Effective Screening of Antagonistic Bacteria Control of Torch Ginger Wilt Disease Caused by *Ralstonia solanacearum*

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Abstract

This study focused on selecting and identifying antagonistic bacteria for controlling torch ginger wilt disease caused by *Ralstonia solanacearum*. Soil samples were collected from torch ginger fields in Yala and Narathiwat Provinces. Screening for endospore-forming bacteria yielded 97 isolates, and their abilities to inhibit the growth of *R. solanacearum* were evaluated using the agar disk diffusion method. Five highly inhibitory isolates were selected. Analysis of the 16S rRNA gene sequences identified the five isolates as TTYL-14, TTYL-18, and BTYL-5 similar to *Bacillus subtilis* and BTYL-4 and MGYL-1 similar to *Bacillus amyloliquefaciens* and *Bacillus altitudinis*. Evaluation of the antagonistic ability showed that all the selected isolates had significant potential for controlling torch ginger wilt disease in a greenhouse, with disease severity indices ranging from 40 to 46.67% compared to 80% in untreated controls. These antagonistic bacteria showed the ability to suppress the growth of *Colletotrichum gloeosporioides* (anthracnose), *Corynespora cassiicola* (corynespora leaf disease), and *Fusarium oxysporum* (Fusarium wilt). The antagonistic bacteria coexisted with the important biocontrol fungus *Trichoderma asperellum in* laboratory tests.

Keywords: *antagonistic bacteria, ralstonia solanacearum, bacterial wilt disease, torch ginger*

1. Introduction

Torch ginger (*Etlingera elatior*) is a member of the Zingiberaceae family, along with ginger and galangal. This native plant of the lower southern region of Thailand is widely found across Southeast Asia and Northern Australia. Torch ginger is known by different names such as “Dahla” or “Gahla” in Thailand, “Bunga Kantang” in Malaysia, and “Kecombrang” or “Sikala” in Indonesia. In Thailand, 15 genera of torch ginger have been identified, with five genera explicitly found in the lower southern region (Department of Agriculture, 2019). The plant is commonly consumed as food and also used as a medicinal herb. Studies on the medicinal properties of torch ginger, essential oil extraction, phytochemicals, and biological activities have enhanced its importance. The key bioactive compounds found in torch ginger include phenols, polyphenols, flavonoids, and terpenoids, which play an essential role in various biological activities. These compounds exhibit antioxidant, antimicrobial, antitumor, anti-hyperuricemic, antiallergic, cytotoxicity, and antidiabetic properties that contribute to the prevention of non-communicable diseases (NCDs). Torch ginger shows potential for use in cosmeceutical skincare product applications. The demand for torch ginger is increasing in domestic and international markets, particularly for ornamental and medicinal purposes (Anzian et al., 2020; Ismail, & Ridzuan, 2023). The outbreak of banana wilt disease caused by *R. solanacearum* in the three southern border provinces of Thailand—Yala, Pattani, and Narathiwat—since 2014 has significantly damaged banana production, leading to a decline in yield (Tarasook, 2023). Wilt disease symptoms have also been observed in torch ginger plants grown alongside banana plantations or near banana fields, causing plant deterioration and yield reduction. *R. solanacearum* is a pathogen with a broad host range, particularly affecting economically important crops including potatoes, tomatoes, black peppers, bell peppers, eggplants, peanuts, tobacco, bananas, and ginger in tropical, equatorial, and temperate regions. This soil-borne bacterium resides in both surface and deep soil layers and infects host plants through the roots, making it difficult to control or eliminate using chemical soil treatments (Horita et al., 2023; Rivera-Zuluaga et al., 2023; Nion, &

Koki, 2015). Biological control of bacterial wilt using antagonistic *Bacillus* spp. has been shown to reduce disease damage caused by *R. solanacearum* in ginger (Sussanti et al., 2023; Li et al., 2024; Cui et al., 2024). Therefore, antagonistic bacteria present a promising alternative for managing and preventing wilt disease. This approach can support the production of high-quality, chemical-free torch ginger, ensuring consumer safety and enhancing its economic value in the future.

2. Objectives

1. To collect and isolate *R. solanacearum* causing wilt disease of torch ginger, along with its antagonistic bacteria.
2. To screen the effective antagonistic bacteria for inhibiting *R. solanacearum* and investigate the broad spectrum of selected antagonistic bacteria to inhibit the growth of other plant pathogenic fungi.
3. To identify the collected wilt disease bacteria and the effective antagonistic bacteria using the nucleotide sequence of the 16S Ribosomal RNA (16S rRNA) gene.
4. To evaluate the effectiveness of the selected antagonistic bacteria in controlling bacterial wilt of torch ginger under greenhouse conditions.

3. Materials and Methods

3.1 Isolation of torch ginger wilt disease bacteria

Torch ginger plants exhibiting wilt symptoms were collected from Than To, Bannang Sata, and Mueang Districts in Yala Province, as well as from Waeng and Sukhirin Districts in Narathiwat Province, three samples each where torch ginger is extensively cultivated for commercial purposes. Wilted torch ginger stems, particularly near the base, were cut transversely and soaked in a 2% sodium hypochlorite solution, followed by rinsing in sterile water. The surface-disinfected stem segments were placed in sterile distilled water for 30 minutes to allow white, turbid bacterial exudates (or ooze) to flow from the infected stems. A sterile loop was then dipped into the ooze suspension and streaked onto a triphenyl tetrazolium chloride (TZC) medium (Himedia, India). The streaked plates were incubated at room temperature for 48 hours. After incubation, virulent colonies with a red or pink center and a whitish margin (Kelman, 1954) were observed and transferred to new TZC medium plates, and incubated at room temperature for 48 hours.

3.2 Collection and isolation of endospore-forming bacteria

Rhizosphere soil samples were collected from healthy torch ginger plants at the plantation site. To isolate endospore-forming bacteria, 1 g of each soil sample was mixed with 9 ml of sterile water and heated in a water bath at 80 °c for 10 minutes. Subsequently, 100 ml of heat treatment suspension dilution was plated on TSA medium (Difco™ & BBL™, U.S.A.) and incubated for 4 days at room temperature. After incubation, a single colony of fast-growing bacteria was transferred to a new TSA medium using the streak method and incubated for another 4 days at room temperature. This process allowed differentiation of the colonies formed between each isolate before being maintained on TSA medium in a test tube (Namsena, 2018).

3.3 Screening for antagonistic bacteria against R. solanacearum

The antagonistic properties of bacteria were assessed using the agar disk diffusion method against *R. solanacerum*. A volume of 100 µl from the *R. solanacearum* suspension was uniformly spread on a nutrient agar (NA) (Difco™ & BBL™, U.S.A.) plate. Filter paper disks (5 mm diameter) were soaked and saturated with a pure isolate of collected endospore-forming bacterial suspension. The paper disks were placed on the surface of NA, spread with *R. solanacearum* in three replicates, and incubated at room temperature. After 2 days, the size of the inhibition (clear) zone around the paper disk was determined. The isolates that effectively inhibited *R. solanacearum* were selected and tested using a dual culture technique for broad-spectrum antimicrobial activity against various plant pathogens, kindly provided by Associate Professor Dr. SuwitaSaepaisan, Department of Entomology and Plant Pathology, Faculty of Agriculture, Khon Kaen University. *Colletotrichum gloeosporioides*, *Corynespora cassiicola*, and *Fusarium oxysporum* are responsible for anthracnose, corynespora leaf disease, and Fusarium wilt, respectively.

3.4 Molecular identification of the bacterial isolates

Species identification of the torch ginger wilt disease and antagonistic bacterial isolates was conducted by analyzing the nucleotide sequence of the 16S Ribosomal RNA (16S rRNA) gene. For DNA extraction, pathogenic and antagonistic bacterial colonies were picked directly using a sterile pipette tip to transfer a small volume of bacterial cells into tubes containing 100 µl of TE buffer (Tris 0.01M, EDTA 0.001M, pH 8.0), vortexed for 20 seconds, and allowed to rest for 20 minutes. One microliter of the bacterial DNA suspension was used as a PCR template and transferred into a PCR master mix (Promega, U.S.A.). The following primers were used: 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492 R (5′-GTTACCTTGTTACGACTT-3′) before performing thermocycling (GeneAmp™ 9700, U.S.A.). The PCR products were analyzed using 1.5% agarose gel electrophoresis and visualized under a UV transilluminator. The reliable PCR products were then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, U.S.A.) before being sent to First BASE Laboratories, Malaysia, for nucleotide sequencing. The sequencing results were analyzed for accurate species identification using BLASTN by comparing the sequence similarity with existing records in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

3.5 Evaluation of biocontrol efficacy of antagonistic bacteria in the greenhouse

The experiment followed a completely randomized design (CRD) with six treatments and four replications. A bacterial suspension of *R. solanacearum* at a concentration of 1.0×10^8 CFU/ml was thoroughly mixed with sterilized soil at a volume of 100 ml per 8 kg of soil (Khositcharoenkul, 2008). The infested soil was placed into 28 4-inch diameter pots, each containing 800 g of soil. The pots were exposed to the following treatments: T1 applied with TTYL-14, T2 applied with TTYL-18, T3 applied with BTYL-5, T4 applied with BTYL-4, T5 applied with MGYL-1, and T6 applied with water as a control treatment. Eight-month-old, healthy torch ginger plants were transferred to the prepared plots with *R. solanacearum*-infested soil. A 50 ml suspension of each antagonistic bacteria at a concentration of 1.0×10^8 CFU/ml was poured into the prepared pots around the torch ginger root according to the treatment. Disease progression was recorded every 7 days after inoculation. The severity of the wilt disease was assessed based on the modified scale from Kumvinit et al. (2022) as follows: Disease Severity Scale: 0 = No symptoms, 1 = One or two lower leaves curling or wilting (plant remains green), 2 = More than two lower leaves curling or wilting (plant remains green), 3 = Whole plant wilting and starting to turn yellow, 4 = Whole plant wilting, turning yellow, and showing water-soaked lesions at the base, 5 = Water-soaked lesions at the base, plant collapses and dies. The percentage of disease incidence was calculated as Disease Severity Percentage (DSP) = ∑(Number of affected plants at each level × Disease severity level) / (Total number of tested plants× Maximum disease severity level) x 100

4. Results and Discussion

4.1 Isolation of torch ginger wilt disease bacteria

The collection of torch ginger plants exhibiting wilt symptoms revealed a significant outbreak in Ban Rae Subdistrict, Than to District, Yala Province. Ten samples were also collected from Bannang Sata Subdistrict, Bannang Sata District, Yala Province (Figure 1 A). Six pure isolates were successfully obtained, and the presence of *R. solanacearum* was tested using the semi-selective TZC medium. The bacterial colonies displayed irregular shapes, with opaque white edges and a light pink to pale red mucoid center surrounded by a white slime layer (Figure 1 B). These preliminary morphological characteristics corresponded to *R. solanacearum*, which Kelman (1954) classified as a virulent strain.

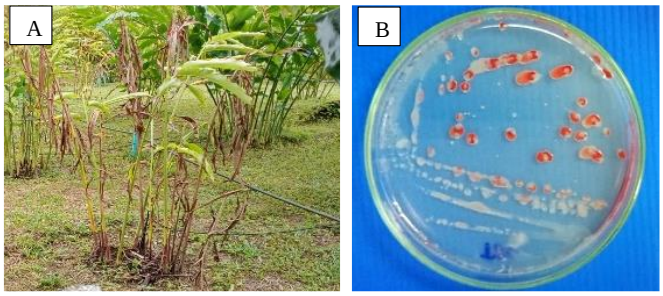

Figure 1 A) Symptom of bacterial wilt disease in torch ginger showing wilting of leaves at the ends of branches, and B) Colony morphology of virulent *R. solanacearum* strains on TZC medium.

4.2 Collection and isolation of endospore-forming bacteria

Soil samples collected from torch ginger fields showed no signs of wilt disease at five sites in Yala Province (Than To, Bannang Sata, and Mueang Yala District, five samples each) and Narathiwat Province (Waeng and Sukhirin Districts, five samples each). Twenty-five rhizosphere soil samples from healthy torch ginger plants were use to isolate the endospore-forming bacteria, the heat treatment method was applied with the soil dilution spread plate method on TSA (Tryptic Soy Agar) medium (TSA, Difco™ & BBL™, U.S.A.). Pure bacterial isolates (97 in total) were successfully obtained from Than to District - 49 isolates, Bannang Sata District - 17 isolates, Mueang Yala District - 11 isolates, Waeng District - 6 isolates and Sukhirin District - 14 isolates. Endospore-forming bacteria can withstand harsh environmental conditions, including extreme temperatures, radiation, and nutrient scarcity (McKenney et al., 2013), a crucial property for producing microbial pesticides.

4.3 Screening for antagonistic bacteria against R. solanacearum

The selected bacteria of 97 isolates were evaluated using the paper disc diffusion method in the laboratory. Isolates that produced a clear inhibition zone were considered effective in suppressing *R. solanacearum*; 33 isolates exhibited inhibition activity of ≥30%, accounting for 34% of the total isolates (Figure 2). The remaining isolates showed no inhibition or only weak inhibition ability. The percentage inhibitory effects against *R. solanacearum* by the five antagonistic bacteria were MGYL-15 (59.83%), TTYL-14 (53.83%), BTYL-5 (50.83%), TTYL-18 (51.00%), and BTYL-4 (50.83%). A preliminary characterization of the colony morphology of the five potent antagonistic bacterial isolates was conducted, with results presented in Figure 3 and summarized in Table 1.

These five potent antagonistic bacterial isolates were subjected to greenhouse trials to confirm their effectiveness in controlling wilt disease in torch ginger.

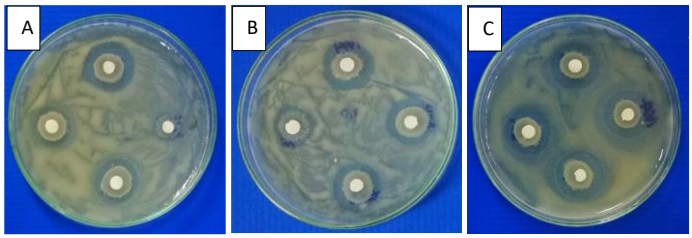

Figure 2 The paper disc diffusion method of antagonistic bacteria showing inhibitory effects (clear zone) against *R. solanacearum* (A) BTYL-4, (B) TTYL-14, (C) MGYL-15.

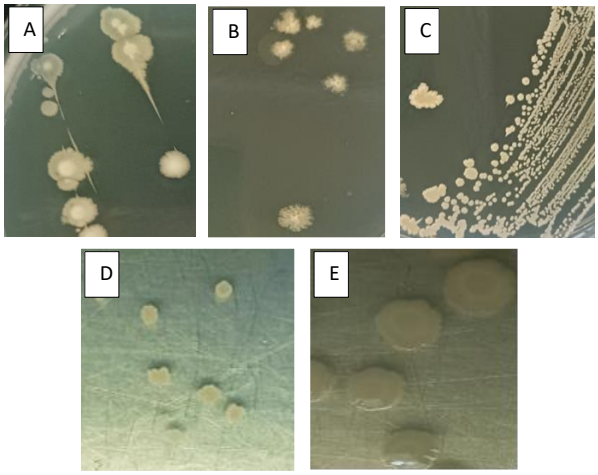

Figure 3 Colony characteristics of antagonist bacterial isolates cultured on nutrient agar and incubated at 37°C for 48 hours. (A) TTYL-14, (B) TTYL-18, (C) BTYL-5, (D) BTYL-4,(E) MGYL-1.

Table 1 Colony characteristics of antagonist bacterial isolates cultured on nutrient agar and incubated at 37°C for 48 hours.

Characteristic	Isolates				
	TTYL – 14	**TTYL – 18**	**BTYL-5**	**BTYL-4**	**MGYL-1**
Colony shape	Irregular	Irregular	Irregular	Irregular	Circular
Elevation	Raised	Raised	Crateriform	Flat	Raised
Margin	Undulate	Undulate	Undulate	Undulate	Undulate
Size	Medium	Medium	Medium	Medium	Medium
Color	White	White	White	White	White
Opacity	Translucent	Translucent	Opaque	Translucent	Translucent

Broad-spectrum antimicrobial activity testing of the five antagonistic bacteria to suppress the growth of plant pathogenic fungi *Corynespora cassiicola, Colletotrichum gloeosporioides,* and *Fusarium oxysporum* was performed using the dual culture method. Results showed that isolates BTYL-5 and BTYL-4 were highly effective in inhibiting the growth of *Corynespora cassiicola*, *Colletotrichum gloeosporioides*, and *Fusarium oxysporum*. The next best isolate, TTYL-18, had suppressive activity over *Corynespora cassiicola* and *Colletotrichum gloeosporioides* but only slightly inhibited *Fusarium oxysporum*. Isolates TTYL-14 and MGYL-1 did not inhibit the growth of these three fungal pathogens (Figure 3), consistent with the findings of Romeiro et al. (2022) and Zhiqiong et al. (2013). They investigated *Bacillus cereus* isolate UFV-101, which enhanced tomato resistance to *Pseudomonas syringae* pv., *Xanthomonas vesicatoria*, *Alternaria solani,* and *Corynespora cassiicola*. Similarly, *Bacillus subtilis* B25 isolated from banana rhizomes was highly effective as an antagonist against *Fusarium oxysporum* f. sp. *cubense*, *Corynespora cassiicola, Alternaria solani*, *Botrytis cinerea*, and *Colletotrichum gloeosporioides*. Testing the interaction of the five antagonistic bacterial isolates with the antagonist fungus *Trichoderma asperellum* showed no growth inhibition between the antagonistic bacteria and *T. asperellum*. This result suggested that both antagonists were compatible and could be combined to increase the efficiency in controlling plant pathogenic microorganisms. Kipngeno et al. (2015) used *Bacillus subtilis* and *T. asperellum* to coat tomato seeds in Kenya to prevent infection by *Pythium aphanidermatum* in greenhouse trials. They found the coated seeds with either *B. subtilis* or *T. asperellum* at a concentration of 10^6 CFU/ml geminated in *P.*

Aphanidermatum inoculated media, resulting in a remarkable reduction of the pre-emergence damping-off to 20.19% and 24.07%, respectively, while the non-coated seeds had 65.89% seedling mortality.

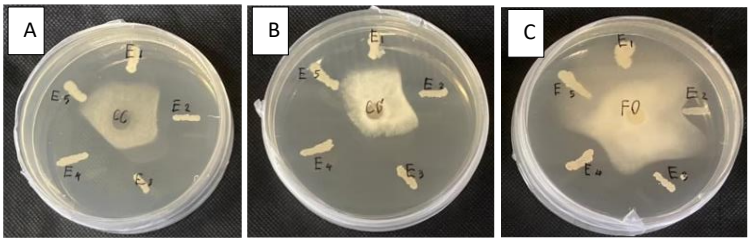

Figure 4 Agar disk diffusion of five antagonistic bacteria TTYL-14; (E_1), TTYL-18 (E_2), BTYL-5 (E_3), BTYL-4 (E_4), and MGYL-1 (E_5) against pathogenic fungi (A) *Corynespora cassiicola*, (B) *Colletotrichum gloeosporioides* and (C) *Fusarium oxysporum*.

4.4 Molecular identification of pathogenic and antagonistic bacteria

The identification of the bacterial wilt pathogen isolated from antagonistic bacteria was performed using 16S ribosomal RNA (16S rRNA) gene sequencing. The primers 27F and 1492R were used for PCR amplification. The expected gene fragment of 1490 bp was successfully detected. Results showed that the bacterial wilt isolate RSYL-1 had 99.79% similarity to *Ralstonia solanacearum* (Accession number MN508417.1), and isolate RSYL-2 had 99.86% similarity to *Ralstonia solanacearum* (Accession number MN508408.1). Species identification and nucleotide sequence similarity analysis of the 16S rRNA gene of the five antagonistic bacterial isolates revealed that isolate TTYL-14 showed 99.58% similarity to *Bacillus subtilis* (Accession number EF563825.1), isolate TTYL-18 showed 99.86% similarity to *Bacillus subtilis* (Accession number MN945444.1), isolate BTYL-5 showed 98.71% similarity to *Bacillus subtilis* (Accession number LC543400.1), isolate BTYL-4 showed 96.99% similarity to *Bacillus amyloliquefaciens* (Accession number MT678828.1), and isolate MGYL-1 showed 98.51% similarity to *Bacillus altitudinis* (KT719885.1) (Table 2).

Table 2 Similarity of screened isolate sequences with the NCBI GenBank database.

Isolate	Gene similarity in the NCBI database	% Similarity	Accession number
RSYL-1	*Ralstonia solanacearum* strain *HN4B 16S ribosomal RNA* gene	99.79	MN508417.1
RSYL-2	*Ralstonia solanacearum* strain *YS-Y 16S ribosomal RNA* gene	99.86	MN508408.1
TTYL-14	*Bacillus subtilis* strain *Bobby2007 16S ribosomal RNA* gene	99.58	EF563825.1
TTYL-18	*Bacillus subtilis* strain *BSFT-39 16S ribosomal RNA* gene	99.86	MN945444.1
BTYL-5	*Bacillus subtilis* strain *HCM8-3* gene for 16S rRNA	98.71	LC543400.1
BTYL-4	*Bacillus amyloliquefaciens* strain *HJ1 16S ribosomal RNA* gene	94.05	MT678828.1
MGYL-1	*Bacillus altitudinis* strain *MSL_ 3055 16S ribosomal RNA* gene	98.51	KT719885.1

4.5 Evaluation of biocontrol efficacy of antagonistic bacteria in the greenhouse

A greenhouse experiment was conducted to evaluate the effectiveness of antagonistic bacteria against torch ginger wilt disease. The treated plant symptoms appeared three weeks after transplant to soil infested with *R. solanacearum*. Leaf curling showed that the isolated wilt disease agent, *R. solanacearum*, was a virulent strain. At four weeks, disease severity was statistically analyzed. All treatments with antagonistic bacteria significantly reduced disease severity compared to the control group (Figure 5). The

control treatment (without applied antagonistic bacteria) had an average disease severity index of 80%, while treatments containing the antagonistic bacteria *B. subtilis* (TTYL – 14, TTYL – 18, BTYL – 5), *B. amyloliquefaciens* (BTYL-4), and *B. altitudinis* (MGYL – 1) reduced the severity to 43.33– 46.67% (Table 3). This result aligned with Khositcharoenkul et al. (2008), who found that *B. subtilis* isolated from tobacco rhizosphere soil effectively controlled ginger wilt disease, reducing disease spread by 62– 65% in field trials. Another study by Khositcharoenkul et al. (2006) demonstrated that integrating soil management with urea and lime (80:800 kg/rai) alongside the antagonistic bacterial strain 4415 effectively controlled torch ginger wilt disease by reducing disease incidence to 10%.

Table 3 Disease incidence scale and torch ginger wilt disease (*R. solanacearum*) severity percentage controlled by antagonistic bacteria under greenhouse conditions.

Treatment	Disease Incidence Scale	Disease Severity Percentage
T1. *R. solanacearum*-infested soil + *B. subtilis* (TTYL – 14)	2.50[a]	43.33[a]
T2. *R. solanacearum*-infested soil + *B. subtilis* (TTYL – 18)	2.50 [a]	46.67 [a]
T3. *R. solanacearum*-infested soil + *B. subtilis* (BTYL – 5)	2.33 [a]	43.33 [a]
T4. *R. solanacearum*-infested soil + *B. amyloliquefaciens* (BTYL-4)	2.67 [a]	46.67 [a]
T5. *R. solanacearum*-infested soil + *B. altitudinis* (MGYL – 1)	2.33 [a]	43.33 [a]
T6. *R. solanacearum*-infested soil + water (control)	4 [b]	80 [b]

Means followed by the same letters in columns are not statistically significantly different according to DMRT at P = 0.05

Figure 5 Biocontrol efficacy of antagonistic bacterial isolates against wilt diseases of torch ginger: (A) *R. solanacearum*-infested soil + *B. subtilis* (TTYL – 14), (B) *R. solanacearum*-infested soil + *B. subtilis* (TTYL – 18), (C) *R. solanacearum*-infested soil + *B. subtilis* (BTYL – 5), (D) *R. solanacearum*-infested soil + *B. amyloliquefaciens* (BTYL – 4), (E) *R. solanacearum*-infested soil + *B. altitudinis* (MGYL – 1), (F) *R. solanacearum*-infested soil (Control) at 28 days after inoculation.

5. Conclusion

This study successfully evaluated five antagonistic bacterial isolates with the potential to control torch ginger wilt disease caused by *Ralstonia solanacearum*. The potent isolates were identified as *Bacillus subtilis* (TTYL-14, TTYL-18, BTYL-5), *Bacillus amyloliquefaciens* (BTYL-4), and *Bacillus altitudinis* (MGYL-1), which demonstrated inhibitory effects against the pathogen in laboratory tests. TTYL-18, BTYL-5, and BTYL-4 exhibited antifungal activity against *Colletotrichum gloeosporioides*, *Corynespora cassiicola*, and *Fusarium oxysporum*. All the isolates were able to coexist with *Trichoderma asperellum*, indicating their potential for biological control. Greenhouse experiments confirmed the efficacy of these bacterial isolates in reducing the incidence and severity of torch ginger wilt disease. These promising results highlight the potential application of bacteria as biocontrol agents for sustainable disease management. However, further field trials are necessary to maximize the effectiveness of controlling this disease by focusing on using individual antagonistic bacterial isolates or a combination of more than one isolate and applying multiple doses every 2-3 weeks. Research on large-scale spore production is also necessary to develop a commercial biocontrol powder product. Our findings provide a foundation for eco-friendly and sustainable approaches to managing bacterial wilt and other fungal diseases in torch ginger cultivation. Biocontrol agents have slow disease control potential compared to chemical pesticides. It should be applied before disease outbreaks for a high level of prevention.

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