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Biocontrol Efficacy of *Bacillus subtilis* and *Pseudomonas fluorescens* Isolates against Bacterial Wilt Disease of Tomato under Protected Conditions

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ABSTRACT

Bacterial wilt, caused by *Ralstonia solanacearum*, remains one of the most destructive soil-borne diseases of tomato in protected cultivation systems. This study assessed the greenhouse performance of two rhizobacterial isolates, *Bacillus subtilis* (B-12) and *Pseudomonas fluorescens* (P-05), as potential biocontrol agents against the pathogen. Both isolates inhibited pathogen growth in vitro, producing identical inhibition zones of 18.4 ± 1.2 mm. Functional screening showed that B-12 expressed stronger siderophore production (+++; halo ratio 2.8) and higher protease activity (++) than P-05 (++ and +, respectively), whereas neither isolate showed detectable cellulase activity. Under greenhouse conditions, preventive soil application of either isolate significantly reduced disease incidence from 95.0 ± 4.2% in the pathogen-inoculated control to 35.0 ± 5.1%, corresponding to a biocontrol efficiency of 63.2%. Despite the similar effect on incidence, the two isolates differed in their capacity to limit symptom progression. *Bacillus* sp. B-12 lowered disease severity to 1.2 ± 0.3, compared with 2.1 ± 0.4 for P-05 and 3.6 ± 0.2 for the infected control. B-12 also restored plant height and chlorophyll status to levels approaching those of healthy plants, while P-05 showed a comparatively stronger effect on root dry weight. The data indicate that the two isolates express distinct but potentially complementary modes of action. *B. subtilis* B-12 appears particularly promising for limiting vascular symptom development, whereas *P. fluorescens* P-05 may contribute more strongly to rhizosphere fitness and root growth. These findings support further characterization of both isolates and provide a sound basis for testing single-strain and consortium-based biocontrol strategies for tomato bacterial wilt management under protected conditions.

فاعلية العزلات البكتيرية من *Bacillus subtilis* و *Pseudomonas fluorescens* في المكافحة الحيوية لمرض الذبول البكتيري في نبات الطماطم تحت الظروف المحمية

محمد سالم بو هدمة

يظل مرض الذبول البكتيري، الناتج عن العامل الممرض *Ralstonia solanacearum*، واحدًا من أكثر أمراض التربة تدميرًا لمحصول الطماطم في نظم الزراعة المحمية. هدفت هذه الدراسة إلى تقييم أداء عزلتين من البكتيريا الجذرية، وهما *Bacillus subtilis* (B-12) و*Pseudomonas fluorescens* (P-05)، كعوامل محتملة للمكافحة الحيوية ضد هذا الممرض تحت ظروف الصوبة الزراعية. أظهرت النتائج أن كلتا العزلتين تثبطان نمو الممرض في الظروف المعملية، حيث أنتجتا مناطق تثبيط متماثلة بلغت 18.4 ± 1.2 ملم. وكشفت الفحوصات الوظيفية أن العزلة B-12 تميزت بإنتاج أقوى للسيدروفورات (+++؛ نسبة الهالة 2.8) ونشاط بروتيزي أعلى (++) مقارنة بالعزلة P-05 التي سجلت (++ و + على التوالي)، في حين لم يُكتشف أي نشاط للسليولاز في أي من العزلتين. تحت ظروف الصوبة الزراعية، أدى التطبيق الوقائي للعزلتين في التربة إلى خفض معنوي في معدل الإصابة بالمرض، من 95.0 ± 4.2% في معاملة التحكم الملقحة بالممرض إلى 35.0 ± 5.1%، بما يعادل كفاءة مكافحة حيوية بلغت 63.2%. وعلى الرغم من التشابه في تأثير العزلتين على معدل الإصابة، فقد اختلفتا في قدرتهما على

الحد من تطور الأعراض المرضية. حيث خفضت العزلة B-12 شدة المرض إلى 1.2 ± 0.3، مقارنة بـ 2.1 ± 0.4 للعزلة P-05و3.6 ± 0.2 لمعاملة التحكم المصاب.كما ساهمت العزلة B-12 في استعادة طول النبات وحالة الكلوروفيل إلى مستويات تقترب من تلك المسجلة في النباتات السليمة، في حين أظهرت العزلة P-05 تأثيرًا نسبيًا أقوى على الوزن الجاف للجذور. وتشير هذه البيانات إلى أن العزلتين تعبران عن آليات عمل متميزة ولكنها قد تكون متكاملة. وتبدو العزلة B-12 واعدة بشكل خاص في الحد من تطور الأعراض الوعائية، في حين قد تساهم العزلة P-05 بشكل أكبر في تعزيز لياقة الريزوسفير ونمو الجذور.وتدعم هذه النتائج الحاجة إلى مزيد من التوصيف لهاتين العزلتين، وتوفر أساسًا علميًا رصينًا لاختبار استراتيجيات المكافحة الحيوية باستخدام سلالة مفردة أو مجتمعات ميكروبية متكاملة لإدارة مرض الذبول البكتيري في الطماطم تحت ظروف الزراعة المحمية.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is among the most important vegetable crops worldwide because of its nutritional value, market demand, and central role in protected horticulture. However, intensive tomato production is frequently constrained by soil-borne diseases that are difficult to suppress once established in the rhizosphere. Among these diseases, bacterial wilt caused by *Ralstonia solanacearum* is especially damaging because the pathogen survives in soil and water, invades through roots, colonizes xylem tissues, and rapidly causes irreversible wilting. *R. solanacearum* is widely recognized as one of the most important bacterial

plant pathogens globally, and severe outbreaks may result in major yield losses under favorable environmental conditions (Mansfield *et al*., 2012; Wang *et al*., 2023).

Disease development depends on a complex sequence of events that includes root entry, motility, vascular multiplication, secretion of virulence factors, and extracellular polysaccharide production that restricts water transport. The diversity and ecological adaptability of the *Ralstonia solanacearum* species complex complicate disease control, particularly in protected systems where temperature, humidity, irrigation practices, and repeated cropping may favor pathogen persistence (Wang *et al*., 2023). Chemical bactericides and soil disinfestation measures generally provide inconsistent control, and their repeated use raises concerns regarding cost, environmental safety, and disruption of beneficial soil microbiota. Consequently, interest has increased in biologically based approaches that suppress the pathogen while improving overall plant performance.

Plant growth-promoting rhizobacteria (PGPR) represent one of the most promising biological options for bacterial wilt management. In particular, *Bacillus* and *Pseudomonas* species have received sustained attention because they combine rhizosphere competence with multiple antagonistic mechanisms. *Bacillus* spp. are attractive candidates because they produce resistant spores, tolerate variable environmental conditions, and synthesize diverse antimicrobial compounds and extracellular enzymes. In addition, successful biocontrol by *Bacillus* spp. is often associated with biofilm formation and the activation of plant defense responses (Chen *et al*., 2012; Karačić *et al*., 2024). *Pseudomonas* spp., in contrast, are well known for rapid root colonization, metabolic versatility, and strong iron competition through siderophore production. Recent work has highlighted the importance of siderophore-mediated interactions in suppressing *R. solanacearum* in tomato-associated microbiomes and consortia (Gu *et al*., 2020; Shao *et al*., 2024).

Although the general value of PGPR-based suppression is well established, the performance of candidate strains remains context dependent. Efficacy is influenced by isolate identity, greenhouse or field conditions, and compatibility with local pathogen populations. For this reason, evaluation of locally recovered isolates under relevant protected-cropping conditions remains scientifically and practically important. The present study therefore examined two bacterial isolates, *Bacillus subtilis* B-12 and *Pseudomonas fluorescens* P-05, with the following objectives: (i) to determine their in vitro antagonistic activity against *R. solanacearum*; (ii) to assess selected phenotypic traits associated with biocontrol; (iii) to quantify their effects on bacterial wilt incidence and severity under greenhouse conditions; and (iv) to examine their influence on selected tomato growth parameters.

MATERIALS AND METHODS

Bacterial isolates and inoculum preparation

The bacterial strain *Ralstonia solanacearum* was obtained from the Microbial Vaccine Centre, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The pathogen was propagated on Nutrient Agar (NA) at 28 °C for 24 h, and the resulting bacterial suspension was

adjusted to a final concentration of 1 × 10^8 colony-forming units per milliliter (CFU/mL).

Two biocontrol isolates were employed in this study: *Bacillus subtilis* (isolate B-12) and *Pseudomonas fluorescens* (isolate P-05), both sourced from the same institution aforementioned. All bacterial isolates were routinely maintained on Nutrient Agar (NA) and Luria-Bertani (LB) agar, respectively, and stored at 28 ± 2 °C. Working suspensions were prepared in sterile distilled water and standardized spectrophotometrically at an optical density of 600 nm (OD_{600}) to achieve a final concentration of 1 × 10^8 CFU/mL prior to all experimental procedures.

In Vitro Antagonistic Activity

Antagonistic activity against *R. solanacearum* was evaluated on nutrient agar using an agar diffusion assay. A standardized pathogen suspension (10^8 CFU mL-1) was evenly spread over the plate surface. Sterile 6-mm paper discs loaded with the antagonist suspensions were then placed on the inoculated medium. Discs containing sterile distilled water served as the negative control. Plates were incubated at 28°C for 48 h, after which inhibition zones were measured in millimeters. The assay was conducted in triplicate and repeated independently to confirm reproducibility.

Detection of Biocontrol Mechanisms

Qualitative assays were used to examine three traits relevant to antagonistic performance. Siderophore production was assessed on Chrome Azurol S agar and scored according to the size of the orange halo relative to colony diameter. Protease activity was determined on skim milk agar based on the formation of a clear zone around colonies. Cellulase activity was assessed on carboxymethylcellulose agar after Congo red staining and NaCl destaining, where a clear halo indicated cellulose degradation. Trait expression was recorded as absent (-), weak (+), moderate (++), or strong (+++), together with the halo ratio where applicable.

Greenhouse Experiment

The greenhouse experiment was carried out under protected conditions at 28-32°C during the day, approximately 25°C at night, 70-80% relative humidity, and a 16-h photoperiod. Four-week-old seedlings of a susceptible tomato cultivar were transplanted into 20-cm plastic pots containing a sterilized peat: sand mixture (1:1, v/v). Four treatments were evaluated: healthy control, pathogen-inoculated control, *Bacillus subtilis* (B-12) + pathogen, and *Pseudomonas fluorescens* (P-05) + pathogen. Antagonists were applied preventively as soil drenches (50 mL pot-1 at 10^8 CFU mL-1) 48 h before pathogen inoculation. The pathogen was then applied at the same volume and concentration. Each treatment consisted of five plants per replicate in three randomized blocks.

Table 1: In vitro antagonistic activity and mechanism characterization of selected isolates against *R. solanacearum*

Treatment	Inhibition Zone (mm)	Siderophore (CAS)	Protease	Cellulase
Bacillus sp. (B-12)	18.4 [a] ± 1.2	+++ (2.8)	++	-
Pseudomonas sp. (P-05)	18.4 [a] ± 1.2	++ (2.1)	+	-
Negative Control	0.0 [c]	-	-	-

Values represent mean ± standard deviation. Different letters indicate significant differences at $p \leq 0.05$ (LSD test). (+++) strong production, (++) moderate, (+) weak, (-) negative.

Disease and Growth Assessment

Plants were assessed 30 days after pathogen inoculation. Disease incidence was calculated as the percentage of symptomatic plants per treatment. Disease severity was rated on a 0-4 scale, where 0 = no visible symptoms, 1 = wilting of one or two leaves, 2 = 25-50% wilted foliage, 3 = 51-75% wilted foliage, and 4 = complete collapse or plant death. Phiri *et al*. (2024). Plant height was measured from the substrate surface to the shoot apex. Root dry weight was determined after oven drying at 70°C to constant mass. Leaf chlorophyll status was estimated with a SPAD-502 meter using three readings per plant from fully expanded leaves.

Statistical Analysis

The experiment was conducted using a completely randomized design (CRD) with three replicates per treatment. Data were subjected to one-way analysis of variance (ANOVA) using the appropriate statistical software. Treatment means were separated using Fisher's Least Significant Difference (LSD) test at $p \leq 0.05$. Results are presented as means ± standard deviation, and means followed by different letters within the same column indicate significant differences among treatments. Statistical analyses were conducted using CoStat statistical software (version 6.4).

RESULTS AND DISCUSSION

In Vitro Antagonistic Activity and Biocontrol Mechanisms

Both isolates B-12 and P-05 exhibited strong and statistically significant inhibitory activity against *R. solanacearum*, producing identical mean inhibition zone diameters of 18.4 ± 1.2 mm. No inhibitory was recorded for the negative control (0.0 mm). Despite this equivalence in inhibitory zone size, the two isolates differed in their phenotypic profiles. Isolate B-12 demonstrated the highest siderophore production intensity (+++ with halo ratio 2.8), while P-05 displayed moderate production (++ with ratio 2.1). B-12 also exhibited moderate protease activity (++), whereas P-05 showed only weak protease activity (+). Neither isolate produced detectable cellulase activity under the test conditions. This mechanistic divergence despite equivalent in vitro suppression strongly implies that the two isolates achieve comparable pathogen inhibition through distinct biochemical pathways, a finding with important implications for their potential complementary use.

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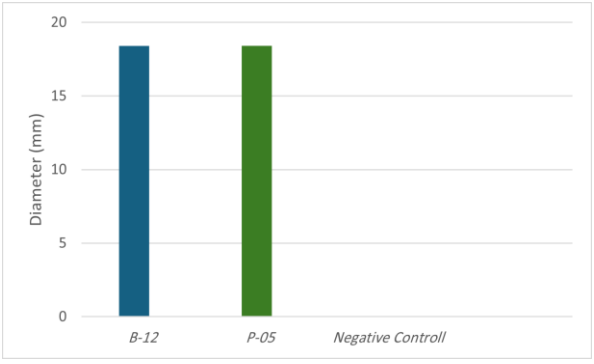

Figure 1: Comparison of inhibition zone diameters (mm) of bacterial isolates against *R. solanacearum*

Suppression of bacterial wilt under greenhouse conditions

The pathogen-inoculated control developed severe disease, confirming successful infection pressure in the greenhouse assay. Disease incidence reached 95.0 ± 4.2%, and the mean severity score was 3.6 ± 0.2. Preventive treatment with either antagonist reduced disease incidence to 35.0 ± 5.1%, equivalent to an overall biocontrol efficiency of 63.2%. However, the isolates differed clearly in their ability to limit disease progression after infection. *B. subtilis* B-12 reduced disease severity to 1.2 ± 0.3, whereas *P. fluorescens* P-05 reduced it to 2.1 ± 0.4. Thus, although both isolates lowered the proportion of diseased plants to a similar extent, B-12 provided stronger suppression of symptom expression within affected plants.

Table 2: Effect of biocontrol treatments on bacterial wilt disease indices and tomato growth parameters

Pathogen challenge markedly impaired tomato growth. Compared with healthy plants, infected controls were shorter, had lower root dry weight, and exhibited reduced SPAD values. Treatment with B-12 markedly improved shoot-related variables, raising plant height to 42.5 ± 3.1 cm and chlorophyll status to 38.4 ± 2.1 SPAD units, both close to the values recorded for the healthy control. By contrast, P-05 produced a more moderate recovery in shoot growth but yielded the strongest improvement in root dry weight (1.25 ± 0.12 g), exceeding the infected control. These contrasting responses suggest that the two isolates may influence host performance through partially different ecological or physiological pathways. **Treatment**	**Incidence (%)**	**Disease Severity (0-4)**	**Plant Height (cm)**	**Root Dry Weight (g)**	**SPAD**
Negative Control (Healthy)	0.0 ^{c} ± 0.0	0.0 ^{c} ± 0.0	48.6 ^{a} ± 2.8	2.10 ^{a} ± 0.18	42.5 ^{a} ± 1.9
Positive Control (Pathogen)	9.5 ^{a} ± 4.2	3.6 ^{a} ± 0.2	18.2 ^{c} ± 2.4	0.65 ^{c} ± 0.08	21.5 ^{c} ± 3.4
B-12 + Pathogen	35.0 ^{b} ± 5.1	1.2 ^{b} ± 0.3	42.5 ^{ab} ± 3.1	0.65 ^{c} ± 0.08	38.4 ^{ab} ± 2.1
P-05 + Pathogen	35.0 ^{b} ± 5.1	2.1 ^{b} ± 0.4	32.1 ^{b} ± 2.9	1.25 ^{bc} ± 0.12	30.2 ^{b} ± 2.8

Values represent means ± standard deviation (n=3). Different letters within a column indicate significant differences at p = 0.05 (LSD). Disease severity scale: 0 = healthy, 4 = dead plant (30 days post-inoculation).

Effects on Plant Growth Parameters

Pathogen challenge markedly impaired tomato growth. Compared with healthy plants, infected controls were shorter, had lower root dry weight, and exhibited reduced SPAD values. Treatment with B-12 markedly improved shoot-related variables, raising plant height to 42.5 ± 3.1 cm and chlorophyll status to 38.4 ± 2.1 SPAD units, both close to the values recorded for the healthy control. By contrast, P-05 produced a more moderate recovery in shoot growth but yielded the strongest improvement in root dry weight (1.25 ± 0.12 g), exceeding the infected control. These contrasting responses suggest that the two isolates may influence host performance through partially different ecological or physiological pathways.

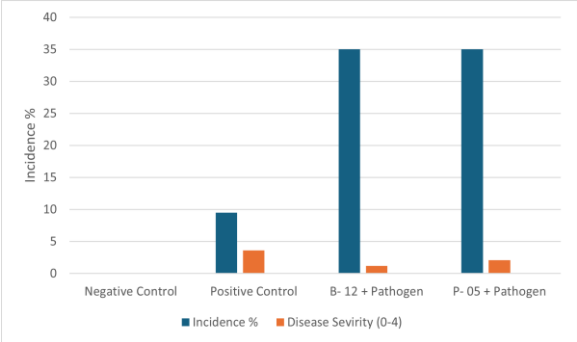

Figure 2: Effect of biocontrol treatments on disease incidence (%) and severity (0-4)

The results of this study unequivocally demonstrate the high efficacy of isolated *Bacillus subtilis* (B-12) and *Pseudomonas fluorescens* (P-05) in mitigating the devastating impact of bacterial wilt on tomato, thereby corroborating global trends favoring biological control as a sustainable pillar of Integrated Pest Management (IPM). The observed 63.2% reduction in disease incidence aligns closely with the efficacy ranges reported in comparable international studies. For instance, Mekonnen *et al*. (2022) reported similar suppression levels using rhizobacteria, while Yendyo *et al*. (2018) documented comparable efficacy for *P. fluorescens* and *B.subtilis* isolates against *Ralstonia solanacearum*.

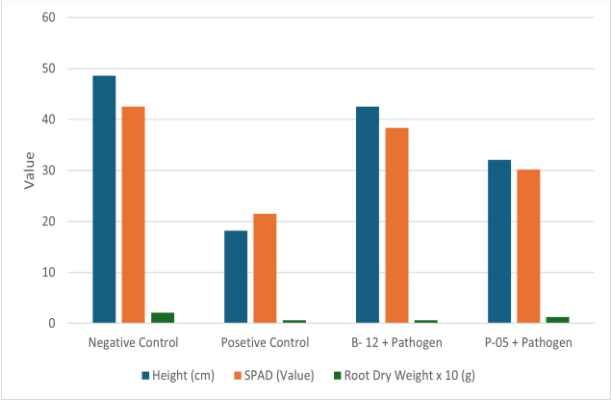

Figure 3: Effect of treatments on plant growth parameters

The strong performance of B-12 is consistent with the broader literature on *Bacillus*-based biocontrol. *Bacillus* spp. can suppress bacterial wilt through a combination of antibiosis**,** competitive root colonization, biofilm formation, and stimulation of host defenses. Chen *et al*. (2012) showed that effective suppression of tomato wilt by *Bacillus subtilis* depended on conserved genes involved in biofilm formation, highlighting the importance of stable root association rather than simple antagonism alone. More recently, Sun *et al*. (2023) reported that *Bacillus subtilis* strain R31 significantly reduced bacterial wilt in tomato, while Samaras *et al*. (2021) documented concurrent disease suppression and growth promotion by *B. subtilis* MBI600 in tomato. In the present study, the stronger siderophore signal and higher protease activity observed for B-12, together with its superior reduction of severity, support the interpretation that this isolate possesses a broader functional profile than P-05 under the tested conditions.

The in vitro analyses revealed a fascinating divergence in mechanistic profiles despite identical inhibitory zone diameters. The potent inhibition exhibited by *Bacillus* sp. B-12 is likely attributable to the production of lipopeptide antibiotics — such as iturin, fengycin, surfactin, or difficidin — which are well-documented primary antimicrobial agents in this genus capable of disrupting bacterial cell membrane integrity. The elevated siderophore production and concurrent protease activity observed in B-12 further broaden its inhibitory toolkit, enabling it to simultaneously deprive the pathogen of essential iron and potentially degrade exoenzymes or structural proteins of the pathogen. This multiplicity of mechanisms aligns with the findings of Karačić *et al*. (2024) and Chen *et al*. (2012), who emphasized that the efficacy of *Bacillus* spp. is often driven by a synergistic convergence of multiple suppressive pathways, which likely contributes to the robust in planta protection observed subsequently in our greenhouse assay.

Conversely, the antagonistic activity of Pseudomonas fluorescens (P-05) appears to be driven primarily by siderophore-mediated iron sequestration. In the iron-impoverished rhizosphere micro-environment, where soluble Fe^{3+} availability is generally below 10^{-18} M, the secretion of high-affinity siderophores (such as pyoverdine) confers a decisive competitive advantage to Pseudomonas by outcompeting pathogens for this limiting nutrient.

Pseudomonas spp. are widely recognized for efficient rhizosphere colonization and iron competition, and siderophore-mediated interactions are now considered a major determinant of pathogen suppression in tomato-associated bacterial communities (Gu *et al*., 2020; Shao *et al*., 2024). The moderate siderophore activity of P-05 observed here is therefore biologically meaningful, even though other unmeasured mechanisms may also have contributed. The relatively better root response under P-05 may reflect enhanced rhizosphere fitness, improved

nutrient acquisition, or subtle modulation of root development.

The findings of Deb *et al*. (2024) further confirm the broad relevance of siderophore-producing bacteria as biocontrol agents. These collective insights provide a robust mechanistic context for P-05's strong inhibitory activity despite its comparatively limited enzymatic arsenal.

A critical finding of this study was the superior capability of isolate B-12 to reduce disease severity (66.7% reduction vs 41.7% for P-05). This enhanced protection likely stems from mechanisms beyond direct antibiosis, such as the induction of systemic resistance (ISR) and the formation of stable, protective biofilms on root surfaces. The pivotal study by Chen *et al*. (2012) elegantly demonstrated that in *Bacillus subtilis*, surfactin production functions not only as an antimicrobial mechanism but also as a signaling cue that triggers biofilm matrix assembly — a synergistic relationship essential for effective root colonization and plant protection. Sun *et al*. (2023) reported a control effect of 85.6% for *B. subtilis* R31 at 10^8 CFU/mL; our B-12 achieved a 66.7% severity reduction under comparable experimental conditions, confirming that the isolate's performance is consistent with published benchmarks. Samaras *et al*. (2021) additionally documented the capacity of *B. subtilis* strains to concurrently suppress *R. solanacearum* while promoting overall plant vigor, corroborating our parallel observations on shoot growth restoration.

In contrast, *Pseudomonas fluorescens* P-05 distinguished itself by promoting significantly higher root dry weight compared to B-12 and the positive control. This result is consistent with the well-documented capacity of *Pseudomonas* spp. to stimulate lateral root proliferation and root elongation through the secretion of auxins, specifically indole-3-acetic acid (IAA). Furthermore, P-05's siderophore activity may have concurrently improved rhizosphere iron nutrition, thereby supporting enhanced root metabolic activity. Chandrasekaran *et al*. (2016) highlighted this dual role of *Pseudomonas* — as both pathogen suppressor and plant growth promoter — particularly noting its rhizosphere fitness and hormone-mediated growth stimulation. This specific stimulation of root architecture suggests that P-05 may be particularly valuable in the early stages of crop establishment or in soil conditions where root development is constrained.

An intriguing observation from our data was that while B-12 excelled at protecting aerial parts, it did not improve root dry weight beyond the level of the positive control. This anomaly warrants careful interpretation. The most plausible explanation is that *R. solanacearum* colonization of root tissues had indeed occurred in the 35% disease-incidence fraction before the full protective effects of B-12 could be established, causing localized and irreversible root biomass loss. Critically, however, B-12 appears to have effectively intercepted systemic vascular spread — preventing the pathogen's upward migration through xylem vessels — thereby preserving the integrity of aerial plant organs despite concurrent root-level infection. This distinction between root protection and systemic defense interception has important mechanistic implications and represents a potentially novel aspect of B-12's biocontrol profile that warrants further molecular investigation.

The complementary mechanistic profiles and spatially differentiated protective effects identified in this study — with B-12 providing superior systemic and shoot-level protection, and P-05 delivering intensive rhizosphere iron competition and root stimulation — suggest that a consortium formulation combining both isolates could yield synergistic biocontrol outcomes exceeding either isolate alone. This integrated consortium approach aligns with the growing consensus in the field (Zheng *et al*., 2024; Shao *et al*., 2024) that multi-strain preparations outperform single-strain interventions by exploiting functional complementarity.

CONCLUSIONS

Under protected conditions, both *Bacillus subtilis* (B-12) and *Pseudomonas fluorescens* P-05 significantly reduced tomato bacterial wilt caused by *Ralstonia solanacearum*. The two isolates were equally effective in lowering disease incidence, but *B. subtilis* B-12 provided stronger suppression of disease severity and better restoration of shoot growth and leaf chlorophyll status. *P. fluorescens* P-05, while less effective in reducing symptom severity, was associated with improved root dry weight. These results indicate that the isolates do not act identically and may offer complementary strengths for future biocontrol development.

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