POPULATION OF ENDOPHYTE AND RHIZOSPHERE BACTERIA ISOLATED FROM DIFFERENT *CURCUMA* SPECIES

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Abstract. Turmeric (*Curcuma* spp.) is widely used as a spice and herbal medicine in the world. Several groups of microorganisms, such as endophytic bacteria increase growth and quality of plants. This study determined bacterial population in nine different turmeric species cultivated with or without fertilizer application. The bacterial population in leaves of turmeric species cultivated without fertilizer were: *C. zedoaria* (Ze), 4×10^4*;* *C. zanthorrhiza* (Za), 0; *C. amada* (A), 0; *C. longa* strain 1 (L1), 13.3×10^4; *C. longa* strain 2 (L2), 272.5×10^4; *C. longa* strain 3 (L3), 217.5×10^4; *C. longa* strain 4 (L4), 96.5×10^4; *C. longa* strain 5 (L5), 171.5×10^4; *C. longa* Ryudai gold (RG), 13.5×10^4. While the bacterial population in leaves with fertilizer were: Ze, *0;* Za, 2.3×10^4; A, 16.3×10^4; L1, 8.3×10^4; L2, 37.9×10^4; L3, 13.3×10^4; L4, 35.7×10^4; L5, 4.2×10^4; RG, 1.7×10^4. The population in stems without fertilizer were: *Ze*, 5×10^5*; Za*, 78×10^5; A, 23×10^5; L1, 165×10^5; L2, 300.5×10^5; L3, 11.7×10^5; L4, 8.5×10^5; L5, 33.4×10^5; RG, 5×10^5. The population in stems with fertilizer were: Ze, 2.4×10^5*;* Za, 2×10^5; A 28.2×10^5; L1, 63.3×10^5; L2, 64.3×10^5; L3, 13.7×10^5; L4, 18.9×10^5; L5, 41.3×10^5; RG, 37.7×10^5. The population of rhizosphere bacteria without fertilizer were: Ze, 8.7×10^6*;* Za, 14.7×10^6; A, 15.3×10^6; L1, 23.5×10^6; L2, 26×10^6; L3, 11×10^6; L4, 9.8×10^6; L5, 12.4×10^6; RG, 8×10^6. While the population of rhizosphere bacteria with fertilizer were: Ze, 9.4×10^6*;* Za, 9×10^6; A, 18.5×10^6; L1, 25.5×10^6; L2, 27.3×10^6; L3, 12.3×10^6; L4, 13.5×10^6; L5, 15.3×10^6; RG, 14×10^6. Overall results indicated that bacterial population differed with the turmeric species/strains, plant parts and fertilizer may be due to the differences of chemical compositions in the turmeric species/strains and parts, which could be determined in future studies.

Keywords: *turmeric species, bacteria population, endosymbiont bacteria, plant growth bacteria, fertilizer effect*

Introduction

The genus *Curcuma* L., a member of the Zingiberaceae family, is distributed throughout tropical and subtropical regions worldwide (Sun et al., 2017). Turmeric (*Curcuma* spp.) is widely used as a colorant, traditional medicine, and spices. There are more than 90 species of *Curcuma* which are different in morphological characteristics and chemical properties (Akter et al., 2018). Rhizome of this plant is the most widely used part as herb because it contains volatile oils and curcuminoids consisting of curcumin, demethoxycurcumin, and bidemethoxy-curcumin (Dosoky and Setzer, 2018; Kotha and Luthria, 2019). Curcumin is known to have many pharmacological activities (Akter et al., 2018; Urosevic et al., 2022). Many common agronomic practices have been developed to increase growth and yield of turmeric (Akamine et al., 2007; Hossain and Ishimine, 2007), but no biological method has yet been developed for turmeric production.

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Endophytes are endosymbiotic microorganisms found in plant tissues both intracellular and intercellular, that do not cause disease in plants (Miliute et al., 2015). Endophytes are thought to have an important role in plant health and productivity. Several studies have evaluated the ability of plant-related microbes to influence important traits such as growth, disease resistance, abiotic stress tolerance, water retention, and synthesis of plant growth-promoting hormone (Huang et al., 2018). Endophytic microbes produce a large number of new-secondary metabolites and bioactives which are beneficial for host plant growth as well as economically important for increasing yield and quality of agricultural production and medicinal plants (Strobel, 2013; Ek-Ramos et al., 2019). The recent research showed that the secondary metabolites content of medicinal plant species differed with cultivation locations which could partly be associated with different microbial compositions (Egamberdieva et al., 2017). The symbiosis between microbes and plants is not limited to endophytic bacteria in plants, where other microbial communities outside the plant tissue also influence plant growth. Plant growth promoting rhizobacteria (PGPR) is a group of bacteria that actively colonize plant roots and live freely around or inside roots. PGPR has a role in increasing plant growth and providing protection against specific pathogens (Backer et al., 2018).

In turmeric plants, endophytic bacteria have several biological activities, such as increasing curcumin content, anti-microbial activity, and biocontrol agent of plant diseases (Singh et al., 2017; Vinayarani and Prakash, 2018). It is important to evaluate interaction between endophyte and rhizosphere bacteria with turmeric plant for understanding population and association of microorganisms which could be selected as the biofertilizers in future for promoting growth, yield and quality of turmeric. It is very common that different turmeric species and strains possess different chemical compounds (Akter et al., 2018), which may influence associations and populations of microorganisms. In addition, chemical and organic fertilizers are usually used for promoting growth and yield of turmeric, which may influence populations and association of microorganisms. Considering the above facts, present study has been conducted to evaluate population and associations of microorganisms in different turmeric species and strains cultivated with or without chemical fertilizer application.

Materials and methods

Turmeric species/strains

Four turmeric species, *Curcuma longa* (cultivar Ryudai gold, and 5 strains), *C. zedoaria, C. zathorrhiza and C. amada* were used in this study. The cultivar Ryudai gold (RG) and five strains belonged to the species of *Curcuma longa* are called *C. longa* strain 1 (L1), *C. longa* strain 2 (L2), *C. longa* strain 3 (L3), *C. longa* strain 4 (L4) and *C. longa* strain 5 (L5). The turmeric species, cultivar and strains are different in rhizome size, shape, and color (*Fig. 1*). In addition, they are different in chemical properties, flavor, taste, and physiological and morphological characteristics of shoot (data not published). According to yield performance, the turmeric species and strains could be commercially cultivated.

Turmeric cultivation

The pot experiment was conducted in a plastic house from May 11, 2020 to February 3, 2021. Air-dried dark-red soil of 3.5 kg and cultured soil (commercial name:

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Hanasakimonogatari) of 2.5 kg were mixed properly and placed in each Wagner pot (0.05 m^2). As the rhizome sizes were different with the turmeric species and strains, the best seed-rhizomes were selected for each species and strains. One seed-rhizome per pot was planted at the depth of 6 cm. The two experiments conducted in this study were "without fertilizer application (experiment 1)" and "with fertilizer application (experiment 2)." We planted three plants for each species/strain in both the experiments. The pots were placed in the house randomly. Outdoor environment was maintained in the house by keeping the windows opened, but the windows were closed during typhoon. Water was applied regularly as required to maintain optimum soil moisture level for proper seedling emergence and plant growth. The chemical fertilizer of 3.6 g (N = 0.9 g, P = 0.9 g and K = 1.8 g) was applied per pot on August 10 for the experiment 2.

Sample collection

We collected both the plant (leaf and stem) and rhizosphere samples from December 1 to 15, 2020 when the plants were still green. The plants were cut at the soil surface and the leaves were separated from the stems. The rhizosphere soil was collected from each pot, and composite soil sample was prepared for each turmeric species or strains (*Fig. 1*).

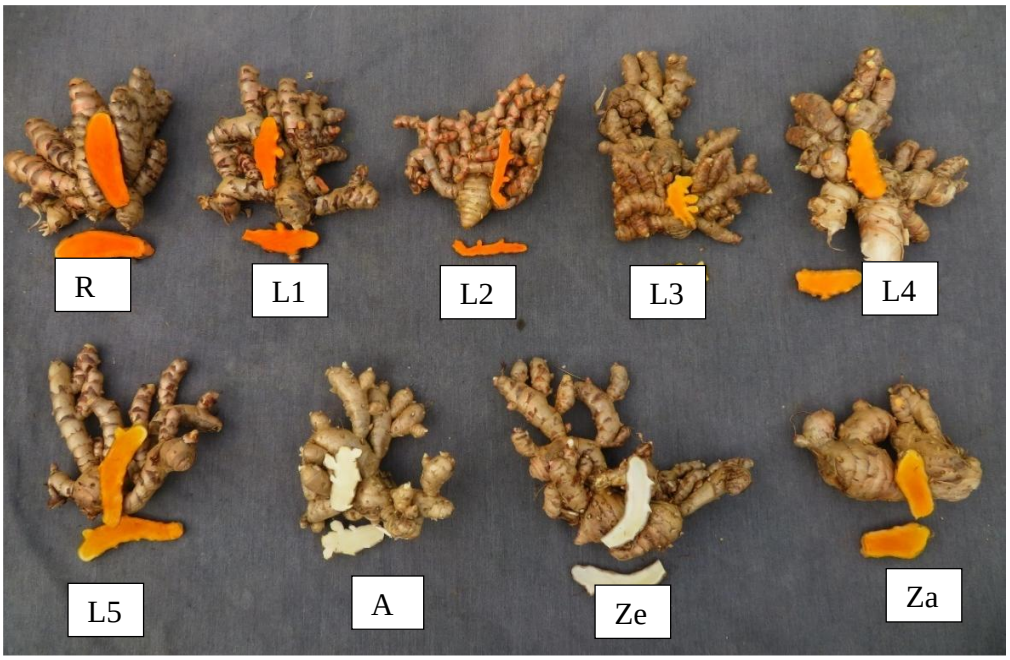

Figure 1. *Differences in rhizome shapes, sizes and colors of turmeric species and strains used in the experiments. RG, C. Ryudai gold; L1, C. longa strain 1; L2, C. longa strain 2; L3, C. longa strain 3; L4, C. longa strain 4; L5, C. longa strain 5; A, C, amada; Ze, C. zedoaria; Za, C. zanthorrhiza*

Isolation and enumeration of endophytic bacteria

Plant samples (stems and leaves) were cleaned by washing in running water (tap water) and cut into pieces. The surface of the sample was washed and sterilized to get free from microbes as follows. The leaves or stems were washed with sterile distilled water and then with 70% ethanol solution for 1 min. The leaves or stems were then washed with 5% sodium hypochlorite solution (Nacalai Tesque, Kyoto, Japan) for

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3 min, and finally rinsed with sterile distilled water for three times. The samples were then dried using sterile tissue paper. Surface sterilization of the samples was performed by spreading 0.1 mL of distilled water on Nutrient Agar (Difco) media, then the petri dishes were incubated (Sanyo MIR-152) at 28°C for 14 days, and it was ensured that no colonies appeared. Surface sterilized leaves or stems are crushed using a sterile mortar and pestle. The crushed sample was put into a test tube containing 9 ml of sterile physiological NaCl solution (0.85%) then serially diluted, and 0.1 mL of each dilution was plated on a Nutrient Agar (Difco) media that had been added with 50 g/mL nystatin to inhibit the growth of the fungus. Sample from each dilution was taken in three petri dishes, and the petri dishes were incubated at 28°C for 14 days. Colonies that appeared on petri dishes were counted every day for 14 days.

Isolation and enumeration of rhizosphere bacteria

Rhizosphere soil samples were taken by removing the Curcuma plant from the pot and carefully shaking the rhizome to remove loose and non-adherent soil. The soil that was still attached to the roots and rhizomes was then collected using a sterile spatula, and the rhizosphere soil obtained was then put into a sterile plastic bag. Each soil composite sample of 10 g was taken and then put into 90 mL of physiological NaCl solution (0.85%). It was homogenized using an orbital shaker for 30 min at a speed of 150 rpm, followed by serial dilutions. A total of 0.1 mL of soil suspension from each dilution was spread on Nutrient Agar (Difco) media. Sample from each dilution was taken in three petri dishes, and the petri dishes were incubated at room temperature (22-27°C) for seven days. Bacterial colonies that appeared were counted every day.

Data analysis

The data of this study were analyzed qualitatively and quantitatively. The data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using the one-way Analysis of Variance (ANOVA) then followed up with the Tuckey test using the SPSS (Statistical Program Software System) program version 16.0. Significant differences were those which $P < 0.05$ or $P < 0.01$.

Results

Population of endophyte bacteria on leaves

Population of endophytic bacteria in the leaves of all Curcuma species/strains showed that each species or strains cultivated with fertilizer application or not had a different bacterial population. The population of endophytic bacteria in leaves without fertilizer application ranged from 0 ± 0 - $272.5 \pm 2.5 \times 10^4$ CFU/g, and with fertilization ranged from 0 ± 0 - $37.9 \pm 1 \times 10^4$ CFU/g (*Fig. 2*). Bacteria was not found in leaf of *C. amada and C. zanthorrhiza* when cultivated without fertilizer, whereas *C. zedoaria* did not show any bacteria when cultivated with fertilizer. Bacterial population in leaves of all *Curcuma* species or stains were influenced by fertilizer. Curcuma longa strains L2, L3, L4, and L5 in the experiment without fertilizer and Curcuma longa strains L2 and L4 in the experiment with fertilizer showed significantly higher bacterial population in leaves than other Curcuma species. The highest bacterial population was found in the turmeric strain of L2 ($272.5 \pm 2.5 \times 10^4$ CFU/g for without fertilizer application, and $37.9 \pm 1 \times 10^4$ CFU/g for with fertilizer application).

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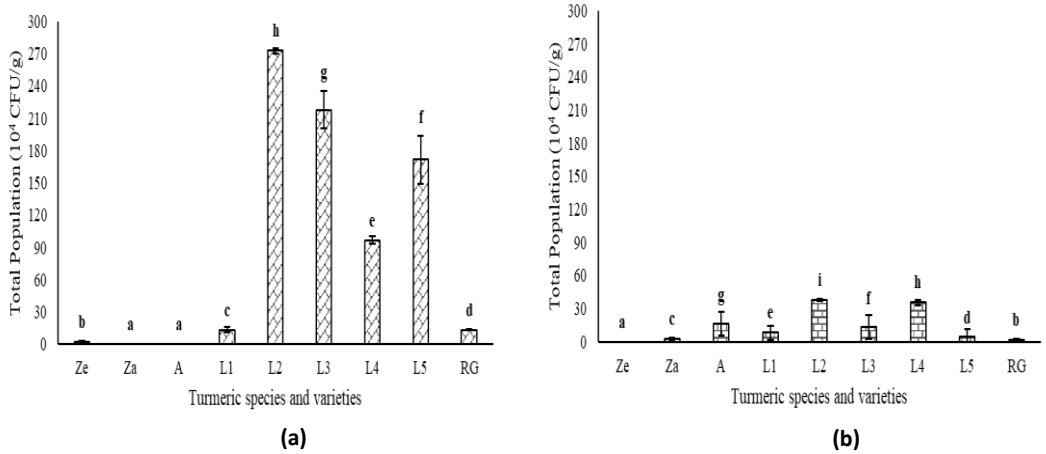

Figure 2. *Bacterial population in the leaves of turmeric cultivated without fertilizer application (a) and with fertilizer application (b). The different letters above the bar represent that the data were significantly different based on the Tuckey test at $P < 0.05$*

Differences in the diversity of bacterial colonies were also observed in each turmeric species and strains, indicated by the size, shape, and pigmentation of the colonies. The results showed that the size of the colonies obtained ranged from punctiform to medium. Circular and irregular colony forms are the most common forms of colonies found. Pigmentation of colonies that have been isolated include non-pigmented colonies such as transparent, cream, white, and milky white and colonies with pigments such as yellow and transparent yellow (*Fig. 3*).

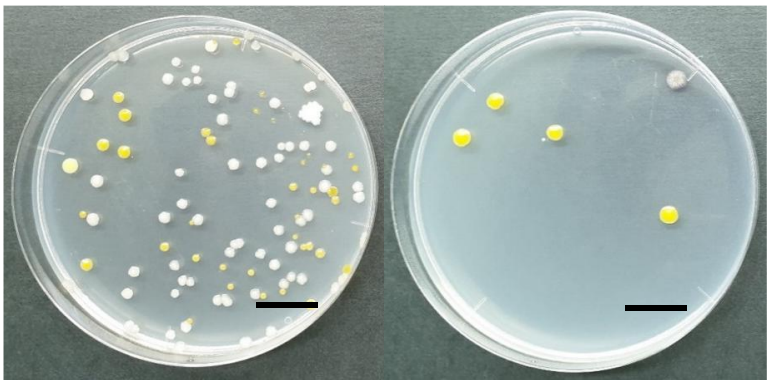

Figure 3. *Color and size of bacterial colonies isolated from leaves of turmeric species Za (C. zanthorrhiza) in nutrient agar. (a) Without fertilizer application (10-2). (b) With fertilizer application (10-2). Bar = 10 mm*

Population of endophyte bacteria in stems

The population of endophytic bacteria in the stems differed significantly with the turmeric species or strains (*Fig. 4*). The results showed that bacterial population in the stems without fertilizer application ranged from 5 ± 1 - $300.5 \pm 23.5 \times 10^5$ CFU/g, and with fertilizer application ranged from 2 ± 1 - $64.3 \pm 5.5 \times 10^5$ CFU/g (*Fig. 4*). The turmeric strain of L2 had the highest population of $300.5 \pm 23.5 \times 10^5$ CFU/g when

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cultivated without fertilizer application and $64.3 \pm 5.5 \times 10^5$ CFU/g with fertilizer application, followed by L1. The bacterial population in the stems of each turmeric species or strains were different between without fertilizer application and with fertilizer application (*Fig. 4*). The *C. longa* strain 1 (L1) and *C. longa* strain 2 (L2) had significantly higher bacterial population compared to other strain in both experiments. In the stems, it was also observed that there was a diversity of bacterial colonies in each species/strain based on colony size, shape, and pigmentation. The size of the colonies found ranged from punctiform to medium with dominated by circular colonies, and some non-pigmented colonies such as transparent, cream, white and milky white, and pigmented colonies such as yellow, transparent yellow and orange (*Fig. 5*).

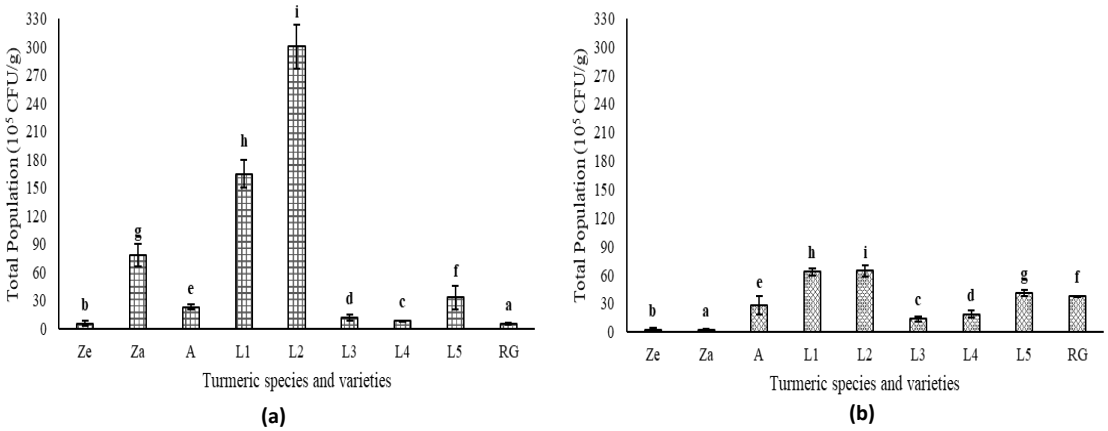

Figure 4. *Bacterial population in the stems of turmeric species or strains cultivated without fertilizer application (a) and with fertilizer application (b). The different letters above the bar represent that the data were significantly different based on the Tuckey test at $P < 0.05$*

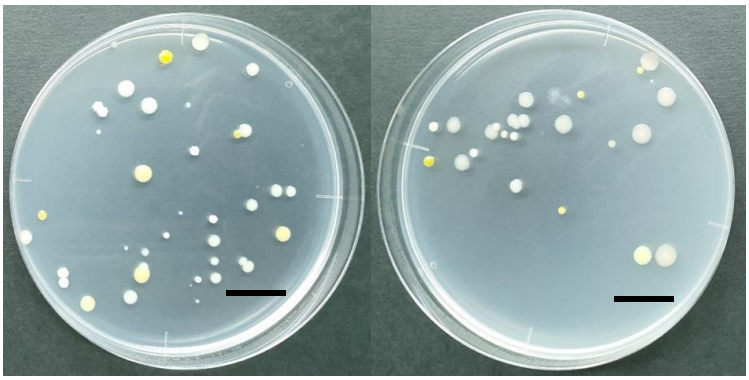

Figure 5. *Color and size of bacterial colonies isolated from stems of turmeric species Ze (C. zedoaria) in nutrient agar. (a) Without fertilizer application (10^{-3}). (b) With fertilizer application (10^{-3}). Bar = 10 mm*

Population of endophyte bacteria in rhizosphere soil

The bacterial population in the rhizosphere soil of turmeric species or strains cultivated without fertilizer application was 8 ± 4 - $26 \pm 2 \times 10^6$ CFU/g, and with fertilizer application was 9 ± 2 - $27.3 \pm 4 \times 10^6$ CFU/g (*Fig. 6*). The bacterial population

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differed in the rhizosphere soils with the turmeric species or strains. The rhizosphere soil of turmeric strain L2 had the highest bacterial population of $26 \pm 2 \times 10^6$ CFU/g without fertilizer application and $27.3 \pm 4 \times 10^6$ with fertilizer application, followed by L1. Influence of fertilizer application on bacterial population in the rhizosphere soil was not clearly observed. In the rhizosphere soil samples, differences in the diversity of bacterial colonies were observed based on the colonies' size, shape, and pigmentation. The shapes of the colonies found were irregular and dominantly circular (*Fig. 7*). Non-pigmented colonies such as transparent, cream, white and milky white, and pigmented colonies such as yellow, transparent yellow and orange were found (*Fig. 7*).

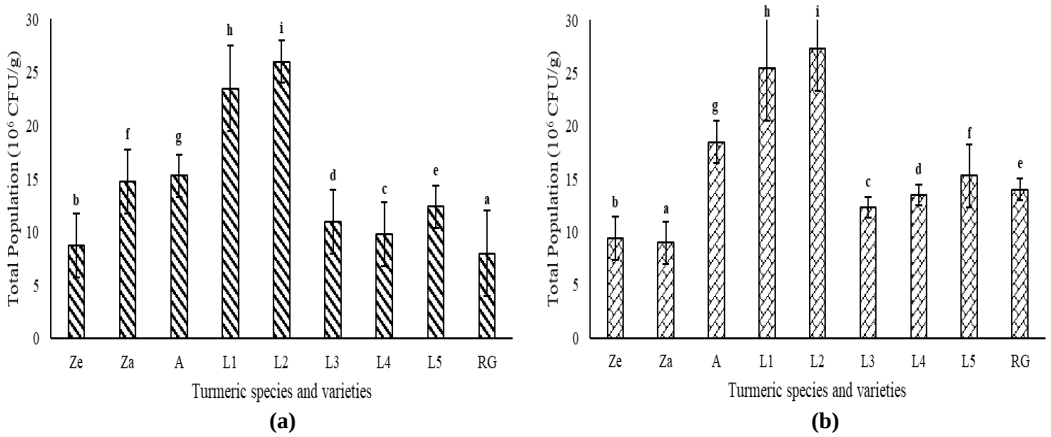

Figure 6. *Bacterial population in the rhizosphere soil of turmeric cultivated without fertilizer application (a) and with fertilizer application (b). The different letter above the bar represents that the data were significantly different based on the Tuckey test $P < 0.05$*

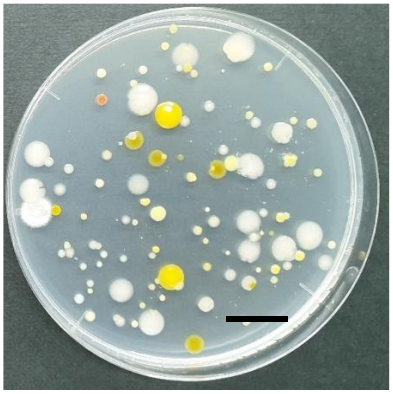

Figure 7. *Population of rhizosphere bacteria in Nutrient agar medium (10^{-2}). Bar = 10 mm*

Discussion

Endophytic bacteria are known to provide benefits to plants both directly and indirectly. The direct benefits of plants include helping plants get nutrients and produce growth hormones to increase plant growth (Ma et al., 2016). The ability of endophytic bacteria to produce antimicrobial compounds and lytic enzymes to protect plants from pathogen attack is an indirect advantage for plants. So its existence is essential for plants (Miliute et al., 2015).

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In this study, the isolation results from leaf samples showed differences in endophytic bacterial populations among the plant species or strains cultivated without fertilizer and with fertilizer application. The same results were also shown in the stems. Different endophytic bacterial populations in a plant are influenced by various factors, one of which is plant species or strains. Germida et al. (1998) reported that canola and wheat planted on the same land had different endophytic populations. A study conducted by Monika et al. (2018) reported that the population densities of endophytic bacteria were different with the different varieties of *Lycopersicum esculentum* (10^3-10^4 CFU/g). In addition, Graner et al. (2003) also reported that four *Brassica napus* cultivars grown under the same conditions had different endophytic bacterial populations which showed differences in resistance to pathogens in each cultivar. The differences in their abilities against pathogens indicated that differences in endophytic bacterial populations with the plant species.

This study also showed that the population density of endophytic bacteria in the turmeric leaves was 10^4 CFU/g. Compared to the leaves, the endophytic bacteria population in the stems was higher (10^5 CFU/g). The difference in endophytic bacterial populations in leaves and stems was because each plant organ has a different bacterial community. It is also thought that the differences of endophytic bacteria population in the leaves and stems were due to the differences of compounds in the leaves and stems. Several studies reported that compounds differ with different organs in a plant species (Meng et al., 2018). The results of a research conducted by Monika et al. (2018) also showed that the population density of endophytic bacteria in the stems and leaves of *Lycopersicum esculentum* plants were different. Similarly, Sobral et al. (2005) reported that the population of endophytic bacteria in the stems (10^4 CFU/g) and leaves (10^2 CFU/g) of soybean plants were different.

The data shows that the L2 strain has the highest population in stems and leaves. In contrast, it is also observed that the population of endophytic bacteria in stems has a higher population than leaves. This shows a relationship between bacterial populations in stems and leaves. Endophytic bacteria in the leaves and stems are bacteria originating from the roots. The position of the roots in the soil is the main route for the entry of endophytic bacteria into plant tissues. However, only bacteria that can attach to the roots and escape from the plant's immune system can enter the roots, so only a small number of soil bacteria can enter the plant tissue (Lundberg et al., 2012; Reinhold-Hurek et al., 2015). After entering the plant, the processes of bacterial migration in plant tissues and organs are supported by bacterial flagella and plant transpiration flow (Compant et al., 2005; James et al., 2002; Shelud'ko et al., 2010). In addition, cell wall-degrading enzymes such as cellulases and pectinases support migration between cells. However, movement through the xylem allows bacteria to move through large pores so that they do not require cell wall-degrading enzymes (Compant et al., 2010). In addition to the root, leaf tissue is the entry point for other endophytic bacteria. Endophytic bacteria in leaf tissue mainly come from plant roots, but like phytopathogenic bacteria, endophytic bacteria can enter leaves from the phyllosphere through leaf stomata. Open tissue due to injury to plant organs is also one of the entry routes for endophytic bacteria into plant tissue (Senthilkumar et al., 2011; Oukala et al., 2021). It is thought that physiological and morphological characterustiics in leaves, stems and roots of the turmeric species/strains were different which influenced endophytic bacterial population.

The differences in endophytic bacterial populations among plant species may be attributed to the variations in chemical compounds present in each species (Meng et al.,

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2018). It is very common for the chemical composition and compounds to differ among turmeric species or strains (Akter et al., 2018), which leads to variations in their endophytic bacterial populations. Additionally, the populations of endophytic bacteria in both the leaves and stems of all tested turmeric species and strains were influenced by fertilizer application. This is probably due to the changes in the chemical compositions and compounds of turmeric of different turemirc species/strains (Akamine et al., 2007; Hossain and Ishimine, 2007).

The bacterial population in the rhizosphere differed with the turmeric species or strains. The results of this study showed that the density of bacteria in the rhizosphere was 10^6 CFU/g. Bulgarelli et al. (2013) stated that the bacterial population in the rhizosphere ranged from 10^6-10^9 CFU/g. A study by Cavaglieri et al. (2009) reported that the population density of rhizosphere bacteria in maize was 10^9 CFU/g. Germida and Siciliano (2001) reported that rhizosphere bacterial population in three wheat cultivars were different. In addition, it was also known that the rhizosphere of old wheat cultivars was colonized by more diverse rhizobacteria, while Proteobacteria dominated the rhizosphere of modern cultivars. Another study on the rhizosphere of five Acacia species conducted by Yuan et al. (2022) showed that each rhizosphere sample had a different microbial population and community. It is known that the rhizosphere is the area around plant roots with a narrow coverage area with high biological activity. Plant roots secrete various compounds that can attract microbial colonization in the rhizosphere. Root exudate affects bacterial colonization in the rhizosphere (Upadhyay et al., 2022). The results of carbon fixation in plant photosynthesis are partially translocated to the root region and released as root exudates (Vives-Peris et al., 2019). The exudate may attract microbes to approach it and colonize the rhizosphere (Lugtenberg and Kamilova, 2009; Mavrodi et al., 2021). Similarly, it is thought that different turmeric species or strains released different exudates which affect the bacterial population in rhizosphere.

Conclusion

Based on the results of the isolation of endophytic bacteria in the leaves and stems of different turmeric species and strains cultivated without fertilizer application and with fertilizer application, it is known that there are differences in endophytic bacterial populations in each *Curcuma* species. Endophytic bacteria population was higher in both the leaves and stems when turmeric species and strains were cultivated without fertilizer application. The population of rhizosphere bacteria were also different with the *Curcuma* species and strains. This study indicates that endophytic bacterial are avail in *Curcuma* plants, but the bacterial populations differ with the *Curcuma* species and strains, which may be due to the differences of chemical compositions and compounds, as well as physiological and morphological properties. Further studies are required to elucidate the chemical compositions and compounds of the *Curcuma* species and stains, and their effects on the endophytic bacteria species/strains and population.

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Conflict of interests. The authors declare that they have no conflict of interests.

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