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# EVALUATION OF MICROBIAL POTENTIAL OF RHIZOBACTERIAL ISOLATES ASSOCIATED WITH SPENT MUSHROOM COMPOST AGAINST BACTERIAL WILT OF TOMATO

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## ABSTRACT

Bacterial wilt, caused by the soil-borne pathogen *Ralstonia solanacearum*, poses a significant threat to tomato crops worldwide. This study aims to assess the microbial potential of rhizobacterial isolates obtained from spent mushroom compost in suppressing bacterial wilt of tomatoes. Spent mushroom compost is a byproduct of mushroom cultivation and is known to harbor diverse microbial communities with potential plant-beneficial properties. Tomato leaves that were contaminated were collected from a number of places in the Rawalpindi Area, Punjab, Pakistan. Rhizobacterial isolates were collected from the rhizosphere of tomato plants grown in the presence of spent mushroom compost. These isolates were then subjected to laboratory evaluations for their antagonistic activity against *R. solanacearum*. Selected rhizobacterial isolates were further characterized for their plant growth-promoting traits. The potential of these isolates to enhance tomato plant growth and confer resistance against bacterial wilt was evaluated through greenhouse experiments. Results indicated that certain rhizobacterial isolates exhibited substantial antagonistic activity against *R. solanacearum*. Additionally, these isolates demonstrated multiple plant growth-promoting traits, suggesting a potential dual role in both pathogen suppression and plant enhancement. Greenhouse experiments revealed a significant reduction in the incidence of bacterial wilt in tomato plants and increase in the growth promotion traits were observed while treated with the selected rhizobacterial isolates alone and in combination compared to control groups. The findings from this study highlight the promising role of rhizobacterial isolates associated with spent mushroom compost in managing bacterial wilt in tomatoes.

**Keywords:** Bacterial wilt; Soil born pathogen; Spent mushroom compost

## INTRODUCTION

One of the most popular vegetables in the world, the tomato (*Lycopersicon esculentum*) is grown and eaten by a lot of people. The tomato is in the Solanaceae family (Pritesh *et al*., 2011). With over 160 million tonnes, tomatoes make up about 15% of all the vegetables grown in the world. In Pakistan, a number of low-yielding cultivars have trouble growing in these conditions (Hadian *et al*., 2011). Bacterial wilt, caused by the soil-borne pathogen *Ralstonia solanacearum*, represents a significant threat to tomato crops globally, leading to substantial economic losses in agriculture. Traditional control methods often involve the use of chemical pesticides, which can have adverse environmental effects and contribute to the development of resistant pathogen strains. In recent years, there has been an increasing interest in exploring sustainable and eco-friendly alternatives for disease management, including the use of beneficial microorganisms (McGovern, 2015; Rashid *et al*., 2011). Bacterial wilt is the second most important potato disease in tropical and subtropical parts of the world, after late blight (Champoiseau *et al*., 2010). These bacteria might be a soil-borne pathogen that kills many types of plants by causing shrivel diseases (Chu *et al*., 2008).

Spent mushroom compost, a byproduct of mushroom cultivation, is known for its rich microbial diversity. The compost, primarily consisting of mushroom mycelium, straw, and other organic materials, provides a favorable environment for the proliferation of various microorganisms, including

bacteria with potential plant-beneficial properties. The potential of these microbial communities associated with spent mushroom compost to suppress soil-borne pathogens and enhance plant health presents an intriguing avenue for sustainable agriculture (Koberl *et al*., 2013).

Rhizobacteria, residing in the rhizosphere of plants, have been recognized for their multifaceted roles in promoting plant growth and inducing systemic resistance against pathogens. In this context, the current study focuses on evaluating the microbial potential of rhizobacterial isolates obtained from the rhizosphere of tomato plants grown in the presence of spent mushroom compost. Specifically, the research aims to investigate the antagonistic activity of these isolates against *R. solanacearum* and their ability to mitigate bacterial wilt in tomatoes (Altaf and Inam-ul-Haq, 2020; Goudjal *et al*., 2014; Jain *et al*., 2015).

Utilizing biocontrol agents presents a promising alternative to synthetic chemical-based formulations for effective disease management. In investigating the combined efficacy of antagonist rhizobacteria as biocontrol agents and spent mushroom compost as a biofertilizer against the bacterial wilt pathogen, among the twenty tested antagonistic rhizobacterial isolates, only three, namely Rh10, Rh12, and Rh15, exhibited the highest inhibitory effects against *R. solanacearum* (Altaf and Inam-ul-Haq, 2020).

The growth of cucumber seedlings was notably enhanced, and the population of *Fusarium oxysporum* f. sp. *cucumerinum* (FOC) was significantly suppressed ($p < 0.05$) when three different spent mushroom substrate (SMS) amendments were applied, as compared to the control. Notably, among the three SMS amendments, the utilization of spent substrate from *F. velutipes* exhibited a particularly pronounced effect. This specific SMS amendment led to a notable increase in the presence of beneficial microbes, including species such as *Bacillus* spp., *Pseudomonas* spp., and *Lysobacter* spp. (Wang *et al*., 2020).

The objectives of the current study was to evaluate the effects of microbial population associated with spent mushroom compost against bacterial wilt pathogen.

## MATARIALS AND METHODS

**Pathogen:** To isolate *Ralstonia solanacearum* from infected tomato plants, diseased plant tissues were collected, and surface sterilization was carried out using sequential immersions in 70% ethanol and 1-2% sodium hypochlorite. The sterilized tissues were homogenized, and the resulting suspension was subjected to serial dilution. The dilutions were then plated onto TTC medium, following an incubation period at 28-30 °C for 48-72 hours. Colonies with characteristic appearances indicative of *Ralstonia solanacearum* were observed and subjected to further characterization (Begum *et al*., 2012).

**Pathogenicity:** For the pathogenicity test, tomato plants were selected as the host for inoculation. The bacterial isolates were cultured in nutrient-rich medium. Tomato plants at the seedling stage were carefully uprooted and roots were dip-inoculated in the bacterial suspension containing a standardized concentration of *R. solanacearum*. Control plants were similarly treated with a sterile medium. Inoculated plants were transplanted into sterile soil and placed in a controlled environment with optimal conditions for bacterial infection. Disease progression was monitored daily by assessing symptoms such as wilting, yellowing, and vascular discoloration. After an appropriate incubation period, the plants were uprooted, and the pathogen was re-isolated from symptomatic tissues.

**Biochemical confirmation of *R. solanacearum:*** To biochemically identify *Ralstonia solanacearum*, bacterial isolates were initially cultured in nutrient-rich media to obtain a pure culture. Then gram-staining, potassium hydroxide (KOH) test (Chaudhry and Rashid, 2011), catalase oxidase test (Derib *et al*., 2013), levan production from sucrose, kovacs oxidase test (Hossain *et al*., 2007), race identification test (Janse, 1991) and production of fluorescent pigment was employed for the identification of bacterial isolates.

**Isolation of plant growth promoting rhizobacteria from SMC:** Six-month-old SMC samples were collected from the NARC National Agriculture Research Center, and rhizospheric soil adhering to the roots of plants grown in SMC-amended soil was carefully collected. Serial dilutions of the soil suspension were prepared, and aliquots were spread onto King's B and nutrient agar media. The plates were incubated at 28 ± 2 ℃, and distinct bacterial colonies were subcultured to obtain pure isolates.

***In vitro* evaluation of rhizobacteria:** Pure cultures of bacterial isolates collected from SMC were prepared and assessed for their antagonistic potential against *R. solanacearum*. Dual-culture confrontation assays were conducted on nutrient agar, where the rhizobacterial isolates were co-inoculated with *R. solanacearum*. Inhibition zones and interactions between the bacterial colonies were observed.

Additionally, the production of antimicrobial metabolites was assessed by measuring zones of inhibition on agar plates containing *R. solanacearum*.

**Evaluation of Rhizobacterial properties of PGPRs:** Furthermore, the selected rhizobacterial isolates were characterized for plant growth-promoting traits, including hydrogen cyanide (HCN) production, phosphate solubilization, and siderophore production. The combined results from these in vitro assays aimed to identify rhizobacterial isolates with robust antagonistic activity against *R. solanacearum* and potential plant growth-promoting capabilities, providing valuable insights for their application in biocontrol strategies against bacterial wilt in tomato crops.

***In vivo* evaluation of rhizobacteria:** Tomato plants were selected as the host for the study, and rhizobacterial isolates previously identified for their antagonistic activity against *R. solanacearum* were applied. Prior to plant inoculation, the rhizobacterial isolates were cultured, and their concentrations were standardized. A completely randomized design was employed, with treatments including the selected rhizobacterial isolates and a control group. Tomato seedlings were inoculated with the rhizobacteria by soil drenching and root dipping methods. The plants were transplanted into greenhouse pots containing sterile soil, and environmental conditions conducive to the development of bacterial wilt were maintained. Disease progress and plant growth parameters were monitored over time. The greenhouse experiment aimed to assess the efficacy of the selected rhizobacterial isolates in suppressing bacterial wilt and promoting the growth of tomato plants under controlled conditions, providing insights into the potential practical application of these isolates as biocontrol agents

## RESULTS

**Pathogenic Bacteria:** A total of 22 isolates were isolated from soil samples, using single colonies that formed after serial dilution of infected plants on TTC media. For confirmation and pathogenicity examinations, cultures were grown on TTC medium and treated with sterile distilled water. The hue of purified cultures of bacteria was rosy pink (Figure 1).

Figure 1. Confirmation of *R. solanacearum* on TTC media

**Pathogenicity test:** During pathogenicity a total of seven isolates, including I-3, I-7, I-10, I-12, I-15, I-19, and I-21 exhibited symptoms to varied degrees. These findings demonstrate that these isolates were pathogenic. The isolate I-3 caused plant mortality within 5-6 days of inoculation, whereas I-15 and I-19 caused plant death within 10 days due to severe infection. These investigations demonstrate that a disease has inhibited plant growth and that plants have not reached their genetic potential. Isolates I-7, I-10, I-12, and I-21 exhibited moderate symptoms, but they were unable to overcome the plant's defences (Table 1).

**Biochemical Identification:** The results of the biochemical identification of virulent seven pathogenic isolates revealed distinctive metabolic characteristics indicative of the pathogen. Initial gram staining confirmed the bacterium's Gram-negative nature. Subsequent biochemical tests demonstrated positive reactions for oxidase and catalase activities (Table 2), while levan formation was not shown when cultured with sucrose. Selective media also supported the growth of *R. solanacearum* colonies, further confirming its identity.

Table 1: Virulence of pathogenic isolates on tomato plants.

| Isolate | Virulence* | Isolate | Virulence* |
|---|---|---|---|
| I-1 | + | I-12 | ++ |
| I-2 | + | I-13 | + |
| I-3 | +++ | I-14 | + |
| I-4 | + | I-15 | ++ |
| I-5 | + | I-16 | ++ |
| I-6 | + | I-17 | + |
| I-7 | ++ | I-18 | + |
| I-8 | + | I-19 | + |
| I-9 | + | I-20 | + |
| I-10 | ++ | I-21 | ++ |
| I-11 | + | I-22 | + |

Weakly virulent (+), moderately virulent (++) and *highly virulent (+++)

Table 2: Biochemical identification of pathogenic isolates.

| S. No | Isolates | KOH (Loop Test) | Gram Staining | Catalase oxidase | Levan Production |
|---|---|---|---|---|---|
| 1 | I-3 | + | - | + | - |
| 2 | I-7 | + | - | + | - |
| 3 | I-10 | + | - | + | - |
| 4 | I-12 | + | - | + | - |
| 5 | I-15 | + | - | + | - |
| 6 | I-19 | + | - | + | - |
| 7 | I-21 | + | - | + | - |

**Biovar identification test**

Biovar confirmation test was performed and kept under observation for 15 days for color change in lactose, maltose, and cellobiose, revealing that all the seven isolates belonged to biovar 2 (Table 3).

**Rhizobacteria isolated from SMC**

A total of 25 rhizobacterial isolates were collected from the serial dilutions of spent mushroom compost samples. Each individual isolate has specific colony features. Colony characteristics of distinct bacterial species differ, although strains with similar characteristics differed insignificantly. Each isolate's colony size, shape, and colour were observed and documented (Table 4).

Table 3: Determination of biovar.

| Isolates | C | Mal | Lac | Sorb | Celbo | Mant | Dul | Biovar |
|---|---|---|---|---|---|---|---|---|
| I-3 | - | + | + | - | + | - | - | 2 |
| I-7 | - | + | + | - | + | - | - | 2 |
| I-10 | - | + | + | - | + | - | - | 2 |
| I-12 | - | + | + | - | + | - | - | 2 |
| I-15 | - | + | + | - | + | - | - | 2 |
| I-19 | - | + | + | - | + | - | - | 2 |
| I-21 | - | + | + | - | + | - | - | 2 |

C: Control, Mal: Maltose, Lac: Lactose, Sorb: Sorbitol, Mant: Manitol, Celbo: Cellobiose, Dul: Dulcitol

***In vitro* evaluation of rhizobacterial anatagonistic activity against *R. solanacearum:*** From all the isolates Rh-7 and Rh-20 shown considerable antagonistic activity in dual culture assay against *R. solanacearum.* Rh-20 produced superior outcomes compared to Rh-7, as they independently inhibited the growth of pathogenic isolate at 14 mm and 11 mm zone of inhibition (Figure 2).

**Evaluation of rhizobacterial properties:** Both Rh-7 and Rh-20 were thought to be conclusive evidence of siderophore age (Table 5). Rh-7 altered the colour of cocoa to an orangey red, while Rh-20 brought about a yellowish green. Hydrogen cyanide-producing bacteria were classified into four ability levels, from very high to medium to low. Compared to Rh-7, Rh-20 has a high HNC-generating capacity

(Table 5). Each disconnect clearly responded when corona zones were generated in this experiment. This proves that the segregates have a solubilizing effect on phosphorus (Table 5). Because the Rh-7 radiation zone was so much wider, its results were far more impressive than those of the Rh-20 radiation zone. This revealed that Rh-7's 15-mm-wide radiance zone could solubilize more Ca-P than Rh-20's 8-mm-wide corona zone.

Table 4: Rhizobacteria colony characteristics.

| S. No | Isolates | Color of Colony | Shape of Colony | Size of Colony (mm) |
|---|---|---|---|---|
| 1 | Rh-1 | off white | Large, flat, wavy, margin | 2-3 |
| 2 | Rh-2 | pale yellow | Wavy, medium size, flat | 1-3 |
| 3 | Rh-3 | creamy white | Round, medium, sized, slightly raised | 2-0 |
| 4 | Rh-4 | pale yellow | Wavy, medium size, flat | 1-0 |
| 5 | Rh-5 | reddish | Circular, entire margin | 1-5 |
| 6 | Rh-6 | off white | Large, flat, wavy, margin | 3-4 |
| 7 | Rh-7 | creamy white | Round, medium, sized, slightly raised | 3-5 |
| 8 | Rh-8 | whitish | Flat, circular and margin undulated | 0.7 |
| 9 | Rh-9 | pale orange | Large, round, flattened helical | 0.5 |
| 10 | Rh-10 | white colony | Round, small, raised | 2-3 |
| 11 | Rh-11 | pale yellow, shiny | Round, small, smooth | 1-0 |
| 12 | Rh-12 | light brown | Circular, convex with entire margin | 2-0 |
| 13 | Rh-13 | reddish | Circular, entire margin | 3-0 |
| 14 | Rh-14 | yellow | Small, round convexly raised | 3-5 |
| 15 | Rh-15 | pale orange | Large, round, flattened helical | 2-3 |
| 16 | Rh-16 | pale yellow, shiny | Round, small, smooth | 1-5 |
| 17 | Rh-17 | whitish creamy | Flat, larger, circular and margin undulated | 0-5 |
| 18 | Rh-18 | pale yellow | Wavy, medium size, flat | 0-8 |
| 19 | Rh-19 | off white | Large, flat, wavy, margin | 2-3 |
| 20 | Rh-20 | yellow | Small, round convexly raised | 3-4 |
| 21 | Rh-21 | light brown | Circular, convex with entire margin | 1-5 |
| 22 | Rh-22 | off white | Large, flat, wavy, margin | 0-5 |
| 23 | Rh-23 | white colony | Round, small, raised | 3-4 |
| 24 | Rh-24 | white colony | Round, small, raised | 3 |
| 25 | Rh-25 | whitish | Flat, circular and margin undulated | 2-5 |

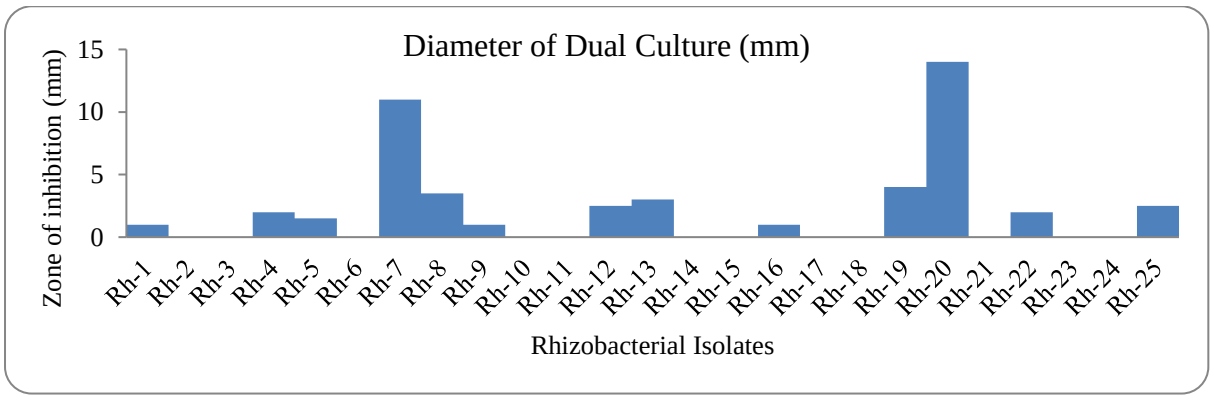


Figure 2: In vitro evaluation of rhizobacterial isolates activity against *R. solanacearum.*

Table 5. Response of the antagonistic rhizobacteria to rhizobacterial properties.

| Isolates | Siderophore Production test | HCN Production | Phosphorus Solubilization |
|---|---|---|---|
| Rh-7 | - | + | + |
| Rh-20 | + | + | + |

***In vivo* evaluation of antagonistic rhizobacteria against *R. solanacearum***

**Assessment of disease:** The results from the eight treatments showed that T5 had the greatest outcomes (16% disease incidence), followed closely by T6 (20%). Of all the treatment concentrations tested T2 (Rh-7 + Rs ($10^7$)) was the least effective either showing 50% disease incidence as compared with positive control (78%) (Table 6).

**Pathogen population measurement (cfu/ml)**

The bacterial population (cfu/ml) in response to the control and other treatments was determined by cutting down stem sections from uprooted plants and isolating the pathogen and it was observed that the maximum bacterial population was measured in T2 that was 47 cfu/ml as compared with control T7 (78 cfu/ml) while minimum bacterial population was measured in T5 that was 21 cfu/ml (Table 7).

Table 6. Effect of antagonistic rhizobacterial isolates against disease incidence percentage of tomato bacterial wilt incidence.

| Treatments | | Disease incidence (%) |
|---|---|---|
| T1 | Rh-7 + Rs ($10^5$) | 23 |
| T2 | Rh-7 + Rs ($10^7$) | 50 |
| T3 | Rh-20 + Rs ($10^5$) | 23 |
| T4 | Rh-20 + Rs ($10^7$) | 34 |
| T5 | Rh-7 + Rh-20 + Rs ($10^5$) | 16 |
| T6 | Rh-7 + Rh-20 + Rs ($10^7$) | 20 |
| T7 | Positive Control [Rs ($10^7$)] | 78 |
| T8 | Control (Distilled Water) | 0 |

Table 7. Mean cfu/ml pathogen population recovered from all the treatments.

| Treatments | | Mean Value CFU/ml |
|---|---|---|
| T1 | Rh-7 + Rs ($10^5$) | 25 |
| T2 | Rh-7 + Rs ($10^7$) | 47 |
| T3 | Rh-20 + Rs ($10^5$) | 27 |
| T4 | Rh-20 + Rs ($10^7$) | 39 |
| T5 | Rh-7 + Rh-20 + Rs ($10^5$) | 21 |
| T6 | Rh-7 + Rh-20 + Rs ($10^7$) | 24 |
| T7 | Rs ($10^7$) | 78 |
| T8 | DW | 0 |

**Plant Growth Parameters Assessment**

**Effect on plant height:** Maximum shoot length and root length of 41.1 cm and 37.3 cm were recorded in T5 when both the bacteria Rh-7 and Rh-20 used in combination with pathogenic bacterial concentration 1 x $10^5$ cfu/ml, respectively as compared with positive control that was 16.8 cm shoot length and 14.6 cm root length (Figure 3).

**Effect on plant (fresh and dry) weight":** The shoot fresh weight of plants treated with Rh-20 in combination with Rh-7 was 52.3g at both pathogen concentrations ($1\times10^5$cfu/ml and $1\times10^7$ cfu/ml), and the root fresh weight of plants treated with Rh-20 in combination with Rh-7 was 9.8g at $1\times10^5$ cfu/ml while 8.9g at the concentration of $1\times10^7$ cfu/ml of pathogenic bacteria, both with highly significant differences compared with the pathogen-infected control and other treatments (Figure 4).

## DISCUSSION

The evaluation of the microbial potential of rhizobacterial isolates associated with spent mushroom compost against bacterial wilt of tomato represents a significant step towards developing sustainable and eco-friendly strategies for disease management in agriculture. The study aimed to harness the diverse microbial communities present in spent mushroom compost, with a focus on their

antagonistic activity against *Ralstonia solanacearum*, the causative agent of bacterial wilt in tomatoes (Andrews *et al*., 2003).

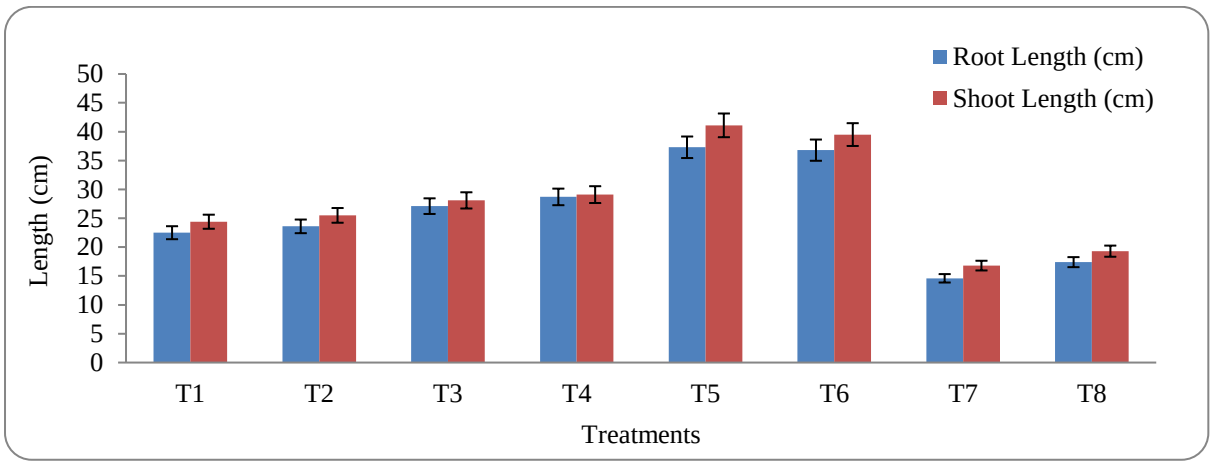


Figure 3. Effect of treatments on plants root length and shoot length.

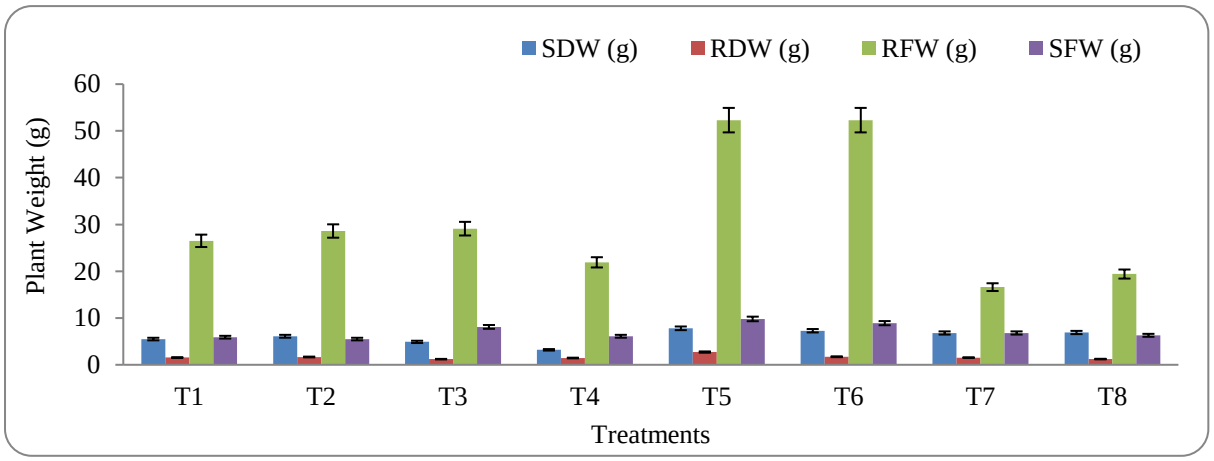


Figure 4. Effects of treatments on Plants SFW, RFW, SDW and RDW.
Rs = *Ralstonia solanacearum*; SDW = Shoot dry Weight; RDW = Root Dry Weight; SFW = Shoot Fresh Weight; RFW = Root Fresh Weight

The results of the study revealed that certain rhizobacterial isolates obtained from the rhizosphere of tomato plants grown in the presence of spent mushroom compost exhibited substantial inhibitory effects against *R. solanacearum*. The two selected isolates, Rh7, and Rh20, demonstrated maximum antagonistic activity, highlighting their potential as biocontrol agents. This finding is crucial for mitigating bacterial wilt, a notorious soil-borne pathogen affecting tomato crops worldwide. Moreover, the study results were parallet with the findings of the previous studies as explored the impact of rhizobacterial isolates on the microbial ecology of the rhizosphere. The use of spent *F. velutipes* substrate, in particular, resulted in an increased abundance of beneficial microbes such as *Bacillus* spp., *Pseudomonas* spp., and *Lysobacter* spp. This dual functionality, suppressing the pathogen and enhancing beneficial microbial communities, suggests a holistic approach to disease management (Wang *et al*., 2020; Wandersman *et al*., 2004; Asghar *et al*., 2000).

The greenhouse experiments further supported the efficacy of the selected rhizobacterial isolates in reducing the incidence and severity of bacterial wilt in tomato plants. The observed results provide valuable insights into the practical application of these isolates in real-world agricultural conditions. The findings of this study have broader implications for sustainable agriculture by emphasizing the potential of utilizing waste materials, such as spent mushroom compost, as a reservoir for beneficial microorganisms. The dual role of these rhizobacterial isolates in pathogen suppression and plant growth promotion underscores their significance in integrated pest management and sustainable crop production practices (Glick *et al*.,

2007). Future research should delve into the molecular mechanisms underlying these observed effects and explore optimal strategies for field application, considering environmental and agronomic factors (Boukhalfa *et al*., 2002; Haas *et al*., 2003; Egamberdieva*et al*., 2009). This study was done to find out what happened when antagonistic rhizobacteria were added to tomato plants to stop bacterial wilt from happening. An in vivo study was also started. So, the isolates Rh-20 and Rh-7 greatly decreased the amount of wilt compared to the control. When pathogens were present, rhizobacterial strains made plants much more resistant and helped them grow more than plants that had not been treated with pathogens. In line with this study, our findings showed that rhizobacteria may be able to lower the number of tomato plants that get bacterial wilt disease and also make the plants taller and heavier.

## CONCLUSION

The evaluation of rhizobacterial isolates associated with spent mushroom compost presents a promising avenue for the development of biocontrol strategies against bacterial wilt in tomatoes. The findings hold implications for sustainable agriculture, emphasizing the potential of leveraging microbial communities for enhanced crop protection and overall plant health.

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