



Growth promotion and alleviation of *Ralstonia pseudosolanacearum*-induced wilt symptoms in tomato (*Solanum lycopersicum*) by *Streptomyces nigr*

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Abstract

Bacteria of the *Streptomyces* genus colonize diverse ecological niches and can exhibit diverse traits of potential applied value. With regards to crop production, some species can induce plant diseases while others are capable of promoting plant growth or controlling diseases. To identify Taiwanese *Streptomyces* strains suitable for crop growth promotion and disease management, this study obtained 49 potato-isolated, *Streptomyces*-like strains from a culture collection and screened for bacteria showing promise for stimulating plant growth and controlling soil-borne phytopathogenic bacteria. The tested strains were examined for their effects on radish seedling growth, and strains exhibiting detrimental effects were screened out. Antimicrobial activities of the remaining strains against the soil-borne phytopathogenic bacteria *Ralstonia pseudosolanacearum* and *S. scabiei* were determined with agar diffusion tests, and only 8 strains were found capable of inhibiting both pathogens. Results from detection of genes associated with pathogenicity (*txtAB*, *tomA* and *necI*) and multilocus sequence analysis (MLSA) indicated that all 8 strains were unlikely to harm plants. The strains' ability to promote plant growth were examined using tomato plants in pot culture assays, and after two trials, strain 94-b, identified as *S. nigr*, exhibited the most consistent plant growth promotion activity. Strain 94-b also significantly reduced the severity of symptoms caused by *R. pseudosolanacearum* on tomato in a biocontrol assay. Further testing revealed that the strain produced amylase, lipase, siderophore, indole-3-acetic acid (IAA), and solubilized phosphate. This is the first report of *S. nigr* capable of both improving plant growth and controlling a soil-borne bacterial disease.

Keywords Bacterial wilt · Biological control · Plant growth-promoting bacteria · Thaxtomin

Introduction

Streptomyces species are Gram-positive, filamentous bacteria that are ubiquitous in the environment (Alam et al. 2022). These bacteria can produce a wide range of extracellular enzymes and metabolites that allow them to thrive in diverse niches, including those in agroecological systems (Alam et al. 2022; Dow et al. 2023; Viaene et al. 2016). Studies have shown that streptomycetes can interact with surrounding plants and microbes, leading to repercussions in crop production (Kinkel et al. 2012; Viaene et al. 2016).

Many streptomycetes can facilitate plant growth and are designated as plant growth-promoting bacteria (PGPB; Omar et al. 2022; Timofeeva et al. 2022). Mechanisms contributing to growth promotion activity include the production of phytohormones like indole-3-acetic acid (IAA; Myo et al. 2019), solubilization of phosphate, and secretion of iron-sequestering siderophores (Omar et al. 2022; Timofeeva et al. 2022). Studies have also suggested that extracellular enzymes produced by *Streptomyces* strains (such as amylase and lipase) could be associated with plant growth promotion (Sousa et al. 2008). Capabilities in producing extracellular enzymes can be assessed using media including different individual substrates (Praveen Kumar et al. 2015). Other than facilitating plant growth, *Streptomyces* species have also been exploited for controlling plant pathogens (Kumar et al. 2022; Vurukonda et al. 2018). Some streptomycetes produce enzymes or metabolites that

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can either directly inhibit plant pathogens or suppress their growth by limiting nutrient sources (Kumar et al. 2022; Vurukonda et al. 2018). To identify strains capable of managing plant diseases, studies typically conducted agar-based in vitro antagonism tests, followed by in vivo plant assays (Ling et al. 2020).

Crops can be exposed to many stressors during cultivation (Gonzalez Guzman et al. 2022; Shi et al. 2023). Among them, soil-borne pathogenic bacteria are considered some of the most important limiting factors of crop production (Shi et al. 2023). For instance, members of the *Ralstonia solanacearum* species complex (RSSC; *R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii*) can invade plant vascular tissues and induce severe wilting in diverse plant species, causing annual global crop losses of approximately USD 950 million (Ahmed et al. 2022; Shi et al. 2023). When managing soil-borne pathogens like RSSC, the effectiveness of individual disease control measures is often limited (Dickstein et al. 2024). Specifically, efficacies of chemical control have been shown to be variable (Dickstein et al. 2024; Locci 1994), and the selection for resistant populations in the field can further compromise its effectiveness (Liu et al. 2014). Various cultural practices can sometimes reduce pathogen loads in the soil (Dickstein et al. 2024), but soil-borne pathogens may also behave as saprophytes and survive for a long time in the field (Genin and Boucher 2004), making them difficult to eradicate. Additionally, the broad host range and genetic diversity of some pathogens (Dickstein et al. 2024) may further complicate disease management strategies. Therefore, there is constant need for alternative measures for controlling soil-borne bacterial diseases.

The diversity of physiological characteristics among streptomycetes makes them promising for exploration of plant growth-promoting strains that can also alleviate plant diseases (Le et al. 2021; Pang et al. 2022). However, when considering streptomycetes for such applications, it is essential to distinguish beneficial species/strains from those that can produce phytotoxins and induce diseases. For example, *S. scabiei*, one of the most well-characterized phytopathogenic streptomycetes, produces a cellulose-biosynthesis inhibitor known as thaxtomin A (Li et al. 2019), and causes scab symptoms on potato tubers (*Solanum tuberosum* L.). Previous studies showed that thaxtomin A induces stunting and swelling of hypocotyls and roots in seedlings of various plant species (Li et al. 2019; Loria et al. 1997). Bioassays using radish seedlings (*Raphanus sativus* L.) is an effective method for identifying strains capable of producing thaxtomin-like phytotoxins (Leiner et al. 1996). Detection of pathogenicity-associated genes such as *txtAB*, *tomA* and *nec1* is also a common strategy for assessing the pathogenicity of *Streptomyces* strains towards plants (Bukhalid et

al. 1998; Wanner 2006). In general, strains lacking these genes were assumed to be non-pathogenic, with the absence of *txtAB* being particularly indicative (Wanner 2006). Another approach for identifying phytopathogenic streptomycetes is through molecular identification; analysis of sequences conserved within the genus has been used to determine whether a strain belonged to one of the known phytopathogenic species (Henao et al. 2022). Multilocus sequence analysis schemes (MLSA) previously developed for species identification within the *Streptomyces* genus (Guo et al. 2008) could be used for such purposes. By avoiding potential pathogenic strains and screening for beneficial traits, it is likely that more promising streptomycetes could be obtained from the environment.

In a previous study on the genetic and phenotypic diversity of streptomycetes causing potato scab in Taiwan, strains morphologically similar to streptomycetes were isolated from surfaces of potato tubers collected across different counties and cities (Wu 2021). Among them, only a portion of the strains were identified as potential plant pathogens, and the remaining strains were assumed to be non-pathogenic (Wu 2021). Preliminary radish seedling bioassays showed that these potentially harmless strains did not exhibit significant negative effects on the seedlings' hypocotyl growth (Wu 2021). However, other characteristics of these strains, such as their effects on root growth, and their ability to antagonize plant pathogens, have not yet been evaluated. The aim of this study was to determine whether any of these strains could be used for crop growth promotion and disease control. A total of 49 strains were screened using radish seedling bioassays and agar diffusion tests, and the selected strains were further subjected to pathogenicity-associated gene detection and phylogenetic analysis. Plant growth promotion tests and biological control assay using tomato (*Solanum lycopersicum* L.) were also conducted.

Materials and methods

Source and culturing of bacterial strains

All *Streptomyces*-like strains screened in this study belonged to a culture collection preserved in National Chung Hsing University, Taiwan. The collection was established in a previous study (Wu 2021). Forty-nine *Streptomyces*-like strains were tested, all of which were isolated from skin/surface samples of potato tubers collected in Taiwan (Table 1). Unless otherwise stated, all strains were cultured on International *Streptomyces* Project medium 4 (ISP4 medium; Shirling and Gottlieb 1966) for 7 days at 28 °C prior to experimental testing.

Table 1 Information on the *Streptomyces*-like strains used in this study

Collection sites	Potato cultivar	Strain name(s) ¹
Xiaying, Tainan	Kennebec	1-b, 4-a, 4-b, 7-a, 7-b, 10-a, 14-a, 14-b, 16-a, 22-a, 26-a
Taibao, Chiayi	Kennebec	11-b, 12-a, 13-b, 20-a, 24-a
Xikou, Chiayi	Kennebec	30-a, 30-b, 31-a
Taibao, Chiayi	Kennebec	43-a, 43-c, 54-2a, 55-b
Xingang, Chiayi	Tainong No.1.	65-a, 65-b
Huwei, Yunlin	Kennebec	33-a, 33-c, 40-a, 40-b, 41-a, 42-a, 50-b, 81-b, 81-b2, 81-c
Huwei, Yunlin	Unknown	36-d, 100-a
Erlun, Yunlin	Kennebec	94-a, 94-b, 94-c, 94-d
Dounan, Yunlin	Kennebec	77-a
Houli, Taichung	Kennebec	88-a, 88-b, 93-a, 0512c
Sanyi, Miaoli	Kennebec	84-b
Beinan, Taitung	Unknown	112-a, 113-a

¹the strains belonged to a culture collection preserved in National Chung Hsing University, Taiwan (Wu 2021)

In addition to the strains subjected to screening, *S. scabiei* strain 18-c and *R. pseudosolanacearum* (*R. solanacearum* phylotype I) strain PS152, isolated in previous studies (Ding 1995; Lin 2021; Wu 2021), were also included, serving either as target pathogens in agar diffusion and biological control assays, or a reference strain in an IAA production test. Throughout this study, strain 18-c was cultured on ISP4 medium plates, and strain PS152 was grown on either NA, triphenyl tetrazolium chloride (TTC) or casamino acid-peptone-glucose (CPG) medium plates (Kelman 1954), depending on the assays (described below).

Radish seedling bioassay

Because the effects of the tested *Streptomyces* strains on root growth have not been previously examined, bioassays evaluating changes in both hypocotyl and root length were conducted. The bioassays were completed as previously described (Leiner et al. 1996) with slight modifications. Specifically, one loopful of each tested bacterial strain (spores and aerial mycelium) was transferred to 5 ml of oatmeal broth (International *Streptomyces* Project medium 3; ISP3 medium; Shirling and Gottlieb 1966) and incubated at 28 °C for 5 d under 125 rpm agitation. To obtain the plant samples for the bioassays, radish seeds ‘Yongxiang’ were washed with 1% sodium hypochlorite (in 0.1% Tween 20) for 3 min, and washed twice with sterile water. The seeds were then placed on top of water agar plates and incubated at 24 °C in the dark for one day. Emerged seedlings were separately placed in a well of a 24-well plate containing 1 cm³ of 1% water agar, and 125 µl of 5-day-old liquid bacterial culture of each strain was added to each well. Each strain included 7–8 replicates. Due to the large number of strains assayed, the strains were tested in three separate batches. In addition to the tested strains, each batch also included the pathogen *S. scabiei* strain 18-c, which has been shown capable of inhibiting radish seedling growth (Wu 2021); this

strain served as a positive control. For these assays, bacteria-free oatmeal broth was used as negative controls. The 24-well plates were placed in a growth chamber set at 24 °C (12 h light period). After 3 d, the lengths of the seedlings’ hypocotyls and roots were recorded and compared using Kruskal-Wallis tests and uncorrected Dunn’s multiple-comparison tests. Treatments that resulted in shorter hypocotyl or root lengths compared to those of the negative controls (oatmeal broth only) were identified, and strains that inhibited hypocotyl or root growth were screened out. For this assay, the uncorrected *P*-value cutoff of 0.1 was adopted to avoid accidentally selecting for harmful streptomycetes reducing plant growth.

Agar diffusion assays

To identify streptomycetes exhibiting potentials for biological control, strains that did not significantly inhibit radish seedling growth were subjected to agar diffusion assays against two soil-borne bacterial pathogens, the Gram-negative bacterium *R. pseudosolanacearum* and the Gram-positive species *S. scabiei*.

In assays against *R. pseudosolanacearum*, one loopful of each tested streptomycete was separately grown in 5 ml of oatmeal broth for 5 d at 28 °C (125 rpm). The culture of each tested strain was loaded into Oxford cups (30 µl per cup) placed on top of TTC plates (Kelman 1954). TTC medium is a standard differential medium for *R. pseudosolanacearum* (Kelman 1954), and was used to maintain the typical morphology of the bacterium during the assay. Each strain included 3 replicates, and bacteria-free oatmeal broth was used for the controls. After incubation at 28 °C for 2 d, the plates were sprayed with suspensions of *R. pseudosolanacearum* strain PS152 (in 10 mM MgCl₂; OD₆₀₀=0.3; around 3.8×10⁸ CFU/ml) prepared from 2-d-old cells grown on TTC plates. Each plate received 20 sprays using a 100-ml glass atomizer containing the PS152 suspension.

After another day of incubation at 28 °C, the plates were examined for the production of inhibitory zones around the colonies of the tested streptomycetes.

For assays targeted against *S. scabiei*, the tested strains and *S. scabiei* strain 18-c were grown in 5 ml of oatmeal broth for 5 d at 28 °C (125 rpm), and 18-c's liquid culture was spread onto ISP3 medium plates (100 µl per plate). Oxford cups were subsequently placed on top of the plates and loaded with liquid culture of each tested strain (30 µl per cup). Again, every strain included 3 replicates, and sterile oatmeal broth was used for the controls. After 3 d of incubation at 28 °C, the plates were examined for the presence of inhibitory zones.

Strains that were able to inhibit both *R. pseudosolanacearum* and *S. scabiei* were selected for further testing.

Detection of pathogenicity-associated genes using polymerase chain reaction assays

Polymerase chain reaction (PCR) tests targeting pathogenicity-related genes *txtAB*, *tomA* and *nec1* (Bukhalid et al. 1998; Wanner 2006) were conducted on streptomycetes selected from previous assays using gene-specific primers (Table 2). Bacterial DNA were first extracted using the Quick-DNA Fungal/Bacterial Kit (Zymo Research, Orange, CA, USA) and examined with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For all of the assays, the reaction volume was 50 µl. Each reaction included 25 µl of GoTaq Green Master Mix (Promega, Madison, WI, USA), 0.5 µM of each primer and 15 ng of template DNA. In all of the tests, DNA sample extracted from *S. scabiei* strain 18-c was included as a positive control. The thermocycling conditions for the assays were 3 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at each primer pair's respective annealing temperature, 35 s at

72 °C, and a final extension step for 7 min at 72 °C. The annealing temperatures for assays targeting *txtAB*, *tomA* and *nec1* were 46 °C, 54 °C and 57 °C, respectively. The PCR results were analyzed using electrophoresis.

Identification of bacterial strains using multilocus sequence analysis (MLSA)

To determine the phylogenetic placement of the *Streptomyces* strains selected from previous assays and confirm whether they were associated with phytopathogenic species/strains, MLSA targeting five housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*) were conducted based on previously described methods (Guo et al. 2008). Information on the primers used are shown in Table 2. In all of the assays, each 50 µl reaction included 25 µl of GoTaq Green Master Mix, 0.4 µM of each primer and 60 ng of template DNA. The PCR conditions were 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at each primer set's respective annealing temperature (*atpD*, 63 °C; *gyrB*, 65 °C; *recA*, 60 °C; *rpoB*, 65 °C; *trpB*, 66 °C), 1.5 min at 72 °C, and a final extension step for 10 min at 72 °C. Electrophoresis was conducted and the amplified products were recovered with a Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA) and subjected to Sanger sequencing. The sequences were imported to Geneious R10 (Biomatters Inc., Auckland, New Zealand) and trimmed to intervals matching those in the “*Streptomyces* isolates database” in PubMLST (<https://pubmlst.org/organisms/streptomyces-spp>; *atpD*, 495 bp; *gyrB*, 405 bp; *recA*, 504 bp; *rpoB*, 540 bp; *trpB*, 567 bp; Jolley et al. 2018). The sequences of the five housekeeping were then concatenated with Geneious R10. The concatenated sequence of each selected strain in this work was searched against the “*Streptomyces* isolates database” (Jolley et al. 2018), which included MLSA profiles of 974 strains (using

Table 2 Primers used in this study

Target gene	Primer name	Primer sequence (5' to 3')	Reference
<i>atpD</i>	atpDPF	GTCGGCGACTTCACCAAGGGCAAGGTGTTCAACACC	(Guo et al. 2008)
	atpDPR	GTGAAGTCTTGGCGACGTGGGTGTTCTGGGACAGGAA	
<i>gyrB</i>	gyrBPF	GAGGTCGTGCTGACCGTGCTGCACGCGGGCGCAAGTTCGGC	
	gyrBPR	GTTGATGTGCTGGCCGTCGACGTCGGCGTCCGCCAT	
<i>recA</i>	recAPF	CCGCRCTCGCACAGATTGAACGSCAATTC	
	recAPR	GCSAGGTCGGGGTTGTCCTTSAGGAAGTTGCG	
<i>rpoB</i>	rpoBPF	GAGCGCATGACCACCCAGGACGTCGAGGC	
	rpoBPR	CCTCGTAGTTGTGACCCTCCCACGGCATGA	
<i>trpB</i>	trpBPF	GCGCGAGGACCTGAACCACACCGGCTCACACAAGATCAACA	
	trpBPR	TCGATGGCCGGGATGATGCCCTCGGTGCGCGACAGCAGGC	
<i>txtAB</i>	TxtAB1	CCACCAGGACCTGCTCTTC	(Wanner 2006)
	TxtAB2	TCGAGTGGACCTCACAGATG	
<i>tomA</i>	Tom3	GAGGCGTTGGTGGAGTTCTA	
	Tom4	TTGGGGTTGTACTCCTCGTC	
<i>nec1</i>	Nf	ATGAGCGCGAACGGAAGCCCCGGA	(Bukhalid et al. 1998)
	Nr	GCAGGTCGTCACGAAGGATCG	

BLASTN). For each strain, the hits with the top 10 highest Bit-Scores were retrieved for phylogenetic analysis. Additionally, sequences of type strains of phytopathogenic streptomycetes that were not present in the database were also retrieved from GenBank and added to the dataset. The sequence of *Kitasatospora atroaurantiaca* DSM 41,649^T was included as an outgroup (Table S1). After aligning the sequences with ClustalW, a maximum-likelihood tree was reconstructed with MEGA 12 (Kumar et al. 2024) using the T3+G+I model, and the analysis included 1,000 bootstrap replicates.

Determination of plant growth promotion efficacies in selected *Streptomyces* strains

Streptomyces strains selected from previous assays were examined for their efficacies in promoting the growth of potted tomato (*S. lycopersicum* L. 'Known-you 301'). Specifically, 7-d-old bacterial cells grown on ISP4 medium plates were suspended in 0.1% Tween 20 ($OD_{600}=0.5$; on average 7.5×10^7 CFU/ml), and 2 ml of suspension prepared from each strain was added to 200 ml of oatmeal broth in 500 ml glass media bottles and incubated at 28 °C (150 rpm) for 5 d before use. As negative controls, 2 ml of bacteria-free 0.1% Tween 20 were also added to 200 ml of oatmeal broth and incubated under the same setting.

In terms of the plant materials, tomato seeds were surface-disinfected with 0.6% of NaClO for 5 min and washed three times with sterile water. The seeds were planted in a planting substrate (peat moss : perlite : vermiculite=6:1:1; pH=5.91) inside a seedling tray and grown in a growth chamber (28 °C/26°C; day/night; 14-photoperiod). After 7 d, a single plant was kept for each well, and plants with similar growth vigor were subjected to further testing.

Treatment of the plants with different bacterial strains were conducted by drenching each tray cell with 5 ml of a bacterial culture 7 and 14 d after planting. Each treatment group (bacterial strain) and the control group included 14–15 replicates. Two days after the second drenching treatment, the plants were transplanted to plastic pots (9 cm in diameter) containing the same planting substrate as in the seedling tray. After 21 d, the lengths of the main stems and longest roots, as well as the wet and dry weights of the aboveground and belowground tissues were determined. All statistical analyses were completed using SPSS (IBM corp., Armonk, NY, USA). Different transformation and statistical methods were used based on the characteristics of each dataset. One-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) tests were used to analyze root length and $\log_{10}$ -transformed stem length data. For data on the wet and dry weights, Kruskal-Wallis tests

and uncorrected Dunn's multiple-comparison tests were conducted. The experiment was repeated two times.

Biological control assay

The *Streptomyces* strain that exhibited the most consistent plant growth promotion efficacy was selected for assessment of its ability to control bacterial wilt caused by *R. pseudosolanacearum*. For this assay, tomato plants were grown as previously described and divided into 3 groups, each including 15 plants. Plants in Group 1 were first treated with liquid culture of the *Streptomyces* strain selected from previous assays and then inoculated with *R. pseudosolanacearum* strain PS152. Group 2 was only inoculated with the pathogen, and Group 3 was not treated with any of the two bacteria.

For Group 1, liquid culture of the selected *Streptomyces* strain was prepared and treated to the plants as in the plant growth promotion assay. Two days after the second drenching treatment, the plants were inoculated with strain PS152 using previously described methods (Chen 1996) with modification. Briefly, the plants were transplanted to pots (9 cm in diameter) containing planting substrate mixed with 2-d-old PS152 cells grown on CPG medium (28 °C; Kelman 1954). For every 100 g of planting substrate, 70 ml of an inoculum was added; the inoculum was prepared by a 2-fold dilution of a PS152 cell suspension (undiluted $OD_{600}=0.3$; approximately 3.6×10^8 CFU/ml; in 10 mM $MgCl_2$). For Group 2, the liquid culture of the tested streptomycete was replaced with bacteria-free oatmeal broth containing 0.1% of Tween 20, and PS152 was inoculated as in Group 1. Finally, for Group 3, the liquid culture of the tested streptomycete and the pathogen suspension were respectively replaced with oatmeal broth containing 0.1% of Tween 20 and 10 mM $MgCl_2$. All plants were grown in a walk-in growth chamber set at 28 °C/26°C (day/night; 14-photoperiod). After 18 days, disease symptoms were graded following previously described methods. Specifically, symptomless plants were graded '0', plants with only a single wilted leaf were graded '1', plants with two to three wilted leaves were graded '2', plants with four or more wilted leaves but were not completely wilted were graded '3'. Plants that were completely wilted were graded '4' (Le et al. 2020). The disease index value of each treatment group was calculated using the formula $[(0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4) / 4 \times N_T] \times 100$, where N_0 – N_4 were the number of plants with different gradings (0 to 4), and N_T was the total number of plants included in each group (Winstead and Kelman 1952). The experiment was carried out three times, each of which obtained a disease index value for each group. Disease index values of Group 1 and Group 2 were compared using a paired *t*-test, which removes the between-experiment

variation and focuses on the treatment effect. Biological control efficiency (BCE) was also calculated separately for each repeat, using the formula $BCE (\%) = [(DI_Group\ 2 - DI_Group\ 1) / DI_Group\ 2] \times 100$, where DI_Group 1 and DI_Group 2 represent the disease index values of the *Streptomyces*-treated plants (*Streptomyces* + PS152) and the infected control (PS152 only), respectively.

Additional phenotypic characterization of the selected *Streptomyces* strain

Quantitative IAA production assay

The tested strain's ability to produce IAA was quantified spectrophotometrically using previously described methods with modification (Harikrishnan et al. 2014). For this assay, *S. scabiei* strain 18-c was included as a positive control. Bacterial cells were suspended in 0.1% Tween 20 and the suspension was adjusted to $OD_{600} = 0.5$. Thereafter, 200 μ l of the suspension was added to 5 ml of yeast extract-malt extract broth (Pridham et al. 1957; Shirling and Gottlieb 1966) including 5 mM of L-tryptophan. For the negative controls, the suspension was replaced with 0.1% Tween 20. After 7 d of culturing at 28 °C (125 rpm) the culture was centrifuged (11,000 $\times$ g, 4 °C, 15 min) and 1 ml of the supernatant was mixed with 2 ml of Salkowski reagent (1 ml of 0.5 M $FeCl_3$ added to 49 ml of 35% $HClO_4$ solution) and incubated in the dark at 25 °C for 30 min. A positive reaction was indicated by the production of a pink color or higher absorbance at 530 nm compared to the controls (Harikrishnan et al. 2014). Each group included 3 replicates. To quantify the amounts of IAA produced by the tested strains, a standard curve was constructed using a serial dilution of a 50 μ g/mL IAA solution (in yeast malt extract broth added with 5 mM L-tryptophan and 0.0038% Tween 20), and adding 1 ml of each diluted solution to 2 ml of Salkowski reagent. The absorbance values at 530 nm were recorded for each solution, and the amounts of IAA produced by the tested strains were calculated based on the standard curve.

Qualitative agar plate-based assays

To further characterize the best-performing *Streptomyces* strain selected from previous assays, multiple agar plate-based tests were conducted with 7-d-old culture of the bacterium. Results obtained from these assays were recorded qualitatively as positive or negative.

The strain's abilities to degrade starch and lipid (Tween 80), and solubilize phosphate were tested by respectively transferring the bacterium to starch agar, Tween 80 medium plates (Schaad et al. 2001), and Pikovskaya's agar (M520, HIMEDIA, Mumbai, India; PVK; Table S2; Sundaro Rao

and Sinha 1963), and examining the results after 7 d. Formation of a clear area around a colony on starch agar after staining with an iodine solution indicates that the tested strain degraded starch. Observation of white precipitation around a colony on Tween 80 medium indicated that the bacterium exhibited lipase activity. Production of clear zones around bacterial colonies on PVK agar indicated positive results for the ability to solubilize phosphate. To determine whether the tested strain produced siderophores, one loopful of bacteria was suspended in 0.1% Tween 20, and 30 μ l of the suspension was dropped onto a paper disc placed on top of chrome azurol S (CAS) medium plates (Table S2; Schwyn and Neilands 1987). The plates were incubated at 28 °C for 7 d, and a positive reaction was indicated by production of an orange or yellow area around a colony. Details regarding the specific media and reagents used in the above phenotypic tests are listed in Table S2. Additional bacterial strains, serving as positive and negative controls, were also included for these tests (Table S3).

Results

Radish seedling bioassay

Compared to the negative controls, four tested strains resulted in notable reduction in hypocotyl growth of radish seedlings (uncorrected Dunn's multiple-comparison tests following Kruskal-Wallis tests; $P < 0.1$); these strains were 4-b, 13-b, 65-a and 77-a. Three tested strains, 65-a, 77-a, and 113-a, inhibited root growth (Fig. 1; $P < 0.1$). In all of the tests, *S. scabiei* strain 18-c inhibited the growth of both the hypocotyls and roots of radish seedlings (Fig. 1). Based on the results, strains 4-b, 13-b, 65-a, 77-a, and 113-a were excluded from further testing.

Agar diffusion assays

Tests conducted on the 44 *Streptomyces* strains that were retained in the radish seedling bioassays showed that 41 of them consistently produced inhibitory zones against *R. pseudosolanacearum* strain PS152 (Fig. 2). Among the three strains that did not produce consistent results in all three replicates, strain 20-a only had two replicates producing an inhibitory zone, whereas strains 7-b and 88-a failed to inhibit PS152 growth in all three replicates. In terms of the assays against *S. scabiei*, only 8 strains produced inhibitory zones in all three replicates; these strains were 26-a, 31-a, 33-a, 42-a, 81-b, 84-b, 94-a, and 94-b. Because these 8 strains were also able to inhibit the growth of PS152, they were selected for further testing.

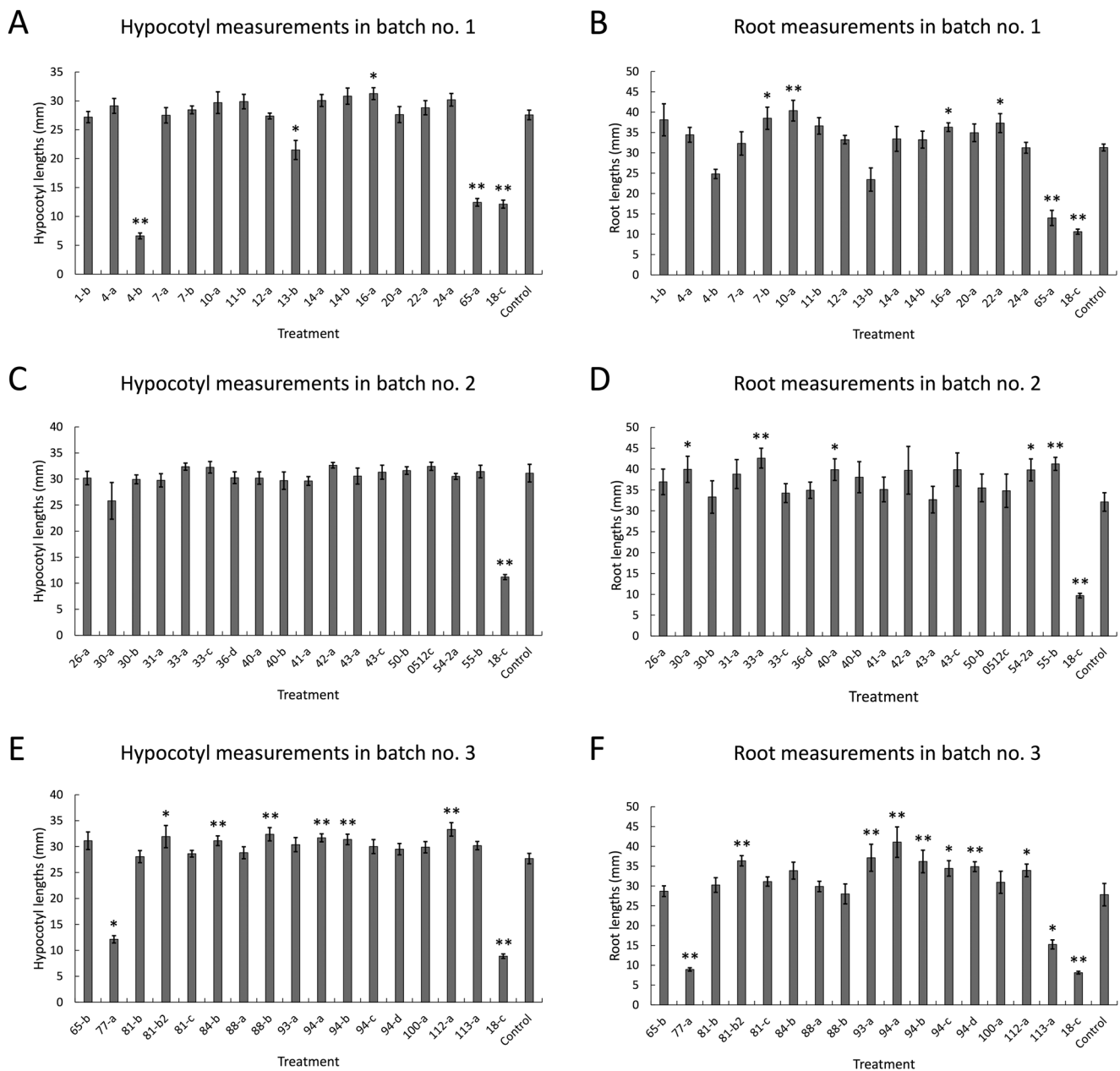


Fig. 1 Effects of different *Streptomyces* strains on the growth of radish seedlings. Because of the large number of strains tested, the assays were conducted in three batches [batch no. 1 (A–B), batch no. 2 (C–D) and batch no.3 (E–F)]. The lengths of the hypocotyls and roots were measured for each batch, and the recorded values were analysed using Kruskal-Wallis tests and uncorrected Dunn’s multiple-comparison

Detection of pathogenicity-associated genes using polymerase chain reaction assays

Tests on DNA extracted from strains 26-a, 31-a, 33-a, 42-a, 81-b, 84-b, 94-a, and 94-b failed to amplify fragments of pathogenicity-associated genes *txtAB*, *tomA*, and *necl* (Fig. 3). The positive control (DNA extracted from *S.*

tests. In each assay, *S. scabiei* strain 18-c was included as a reference, and oatmeal broth served as the negative control (the rightmost bar in all panels). Significant differences between each strain and the negative control are indicated by one or more asterisks (*: $0.05 < P < 0.1$; **: $P < 0.05$)

scabiei strain 18-c) produced the expected amplicons for all three genes (Fig. 3).

Identification of bacterial strains using multilocus sequence analysis (MLSA)

Sequences of housekeeping genes *atpD*, *gyrB*, *recA*, *rpoB*, and *trpB* were successfully obtained for strains 26-a, 31-a,

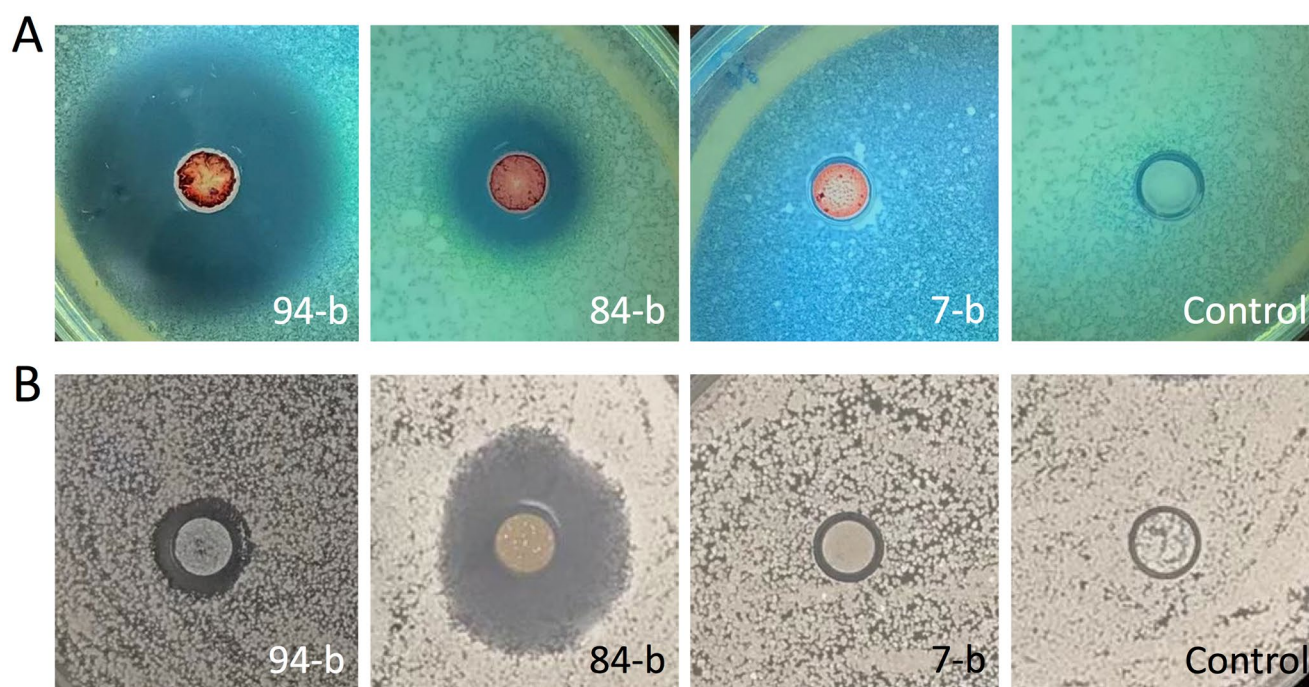


Fig. 2 Results of agar diffusion assays. **(A)** Tests against *Ralstonia pseudosolanacearum* strain PS152 were conducted on triphenyl tetrazolium chloride (TTC), while assays against *Streptomyces scabiei* strain 18-c were completed using International *Streptomyces* Project medium 3 (ISP3 medium) plates. Representative results of strains

94-b, 84-b and 7-b are shown. Plates added with oatmeal broth served as negative controls. The results indicated that strains 94-b and 84-b, but not 7-b were able to inhibit the growths of phytopathogenic strains PS152 and 18-c

33-a, 42-a, 81-b, 84-b, 94-a, and 94-b. All sequencing data have been deposited in GenBank under accession Nos. PV233829-PV233868.

Phylogenetic analysis based on the five-gene-concated sequences revealed that none of the selected strains were closely related to recognized phytopathogenic *Streptomyces* species (Fig. 4). Strain 84-b clustered with multiple *Streptomyces* strains; among the reference strains with species epithets, it was most closely related to *S. coelicolor* A3(2) (99.8% sequence identity), which has historically been designated as either *S. anthocyanicus* or *S. violaceoruber* (Komaki and Tamura 2021; Mahasneh et al. 2011). Strains 26-a and 81-b were more distantly related to the reference strains included in the analysis (separated by longer branches); among strains with complete species names, they were most similar to *S. coelicoflavus* SID14163 (98.17% and 98.25% sequence identity for strains 26-a and 81-b, respectively). Strain 31-a was most closely related to *Streptomyces* sp. strains NA02536 and SLBN-134; among reference strains identified to the species level, it shared the highest sequence identity with *S. griseoflavus* JCM 4479^T (97.41%). Strains 33-a, 42-a and 94-a had identical sequences, and were closely related to *S. viridodiataticus* JCM 4536^T (now identified as *S. albogriseolus*; 99.72% sequence similarity). Finally, strain 94-b clustered with *S.*

nigra 452^T, and the two strains differed by only one base (99.96%; 2,510/2,511 bp).

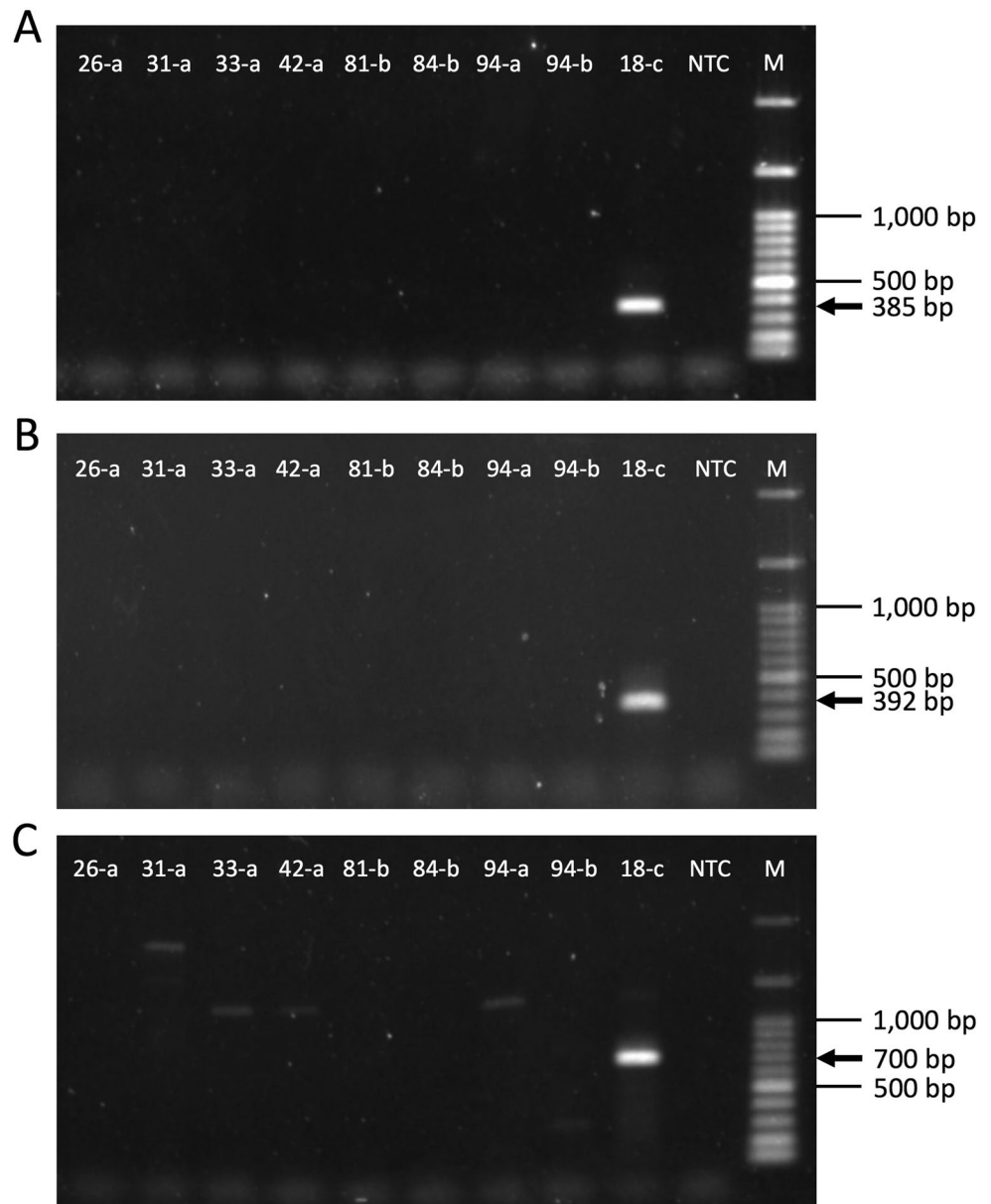
Based on the results, only strains representing unique genotypes were retained for further testing. Because 33-a, 42-a, and 94-a shared identical sequences (Fig. 4), strains 33-a and 42-a were excluded.

Determination of plant growth promotion efficacies in the selected *Streptomyces* strains

Six *Streptomyces* strains retained after previous assays and sequence analyses were subjected to two independent plant growth promotion test trials on tomato plants. For aboveground tissues, strains 26-a, 81-b, 84-b, and 94-b significantly increased tomato stem lengths in both trials (Fig. 5A), whereas strains 31-a and 94-a only increased the lengths in one trial (Fig. 5A). All 6 strains significantly increased fresh weights of aboveground tissues in both trials (Fig. 5B). Strains 94-a and 94-b increased the dry weights of aboveground tissues in both trials, while 26-a, 31-a and 84-b increased the values only in one trial (Fig. 5C).

In terms of root tissues, no significant difference was observed in root length values among treatment groups in both trials (ANOVA, $P > 0.05$; Fig. 5D). Strains 94-a and 94-b increased fresh root weights in two trials, while strains 26-a, 31-a, 81-b, and 84-b increased the weights only in one

Fig. 3 Detection of pathogenicity-associated genes in different *Streptomyces*-like strains using polymerase chain reactions. The results for genes (A) *txtAB*, (B) *tomA* and (C) *necI* are shown. The expected amplicon size for *txtAB*, *tomA* and *necI* were around 385, 392, and 700 bp, respectively. As a positive control, *S. scabiei* strain 18-c was included in each test. Nuclease-free water served as no-template controls (NTC). M: DNA marker



trail (Fig. 5E). Treatments with 94-a or 94-b significantly increased root dry weights in both trials, whereas treatments with 26-a, 31-a or 84-b only increased the weights in one trial (Fig. 5F).

Overall, strain 94-b exhibited the most consistent plant growth promotion efficacy; tomato plants treated with the strain exhibited the highest average values for all recorded parameters in at least one trial (although not all differences were statistically significant). Accordingly, strain 94-b was selected for further testing.

Biological control assay

The average disease index value for plants belonging to Group 1 (treated with both *S. nigra* strain 94-b and *R.*

pseudosolanacearum strain PS152) and Group 2 (treated with PS152 alone) was 32.78% and 57.78%, respectively (Fig. 6). A paired *t*-test on the disease index values revealed that there was significant difference between the two groups ($P=0.019$). None of the plants in Group 3 (not treated with either strain 94-b or PS152) produced observable symptoms (Fig. 6). The corresponding biological control efficiency averaged 45.2% across repeats.

Additional phenotypic characterization of the best-performing *Streptomyces* strain

Strain 94-b was able to degrade starch and lipid (Tween 80). The strain was also capable of producing siderophore and solubilizing phosphate.

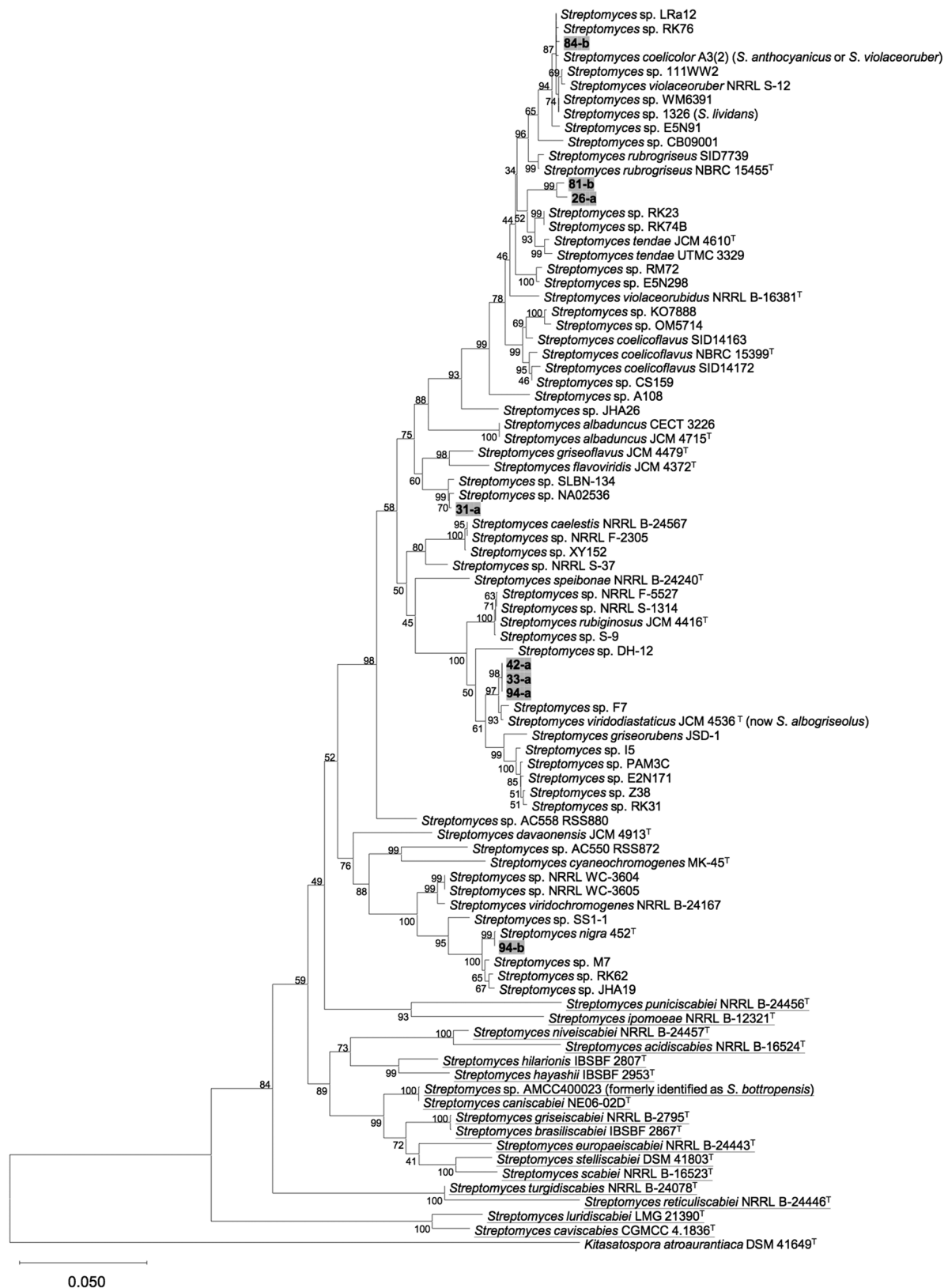


Fig. 4 Phylogenetic tree reconstructed from concatenated sequences of genes *atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*. The analysis included sequences from strains 26-a, 31-a, 33-a, 42-a, 81-b, 84-b, 94-a and 94-b (highlighted in gray shading and bold font), their top hits in PubMLST, and sequences of recognized phytopathogenic strepto-

mycetes (retrieved from PubMLST or GenBank; names underlined). *Kitasatospora atroaurantiaca* strain DSM 41,649^T served as the out-group. The analysis was conducted with the maximum-likelihood method using the T3 + G + I model. Reliability of the clades was examined with 1,000 replicates of bootstrap analysis

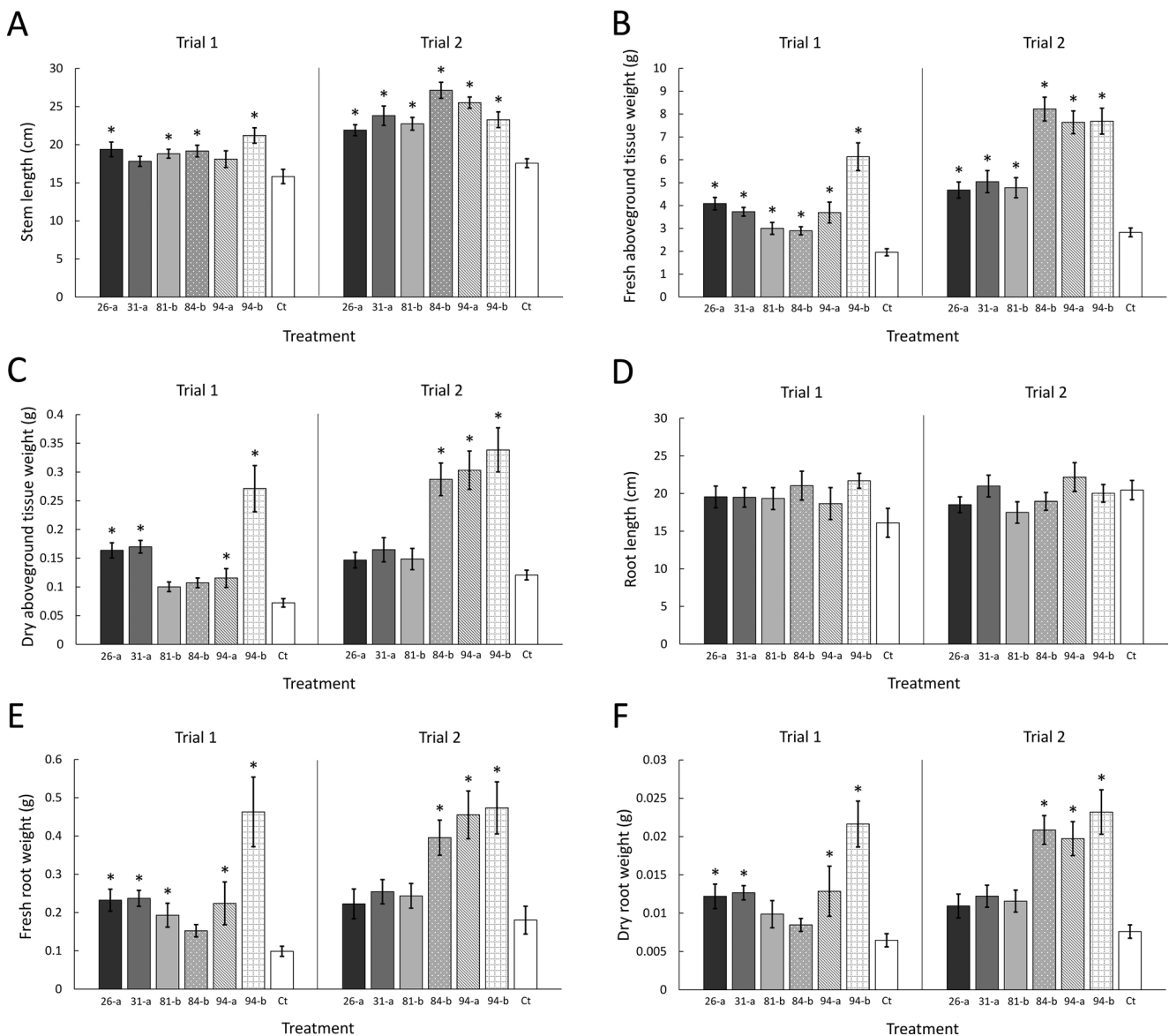


Fig. 5 Results of the plant growth promotion assays conducted on the isolated bacterial strains. Two independent trials were performed. The effects of the bacterial treatments on (A) stem lengths, (B) fresh aboveground tissue weights, (C) dry aboveground tissue weights, (D) root lengths, (E) fresh root weights, and (F) dry root weights of tomato plants were evaluated. Stem length data were $\log_{10}$ -transformed and analyzed using one-way analysis of variance (ANOVA) followed

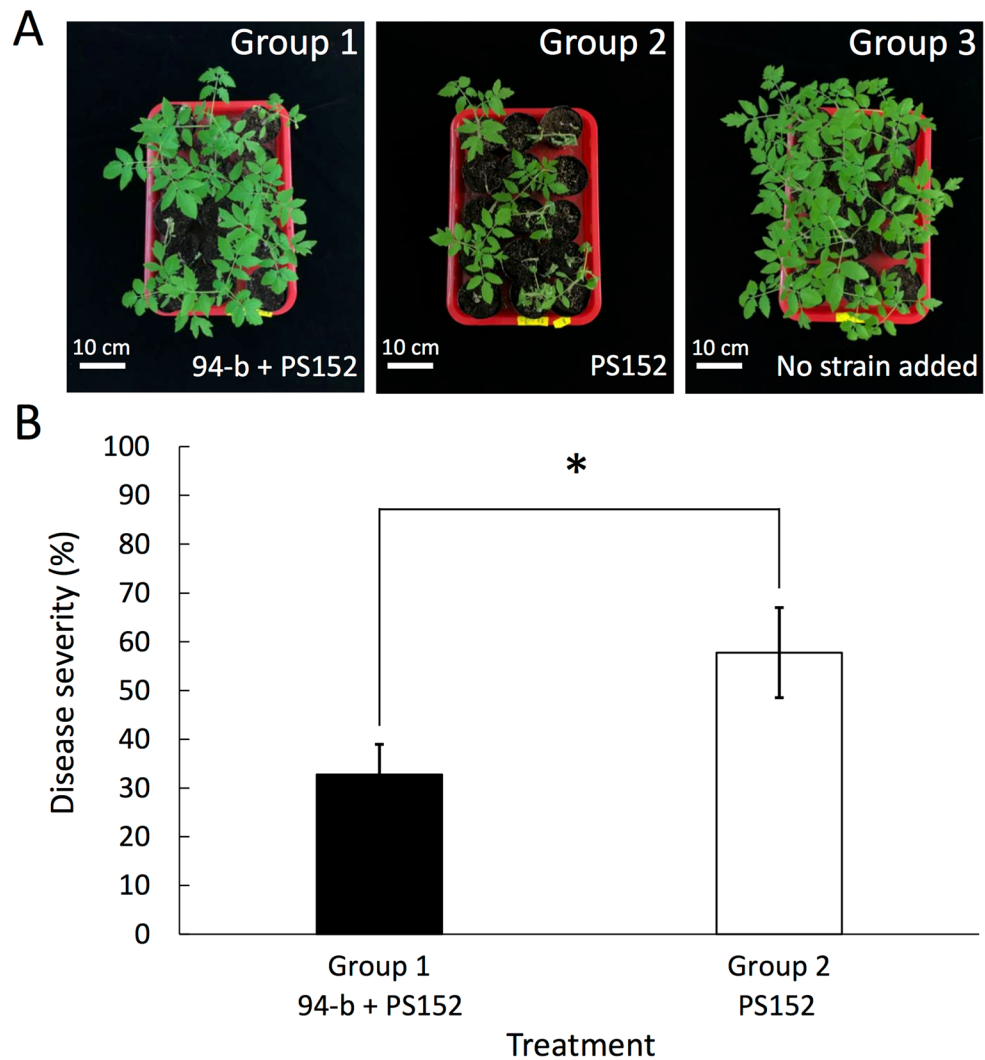
by Fisher's least significant difference (LSD) tests. Root length data were analyzed using one-way ANOVA and Fisher's LSD tests. Data for fresh and dry aboveground tissue weights, and fresh and dry root weights were analyzed using Kruskal-Wallis tests and uncorrected Dunn's multiple-comparison tests. Significant differences relative to the negative controls (Ct) are indicated by asterisks ($P < 0.05$)

After 7 d of culturing, 94-b was found to produce IAA ($1.53 \pm 0.101 \mu\text{g/ml}$), albeit the amount of IAA detected was less than that in *S. scabiei* strain 18-c ($6.96 \pm 1.1 \mu\text{g/ml}$; $P = 0.008$, *t*-test).

Discussion

When exploiting *Streptomyces* strains for agricultural applications, it is necessary to ensure that the selected bacteria do not pose adverse effects on the crops. The radish seedling bioassay has been considered a widely accepted and reliable method for identifying plant symptom-inducing *Streptomyces* strains, with strains not reducing seedling growth being considered nonpathogens overall (Kang et al. 2022). Although the 49 *Streptomyces* strains examined were

Fig. 6 Efficacy of *Streptomyces nigra* strain 94-b on controlling bacterial wilt of tomato. Plants that were treated with strain 94-b and subsequently inoculated with *R. pseudosolanacearum* strain PS152 were designated as Group 1; plants that were only inoculated with PS152 were designated as Group 2; plants that were not treated with PS152 or 94-b belonged to Group 3. (A) Wilt symptoms in Group 1 were less severe compared to those in Group 2; none of the plants in Group 3 were symptomatic. (B) Results from the biological control assay showed that treatment of strain 94-b significantly reduced disease severity (paired *t*-test, $P=0.019$). The asterisk above the bars indicates significant difference



previously assumed to be harmless to plants (Wu 2021), five of them inhibited radish seedling growth in assays conducted in this work (Fig. 1). Test results also revealed that some of these strains reduced either hypocotyl growth or root growth, but not both (Fig. 1). A study indicated that at low concentration, thaxtomin A only inhibited hypocotyl growth and not root growth (Leiner et al. 1996). Another work focusing on a lineage of streptomycetes revealed that some strains only reduced root growth but not hypocotyl growth (Weisberg et al. 2021). It is thus possible that the effects observed in this work were influenced by the amounts of phytotoxins to which the seedlings were exposed, or phenotypic variations among strains of different lineages. Such findings highlight the potential variabilities in test results of large-scale bioassays, and the necessity for recording both hypocotyl and root lengths when screening streptomycetes using radish seedlings.

Agar diffusion assays conducted on 44 *Streptomyces* strains retained from the radish seedling bioassays identified

8 strains capable of inhibiting both *R. pseudosolanacearum* and *S. scabiei*, indicating their potential for managing phylogenetically distant phytopathogenic bacteria. To ensure that the selected strains posed minimal risk to plant health, additional examination of the presence of pathogenicity-associated genes was conducted. The targeted genes, *txtAB*, *tomA* and *necI*, are widely accepted molecular markers used to distinguish pathogenic *Streptomyces* strains from saprophytic or beneficial ones (Dees et al. 2013; Uysal et al. 2025; Webster et al. 2026), and none of them were detected in the selected strains (Fig. 3). Consistently, phylogenetic analysis also showed that none of them were closely related to strains of recognized phytopathogenic *Streptomyces* species (Fig. 4). Instead, the analysis revealed that many of the tested strains were related with streptomycetes that have been explored for agricultural applications. These included *S. anthocyanicus*, whose strain exhibited antimicrobial activities against phytopathogenic bacteria (Polivtseva et al. 2023); *S. violaceoruber*, which is capable of promoting

germination and growth of tomato (Faddetta et al. 2023); *S. coelicoflavus*, which has been reported to inhibit the growth of *R. solanacearum* (Su et al. 2022); *S. griseoflavus*, whose strains have either been found to increase production yields of leguminous crops when applied as a biofertilizer with *Bradyrhizobium* species (Htwe et al. 2019), or capable of controlling chili anthracnose caused by *Colletotrichum capsici* while increasing chili yield (Yanti et al. 2023); *S. albogriseolus*, which has been found showing potential for promoting spinach growth (Saraylou et al. 2021) or producing compounds capable of killing root-knot nematodes (Zeng et al. 2013); and finally *S. nigra*, which includes a strain with the ability to degrade herbicides (Zhao and Wang 2024). As such, the screening procedures adopted in this work were effective in identifying strains with prospects for agricultural application while avoiding potential plant pathogens.

After further removing genetically redundant strains, examinations of the 6 retained streptomycetes' plant growth promotion efficacies were conducted on tomato plants, and all of them increased at least some parameters of both aboveground and belowground plant growth (Fig. 5). These findings indicate that the ability to promote plant growth are common among the potato-isolated streptomycetes. However, the capability to promote plant growth varied considerably among strains, and between-trial variations were also observed (Fig. 5). Although in vitro screening and greenhouse pot assays are routinely conducted to evaluate plant growth-promoting activity, studies on plant growth-promoting *Streptomyces* in the literature often report only a single pot-trial experiment with internal replicates, without assessing reproducibility between independent trials (Anwar et al. 2016; Thenappan et al. 2024; Zhu et al. 2023). By examining multiple parameters of the treated plants in repeated trials, this study was able to identify a strain, *S. nigra* 94-b, which outperformed the others in terms of plant growth promotion activity.

Characterization of strain 94-b revealed phenotypic properties that may have contributed to its positive effects on plants. Specifically, the strain solubilized phosphate, suggesting that it could make phosphorus more accessible to plants, thereby facilitating biomass accumulation, photosynthesis, respiration, biosynthesis of nucleic acids or membranes and other vital processes (Vance et al. 2003). It secreted siderophores, which can chelate iron and make the nutrient easier for plants to absorb and utilize, potentially contributing to plant biosynthesis of phytohormones and photosynthesis (Timofeeva et al. 2022). Strain 94-b was also capable of degrading starch and lipid, which may also contribute to plant growth promotion (Sousa et al. 2008). Lastly, although to a lesser extent, the strain was able to produce IAA, which is crucial for regulation of plant growth (de

Andrade et al. 2023). Interestingly, studies have suggested that optimizing the expression of some of the detected traits may further improve a bacterium's plant growth promotion efficacies (Kumari et al. 2022; Myo et al. 2019). It is thus possible that fine-tuning of culturing conditions or formulation methods in future studies could further improve 94-b's performances.

Biocontrol assays showed that treatment with 94-b significantly lowered diseases index values in tomato plants inoculated with *R. pseudosolanacearum* (Fig. 6). Considering that 94-b inhibited *R. pseudosolanacearum*'s growth in the agar diffusion assay (Fig. 2) and secreted siderophores, it is possible that 94-b's symptom-alleviating ability was attributed to the strain's direct antagonism of the pathogen and, potentially, chelating of iron that would have been available for the pathogen. Further investigations are needed to validate such hypothesis, and to examine whether more complex interactions with the plant, such as defense gene regulation (Le et al. 2021), are involved in symptom alleviation. Lastly, the ability of 94-b to degrade starch and lipid may have allowed it to better compete in the rhizosphere and enhanced its efficacy as a biological control agent, as suggested in other studies (Bach et al. 2016; Sousa et al. 2008).

Since the identification of *S. nigra*, limited research has been conducted on the species' application potentials (Chen et al. 2018; Zhao and Wang 2024). Studies have shown that strains of the bacterium may exhibit anti-tumour activity (Chen et al. 2018) or have potential applications in melanin production (Cimini et al. 2026). Some strains could also degrade herbicides and potentially inhibit leaf spots (Zhao and Wang 2024). To our knowledge, the present study is one of the first to demonstrate that a strain of *S. nigra* is capable of both promoting plant growth and alleviating soil-borne bacterial diseases. The data from this study showed that even though many members of *Streptomyces* have been characterized for practical use, continued exploration of streptomycetes in the environment could lead to identification of new strains with high application potential. Discovery of these microbes may provide new options for improving crop yield and controlling devastating diseases.

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Data availability The sequencing data supporting the results of this work have been deposited in NCBI's GenBank database under accession nos. PV233829-PV233868.

Declarations

Ethical approval Not applicable.

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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