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Diversity of non-symbiotic nitrogen-fixing bacteria and their potential in andisols affected by the eruption of Mount Sinabung, North Sumatra, Indonesia

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Abstract. *Sembiribg M, Sabrina T. 2021. Diversity of non-symbiotic nitrogen-fixing bacteria and their potential in andisols affected by the eruption of Mount Sinabung, North Sumatra, Indonesia. Biodiversitas 22: 3539-3544.* Nitrogen is the main macro-nutrient that is very important for plant growth. Nitrogen is absorbed by plants in the form of NO^{3-}or NH^{4+} ions from the soil. The andisols affected by the eruption of Mount Sinabung alter the chemical, physical and biological characteristics of the soil. As a result, the population of beneficial microbes in the soil decreased, so soil fertility also decreased. The aim of this research is to determine the diversity of nitrogen-fixing microbes in andisol soil affected by the Mt. Sinabung eruption. The soil samples used in this research were collected from andisols affected by the eruption of Mount Sinabung. The isolation of non-symbiotic nitrogen-fixing bacteria was carried out using a nitrogen-free Jensen medium. The results showed that five non symbiotic N-fixing bacteria can increase the N content in andisols affected by Mount Sinabung eruption. *Enterobacter cloacae* can increase soil N by 111.76% as compared to without microbial application.

Keywords: Andisols, nitrogen-fixing bacteria, soil N, diversity, N fixation

INTRODUCTION

Nitrogen is a nutrient that is easily washed and evaporates, hence its availability in the soil decreases, while its amount in the atmosphere increases. One way to increase the availability of N in the soil is by utilizing environmentally specific nitrogen-fixing microbes. Volcanic ash contains heavy metals and harmful micro-substances which are easy to settle. The volcanic ash that covers the surface over a long period of time settles and hardens. Depending on the thickness levels, ash deposited causes disturbance in soil aeration, which affects microorganisms in the soil. The thicker the volcanic ash, the fewer microorganisms in the soil (Pakolo et al. 2018; Sarah et al. 2015; Munawaroh et al. 2020; Qadaryanty et al. 2020; Zebua et al. 2020)

The pH of soil greatly influences the growth of soil microorganisms. Fungi can tolerate and survive a soil pH range of 4-6.5, while bacteria prefer a soil pH of 6 to 7 (Hanafiah et al. 2009). According to Sinaga et al. (2014) the thicker the ash, the lower the pH of soil. Volcanic ash contains heavy metals and harmful micro-substances which are easily deposited. The pH of Mount Sinabung volcanic ash ranged from 3.3 to 3.5, while the soil pH ranged from 4.4 to 6.5 which inhibited the activity of soil organisms (Balitbangtan 2014; Fatmala et al. 2015; Sinaga et al. 2015; Sembiring and Fauzi 2017; Sembiring et al. 2017b) The eruption of Mount Sinabung reduced organic matter in the soil hence nitrogen content in the soil was low. This happens because exposure to the ash makes it difficult for soil organisms to survive, disrupting the decomposition process (Sinaga et al. 2015). N-total in soil was affected by volcanic ash ranged from very low to low 0.04%-0.20% (Sembiring et al. 2017a; Sembiring et al. 2017b). Sembiring et al. (2016) reported showed that total C-organic and N content in the soil affected by the eruption was 5.74% and 0.56%. This is because the source of nitrogen in the soil is reduced, and the microorganisms that function to fix nitrogen in the air are also reduced (Hanafiah 2014). Nitrogen (N) is one of the most widely distributed elements in nature. The problem in terms of the availability of N in the soil is the nature of nitrogen as it is easily dissolved or washed and evaporated by which the amount in the soil is reduced while the amount in the atmosphere is high. Nitrogen fixation can be of two types namely, symbiotic or non-symbiotic. Microorganisms that play a role in non symbiotic fixation include *Azospirillum*, *Azotobacter*, and *Beijerincka* (active in acid soil conditions), *Bacillus*, *Enterobacter*, etc (Hanafiah 2014).

MATERIALS AND METHODS

The soil used in this research was taken from andisols affected by the eruption of Mount Sinabung, from the Naman Teran Sub-district, Karo District, North Sumatra Province, Indonesia. The research was carried out from June to December 2020.

Sampling

Sampling points were taken from the soil which was distinguished based on several ash thicknesses, i.e.

Location I = cultivated land (0 cm), Location II = soil with < 2 cm ash thickness, Location III = soil with medium ash thickness (2-5 cm), and Location IV = soil with thick ash thickness (> 5 cm). The samples were taken at a depth of 0-20 cm from the soil surface around the rhizosphere area using a hoe. Eight soil samples were taken from each location, which was then composited. Soil temperature and humidity were also recorded in the field at the time of soil sampling. The soil samples were then brought to the laboratory for initial analysis such as Temperature (°C), Humidity (%), pH (electrometry), C-Organic (Walkley and Black), S-Dissolved (Turbidimetry), N-Total (Kjeldahl), and total microbes (Plate Count Method) analysis.

Isolation and identification of bacteria

The Jensen media was used for the isolation of bacteria and the composition of media was as follows: (Sucrose 20g, K2HPO4 1g, MgSO4.7H2O 0.5g, NaCL 0.5g, FeSO4 0.1g, CaCO3 2g, jelly 20g, aquadest 1L). Ingredients used for gram staining include crystal violet solution, iodine solution, 70% alcohol solution, safranin solution.

To obtain nitrogen-fixing bacteria present at the four observation sites, soil bacteria were isolated by multilevel dilution and using the pour plate method. The first step was to take soil from several locations with different thicknesses of ash and then compost it (mixed). Take 10 g of composite soil in a beaker, add 90 mL of sterile distilled water to it, and then homogenize it using a shaker. Furthermore, a dilution technique was carried out on soil suspension, to make a dilution factor of 10-1 to 10-5, take 1 mL of the sample suspension and mix it with 9 mL of distilled water (10-1). Then 1 mL of suspension was pipette out from first dilution factor (10-1) and added to 9 mL of distilled water (10-2), the same treatment was done for rest of the dilution factors (10-3, 10-4, and 10-5). 1 mL of each dilution factor (10-3, 10-4 and 10-5) was poured into the Petridish, then add 20 mL of Jensen medium, mix well and incubated at 30°C for 72 h. After the colony grew, isolated species were identified based on morphological and molecular levels. To identify bacteria at the molecular level, universal primers of 63f (5'CAG GCC TAA CAC ATG CAA GTC 3'), primer 1387r (5' GGG CGG WGT GTA CAA GGC 3') were used to amplify the gene sequence of 16S bacteria rRNA through PCR (Lane 1991).

Potential test of nonsymbiotic fixing bacteria on andisols

The soil used to test the potential of microbes in fixing nitrogen was andisol soil taken from location II which was covered with thin ash (< 2 cm). 50 g of sterilized soil was prepared, then 1 mL (10^8) was mixed with media and incubated for 30 days at room temperature. The parameters observed after the incubation process were: pH (electrometry), N-Total (Kjeldahl), and total microbes (Plate Count Method). The calculation of the bacterial population in the medium was calculated using the formula:

$$\text{Total Bacteria} = \sum \text{Colony} \times \text{Diluting factor}$$

RESULTS AND DISCUSSION

Analysis of soil conditions at several thicknesses of eruption ash

Based on the results of the initial analysis temperature, humidity, soil pH, organic C, N, S, and microbial populations were found to be different at each observation location (Table 1).

The result showed that thicker the volcanic ash covering the soil increased temperature by 31.58% (Location IV), while decreased soil moisture by 36.67%. This indicated that thicker the volcanic ash which covered the soil surface, inhibited aeration and infiltration into the soil. The highest 5.8-6.2 soil pH was recorded from (Location I), and the lowest 3.5 pH was at location IV. The decrease in pH with increasing ash thickness was 77.14% (Location IV). The thicker the volcanic ash, the lower the pH value of the soil. This is because the volcanic ash of Mount Sinabung was dominated by high sulfur content which causes a decrease in pH. Simanjuntak et al. (2015) stated that the factor that affects soil pH is the high sulfur (S) content in volcanic ash, resulting in a very acidic to acidic soil pH. Sembiring et al. (2015) reported that the pH of volcanic ash from the eruption of Mount Sinabung is very acidic (3.3-3.5), this acidic nature of volcanic ash can affect the physical, chemical and biological properties of the soil. The highest S-dissolved was 0.34% at Location IV and the lowest was 0.09% at location I. This indicates that the thicker the volcanic ash on andisol soil, the higher the S-dissolved content. This occurs because volcanic ash contains quite high sulfur, and rainfall also affects the S content in the volcanic ash of Mount Sinabung. Sulfur leached to the soil layer below by the rain, which resulted in S in the soil mixed with volcanic ash. According to Balitbangtan (2014), Mt. Sinabung volcanic ash is containing S elements ranged from 0.05-0.32%. The high sulfur content in volcanic ash affects soil pH.

Table 1. Soil conditions at each sampling location

Sampling location	Temperature (°C)	Humidity (%)	pH	C Organic (%)	N (%)	S dissolved (%)	Microbial population (10^4)
I	19-20	78-82	5.8-6.2	6.29-7.45	0.55-0.65	0.09-0.15	25-33
II	19-21	77-78	5.5-5.8	2.47-5.01	0.20-0.33	0.08-0.12	18-26
III	22-24	70-72	3.8-4.5	1.30-1.87	0.09-0.19	0.09-0.18	9-12
IV	24-25	60-68	3.5-4.3	0.65-1.18	0.07-0.11	0.21-0.34	4-6

Note: Location I: Cultivated land (0 cm), II: soil with < 2 cm ash thickness, III: soil with medium ash thickness (2-5 cm), IV: soil with thick ash thickness (> 5 cm)

The highest (7.45%) C organic content was found at Location I, and the lowest (0.65%) was at Location IV. It was also noted that the C organic content decreased with an increase of ash thickness by 104.62%. According to Sukarman and Dariah (2014), the C-organic content of andisol soil found in Indonesia varies from very low to very high. Simanjunak et al. (2015) mentioned that C-organic soil affected by the Mt. Sinabung eruption ranged from 0.91%-7.19%. Sinaga et al. (2015) stated that volcanic ash that covers the soil surface settle down and harden depending on its thickness level. It affects soil aeration, respiration, oxygen, and organic matter availability in the soil, affecting the life of organisms in the soil.

The highest N-total soil was at Location I by 7.45% and the lowest was at Location IV by 0.07%. It was also noted that increasing ash thickness by 105.43% decreased the N total in the soil. Soil nitrogen value is influenced by soil's organic matter and microorganisms that fix N from the air. This follows the work of Sinaga et al. (2015), which noted that the thickness of volcanic ash covering the soil makes it difficult for plant roots to penetrate in the soil and soil organisms are difficult to survive hence the decomposition process is hampered. Simanjuntak et al. (2015) mentioned that the soil mixed with ash with a depth of 5-20 cm has a nitrogen value ranging from very low to low, i.e. 0.04%-0.20%. Increases in soil temperature and the decreases in pH, humidity and soil C organic affect the population of organisms in the soil. The microbial population at location IV decreased by 72.5% compared to location I. This indicates that environmental conditions greatly affect the number and activity of organisms in the soil. According to Tindaon et al (2016), the amount of sulfur and Al in the soil greatly affects the soil pH, affecting the number of organisms in the soil. The low pH results in disruption of the activities in soil organisms (Hanafiah 2014).

Isolation and identification of bacteria

Results showed that from the 4 locations observed, 5 bacteria isolates of had different colony characteristics, namely N1 from location I, N2 and N3 from location II, N4 from Location III and N5 from Location IV.

The differences in the composition of the nucleotides that make up the 16S rRNA coding DNA for each isolate were different, so a kinship analysis was carried out using the BLAST (Basic Local Alignment Search Tool) program. The results of molecular identification of 5 bacterial isolates coded N1, N2, N3, N4 and N5 are presented in Figure 1.

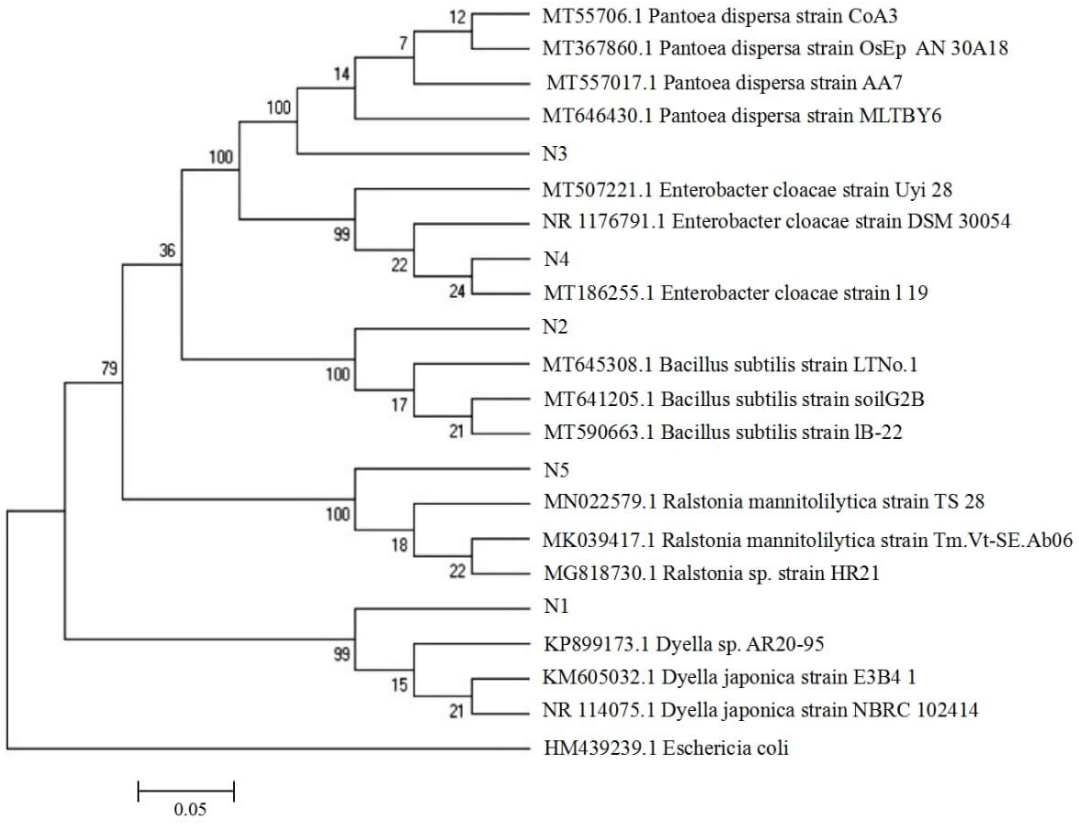

Figure 1. Phylogeny tree of non-symbiotic nitrogen-fixing bacteria sourced from andisols affected by the Mt. Sinabung eruption, North Sumatra, Indonesia

The results of the kinship analysis with the BLAST program were then continued with phylogenetic tree analysis. Based on the identification results (Figure 1), a 16S rDNA sequence showed that the N1 strain was *Dyella japonica*, N2 was *Bacillus subtilis*, N3 was *Pantoea dispersa*, N4 was *Enterobacter cloacae*, and N5 was *Ralstonia mannitolilytica*. The results of research by Xie and Yokota, 2005, found that *D. japonica* is a Gram-negative bacterium capable of fixing N from the air and requires a pH of 6.5-7 and optimum temperature of 25-30ºC. *B. subtilis* fixed nitrogen from the air and its growth decrease at high salt contents (Delgado et al. 1994; Satapute et al. 2012; Jesmi et al. 2017). *B. subtilis* is able to survive at a pH of 5.0-10 and the optimum pH for growth is 7-7.5 (Jadhav et al. 2010). *B. subtilis* able to produce IAA and stimulate plant growth (Salamone et al. 2001; Karadeniz et al. 2006; Ahmad et al. 2016). *P. dispersa* is a PGPR that can increase plant growth in soil conditions with high salinity (Habib et al. 2016; Panwar et al. 2016). *P. dispersa* is a bacterium capable of producing IAA and P solvent in soil (Deshwal and Kumar 2013; Paul et al. 2014). Ji et al. (2010) and Liu et al. (2017) found that *E. cloacae* is a bacterium that can increase nitrogen fixation and increase plant growth. *R. mannitolilytica* is a bacterium capable of fixing nitrogen and dissolving phosphate, especially in land contaminated with heavy metals (Paul and Datta 2016).

Each species of bacteria has different morphological characteristics from one species to another. The difference in these characters can be used as a guide to finding out the taxonomic position. Based on Figure 2, all bacteria cell form was different, which showed that the types of bacteria were also different. The data obtained are consistent with some of the morphological data of nitrogen-fixing bacteria reported in previous studies by Hartono 2014; Xie and Yokota 2005 bacteria that can fix N are generally gram-negative and round cells. This is in accordance with the result of Santoso et al. (2019) that 3 bacterial isolates that namely *Azotobacter*, *Azospirillum* and *Pseudomonas* can freely fix N, all being gram-negative and round cells.

The potential test results showed that nitrogen-fixing bacteria can increase N in the soil Enterobacter cloacae (N4) and increase soil N by 111.76% compared to without microbial application (Table 2). *R. mannitolilytica* bacteria can increase soil N by 11.76%, this shows that the application of nitrogen-fixing microbes at acidic soil pH can increase N content in the soil. The ability of nitrogen-fixing microbes to fix N varies depending on their ability to adapt to their environment. The pH of soil used was ranged from 5.07-5.42 which indicated that the pH was very acidic hence it inhibited the growth and activity of bacteria in the soil. According to Xie and Yokota (2005); Jadhav et al. (2010), 6.6-10 is the optimum pH for bacterial growth and its activity in fixing nitrogen from the air. The results showed that the ability of microbes to survive in the soil used was different depending on the type of bacteria. *B. subtilis* (25.50×10^8) has a higher survival rate than other bacteria. This is because the soil pH is close to the optimum pH of 5.42. The growth of microorganisms in the soil is strongly influenced by soil pH. Bacteria can survive at neutral pH (6-7) and their activity decreases if the pH is too low (Hanafiah et al. 2009).

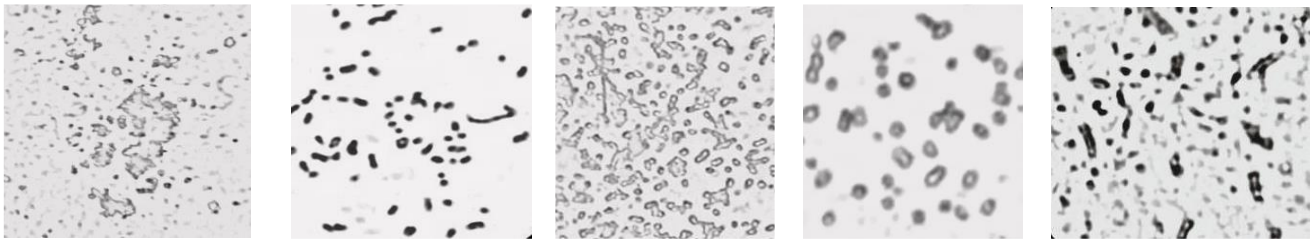

Figure 2. The bacterial cell form of N1. *Dyella japonica*, N2. *Bacillus subtilis*, N3. *Pantoea dispersa*, N4. *Enterobacter cloacae*, and N5. *Ralstonia mannitolilytica*

Table 2. Potential test for nitrogen-fixing bacteria to increase soil N content

Treatment	Soil pH	Soil N (%)	Microbial population (10^8)	IAA
Without Microbes (N0)	5.29	0.17	0.00	-
Dyella japonica (N1)	5.31	0.24	20.00	1.05
Bacillus subtilis (N2)	5.42	0.29	25.50	1.01
Pantoea dispersa (N3)	5.07	0.21	12.50	0.65
Enterobacter cloacae (N4)	5.24	0.36	24.00	2.65
Ralstonia mannitolilytica (N5)	5.26	0.19	24.50	2.45

The results of the analysis showed that the microbes found were able to produce IAA up to 0.65-2.65 ppm indicating that the ability of microbes to fix N and stimulate plant growth would be different. *E. cloacae* has a higher IAA content when compared to other microbes, this indicates that *E. cloacae* has a higher N fixing ability. *E. cloacae* has a high nitrogen fixation activity, and can be found in the plant rhizosphere (Wang et al. 2012; Macedo-Raygoza et al. 2019; Li et al. 2017). According to the results of Liu et al. (2017), *E. cloacae* can increase nitrogen fixation hence plant growth increases. *E. cloacae* application can increase plant growth and affect soil ecology (Kumaran et al. 2010; Ramesh et al. 2014; Khalifa et al. 2016). Nitrogen-fixing microbes can stimulate plant growth and fix nitrogen from the air in high amounts (Paul and Lade 2014; Nabti et al. 2015; Shrivastava and Kumar 2015).

It is concluded that five non-symbiotic N-fixing bacteria were isolated, which increased the N content in andisols affected by the eruption of Mount Sinabung. *Enterobacter cloacae* can increase soil N by 111.76% compared to without microbial application.

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