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Diversity of Shallot Rhizomicrobiome and Twisted Disease Suppression with The Application of *Bacillus* spp. and *Trichoderma asperellum*

Keragaman Rhizomikrobiom Bawang Merah dan Penekanan Penyakit Moler dengan Perlakuan *Bacillus spp.* dan *Trichoderma asperellum*

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ABSTRACT

Twisted disease (*Fusarium* spp.) is an endemic disease that reduces shallot production in the coastal land area of Samas, Bantul, Yogyakarta. The application of *Bacillus* spp. can suppress the twisted disease by secreting secondary metabolites and enhancing soil suppressiveness. This study aimed to determine the effectiveness of adding spraying *Bacillus* spp. on the disease incidence, production of shallots, and their effect on the diversity of rhizomicrobiome by culture microbe approaches. *Bacillus* spp. with a density 10^8 cfu mL^{-1}, *Trichoderma asperellum* 10^6 cfu mL^{-1} was applied by spraying to the shallot. Fungicide chlorothalonil, propiconazole, and prochloraz were used to control the disease. The diversity of rhizobacteria and fungi was analyzed using the ribosomal intergenic spacer analysis (RISA) method. Based on the analysis result, the addition of spraying *B. velezensis* B-27, combination *B. velezensis* B-27 and *B. cereus* RC76, and *T. asperellum* was unable to enhance the suppression of twisted disease, but it was able to enhance the production of shallot bulbs reaching 7.10, 7.80, and 8.43 ton ha^{-1}. Furthermore, the result revealed the diversity of the rhizomicrobiome, spraying *Bacillus* sp. showed 39% differences in bacterial diversity with control while *T. asperellum* caused 43% difference in the diversity. Spraying *Bacillus* spp. has not been able to suppress the incidence of twisted diseases compared to control. However, the similar disease incidence on a spraying *Bacillus* spp. and control showed a higher production until 70% compared to control. This result showed that the addition of spraying *Bacillus* spp. able to increase the tolerance of shallot plants toward twisted disease.

Keywords: disease incidence, diversity, endemic disease, *Fusarium* spp., RISA

ABSTRAK

Penyakit moler (*Fusarium* spp.) merupakan penyakit endemik yang dapat menyebabkan penurunan produksi bawang merah di areal pesisir Pantai Samas, Bantul, Yogyakarta. Aplikasi *Bacillus* spp. dapat menekan penyakit moler melalui sekresi metabolit sekunder dan meningkatkan supresivitas tanah. Penelitian bertujuan untuk menentukan efektivitas *Bacillus* spp. dan *Trichoderma asperellum* yang ditambahkan melalui penyemprotan pada tanaman bawang merah, terhadap insidensi penyakit, produksi bawang merah dan pengaruhnya terhadap keragaman rhizomikrobiom melalui pendekatan kultur mikroba. *Bacillus* spp. dengan kerapatan 10^8 cfu mL^{-1} dan *Trichoderma asperellum* 10^6 cfu mL^{-1} disemprotkan pada tanaman bawang merah. Fungisida klorotalonil, propiconazole dan prokloraz digunakan untuk pengendaliah penyakit. Analisis keragaman rhizobakteri dan cendawan dilakukan menggunakan metode *ribosomal intergenic spacer analysis* (RISA). Penambahan penyemprotan dengan

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B. velezensis B-27, kombinasi *B. velezensis* B-27 dan *B. cereus* RC76, dan *T. asperellum* belum mampu menekan penyakit moler pada bawang merah, tetapi mampu meningkatkan produksi umbi bawang merah mencapai berturut-turut 7.10, 7.80, dan 8.43 ton ha^{-1}. Analisis keragaman rhizomikrobiom menunjukkan penyemprotan *Bacillus* spp. menghasilkan keragaman bakteri yang berbeda 39% dibandingkan dengan kontrol, sedangkan *T. asperellum* menyebabkan perbedaan keragaman 43%. Penyemprotan *Bacillus* spp. belum mampu menekan insidensi penyakit moler, namun dapat meningkatkan produksi lebih dari 70% dibandingkan dengan kontrol. Hal ini menunjukkan bahwa penambahan penyemprotan *Bacillus* spp. mampu meningkatkan toleransi tanaman bawang merah terhadap penyakit moler.

Kata kunci: *Fusarium* spp., insidensi penyakit, penyakit endemik, keragaman, RISA

INTRODUCTION

Application of plant growth-promoting rhizobacteria (PGPR) may induce soil suppressiveness and inhibit the growth of pathogens in the rhizosphere. Therefore, PGPR can be used as bio-immunizer to improve plant health and affects the soil's microbial community. Inoculation of foliar pathogen or biocontrol agent affects root exudates as secrete of shallot plants. Inoculation of the foliar pathogen in *Arabidopsis thaliana* delivered chemical signals below the rhizosphere through root exudate that specifically signals and recruits beneficial rhizobacteria (Rudrappa *et al.* 2008). Based on the research by Rahma *et al.* (2020), *Bacillus velezensis* B-27 can reduce the severity of twisted disease caused by *Fusarium* spp. Spraying *Bacillus* spp. can suppress the growth of pathogens, especially soil-borne pathogens on shallot. Application of *B. amyloliquafaciens* SQR9 affects the root exudates of cucumber plants, which enhances the tryptophan. Tryptophan is a precursor for producing indole acetic acid (IAA) and a signal molecule to attract *B. amyloliquafaciens* SQR9 in colonizing plants (Liu *et al.* 2017). This increase in tryptophan compounds can enhance rhizosphere colonization by PGPR and to produce IAA. Several studies have shown that the rhizosphere bacterial community can be influenced by several factors such as root exudates, soil type, plant genotype, and plant development stage (Zhang *et al.* 2021).

The method for analyzing microbial communities by comparing microbial cultures in different environments or treatments is ribosomal RNA (rRNA) intergenic spacer analysis (RISA) or community fingerprinting. This RISA method is widely used to distinguish bacterial and fungal communities in several soil samples in different locations and vegetation (Ranjard *et al.* 2001). The RISA method involves the amplification of parts of the rRNA gene operon. The amplification in bacteria is carried out between the small subunit (16S) and large subunit (23S), named as the intergenic space, while in fungi is carried out by utilizing the length polymorphism of the nuclear ribosomal DNA (rDNA) region which contains two internal transcribed spacers (ITS) and the 5.8S gene rRNA (ITS1-5.8S-ITS2) (Ranjard *et al.* 2001).

The diversity of bacterial communities in the plant rhizosphere is important to determine the interaction between these microbial communities and soil-borne pathogens. The higher diversity of bacteria in the soil can give the advantage in suppressing plant disease incidence by inhibiting pathogens' growth in the soil (Zhang *et al.* 2021). In addition, highly diverse communities in the soil can also create a suppressive environment, so the deleterious pathogens need to compete for space and nutrients that limiting their survival (Fu *et al.* 2017). Therefore, comparing rhizomicrobiome community structure after spraying *Bacillus* spp. and *T. asperellum* are needed to determine its effect on the incidence of twisted diseases and shallot production under field conditions. The twisted disease is one of the threatening diseases in shallot that cause yield losses of up to 56% (Hadiwiyono *et al.* 2020). The bacteria used in this study were *B. velezensis* B-27, *B. cereus* RC76, and a combination of *B. velezensis* B-27 and *B. cereus* RC76 bacteria.

This research also used *T. asperellum* UGM-LHAF as a comparison, the widely used biocontrol agent in Yogyakarta.

MATERIALS AND METHODS

Experimental Design

Spraying application of *Bacillus* spp. was evaluated to determine its effect on the rhizomicrobiome communities, shallot disease incidence and its production in the coastal area of Samas, Bantul, Special Region of Yogyakarta. The treatments consisted of SB-27 (*B. velezensis* B-27), SRC (*B. cereus* RC76), SC (combination *B. velezensis* B-27 and *B. cereus* RC76), Tricho (*T. asperellum)* and C0 (Control). All plants in each treatment were sprayed with Daconil, a fungicide with the active ingredients of chlorothalonil (75%) and remazole (400 g L^{-1} prochloraz and 20 g L^{-1} propiconazole). Fungicide dose and spray volume were 60 g ha^{-1} and 0.9 L ha^{-1}, respectively.

Application of *Bacillus* spp. and *Trichoderma asperellum* on Shallot Plants

Bacterial colonies were suspended in sterile water and counted at a density of 10^8 cfu mL^{-1} or equivalent to OD 0.3 measured using a spectrophotometer (Genesys 10S UV-Vis, Thermo) at a wavelength of 600 nm (Rahma *et al.* 2020). Treatment with *T. asperellum* was applied at a density of 10^6 spore mL^{-1} (Zhou *et al.* 2021). Treatment with *B. velezensis* B-27, *B. cereus* RC76, and as much as 50 mL of *T. asperellum* was sprayed on plants aged 0, 20 and 40 days after planting (DAP) in each polybag.

Observation Parameters and Data Analysis

The observed variables in this study consisted of pathological parameters (disease incidence and severity) and agronomic parameters (plant height, number of tillers, plant fresh and dry weight, and production of shallot bulb). Disease incidence (DI) was observed every 10 days, by counting the number of diseased plants.

$$DI\ (\%) = \frac{n}{N} \times 100\%, \text{ with}$$

DI, disease incidence (%); n, number of diseased plants; and N, total number of plants.

Observation of the disease severity is carried out every 10 days. Disease severity (DS) is calculated based on (Nugroho 2015):

$$DS\ (\%) = \frac{\sum_{i=0}^{i}(n_i \times v_i)}{N \times V} \times 100\%, \text{ with}$$

DS, disease severity; n, number of infected plants having the same score; v, severity score (0: symptomless, 1: leaf yellowing appears, 2: some leaves dry but not wilt**,** 4: shallot bulb began rot, and 5: plant dies); N, the highest score; and Z, number of plants observed.

The data were then analysed using an analysis of variance (ANOVA) and Duncan multiple range test (DMRT) at confidence levels of α 5%.

DNA Extraction and RISA Fingerprinting of Bacteria Communities

Bacteria derived from the shallot rhizosphere were grown on soil extract agar (SEA) media (Norman 1958). All bacteria's DNA grown in SEA media was extracted and purified according to the Promega's manual protocol (Wizard Genomic DNA purification kit, USA). The intergenic spacer of the bacterial DNA was amplified in a final volume of 10 µL of total rhizobacterial DNA by using universal primers S92Gf (5'-CTYAAAKGAATTGACGG-3') and L189r(5'-TACTGAGATGYTTMARTTC-3'), which anneal to position 910 – 926 of the 16S rRNA gene and position 189 to 207 of the 23S rRNA gene (Joko *et al.* 2012). The mixture reaction contained 1 µL of the purified genomic DNA, 1 µL of each primer, 5 µL GoTaq®Green master mix (Promega, USA), and 2 µL DDH_2O. The PCR (Biorad T100, USA) conditions consisted of an initial denaturing step at 95 °C for 2 min, followed by 30 cycles of denaturing 94 °C for 30 s, annealing 47 °C for 30 s, and an extension at 72 °C at 2 min. After 30 cycles, there was a final extension of 5 min at 72 °C in a thermocycler (Bio-Rad T100, USA) and then cooled and held at 4 °C. Samples were

run on 2% agarose gel stained with SafeGreen for electrophoresis. The PCR product was visualized under UV light for the presence of amplified products.

DNA Extraction and RISA Fingerprinting of Fungi Communities

Fungi from the rhizosphere of shallot were isolated using serial dilutions and incubated for five days. All the grew fungi were extracted and purified using a Genomic DNA mini kit (Geneaid, Taiwan). The length polymorphism of the ITS1-5.8S-ITS2 region was exploited to characterize the fungal community. The primer used to amplify this region represents consensus sequences found at the 3' end of the 18S genes in various organisms (primer 2234C, 5'GTTTCCGTAGGTGAACCTGC-3') and the 5' end of the 28S genes (primer 3126T, 5'-ATATGCTTAAGTTCAGCGGGT-3') (Ranjard *et al.* 2001). The reaction mixture contained 3 µL of the purified genomic DNA, 2.5 µL of each primer, 12.5 µL GoTaq®Green master mix (Promega, USA), and 4.5 µL DDH_2O. The PCR conditions consisted of pre-denaturation at 94 ℃ for 3 min, 25 cycles of denaturation at 94 ℃ for 1 min, annealing at 63 ℃ for 30 s, an extension at 72 ℃ for 1 min, and final extension of 5 min at 72 ℃ in a thermocycler (Bio-Rad T100, USA) and then cooled and held at 4 ℃.

RISA Fingerprint Analysis

The phylogeny tree was formed using the unweighted pair group of arithmetic averages (UPGMA) method. Moreover, the Pearson correlation coefficient was selected. The analysis was performed using the NTSyS-PC version 2.1 T (numerical taxonomy and multivariate analysis system) (Joko *et al.* 2012).

RESULT

The Incidence of Twisted Disease and Symptoms Development

Application of *Bacillus* spp. resulted in a significantly different effect on the incidence of twisted disease in shallot at Samas coastal land area at the beginning of the planting period (Table 1). Application of *Bacillus* spp. and *T. asperellum* as PGPR showed significantly different of disease incidence by 85% compared to control at plant age of 10 to 20 dap. It indicated that these two microbial could suppress the incidence of twisted disease. However, additional fungicide spraying did not increase the suppression of the twisted disease by *Bacillus* spp.

Increase of disease incidence correlates with the increase of disease severity at 20 dap (Table 1 and 2), but disease severity were not significantly different at 30 and 40 dap. Symptoms of the twisted disease at 20 dap are shown by abnormal elongation of the pseudostem, so the plant withered and collapsed (Figure 1). Symptoms on the leaves showed as yellowish leaves turning brown, drying, and swaying (twisting) starting from the top. In severe symptoms, the plant dries and dies. Young plants infected by *Fusarium* spp. failed in producing bulb.

Table 1 The effect of *Bacillus velezensis* B-27, *Bacillus cereus* RC76, a combination of B-27 and RC76, and *Trichoderma asperellum* application on the incidence of twisted disease of shallot

Treatments	Disease incidence (%) on … days after planting			
	10	20	30	40
SB-27 (*B. velezensis* B-27)	0.50 a	2.80 bc	6.50 a	10.20 a
SRC (*B. cereus* RC76)	0.90 a	3.80 ab	6.80 a	10.40 a
SC (B-27 + RC76)	0.50 a	2.70 bc	6.50 a	9.00 a
Tricho (*T. asperellum)*	0.40 a	1.30 a	6.00 a	7.20 a
C0 (control)	2.70 b	4.60 c	7.40 a	11.50 a

Data were analyzed using DMRT; different letters indicate significant differences between treatments (least significant difference test; $P < 0.05$).

Effect of PGPR on the Production of Shallot plants

In general, application of PGPR on shallots in Samas coastal area is not able to suppress the incidence of twisted disease in the field, however, it can improve agronomic characters (Table 3). Treatment with PGPR was able to increase plant height by 25%–32%, the number of tillers reached 37%–81%, increased fresh weight of the plant to 30%–52% and dried weight of plant 34%–52% compared to control. The highest shallot production (8.43 tons ha^{-1}) was found in *T. asperellum* treatment. Furthermore, plants given *Bacillus* spp. treatment had more tiller than control. Treatment of *T. asperellum, B. velezensis* B-27 and combination (*B. velezensis* B-27 and *B. cereus)* resulted in a higher bulb weight compared to the control (Figure 2).

Figure 1 Symptom of twisted disease on shallot under field condition at 20 days after planting

RISA profiles of Rhizobacterial and fungal communities

Application of *Bacillus* spp. affected the rhizobacteria community under field conditions. Based on the composition of DNA, 3 to 5 DNA band profiles representing a group of bacteria was produced from samples taken from Bacillus treatment. The profiling of the DNA band that appeared on all treatments had rhizobacterial diversity varied between 400 bp, 600 bp, and 1000 bp (Figure 3a).

Table 2 The effect of *Bacillus velezensis* B-27, *Bacillus cereus* RC76, a combination of B-27 and RC76, and *Trichoderma asperellum* application on the severity of twisted disease of shallot

Treatments	Disease severity (%) on … days after planting			
	10	20	30	40
SB-27 (*B. velezensis* B-27)	0.10 a	1.14 ab	4.72 a	8.38 a
SRC (*B. cereus* RC76)	0.18 a	1.74 b	4.80 a	9.72 a
SC (B-27 + RC76)	0.28 ab	1.14 ab	4.72 a	8.00 a
Tricho (*T. asperellum)*	0.10 a	0.44 a	4.22 a	5.50 a
C0 (control)	0.42 b	1.86 b	6.30 a	10.36 a

Data were analyzed using DMRT; different letters indicate significant differences between treatments (least significant difference test; P <0.05).

Table 3 The effect of *Bacillus velezensis* B-27, *Bacillus cereus* RC76, a combination of B-27 and RC76, and *Trichoderma asperellum* application on the agronomic parameters of shallot

Treatments	Plant height (cm)	Number of tillers	Fresh weight (kg)	Dry weight (kg)	Bulb production (ton ha^{-1})
SB-27 (*B. velezensis* B-27)	34.03 a	8.05 a	1.14 ab	0.90 a	7.10 a
SRC (*B. cereus* RC76)	33.20 a	7.70 a	1.07 bc	0.87 a	6.60 ab
SC (B-27 and RC76)	35.05 a	7.33 ab	1.25 ab	0.99 a	7.80 a
Tricho (*T. asperellum)*	34.25 a	6.10 b	1.43 a	1.08 a	8.43 a
C0 (control)	26.48 b	4.45 c	0.82 c	0.65 b	4.95 b

Data were analyzed using DMRT; different letters indicate significant differences between treatments (least significant difference test; P <0.05).

The treatment of *T. asperellum* resulted in distinguished rhizobacteria community from the treatment of *Bacillus* spp. and showed a one-band DNA profile at a fragment length of 400 bp. The control treatment has a DNA band profile ranging widely between 400 bp, 900 bp, and 1000–1100 bp.

Clustering analysis of rhizobacteria diversity showed that identical rhizobacterial community (100%) was found out of the samples from the treatments of *B. velezensis* B-27 and *B. cereus* RC76. Interestingly, the application of bacterial combination (B-27 and RC76) had 71% similarity of rhizobacterial community compared to the single treatment of *B. velezensis* B-27 and *B. cereus* RC76 (Figure 4a).

The analysis of fungal diversity using a culture-dependent method (Figure 3b) showed that it could have been more varied. Almost all treatments of *B. velezensis* B-27, *B. cereus* RC76, combination (B-27 and RC76), *T. asperellum,* and control showed a DNA band profiles in the fragment length of about 500 bp and showed similar fungal communities at 100% (Figure 4b). Furthermore, the treatment of *B. velezensis* had 71% similarity of fungal community compared to the treatments of

Figure 2 The growth of shallot plants given different PGPR treatments. a, *B. velezensis* B-27; b, *B. cereus* RC76; c, Combination of B-27 and RC76; d, *Trichoderma asperellum*; and e, Control.

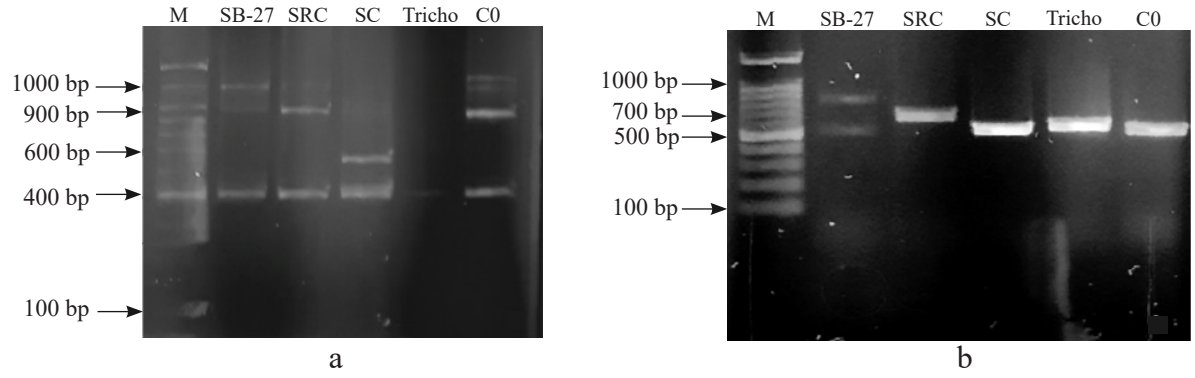

Figure 3 DNA fingerprint of rhizobacteria (a) and fungi (b) from shallot rhizosphere samples based on amplification at 16 -23S rRNA and 18 – 28S rRNA spacer region, respectively. Samples were taken from plants given the treatments *B. velezensis* B-27 (SB-27), *B. cereus* RC76 (SRC), Combination of B-27 and RC76 (SC), *Trichoderma asperellum* (Tricho) and Control (C0). M, Marker DNA 100 bp.

bacterial combination (B-27 and RC76), *T. asperellum,* and control.

DISCUSSION

Application of *Bacillus* sp. and *Trichoderma asperellum* at the beginning of planting induces plant resistance from *Fusarium* sp. infection. Thus, at the age of 10 dap, the treatment of *Bacillus* sp. and *T. asperellum* showed symptomless compared to control. Application of *B. velezensis* B-27 and *B. cereus* RC76 at the beginning of planting can induce plant resistance and suppress twisted disease by enhancing the accumulation of jasmonic acid (Wulan *et al.* 2022). Jasmonic acid can activate plant resistance through the ISR signalling pathway, which causes priming for enhancing host defence gene expressions such as pathogenesis related (PR) 1, PR2, PR5, and plant defensin (PDF 1.2) (Niu *et al.* 2011).

Moreover, the high disease incidence and severity could be caused by the effect of microorganisms' complexity in the field, which can also be an obstacle for PGPR in colonizing plant roots. Root exudates secreted by plants are chemoattractant for microorganisms in the soil because they consist of carbon compounds, amino acids, and other organic compounds needed for their growth. This complexity of microorganisms in the soil can also cause shallot plants to be infected with more than one type of pathogen. Based on the research by Tuhuteru *et al.* (2019), the coastal sandy land of Samas beach, Bantul, is endemic to twisted disease, which can cause incidence up to 61.3%. In addition, purple blotch disease (*Alternaria porri)* and shallot blight (*Stemphylium vesicarium)* were also detected in the same area (Hahuly *et al.* 2018).

Although the addition of biological agents in this research did not enhance disease

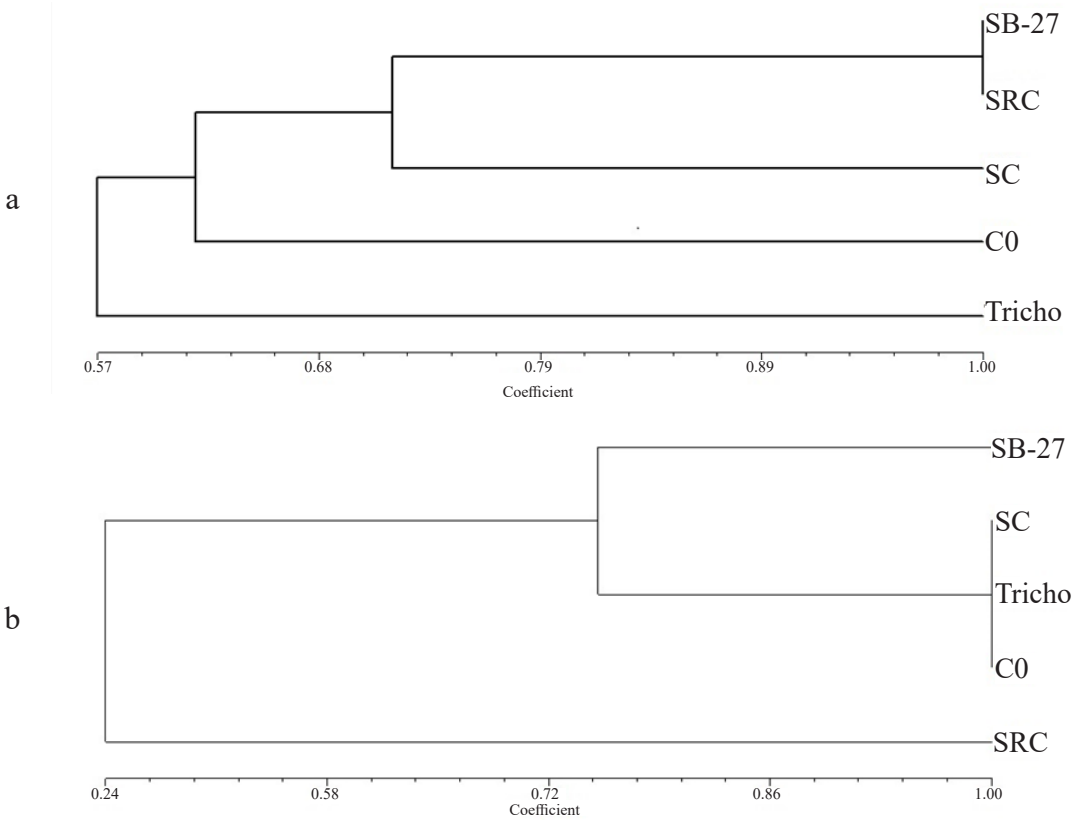

Figure 4 Clustering analysis based on percent similarities of the rhizomicrobiome communities of (a) rhizobacteria and (b) fungal. A dendrogram was built using the UPGMA method. The correlation coefficient of Pearson was chosen. Note: SB-27, Spraying *B. velezensis* B-27; SRC, Spraying *B. cereus* RC76; SC, Combination of B-27 and RC76; Tricho, Treatment with *Trichoderma asperellum*; C0, Control.

suppression, application of biological agents affected several growth variables. This indicated that these biological agents can enhance the tolerance level of plants even though plants show symptoms of twisted disease. *Bacillus* spp. application can enhance the yield of shallots bulb by 33%–58% compared to the control.

Improvement of several growth variables in *Bacillus* spp. treatment may also depend on the enhancement of rhizobacteria. Based on the result, analysis of rhizobacteria diversity indicated the presence of various proteobacteria. Gram-positive bacteria represent groups of bacteria with DNA fragment lengths between 400 bp and 900–1000 bp are represented by groups of proteobacteria (Ranjard *et al.* 2000). The abundance of proteobacteria groups in treatments of *Bacillus* spp. indicated that proteobacteria is an effective root colonizing bacterium in the rhizosphere of various plants and has a high ability to utilize root exudates (Hashimoto *et al.* 2009)

Application of *T. asperellum* resulted in a different rhizobacterial community from *Bacillus* spp. treatment. Although treatment with *T. asperellum* had a rhizobacterial community that was not diverse compared to *Bacillus* spp. and control, this did not negatively affect several agronomic parameters, such as the fresh and dry weight of plant and bulb productions. The results suggest that *T. asperellum* can compete with rhizobacteria in filling the space in the rhizosphere, colonizing plants, and dominating the niche. *T. asperellum* reduces the abundance of rhizobacterial species in shallot plants. This is because *Trichoderma* spp. secretes secondary metabolites that can inhibit the growth of certain bacteria (Artanti *et al.* 2022).

Spraying *Bacillus* spp. on the area that has been applied with fungicides can enhance plant tolerance to twisted disease. It could affect the diversity of rhizobacteria in the shallot rhizosphere based on PCR-RISA. On the other hand, the rhizomicrobiome diversity with this method is not represented the diversity of bacteria or fungi in a whole rhizosphere because the DNA detected is only limited to microorganisms that can grow in the laboratory.

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