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Use of *Sphingomonas yunannensis* to Improve Soil Drought Stress in Chili Plants

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ABSTRACT

The availability of water plays an important role in plant growth. However, water availability depends on the climate and irrigation channels; therefore, there is little water available for plants during the dry season. Bacteria found in soil can produce exopolysaccharides to survive under extreme land conditions, namely, during drought conditions. The research objectives were to (1) isolate and select bacteria originating from dry land for use on chili plants and (2) determine the effect of water supply frequency and select soil bacteria on chili plants. The experiment consisted of two stages. (1) Bacterial selection and characteristics, which included exopolysaccharide bacteria selection on specific ATCC No. media. 14; pathogenicity test (hypersensitivity and hemolysis); characterization and biochemical testing including pH, temperature, salinity, oxidation, and catalase; bacterial functional tests (P and K solvents and nitrogen-fixing); and (2) tests of chili plants in the greenhouse. The treatment consisted of two factors: (a) application of selected bacteria, (b) frequency of water application (every 1, 2, 3, and 5 days), and (3) molecular identification of selected bacteria. The results showed that the best growth of chili plants was obtained by treating them with bacteria and watering them every other day. Molecular identification demonstrated that the selected bacteria was *Sphingomonas yunannensis*, which can grow under environmental conditions affected by drought.

Keywords: exopolysaccharides, drought, *Sphingomonas yunannensis*, chili plants

INTRODUCTION

The area of dry land in Indonesia in 2020 is 8,209.2 ha (BPS 2022). In general, farming on dry land has low productivity because of the lack of nutrients and water in the soil. One effort to increase plant productivity is to maintain the availability of water in the soil, which is sufficient for plant growth. The availability of water in the soil depends on the type of soil and its ability to store water. Water is essential in the vegetative growth phase, which leads to the development of roots and stems (Gardner *et al.* 1991), whereas in the generative phase of plant growth, it leads to flowering, fertilization, and fruit ripening (Vergara 1976). The availability of water depends on climatic conditions far from the dams and agricultural irrigation canals. Plants that experience drought stress can extend their roots to find a water source on the soil surface (Djazuli 2018). In addition to paying attention to watering, microorganisms in the soil, such as bacteria, can help bind and absorb nutrients and spur the growth and physiology of plant roots.

Certain soil bacteria can survive drought conditions by utilizing water stored in soil micropores and survive with minimal metabolic activity (Alikhani *et al.* 2010). According to McMillan (2007), bacteria around plant roots play three main roles: biofertilizers, biostimulants, and bioprotectants. Bacteria around the roots can help in the absorption of nutrients and act as biological control agents in plants. Kloepper (1992) reported that bacteria have several beneficial properties, including providing and facilitating the absorption of various nutrients, including nitrogen and phosphate, in the soil and synthesizing phytohormones that promote growth in plants. Bacteria in the soil can produce exopolysaccharides so that they can survive drought conditions. This is in accordance with Donot *et al.* (2011), who reported that exopolysaccharides produced by bacteria can protect bacteria from a wide variety of environmental stressors.

The objectives of this study were (1) to isolate and select superior bacteria from dry land for use in chili plants and (2) to study and determine the effect of water frequency and superior soil bacteria on the growth of chili plants.

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METHODS

The research was carried out at the Soil Biotechnology Laboratory, Faculty of Agriculture, IPB, while the planting of chili plants was carried out at the Biotech Center Greenhouse, Bogor Agricultural University. Soil sampling for bacterial isolation was carried out in Cikopomayak Village, Jasinga District, Bogor Regency using the random sampling method, where at the point of sampling location were soil that has been planted with chili peppers and soil that has not been planted with chili.

Soil Bacterial Isolation and Selection

Soil bacteria was isolated using the total plate count (TPC) method, including the process of diluting soil samples in a physiological solution of NaCl 0.85%. Suspensions at dilution of 10^{-4}, 10^{-5}, and 10^{-6} were grown on Nutrient Agar (NA) media. The production of exopolysaccharides using a specific medium (ATCC No. 14. Bacterial growth on ATCC No. 14 specific media was characterized by bacterial colonies that form slime on the medium (Tallgren *et al.* 1999). Testing the hypersensitivity reaction of tobacco leaves to bacteria to determine the pathogenicity of isolates in plants marked by the occurrence of necrosis in bacterial suspension infiltration in tobacco leaves. Hemolysis testing was carried out on Blood Agar media by observing clear zones around bacterial colonies which indicated that the isolates could be pathogenic to living things.

Bacteria Characterization

Selected bacterial isolates were tested for their ability to dissolve phosphate on Pikovskaya Medium, potassium dissolution on Alexandrov Medium, and N tethering on NFM Medium. The isolates was biochemically tested using the catalase test, oxidase test, pH, temperature, salinity, and Gram staining.

Testing in the Greenhouse

The greenhouse experiment used a Factorial Group Random Design, with two factors: 1) the administration of bacteria consisting of A1 (with bacteria) and A2 (without bacteria), and 2) the administration of water as much as 200 mL/pot/day, with the frequency: B1 (every day), B2 (every two days), B3 (every three days), and B4 (every five days). The combination of treatments was repeated three times to obtain 24 experimental units (two bacterial treatments × four water frequencies × three replicates). The plant parameters observed during the five weeks after planting were plant height (cm), leaf area, number of leaves, stomatal density, root length, wet weight, and dry weight of roots and upper plants.

Identification of Bacteria Potential Exopolysaccharides

This method was performed using 16S rRNA sequencing analysis. DNA isolation uses the alkaline lysis method, whereas DNA amplification is performed using two universal primary pairs of bacteria (Marchesi *et al*. 1998). The sequencing results were then analyzed on the NCBI *BLAST* (https://blast.ncbi.nlm.nih.gov/Blast.cgi) website and phylogenetic trees were constructed using the MEGA11 application.

Data Analysis

The data from the observation and measurement of plant parameters in this study were analyzed using the analysis of variance. If the treatment showed a significant effect, a further Tukey test (smallest significant difference at the level of α 0.05) was performed using Microsoft Office Excel and SPSS.

RESULTS AND DISCUSSION

Exopolysaccharide Bacteria Isolates

From the results of bacterial isolation, 19 bacterial isolates could grow on the specific medium of ATCC No. 14 with the characteristics of bacterial colonies that were dull and clear in color with a slimy colony texture. This follows the research of Prima *et al*. (2008) and Gofar *et al*. (2019), who found that the growth and activity of exopolysaccharide-producing bacteria are characterized by the ability of bacteria to produce slime or mucus on the specific medium of ATCC No. 14.

In hypersensitivity testing using tobacco plants whose leaves were injected with 19 bacterial isolates, 5 did not show necrosis symptoms, namely isolates 3, 4, 5, 7, and 9 (Table 1, Figure 1). According to Trinayanti (2012), the hypersensitivity reaction is a rapid defence reaction of plants against incompatible pathogens accompanied by rapid cell death in the tissue area injected with a bacterial suspension; thus, its existence does not affect the growth of the host plant. This is in accordance with Fitri *et al.'s* (2023) experiment, which showed that the appearance of necrosis symptoms in tobacco leaves shows that the inoculated bacterial isolate is a pathogenic bacterium to plants.

Hemolysis occurs in blood media, so there are three types, namely alpha hemolysis (α), beta hemolysis (β), and gamma hemolysis (γ). Hemolysis testing of bacterial isolates 3, 4, 5, 7, and 9 revealed that bacterial isolate 9 did not undergo hemolysis on agar blood media (Table 1, Figure 2). Hemolysis is a toxin released by bacteria that forms a clear zone around the colony and grows on the

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Table 1 Isolation and selection of exopolysaccharide-producing potential isolates, hypersensitivity, and hemolysis tests

Isolates	Exopolysaccharides producer	Hypersensitivity test	Hemolysis test
1	√	+	
2	√	+	
3	√	-	α
4	√	-	β
5	√	-	β
6	√	+	
7	√	-	β
8	√	+	
9	√	-	γ
10	√	+	
11	√	+	
12	√	+	
13	√	+	
14	√	+	
15	√	+	
16	√	+	
17	√	+	
18	√	+	
19	√	+	

Remarks: √: Isolate can grow on ATCC No. 14 specific media; +: u The occurrence of necrosis in tobacco leaves; -: does not cause necrosis in tobacco leaves; α: Isolate can hemolyze half of the blood agar medium; β: Isolate can break down blood on the blood agar medium with the whole; and γ: Isolate can grow well by not changing color and there is a clear zone on the blood agar medium.

Figure 1 Hypersensitivity testing in tobacco leaf plants a: no symptoms of necrosis, tobacco leaves b: symptoms of necrosis

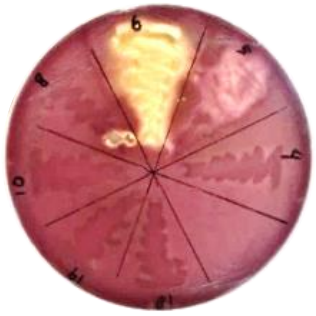

Figure 2 Hemolysis testing on blood media indicating that Bacteria 4 has alpha hemolysis (α), Bacteria 5 has beta hemolysis (β), and Bacteria 9 has gamma hemolysis (γ).

blood agar medium (Rahmi *et al.* 2015). This is by the findings of Sukmadewi *et al.* (2017), that hemolysis zones are formed on the surface *of* the agar blood media in bacterial isolates 3, 4, 5, and 7 because these bacteria can produce extracellular that can lysis red blood cells. Isolate 9, which has been determined to be biologically safe as an isolate that does not have pathogenicity in plants or animals, is tested in subsequent tests until molecular identification tests. The result of the molecular identification of the selected isolate is *Sphingomonas yunannensis*.

Physiological Characteristics of *S. yunnanensis*

The isolate of *S. yunnanensis* grown on various media has a white and slimy colony morphology; thus, when tested on *Pikovskaya, Alexandrov*, and NFM media, which are also white, the isolated bacteria were not visible. Physiological testing of bacteria includes bacterial growth on *Pikovskaya*, NFM, and *Alexandrov media*. Testing on Pikovskaya media determined the ability of bacteria to dissolve phosphate, characterized by the growth of bacterial inoculation on the medium, which can then be applied to the soil. The bacteria can grow by showing clear zones around bacterial growth (Figure 3a); thus, the bacteria have the potential to dissolve phosphate in the soil. Nitrogen (N2) is one of the most important elements for plants, as it is a component of proteins and plays a role in photosynthesis (Leghari *et al.* 2016). The ability of *S. yunnanensis* to act as an N anchor can also be observed from its ability to grow on NFM-specific media (Figure 3c).

Low soil nitrogen can cause plant growth inhibition, yellowing of leaves, small flower and fruit sizes, and low

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yields (Sapalina *et al*. 2022). Nitrogen can be absorbed by plants in the form of ammonium ions (NH4+) or nitrate ions (NO3-), and nitrogen gas in the air is converted into ammonia (NH3), which is then fixed in the soil (Martinez *et al*. 2021). Nitrogen fixation is assisted by nitrogen-fixing bacteria, in which nitrogen (organic and inorganic) in the soil is converted into ammonium and nitrate, which are then absorbed by plants (Sapalina *et al*. 2022).

Biochemical Characteristics of *S. yunnanensis*

Biochemical testing of *S. yunannensis* bacteria included salinity, temperature, pH, oxidase, Gram staining, and catalase activity. The salinity concentrations in the liquid media were 3%, 5%, and 7%. The bacteria grew well at each concentration, especially at a concentration of 5%, which had an optical density of 0.161, whereas 3% and 10% had values of 0.100 and 0.090, respectively (Table 2). At a concentration of 10%, the optical density decreased from 5%, following the results of Wadhwa (2017). If there is an increase in NaCl concentration, the tolerance of bacterial growth isolates will decrease, or it won't be easy to grow.

For temperature testing, the bacteria were stored at different temperatures. The storage temperatures were cold, room, and hot. Good bacterial growth occurs only at room temperature, where turbidity of the liquid medium can be seen, and after being inserted into a spectrophotometer, produces an optical density of 0.070 (Table 2), while at cold and hot temperatures, there is no change in the medium or optical density. The pH test results showed that the bacteria could grow at neutral and alkaline pH at pH 7 and 10, with optical densities of 0.085 and 0.001, respectively. In contrast, for acidic pH, the bacteria could not grow. Torres *et al.* (2012) reported that good exopolysaccharide production occurs at 25–35°C and pH 6.0–8.0. This is following Maalej *et al.* (2015), who found that pH affects the morphology of cell membranes, enzyme activity, exopolysaccharide production, and nutrient absorption by bacteria.

The catalase test (Table 2) showed a positive reaction, indicating that *S. yunnanensis* bacteria contain the catalase. Bacteria that have the catalase can defend themselves from hydrogen peroxide, where the catalase can decompose hydrogen peroxide (H_2O_2), which is toxic to plants and turned to water and oxygen (Panjaitan *et a*l. 2020).

The straining of *S. yunnanensis* bacteria (Table 2) produced a negative Gram, characterized by bacteria binding to safranin dye on the cell wall. In addition to color testing using crystal violet or safranin staining, it can also be seen when isolating the exopolysaccharide producing potential on MacConkey media. MacConkey's medium is selective and only grows in gram-negative bacterial species (Jung *et al.* 2022). S. yunnanensis can grow well in MacConkey media.

Effects of *S. yunnanensis* on Plant Growth

The plant height and weight in the A1B1 treatment or control had good values (Table 3), namely, plant height of 43 cm and plant weight of 17.4 g. In the treatment of A2B2 there was no significant difference from A1B1. A1B1 is a treatment for plants not fed *S. yunnanensis* with daily watering. Watering that is carried out every day causes water availability in the soil to be sufficient so that

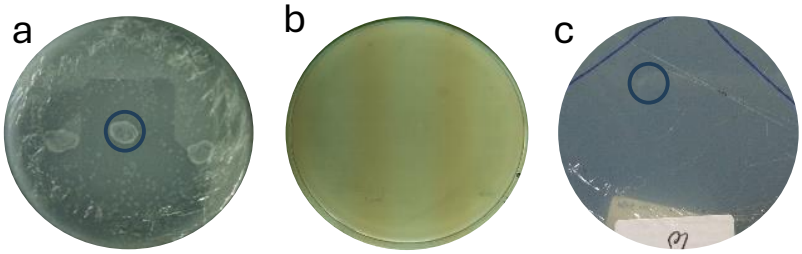

Figure 3 Activity of *Sphingomonas yunnanensis,* on a: P solublization resulting clear zones; b: K solublization resulting anegatif result that does not produce clear zones; c: Isolate can grow on NFM media indicating the bacterium can fix N2

Table 2 Biochemical characteristics of *Sphingomononas yunnanensis*

Parameter	Result	OD (*Optical density)*
Temperature (°C)	25–28	0.070
Salinity (%)	3–7	0.090–0.161
pH	7–10	0.001–0.085
Oxidase	-	
Catalase	Foaming	
Gram staining	Gram negative	

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Table 3 Effect of application of *Sphingomonas yunnanensis* bacteria and fruequency of irrigation in several 5 weeks after planting of chili plants

Treatment	Plant parameter						
	Plant height (cm)	Number of leaf	Area of leaf (cm^2)	Length of root (cm)	Stomata density ($cell/m^2$)	Plant weight (g)	Root weight (g)
A1B1[a]	**43d**	**16.67c**	**51.31ab**	**21.33b**	**600c**	**17.40d**	**1.16e**
A1B2	29a	11.67a	52.81bc	15.17b	292ab	7.97a	0.48a
A1B3	35.67bc	12.67ab	55.48b	17.50b	325ab	13.44bc	0.72bc
A1B4	31ab	13ab	45.92a	12.50b	250a	11.60b	0.69ab
A2B1	30.67ab	13.33ab	54.31b	**41.50a**	325ab	14.94c	0.88bcd
A2B2	**39.33cd**	**15ab**	**65.16c**	35.17a	300ab	**18.47d**	**0.94de**
A2B3	37bcd	14ab	56.27b	16.50b	337ab	13.46bc	0.91cd
A2B4	34.67abc	13.33ab	53.59b	14.50b	**417b**	12.43b	0.77bcd

Remarks: A1: Without administration of *Sphingomonas yunnanensis bacteria*; A2: Administration of *S. yunnanensis* bacteria; B1: Daily watering 200 ml/pot/day; B2: Watering every 2 days 200 ml/pot/day; B3: Watering every 3 days 200 ml/pot/day; B4: Watering every 5 days 200 mL/pot/day.

nutrient absorption can occur properly and plant growth can develop properly. This is by Felania (2017), who stated that water in plants acts as a solvent for organic molecular compounds (nutrients) found in the soil to plant tissues. The A2B2 treatment had an average plant height of 39.33 cm and a plant weight of 18.47 grams, which was not significantly different from A1B1, where A2B2 was treated with the bacteria with watering every 2 d. Thus, *S. yunnanensis* can stimulate plant growth even with insufficient water conditions in the soil. According to Carminati *et al*. (2011), bacteria that produce exopolysaccharides can store and distribute water to plant roots when water in the soil is limited.

The root length in the A2B1 treatment, with a result of 41.5 cm, is significantly different from the A1B1 treatment, which is 21.33 cm, but there is no significant difference from the A2B2 treatment, which is 35.17 cm. The A2B1 treatment is a plant that is fed bacteria with daily watering. The root weight of A1B1, with an average of 1.16 g, was not significantly different from that of the treated root in A2B2, which was 0.94 g. Applying *S. yunnanensis* in the treatment can help dissolve phosphorus and potassium and anchor nitrogen in the soil. Nitrogen plays a role in helping plant vegetative growth (Habibi *et al.* 2017), while phosphorus stimulates root growth as an essential ingredient for protein formation and accelerates the flowering process. Potassium helps proteins and carbohydrates, strengthens plant tissues in leaves, flowers, and fruits, and increases drought resistance (Lingga *et al.* 2001).

The number of leaves and leaf area (Table 3) had the best value, namely, in the A2B2 treatment with an average of 15 leaves and a leaf area of 65.16 cm2, which was significantly different from A1B1 or the control with a result of 16.6 in the number of leaves and 51.31 cm2 in leaf area. A2B4 treatment had a high stomatal frequency of 417 cells/m2, significantly different from the A1B1 treatment. In the A2B4 treatment, plants were treated with *S. yunnanensis* and watered every 5 days. Watering

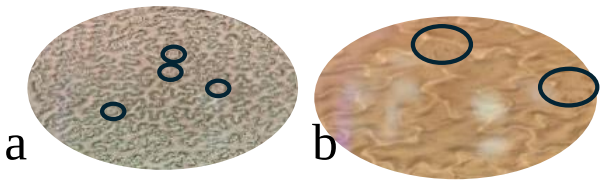

Figure 4 Appearance of stomata on of the leaf with 40× (a) and 100× (b) magnification using a microscope

with different frequencies, namely A2B2 watering every 2 days and A2B4 watering every 5 days by adding *S. yunnanensis* bacteria, can help the plant growth process well, which can be seen from the number and area of leaves in chilli plants. Exopolysaccharide-producing bacteria function to defend themselves from abiotic stress (Donot *et al.* 2011), and exopolysaccharides are produced by bacteria to protect bacterial cells from drought by producing biofilms so that they can help plant growth (Ozturk *et al.* 2010).

The criteria for stomata density can be seen in Table 3 and Figure 4. A2B4 has a moderate stomata density, which is not significantly different from A2B1, A2B2, and A2B3, given by S. yunnanensis bacteria. This is following Rofiah (2010), that the criteria for the value of stomata density if < 300 is said to be low; if it is 300–500, then the stomata density is medium, and if it is > 500, then the stomata density is high. According to Marantika et al. (2021), the density of stomata differs in plant types that are affected by the environment, namely light intensity, temperature, water availability, and CO_2 concentration.

S . yunnanensis applied to plants by regulating the water supply fraction can affect plant growth. The results of plant parameters, namely plant height, number of leaves, leaf area, plant weight, and root weight, in the A2B2 treatment had the highest value after the A1B1 treatment or control, which did not differ significantly from the average root length and stomatal density (Table 3).

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The exopolysaccharides produced by bacteria respond to extreme environmental conditions or at the time of biofilm formation. Putrie (2016) reported that the biofilm produced by exopolysaccharide-producing bacteria on the root surface could form a good soil structure to protect plants from water shortage. Biofilms produced by exopolysaccharide-producing bacteria were reported by Oztruk *et al.* (2010), who reported that exopolysaccharides produced by bacteria to protect bacterial cells from drought, heavy metals, or environmental stresses include responses to immune hosts and to produce biofilms so that bacterial cells can increase their resistance to unique ecological niches.

Molecular Identification of *S. yunnanensis*

Amplification of the DNA gene 16S rRNA isolates potential exopolysaccharide by PCR, shown by the bright and thick DNA band measuring 1525 bp from the of electrophoresis result with agarose gel (Figure 5). The results of nucleotide sequencing from the isolated PCR amplicon, followed by the 16S rRNA sequencing method in BLAST on the NCBI (National Center for Biotechnology Information) database, were identified as *S. yunnanensis* with a homology of 98.6%. *S. yunnanensis* is a bacterium of the genus *Sphingomonas* that can be found in plant samples and soil samples, has a catalase enzyme, can grow at pH 7–7.5 with a room temperature of 25–28°C, and has a negative gram (Zhang et al. 2005). Sphingomonas is a genus whose members are known to play a role in degrading polysilicate aromatic hydrocarbons (PAHs), aiding plant growth, and tolerance to environmental stress (Asaf et al. 2020). According to Wingender et al. (1999), exopolysaccharide-producing bacteria are often found outside the bacterial cell membrane structure in eukaryotes and prokaryotes. In the Harahap study (2018), the number of gram-negative bacteria that produce exopolysaccharides was higher than that of gram-positive bacteria. Luo *et al.* (2019) revealed that *Sphingomonas* sp. can affect the morphological and physiological properties of Arabidopsis plants, which can promote leaf formation and development of root system structure to improve water absorption and reduce drought stress in plants.

Similarity analysis (Table 4) of isolates with exopolysaccharide-producing potential have DNA length is 1349 and *a* 100% query cover for *S. yunnanensis*, with e-value 0.0 a bacterium from Singapore, South Korea, and Portugal, with a percentage identity of 98.52-96.97%. Percent identity to say a bacteria or prokaryote as a species is to use a demarcation of 97.5% or higher to be said to be of the same species (Kucznski *et al.* 2012).

The higher the percent identity value, the higher the level of similarity (Lonthor, 2023). Query cover is the percentage of sequences included in the GenBank database's alignment and alignment with sequences (Newel *et al*. 2013). According to Twindiko *et al*. (2013), if the query cover is more than 90%, it can be declared high for the match value in the sample processed at the NCBI.

Phylogenetic analysis (Figure 6) is an advanced stage performed after DNA sequencing by comparison with other reference sequences. Phylogenetic analysis was

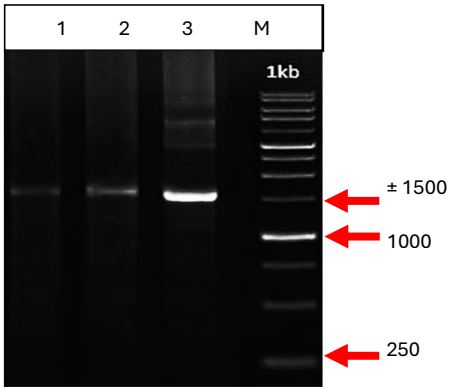

Figure 5 Amplicon PCR product of the 16S rRNA gene of the potential isolate as a exopolysaccharide producer

Table 4 Analysis of nucleotide gene 16S rRNA of potential exopolysaccharide producing bacteria with BLAST in GenBank

Isolate name in Gen*Bank*	Query cover (%)	Identity (%)	Origin of isolate
Bacteria 9	100	98.6	Indonesia
Sphingomonas yunnanensis	100	98.52	Singapore
Sphingomonas yunnanensis	100	97.86	South Korea
Sphingomonas yunnanensis	98	97.9	China
Sphingomonas yunnanensis	100	96.97	Portugal

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carried out to determine the kinship of each bacterial

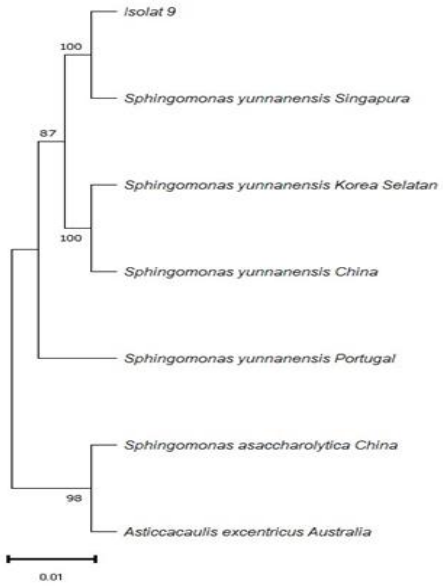

Figure 6 Phylogenetic tree of *Sphingomonas yunnanensis*

species and was visualized in the form of a phylogenetic tree (Dharmayanti 2011). In phylogenetic trees, there is a bootstrap value, which, according to Hillis *et al.* (1993), is a high bootstrap value, indicating that the branching construction in phylogenetic trees has good accuracy and stability. Bootstrap values greater than 50% were considered significant (Kress, 2002). The phylogenetic tree results have two bootstrap groups, namely isolates that produce exopolysaccharides with a bootstrap value of 88-100% similarity to *S. yunnanensis* bacteria in Singapore, South Korea, China, and Portugal. In contrast, the second group, or outside of the *S. yunnanensis* group, *S. asaccharolytica*, and *Asticcacaulis excentricus,* has a 97% resemblance.

CONCLUSION

Isolation and selection of 19 bacterial isolates produced 9 isolates that can be used for the next step exopolysaccharides and can be applied to chilli plants. Isolate 9 was *Sphingomonas yunnanensis* based on morphological, physiological, biochemical, and molecular identification based on the 16S rRNA gene. Bacteria can survive in drought conditions. Bacterial testing on chilli plants showed that the treatment of *S. yunannensis* with watering 200 mL/pot/every 2 days could increase plant height, leaf number, leaf area, plant weight, and root weight.

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