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Diversity and community structure of rhizosphere bacteria in shallot treated with *Rhizophagus intraradices* and *Trichoderma asperellum*

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Abstract. *Artanti H, Joko T, Suryanti. 2023. Diversity and community structure of rhizosphere bacteria in shallot treated with* Rhizophagus intraradices *and* Trichoderma asperellum. *Biodiversitas 24: 6248-6255.* The rhizosphere is an area rich in nutrients and has high microbial activity. The condition of the rhizosphere area can influence plant growth and resistance to pathogens. This study aimed to determine the effect of the application of *Rhizophagus intraradices* and *Trichoderma asperellum* on the diversity and community structure of shallot rhizosphere bacteria as well as on the growth and health of shallots. Metagenomic analysis of the shallot rhizosphere was used to determine the diversity and structure of the bacterial community in shallots treated with *R. intraradices*, *T. asperellum*, control, and bulk soil. The results showed that the application of *R. intraradices* and *T. asperellum* affected the composition and diversity of rhizosphere bacteria and the number of rhizobacteria species. The structure of rhizosphere bacteria was not affected by the application of these two fungi. The diversity and number of rhizosphere bacterial species were able to increase plant growth and resistance, especially triggered by *R. intraradices*.

Keywords: Microbial community, rhizobacteria, *Rhizophagus intraradices*, shallots, twisted disease, *Trichoderma asperellum*

INTRODUCTION

The rhizosphere is the area near the roots where the interaction between soil microorganisms and plant roots is very high. This area is rich in nutrients and has a high diversity and activity of microorganisms. According to Lopez et al. (2012), the rhizosphere is part of the soil where the microorganisms live and are influenced by the plant root system. Interactions in the rhizosphere are mediated by root exudates, which can affect the microorganism community (Joko et al. 2012). Biological and physicochemical processes occur in the rhizosphere due to plant and microbial activities, like root growth, water and nutrient uptake, respiration, and rhizodeposition. The rhizosphere extends from the surface of the plant root to a position in soil that depends on the diffusion rate of exudates and the root's biochemistry and development. Plant type and microbial community composition also affect the rhizosphere extends (Lopez et al. 2012). Xiong et al. (2022) reported that bio-agents caused an increase in microbial community composition and produced a direct solid effect on plant growth.

Microorganism communities in the rhizosphere also affect host plants in different ways, such as improving plant growth, increasing nutrient absorption, and protecting the plants from pathogen infection. The rhizosphere affected by mycorrhizas is called "Mycorrhizosphere" and consists of roots, hyphae, associated microorganisms, and the soil around them (Piliarová et al. 2019). The formation of mycorrhizosphere modifies growth conditions for microorganisms in the root zone of plants, and the plants are protected against harmful microorganisms (Jamiołkowska et al. 2020). Akinola et al. (2021) reported that the maize rhizosphere has a higher diversity and structure community than the bulk soil.

Plant growth promoting fungi (PGPF) are known to stimulate plant growth and influence the rhizosphere microorganism community. Application of *Rhizophagus intraradices* (synonym: *Glomus intraradices*) is known to increase water and nutrient absorption, which has an impact on improving plant growth and resistance to pathogens and induces changes in the composition and structure of the rhizosphere bacterial community (Rodríguez-Caballero et al. 2017). *R. intraradices* was reported to increase the relative abundance of *Bacteroidetes* from 5.25 to 11.74% and *Actinobacteria* from 3.67 to 9.34% in soybean fields (Jie et al. 2021). According to Hao et al. (2021), *Claroideoglomus etunicatum* increased the Shannon index of bacterial communities dominated by *Proteobacteria* by stimulating several beneficial bacteria that can improve plant growth and tolerance to stress. In addition, the genus *Trichoderma* is known to affect the rhizosphere microorganism community. *Trichoderma asperellum*, which can survive in the soil for 60 days, influences plant physiology, the composition and function of soil microorganisms, and inhibits the development of pathogens (Senkovs et al. 2021). Inoculation of *T. asperellum* M45a is capable of regulating nutrient availability and enzyme activity as well as increase the abundance of beneficial bacteria, such as *Spingomonas*, *Pseudomonas*, *Actinomadura*, and *Rhodanobacter* (Zhang et al. 2020). According to Jie et al.

(2022), a total of 905,738 sequence with an average of 226,435 high-quality bacterial sequences were obtained from four rhizosphere soil samples, which clustered into 1946 OTUs at a similarity level of 97%. PCoA analysis based on Bray-Curtis distance shows that soybean rhizosphere soil was separated into four groups, this indicate that the composition of bacterial communities in both non-inoculated and inoculated with *R. intraradices* was distinctly between the two continuous cropping regimes. Also, the heatmap diagram of the bacterial communities at the genus level indicated that the bacterial communities between the two rhizosphere soils were similar.

Interaction between plants and rhizobacteria occurs in the rhizosphere and forms symbiosis that can benefit each other. *Rhizophagus intraradices* and *T. asperellum* can increase plant health and growth. In addition, both fungi can induce direct and indirect changes in the rhizosphere bacterial community. This study aimed to determine the effect of the application of *R. intraradices* and *T. asperellum* on the diversity and community structure of rhizobacteria and the growth of shallots.

MATERIALS AND METHODS

Study area

The research was conducted in Gotakan Village, Panjatan, Kulonprogo, Yogyakarta, Indonesia. Shallots variety Crok Kuning was used for the experiment. Biological control agents (BCA) used as coating materials were *R. intraradices* and *T. asperellum* in powder form with spore populations of 12.19 spore.g^{-1} and 4.6 x 10^{6} CFU.g^{-1}, respectively. The dose of coating material used was 5 g.kg^{-1} of seed, which was applied to the entire surface of the tuber. Effect of *R. intraradices* and *T. asperellum* on shallot plant growth was observed once every two weeks consist of plant height, number of leaves and bulb weight (Amallia et al. 2023). Twisted disease intensity was observed once every two weeks and calculated using the following formula of Rahma et al. (2020):

$$\text{Disease Intensity} = \frac{\sum(\text{nixvi})}{\text{Z x N}} \text{ x } 100\%$$

Where:

n : number of infected plants having the same score
v : severity scores
Z : maximum rating scale number
N : total number of plants observed

Twisted disease symptom category scores as follows:

0 : Symptomless
1 : Leaf yellowing appears
2 : The yellowing leaf area developing and leaves began to wilt
3 : The wilt leaf developing, above half of the leaves yellowed and wilted
4 : The tuber began to rot
5 : The plant dies

Twisted disease incidence was observed once every two week and calculated using the following formula (Fitriani et al. 2019):

$$\text{DI} = \frac{n}{\text{N}} \text{ x } 100\%$$

Where:

DI : disease incidence
n : total number with disease symptoms
N : total number of plants observed

Soil sampling

Soil samples were taken before and after applying *R. intraradices* and *T. asperellum*. Before application, samples were taken as bulk soil from five sampling points taken diagonally at a depth of 10-15 cm and then composited. Soil samples after application of the *R. intraradices* and *T. asperellum* and without application as control treatment were taken from the rhizosphere of shallots aged six weeks after planting. The soil was taken by removing the plant and shaking it off so that the soil remained attached to the roots. The soil attached to the roots was taken without including the roots. The number of samples analyzed was 12 with a weight of 50 g each.

DNA extraction and amplification

DNA genome was isolated from soil using the *Cetyltrimethylammonium bromide* (CTAB) method, then the concentration and purity of the DNA were seen on 1% agarose gel. DNA of each sample was amplified by PCR technique using Phusion® High-Fidelity PCR Master Mix (New England Biolabs, United States) (Trianom et al. 2019).

Preparation of libraries and sequencing

16s rRNA gene region V3-V4 was amplified using specific primers (341F:5'-CCTAYGGGRBGCASCAG-3' and 806R:5'-GGACTACNNGGGTATCTAAT-3'), 30 µL PCR mix consisting of 15 µL Phusion® High-Fidelity PCR Master Mix (New England Biolabs, United State), forward and reverse primers 0.2 µmol L^{-1}, respectively, and ten ng DNA template. Amplification was performed for 30 cycles with a pre-denaturation stage of 98°C for 1 minute, followed by 98°C for 15 seconds, 50°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes. 30 µL PCR product was mixed with loading buffer 1X containing SYBR green and electrophoresed using 2% agarose gel. Samples with bands formed at 400-450 bp were selected for further testing. The selected PCR products were mixed in the same ratio and purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using the TruSeq® DNA Free Sample Preparation Kit (Illumina, USA). The genome library quantification using Qubit and Q-PCR was then sequenced using the Illumina HiSeq2500 PE250 Platform (Beijing Novogene Bioinformatics Technology Co. Ltd. China). The raw data were stored in FASTQ format and in the Sequence Read Archive. The raw sequence data were demultiplexed, filtered, and processed using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Xiao-xiang et al. 2020).

Data analysis

The number of species observed and plant performance were analyzed using a *T*-test; a taxonomy tree was created using R&D software independently; diversity heatmaps were calculated and UPGMA using QIIME software; while PCA and NMDS cluster analyses were applied to reduce variable dimensions using FactoMineR and ggplot2 on R software.

RESULTS AND DISCUSSION

Effects of *R. intraradices* and *T. asperellum* on the diversity and abundance of rhizobacteria community

A total of 37,845 high-quality bacterial sequences were obtained from all the samples. These bacteria were grouped into 6,816 operational taxonomy units (OTUs) with a similarity level of 97%. The application of *R. intraradices* and *T. asperellum* increased the number of rhizobacterial species observed compared to before application and control (Table 1).

The rhizobacteria diversity at the phylum level was dominated by Actinobacteria (21.96%), Firmicutes (45.99%), Nitrospirae (6.21%) and Proteobacteria (20.43%) (Figure 1).

Table 1. Number of rhizobacteria species observed before and after application of *R. intraradices* and *T. asperellum*

Treatments	Number of species observed
Before application	2.968 ns
R. intraradices	3.258 *
T. asperellum	3.231 *
Control	3.158 ns

Note: *: significantly different at $P<0.05$; ns: not significantly different based on the *T*-test

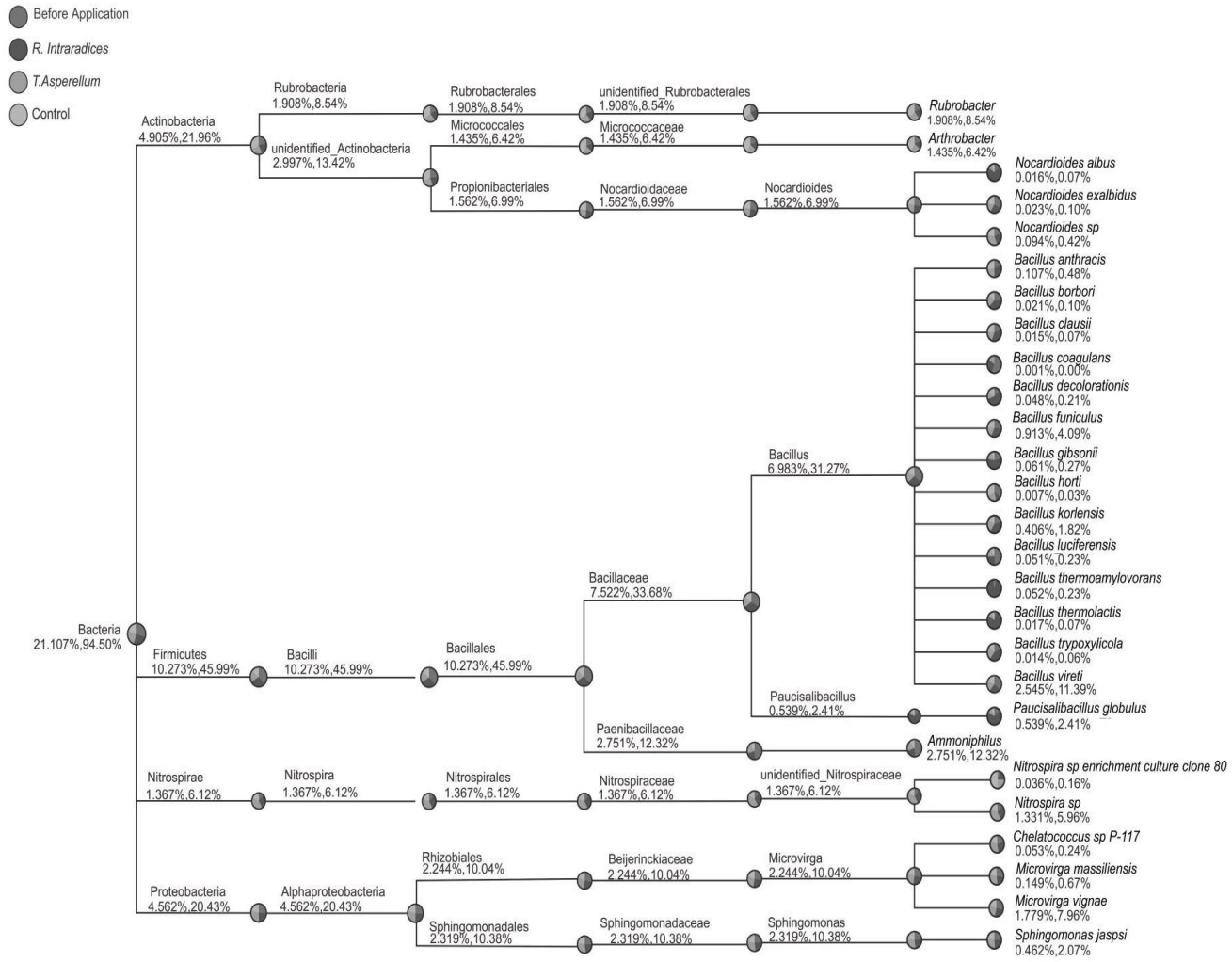

Figure 1. Diversity of rhizobacterial community structure before and after application of *R. intraradices* and *T. asperellum*

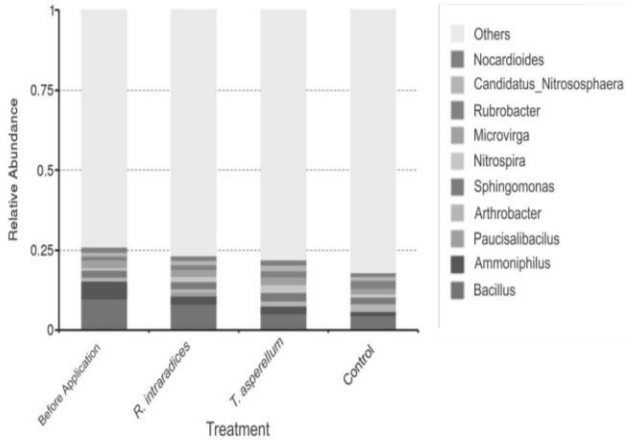

Figure 2. Relative abundance of rhizobacterial communities before and after application of *R. intraradices* and *T. asperellum*

Phylum Actinobacteria included Rubrobacteria (8.54%) and unidentified Actinobacteria (13.42%). Phylum Nitrospirae and Proteobacteria consisted of the genus *Nitrospira* (6.12%), and Alphaproteobacteria (20.43%), respectively. Phylum Firmicutes was dominated by Bacilli (45.99%), which showed the highest diversity compared to other phyla. At the genus level, rhizobacteria diversity consisted of *Bacillus* (31.27%) and *Paucisalibacillus* (2.41%). Genus *Bacillus* included *Bacillus vireti* (11.39%), *Bacillus funiculus* (4.09%), *Bacillus korlensis* (1.82%), *Bacillus gibsonii* (0.27%), *Bacillus thermoamylovorans* (0.23%), *Bacillus lucifferents* (0.23%), *Bacillus decolorationis* (0.21%), *Bacillus anthracis* (0.48%), *Bacillus borbori* (0.10%), *Bacillus thermolactis* (0.07%), *Bacillus clausii* (0.07%), *Bacillus trypoxylicola* (0.06%), *Bacillus horti* (0.03%) and *Bacillus coagulans* (0.01%). Meanwhile, the genus *Paucisalibacillus* was indicated by *Paucisalibacillus globus* (2.41%). In addition, 12.32% of *Ammoniphilus* bacteria were also observed.

Bacillus and *Ammoniphilus* were the dominant genera of the rhizobacterial community in all treatments. The relative abundance of *Bacillus*, *Ammoniphilus*, *Arthrobacter*, and *Sphingomonas* was 5-10%, 1-5%, 1-2%, and 2-3%, respectively. Application of *R. intraradices* and *T. asperellum* decreased the relative abundance of *Bacillus* and *Ammoniphilus* (Figure 2).

Effects of *R. intraradices* and *T. asperellum* on rhizobacterial structure community

Beta diversity based on weighted-unifrac and unweighted-unifrac distances was used to measure the coefficient of difference between samples. The composition of rhizobacteria between samples based on weighted-unifrac and unweighted-unifrac distances was generally relatively small ranged from 0.412-0.533 and 0.092-0.169 (Figure 3). The smaller the distance, the smaller the difference in the composition of rhizobacteria between samples. The application of *R. intraradices* and *T. asperellum* on plant roots has yet to be able to influence differences in rhizobacteria composition based on matrix distance.

The composition of rhizobacterial community based on principal component analysis (PCA) before application of *R. intraradices* and *T. asperellum* treatment had a high similarity compared to the control (Figure 4). At the same time, non-metric multidimensional scaling (NMDS) indicated that the three treatments also had a higher similarity when compared to the control (Figure 5). The application of *R. intraradices* and *T. asperellum* was shown to affect the rhizosphere so that the composition of the rhizobacterial community had a high similarity compared to control at the OUTs level.

UPGMA analysis clustered treatments and control into three groups: the first group consisted of *R. intraradices* treatment and before application, the second group consisted of *T. asperellum* treatment, and the third group consisted of control treatment (Figure 6). The treatment of *R. intraradices* and before application were clustered in one group, which means that these two treatments had high relative abundance similarities in each rhizobacterial phylum.

Effects of rhizobacterial community on health and growth of shallots

Results showed that the presence of a high number of rhizobacteria in the shallot rhizosphere that was treated with *R. intraradices* improved plant resistance to twisted disease. This can be seen from the intensity and incidence of twisted disease, which were 2.92 and 14.58%, respectively (Table 2).

In addition, the presence of rhizobacteria in the rhizosphere also affected plant growth. Plants (shallots) treated with *R. intraradices* had significantly higher plant height, number of leaves, and bulb weight than control. Similarly, plants treated with *T. asperellum* obtained a significantly higher bulb weight than control (Table 3).

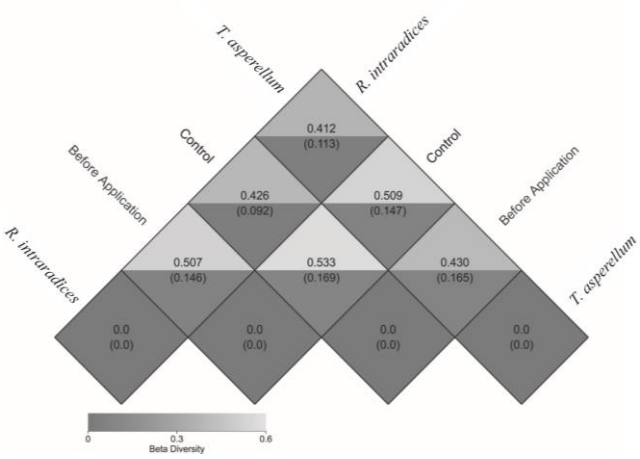

Figure 3. Beta diversity heatmap based on the results of 3 replications for each sample; The top value showed quantitative distance and the bottom value showed qualitative distance

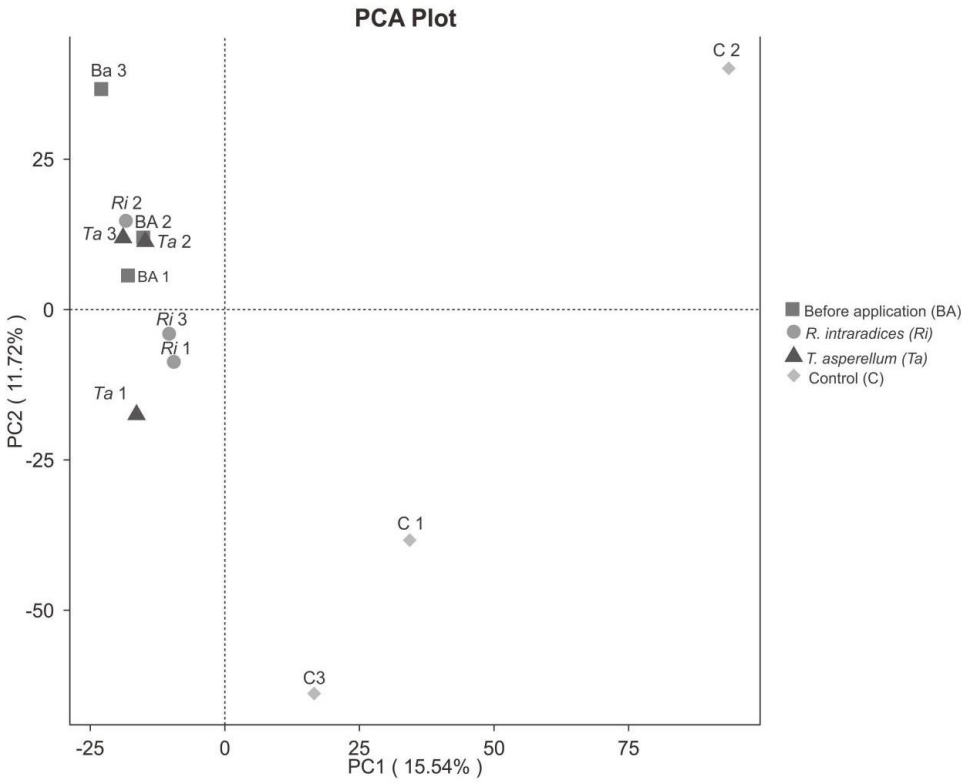

Figure 4. Principal component analysis (PCA) before and after application of *R. intraradices* and *T. asperellum*

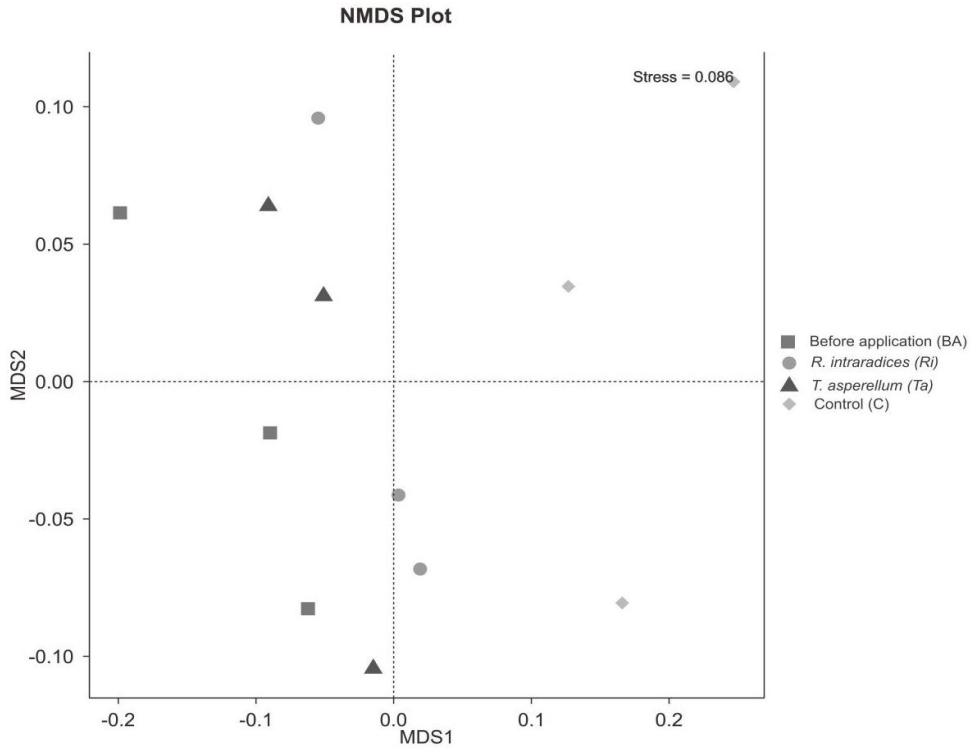

Figure 5. Non-metric multidimensional scaling (NMDS) for rhizobacterial community at OTU level before and after application of *R. intraradices* and *T. asperellum*

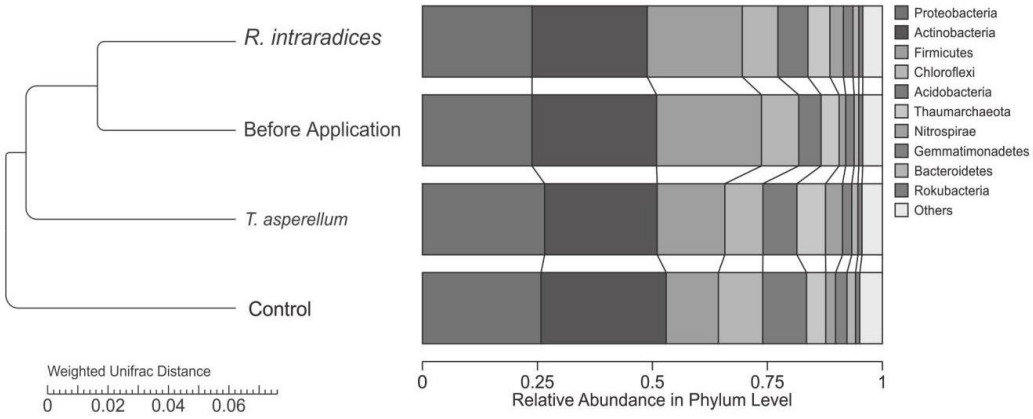

Figure 6. Unweighted pair-group method with arithmetic mean (UPGMA) analysis based on weighted unifrac distance before and after application of *R. intraradices* and *T. asperellum*

Table 2. Disease severity and incidence of twisted disease

Treatments	Disease severity (%)	Disease incidence (%)
R. intraradices	2.92	14.58
T. asperellum	7.08	35.42
Control	25.00	50.00

Table 3. Effects of *R. intraradices* dan *T. asperellum* on plant growth

Treatments	Plant height (cm)	No. of leaves	Bulb weight (g)
R. intraradices	32.97a	25.62a	43.35a
T. asperellum	30.90ab	22.71ab	37.34b
Control	26.99b	18.04b	26.15c

Note: Number in the same column followed by the same letters showed no significant difference according to Duncan Multiple Range Test at 5%

Discussion

Rhizosphere of shallots is known to have low levels of rhizobacteria and this affects for growth and health of shallots. The application of *R. intraradices* and *T. asperellum* on shallot tubers increased the number of rhizobacteria species in the rhizosphere. Carbohydrates secreted by *R. intraradices* hyphae into the environment could significantly affect rhizosphere bacteria's activity and abundance. The secreted carbohydrates are used as a source of energy to grow and develop, creating favorable conditions for rhizosphere bacteria. According to Wang and Feng (2021), arbuscular mycorrhiza fungi (AMF) obtain carbon from the host plant and then use it for their metabolism and release some of it into the environment. Carbohydrates secreted by extraradical hyphae can provide sufficient energy for microbial growth and development. Akyol et al. (2019) reported that AMF was able to influence the abundance of rhizobacteria by stimulating the growth of rhizosphere bacteria. Stimulation is done by providing carbon compounds obtained from the assimilation of the host plant and then secreted through the hyphae. In addition, colonization of beneficial fungi can affect plant physiology, which affects changes in the composition of root exudates. Zin and Badaluddin (2020) reported that the genus *Trichoderma* could increase plant growth by releasing hormones supporting root development and plant growth. In turn, shallots having excellent growth will produce abundant root exudates, thus expanding the availability of nutrients in the rhizosphere area. The additional nutrients in the rhizosphere result in an increase in the population of rhizosphere microorganisms.

Bacillus has more species diversity than other genera, as evidenced by identifying 14 species of the Bacillus genus. The application of *R. intraradices* and *T. asperellum* was able to influence the increase in the diversity of *Bacillus*. Nanjundappa et al. (2019) reported that Bacillus and AMF cross-linked by producing significantly homologous signaling molecular receptors that could establish a mutually beneficial relationship between Bacillus and AMF. AMF hyphae can release organic compounds as a source of energy for rhizobacteria. In addition, the symbiotic relationship between *Bacillus* and AMF occurs through *Mycorrhiza helper bacteria* (MHB). *Bacillus coagulans*, or MHB, can increase spore germination, hyphae growth, and AMF colonization on roots. Wang et al. (2022) reported that applying *T. asperellum* to apple roots changed the composition of root exudates. Nitrofurans, saccharides, phenols, and citrullines contained in apple root exudates significantly positively impacted the genus Bacillus.

There was a significant decrease in the relative abundance of *Bacillus* and *Ammoniphilus* genera in the rhizosphere of shallots treated with *R. intraradices* and *T. asperellum*. This occurs because both fungi and shallot rhizosphere can inhibit the growth of certain bacteria, while bulk soil has more stable conditions for bacterial growth. Jamiołkowska et al. (2017) reported that AMF was able to change the composition and amount of root exudates,

which could affect the development and activity of rhizosphere microbial communities. Inhibition was also shown by *Trichoderma*, as reported by (Keswani et al. 2019), that *T. asperellum* produced trichotoxin, which inhibited the growth of gram-positive and gram-negative bacteria. The inhibition of bacterial growth in the rhizosphere was also reported by Navitasari et al. (2020). Root exudates can influence the structure of the microbial community in the rhizosphere by recruiting beneficial microbes and reducing pathogens. The bacterial genera included in the ten genera with the highest relative abundance were known to be beneficial bacteria. Several genera are known to have the ability to improve plant growth, namely the genera *Bacillus*, *Arthrobacter*, *Sphingomonas*, and *Microvirga*.

The similar composition of the rhizobacterial community based on the resulting matrix distance showed that the application of *R. intraradices* and *T. asperellum* had not been able to affect the differences in rhizobacteria composition in all treatments significantly. The high similarity between treatments was caused by factors of soil conditions and land use history, such as soil texture, soil pH, cultivated plants, and the cultivation method applied. Soni et al. (2017) reported that soil texture, soil type, cultivated plant species, and plant growth phase affected the structure of the rhizosphere microbial community. According to Jie et al. (2021), soybean grown under the same conditions continuously affects plant growth and the composition of the rhizosphere microbial community. AMF colonization can affect plant physiology and impact the exudate released and hyphae, which can affect the development of rhizosphere microbes. The application of *R. intraradices* and *T. asperellum* affected the composition of rhizobacteria at the OTU level and the relative abundance of rhizobacteria. This was because root exudates were affected by *R. intraradices* and *T. asperellum* colonization so that they could influence the composition of rhizobacteria. Huang et al. (2014) reported that changes in the composition of root exudates in plants inoculated with beneficial microbes could affect the differences in the composition of the Arabidopsis rhizosphere bacterial community.

Plants have complex interactions with microorganisms in the rhizosphere. *Rhizophagus intraradices* and *T. asperellum* colonization on the roots influenced improved plant growth and resistance. In addition, bacteria living in the shallots' rhizosphere also improved plant growth and resistance. According to Diagne et al. (2020), AMF can increase plant growth by expanding the area of absorption of nutrients and water so that plants can increase access to nutrient absorption, especially in the form of low ionic concentrations. Furthermore, AMF can produce exudate as carbohydrates secreted through extraradical hyphae. AMF indirectly provides energy for the growth and metabolism of bacteria associated with AMF (Wang and Feng 2021). Interaction between PGPF and rhizosphere bacteria can have a positive effect on plant growth and resistance to soil-borne pathogens. *Rhizophagus intraradices* and *T. asperellum* were able to influence changes in composition and diversity of shallots rhizosphere bacteria and also rhizosphere bacterial communities were able to increase plant growth and resistance especially those triggered by *R. intraradices.*

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