earlier than pathogen attack, as prevention is better than cure. Thus, the study builds a foundation to develop WS-10 based bio-product with dual functions as both growth promoters and biocontrol agents for the tobacco plant. However, the actual mechanism underlying this intermingled interaction among plants, rhizobacteria, and pathogen warrants in-depth research. Further studies will focus on enhancing the control effect of *B. amyloliquefaciens* WS-10 on tobacco bacterial wilt disease through the extraction of antimicrobial compounds and by exploring the effect of *B. amyloliquefaciens* WS-10 on microbial diversity through high-throughput sequencing to understand the underlying biocontrol mechanism better.

Abbreviations

R. solanacearum: *Ralstonia solanacearum*; AWCD: Average well color development; TTC: Kelman's tetrazolium chloride; CPG: Casamino acid-peptone-glucose; LB: Luria–Bertani; CFU: Colony forming units; CK: Control; IAA: Indole-3-acetic acid; MLSA: Multilocus sequence analysis; NF: Nitrogen-free Ashby medium; DI: Disease incidence; PV: Protective value; PGPR: Plant growth-promoting rhizobacteria; PGP: Plant growth-promoter; PF: Potassium feldspar; PVK: Pikovskaya medium.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-022-00527-5.

Additional file 1. Table S1. Amount of fertilizer applied per plant. **Fig. S1**. Colony morphology of isolated bacterial strain Ralstonia solanacearum WS-001 on TTC medium. **Fig. S2**. Symptoms produced by WS-001on tobacco plant. **Fig. S3**. Stem streaming test. **Fig. S4**. Colony morphology of WS-10 on LB medium plates. **Fig. S5**. Phylogenetic tree constructed for molecular identification of WS-001 and WS-10. **Fig. S6**. Indole-3-acetic acid production ability of biocontrol agent WS-10. **Fig. S7**. Co-culture assay.

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Not applicable.

Authors' contributions

ZZ and GJ conceived and designed the experiments. WA, GH, JY, and QL performed the experiments. WA, JY, and QL analyzed the data. WA, SM, and AA wrote the manuscript. All authors contributed to the final draft of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
This material is the author's own original work, which has not been previously published elsewhere and has no conflict of interest.

Declarations

Ethical approval and consent to participate
The paper reflects the author's own research and analysis in a truthful and complete manner. All authors have been personally and actively involved in substantial work leading to the paper and contributed to preparing the final draft of the manuscript and will take public responsibility for its content.

Consent for publication
The manuscript has not been published in whole or in part elsewhere and is not currently being considered for publication in another journal. All the authors have seen the final version of the manuscript.

Competing interests
The authors declared that they have no conflict of interest.

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