Optimal amount of *Bacillus amyloliquefaciens* FH1-RS1 and *Bacillus cereus* R19 to inhibit the growth of *Verticillium dahliae*

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Abstract. Biological control of Verticillium wilt caused by *Verticillium dahliae* is a green and sustainable strategy. However, the antifungal activities of different amounts of biocontrol bacterial are still not understand. This study investigates the antifungal activity of different amount of *Bacillus amyloliquefaciens* FH1-RS1 and *Bacillus cereus* R19 against *Verticillium dahliae* V991 by dual culture plate. The results showed that both the fungal inhibition rate (FIR) and far fungal inhibition rate (FFIR) of *B. amyloliquefaciens* FH1-RS1 elevated with the increasing volume. The highest FIR (77.78%) and FFIR (58.33%) of *B. amyloliquefaciens* FH1-RS1 were both in 22 μL volume (7.2×10^8 CFU/mL). The highest FIR of *B. cereus* R19 was 48.15% in 12, 18 and 20 μL volume (1.2×10^8 CFU/mL). The highest FFIR of *B. cereus* R19 was 38.89% in 6 μL volume. This suggests that not all biocontrol bacteria have higher antifungal activity in higher amounts.

1 Introduction

Verticillium dahliae, a soil-borne pathogen responsible for Verticillium wilt, poses a significant threat to global cotton production [1]. The imperative for sustainable biocontrol strategies in managing this disease underscores the need to explore effective microbial agents. Bacillus species has emerged as a promising biocontrol agent against *V. dahliae*. This gram-positive, non-pathogenic, endospore-forming bacterium, belonging to the group of free-living soil bacteria, exhibits a diverse array of traits, including plant growth promotion and the production of antifungal, antibacterial metabolites, and industrially important enzymes [2]. Among the various species of bacterial species, Bacillus species has garnered substantial research interest and finds wide applications in agriculture, pharmaceuticals, food industry, and environmental sectors [3]. It is routinely surveyed across a range of ecological niches, including soil, animal feces, human food, and aquatic environments, showcasing its adaptability and versatile metabolic prowess. This diversity in screening sources highlights the bacterium's ability to thrive and adapt across a spectrum

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of environments [4]. *Bacillus species* has been recognized for its ability to promote plant growth through various mechanisms [5,6], act as a biocontrol agent against soil-borne plant diseases [7], serve as a widely used biopesticide [8], antagonize plant pathogens by competing for essential nutrients [9], produce antibiotic compounds [10], and induce systemic acquired resistance [11]. However, the antifungal activities of different amounts of biocontrol bacterial are still not understand. In this study we will investigate the antifungal activity of different amount of *Bacillus amyloliquefaciens* FH1-RS1 and *Bacillus cereus* R19 against *Verticillium dahliae* V991 by dual culture plate.

2 Materials and methods

Bacillus amyloliquefaciens strain FH1-RS1, *Bacillus cereus* R19 and *Verticillium dahliae* V991 were obtained from Applied Microbial Ecology Engineering Laboratory (AMEEL) at Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences. *Verticillium dahliae* V991 was pre-cultured on PDA plates for 7 days under dark conditions at 25°C to promote optimal growth. Concurrently, *B. amyloliquefaciens* FH1-RS1 and *B. cereus* R19 were cultivated in PDB medium at 30°C with 180 rpm for 48 h in the dark. The *Bacillus* cultures were centrifuged, the pellets washed, and resuspended. The final concentration of *B. amyloliquefaciens* FH1-RS1 and *B. cereus* R19 was 7.2 $\times 10^8$ CFU/mL and 1.2 $\times 10^8$ CFU/mL, respectively. Put *Bacillus* cultures with different volumes (ranging from 0 μL to 24 μL in 2 μL increments) on the PDA plates, 2cm with the pre-cultured V991. Each treatment set five replicates. Each treatment was replicated three times. The dual cultures plates were incubated at 27°C for 14 days. The antifungal activity was quantified by the Fungal Inhibition Rate (FIR) and Far Fungal Inhibition Rate (FFIR).

$$\textbf{Fungal Inhibition Rate (FIR) (\%)} = ((R - r_c) / R) \times 100\% \quad (1)$$

$$\textbf{Far Fungal Inhibition Rate (FFIR) (\%)} = (R - r_f) / R) \times 100\% \quad (2)$$

where R is the radius of the fungal colony in the blank control plates, and r_c is the radius of fungal colony close to the *Baciilus* spp. and r_f is the radius of fungal colony far from the *Baciilus* spp.

3 Results

3.1 The effects of different amount of *Baciilus spp*. on fungal inhibition rate

The amount of *Baciilus spp*. obviously affected the fungal inhibition rate (FIR) against *Verticillium dahliae* V991. The FIR of *B. amyloliquefaciens* FH1-RS1 fluctuated and elevated with the increasing volume (Figure 1A). The highest FIR of *B. amyloliquefaciens* FH1-RS1 was 77.78% in 22 μL volume.

The highest FIR of *B. cereus* R19 was 48.15% in 12, 18 and 20 μL volume (Figure 1B). There were no obvious differences in antifungal activity among 6-24 μL volume. The FIR of *B. cereus* R19 in 2-4 μL volume was obvious lower than other volumes.

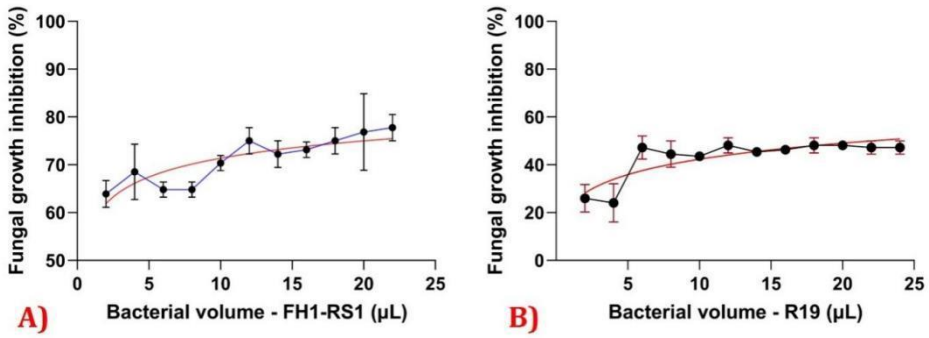

Fig. 1. The effects of different amount of *B. amyloliquefaciens* FH1-RS1 (A) and *B. cereus* R19 (B) on fungal inhibition rate.

3.2 The effects of different amount of *Baciilus spp.* on far fungal inhibition rate

The far fungal inhibition rate (FFIR) of *B. amyloliquefaciens* FH1-RS1 also fluctuated and elevated with the increasing volume (Figure 2A). There was a linear relationship between the FFIR and volume of *B. amyloliquefaciens* FH1-RS1. The highest FFIR of *B. amyloliquefaciens* FH1-RS1 was 58.33% in 22 µL volume.
The highest FFIR of *B. cereus* R19 was 38.89% in 6 µL volume (Figure 2B). The FFIR of *B. cereus* R19 in 10-16 µL volume was obvious lower than other volumes. The FFIR of *B. cereus* R19 in 18-24 µL volume was obvious higher than other volumes except 6 µL volume.

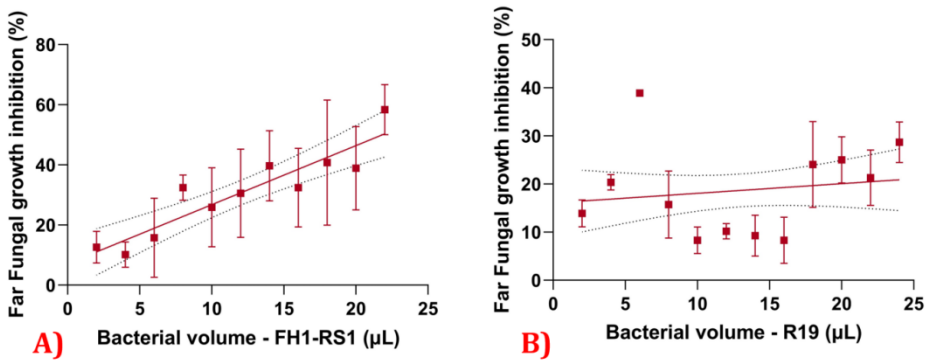

Fig. 2. The effects of different amount of *B. amyloliquefaciens* FH1-RS1 (A) and *B. cereus* R19 (B) on far fungal inhibition rate.

4 Discussion

This study's findings provide compelling evidence for the biocontrol potential of *Bacillus amyloliquefaciens* FH1-RS1 and *Bacillus cereus* R19, demonstrating significant volume-dependent antifungal effects against *Verticillium dahliae* V991. The inhibition of fungal growth by FH1-RS1 not only establishes a clear dose-response relationship but also identifies a saturation point beyond which further increases in bacterial volume do not

translate into increased fungal inhibition. This phenomenon is crucial for determining the optimal concentrations necessary for maximum biocontrol efficacy and is consistent with other reports of dose-dependent responses in biocontrol agents [9].

The variability in inhibition rates by R19 across different bacterial volumes and experimental replications highlights the complex interactions within the biocontrol process. Such variability could be attributed to local environmental conditions, microbial interactions, and the metabolic state of the bacterial strains, which can vary depending on the culture conditions [8]. The saturation effect observed with FH1-RS1 suggests that there is a limit to the biocontrol agent's binding sites or available nutrients, a factor that has been noted in other microbial biocontrol studies [7].

Moreover, the antifungal metabolites and antibiotics produced by these Bacillus strains play a significant role in their inhibitory activity [10]. *B. amyloliquefaciens* is known for its ability to induce systemic acquired resistance in plants, enhancing their defense mechanisms against pathogens [11]. Such mechanisms are integral to the development of sustainable agricultural practices that rely on natural biocontrol agents rather than chemical pesticides.

The current study contributes to a growing body of literature that supports the use of microbial-based solutions for plant pathogen management [3]. By optimizing fermentation parameters and volume concentrations, the antifungal activity of these strains could be maximized, providing an effective and environmentally friendly alternative to traditional chemical fungicides.

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References

1. L. Zhang, Y. Wang, S. Lei, et al., Effect of volatile compounds produced by the cotton endophytic bacterial strain Bacillus sp. T6 against Verticillium wilt. BMC Microbiol **23**, 8 (2023). https://doi.org/10.1186/s12866-022-02749-x
2. H. Liu, V. Prajapati, S. Prajapati, H. Bais, J. Lu, Comparative Genome Analysis of Bacillus amyloliquefaciens Focusing on Phylogenomics, Functional Traits, and Prevalence of Antimicrobial and Virulence Genes. Front. Genet. **12**, 724217 (2021). https://doi.org/10.3389/fgene.2021.724217
3. A. Sharma, T. Satyanarayana, Comparative genomics of Bacillus species and its relevance in industrial microbiology. Genomics Insights **6**, 25–36 (2013).
4. A.M. Earl, R. Losick, R. Kolter, Ecology and genomics of Bacillus subtilis. Trends Microbiol. **16**, 269–275 (2008).
5. J. Shao, S. Li, N. Zhang, X. Cui, X. Zhou, G. Zhang, et al., Analysis and cloning of the synthetic pathway of the phytohormone indole-3-acetic acid in the plant-beneficial Bacillus amyloliquefaciens SQR9. Microbial. Cell Factories **14**, 130 (2015). https://doi.org/10.1186/s12934-015-0323-4

6. Y. Liu, L. Chen, N. Zhang, Z. Li, G. Zhang, Y. Xu, et al., Plant-microbe communication enhances auxin biosynthesis by a root-associated bacterium, Bacillus amyloliquefaciens SQR9. Mol. Plant Microbe Interact. **29**, 324–330 (2016). https://doi.org/10.1094/mpmi-10-15-0239-r
7. S. Tan, Y. Gu, C. Yang, Y. Dong, X. Mei, Q. Shen, et al., Bacillus amyloliquefaciens T-5 may prevent Ralstonia solanacearum infection through competitive exclusion. Biol. Fertility Soils **52**, 341–351 (2016). https://doi.org/10.1007/s00374-015-1079-z
8. L. Wu, H.-J. Wu, J. Qiao, X. Gao, R. Borriss, Novel routes for improving biocontrol activity of Bacillus based bioinoculants. Front. Microbiol. **6**, 1395 (2015).
9. B. Wu, W. Changjun, X. Dihong, Z. Heng, Y. Huan, L. Jinping, et al., Effects of Bacillus amyloliquefaciens ZM9 on bacterial wilt and rhizosphere microbial communities of tobacco. Appl. Soil Ecol. **103**, 1–12 (2016). https://doi.org/10.1016/j.apsoil.2016.03.002
10. S. Srivastava, V. Bist, S. Srivastava, P.C. Singh, P.K. Trivedi, M.H. Asif, et al., Unraveling aspects of Bacillus amyloliquefaciens mediated enhanced production of rice under biotic stress of Rhizoctonia solani. Front. Plant Sci. **7**, 587 (2016).
11. L.C. Ng, M. Sariah, O. Sariam, O. Radziah, M.A. Zainal Abidin, PGPM-induced defense-related enzymes in aerobic rice against rice leaf blast caused by Pyricularia oryzae. Eur. J. Plant Pathol. **145**, 167–175 (2016). https://doi.org/10.1007/s10658-015-0826-1