BIODIVERSITAS
Volume 21, Number 6, June 2020
Pages: 2832-2839

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d210661

Soil properties and sulfur-oxidizing bacterial diversity in response to different planting patterns of shallot (*Allium ascalonicum*)

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Manuscript received: 18 March 2020. Revision accepted: 29 May 2020.

Abstract. *Juwanda M, Sakhidin, Saparso, Kharisun. 2020. Soil properties and sulfur-oxidizing bacterial diversity in response to different planting patterns of shallot* (Allium ascalonicum*). Biodiversitas 21: 2832-2839.* Sulfur is one of the primary elements required by plants for growth and development. Sulfur-oxidizing bacteria (SOB) can oxidize sulfur to sulfate, which is directly taken up by plant roots. This study aims to evaluate the soil properties and SOB diversity in various shallot planting patterns, i.e. PP1 (shallot-dry season-shallot-shallot), PP2 (shallot-dry season-shallot-rice), and PP3 (shallot-pulses-shallot-rice). Soil samples were collected from the rhizosphere of the shallot plant and analyzed for the soil properties based on the standard methods. Bacteria isolation was cultured on Starkey broth and Starkey agar. Bacteria isolate was identified based on the 16S rRNA gene sequence and compared to the GenBank database. The results showed that shallot planting patterns influence soil properties and SOB diversity. The highest content of sulfate (41.31 ppm), organic C (0.957 %), organic matter (1.650%), C/N ratio (9.57), and SOB diversity was obtained in PP3 planting pattern. Three bacterial strains have been successfully isolated i.e. A-3245D, B-3246F, and C-3247C with their closest related to *Burkholderia cepacia, Klebsiella variicola,* and *Klebsiella aerogenes*, respectively. The highest diversity and population density of SOB was in the PP3 planting patterns, i.e *Burkholderia cepacia* (7.45 x 10^5 CFU/mL); *Klebsiella variicola* (1.79 x 10^7 CFU/mL; *Klebsiella aerogenes*: 3.9 x 10^6 CFU/mL). *K. variicola* can be found in three planting patterns of shallot.

Keywords: Bacteria, chemical, shallot, soil, sulfur

INTRODUCTION

Shallot (*Allium ascalonicum* L.) is one of the most important vegetables in Indonesia and commonly used as the main ingredient in numerous traditional cuisines (Hilman et al. 2014). The largest shallot-producing regency in the province of Central Java is Brebes which supplies 75% of the shallots in Central Java and 23% of the national needs (Hartini 2011). Data from the Central Bureau of Statistics (BPS 2018) showed that shallot yield in Brebes regency decreased from 12.23 t/ha in 2013 to 10.19 t/ha in 2017, followed by a decrease in national shallots production. The decline in shallot yield is partially related to the annual water supply. There are three different planting patterns for shallot cultivation by farmers in Brebes, i.e. (i) shallot-dry season-shallot-shallot, (ii) shallot-dry season-shallot-rice and (iii) shallot-pulses-shallot-rice. Due to differences in planting patterns, the use of chemical fertilizers, such as sulfur was varied.

Sulfur is needed by plants in a considerable concentration because sulfur is one of the macro essential nutrients after N, P, and K and required for growth and development (Sriramachandrasekharan 2013; Assefa et al. 2015). Methionine and cysteine, the two primary sulfur-containing amino acids, are both required for protein synthesis. Sufficient level of sulfur in soil is a determinant for the production of shallots and the metabolism of nitrogen in plants (Jaggi and Sharma 2010). Adequate sulfur content in the soil in shallot cultivation results in high yields of bulbs and affects bulb weight per clump, bulb diameter, and bulb yield per hectare (Diriba-Shiferaw et al. 2015; Lasmini et al. 2015). Sulfur level in soil affects soil chemical properties (Losák et al. 2008). The presence of organosulfur corresponds to a distinctive odor of shallot bulbs (Morradi et al. 2013). Since most of the sulfur in soil is present in organic compounds, therefore sulfur can not be absorbed directly by plants (Sridar et al. 2015).

It has been known that sulfur-oxidizing bacteria (SOB) can efficiently convert elemental sulfur and organic carbon of sulfur (unavailable form) into sulfate (available form for the plant) by the sulfur oxidation process (Losák et al. 2008; Fageria 2009; Ullah et al. 2014; Prenafeta-Boldúet al. 2014; Velivelli et al. 2014; Sridar et al. 2015). The oxidation reaction of sulfur to sulfate is as follows (Ryu et al. 2003):

$$S^0 + 3/2\ O_2 + H_2O \longrightarrow 2H^+ + SO_4^{2-}$$

SOB uses sulfur as a source of energy (Ullah et al. 2014). The presence of SOB in soil results in increased sulfate levels in the soil, consequently increased plant growth. Sulfur level and SOB diversity in soil affect soil chemical properties as well as nutrient availability and pH (Sabagh et al. 2014). Furthermore, the physical and chemical properties of soil are primarily related to microbial community and activity. The adequate availability of micro-and macronutrients is also important to improve soil fertility, growth, and yield of plants.

Besides, crop rotation systems by planting different crops can increase yields through improved soil fertility (Riedell et al. 2009; Glab et al. 2013).

Among many bacterial groups, autotrophic and heterotrophic bacteria have been recognized to be involved in the oxidation of sulfur (Hao et al. 2019). Sinha et al. (2008) identified the diversity of SOB especially *Klebsiella* sp. based on the 16S rRNA gene sequence, while Puspitasari et al. (2014) identified the diversity of *Bacillus cereus* in tin-mining soil. *Klebsiella* sp. is SOB that can convert sulfur to sulfate (Behera et al. 2016) and can be used as biological fertilizer to increase plant growth because it can provide nutrients needed by plants (Lin et al. 2015; Sulasih and Widawati 2019). Previous studies by Ryu et al. (2003) showed that SOB *Acidithiobacillus thiooxidans* affects the characteristics of metal content in waste sludge, while SOB *Thiobacillus* sp. affects the levels of sulfate, phosphate, and pH in rice fields, rhizosphere of wheat, industrial wastewater and sewage sludge, and enhance sulfur oxidation in soil and increase soil available sulfate (Mohamed et al. 2014; Ullah et al. 2014). A recent study by Pourbabaee et al. (2020) reported that *Thiobacillus* bacteria in combination with elemental sulfur enhances the oxidation of elemental sulfur resulting in increased nutrient availability in soil, consequently increased plant growth. Therefore, the presence of sulfur oxidizers is crucial to improve sulfur availability to plants at their critical stages to increase crop yields. To date, little is known about soil properties and SOB diversity in the shallot fields. Therefore, this study aimed to determine soil properties and SOB diversity in response to different planting patterns of shallot.

MATERIALS AND METHODS

Study area

This study was conducted at the Laboratory of Agrotechnology, Faculty of Agriculture, University of Jenderal Soedirman, Indonesia from April to September 2018. Soil samples were collected from rhizosphere soils from three different sites with ten replications based on planting patterns, namely Planting Pattern 1 (PP1: shallot-dry season-shallot-shallot), Planting Pattern 2 (PP2: shallot-dry season-shallot-rice) and Planting Pattern 3 (PP3: shallot-pulses-shallot-rice). The type of soils in study sites were vertisol. The experimental field was conducted at Brebes regency, Central Java, Indonesia (Figure 1), that situated at latitude 109° 3’ 24.7608"E and longitude 6° 51’ 59.0688"S for PP1, at latitude 109° 2' 40.4268" E and longitude 6° 53' 13.2288" S for PP2 and latitude 109° 0' 15.084"E and longitude 6° 57' 14.706"S for PP3.

Soil properties analysis

Soil samples were analyzed for physical and chemical properties including organic C, total P_2O_5, total S, and sulfate using the colorimetric method. Other soil properties such as total N (Kjeldahl method), pH (electrometric method), total K_2O (Flame photometry method), bulk density (candle method), particle density (pycnometer method), cation exchange capacity (distillation method), organic matter (conversion method), C/N ratio (calculation method) and porosity (calculation method) were also analyzed (Balittanah 2009).

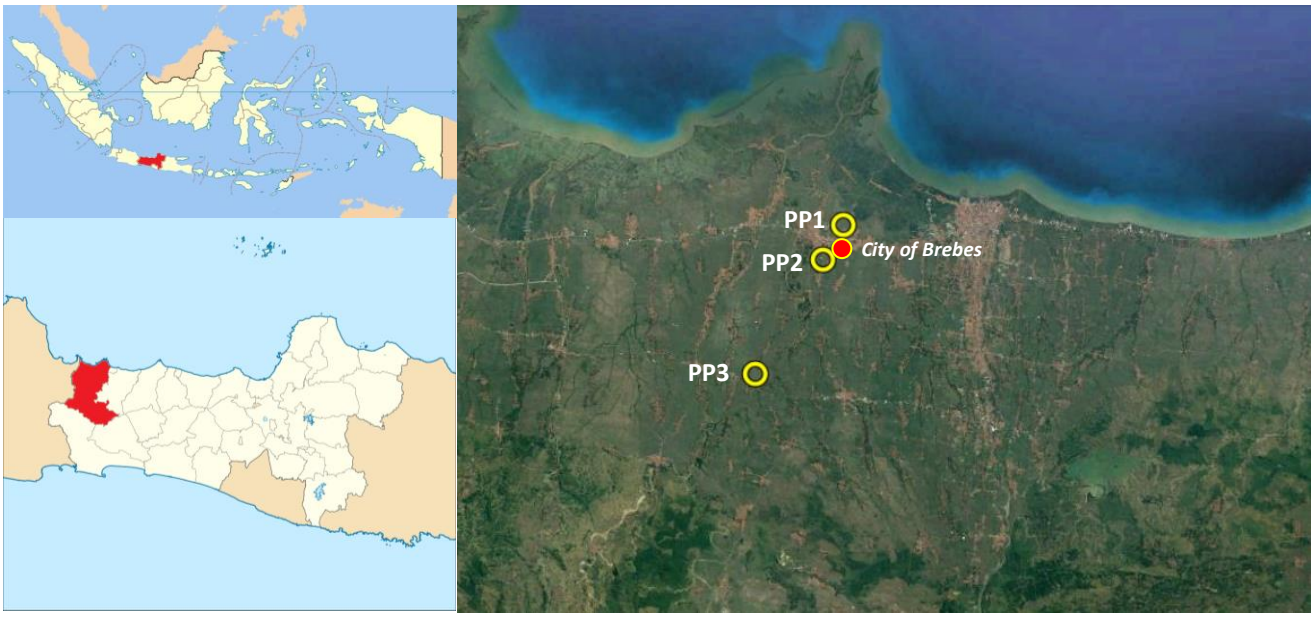

Figure 1. A map of Central Java province, Indonesia showing Brebes District as the study site

Isolation and identification of bacteria

Bacterial samples were collected from rhizosphere soil. Starkey broth medium containing 3.0 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g $CaCl_2.2H_2O$, 0.5 g $(NH_4)_2SO_4$, $FeSO_4$ and 10 g sulfur powder in 1000 mL of distilled water was used for culturing bacteria (Vidyalakshmi and Sridar 2007). Bromocresol purple was used as an indicator. Twenty grams of soil samples from each planting pattern were added into 80 mL sterilized Starkey broth (1:4; pH 4.8) and incubated 32^0C for 24 hours, and Starkey broth without soil sample was used as the negative control. After incubation, broth media were diluted with aqua dest (dilution factor: 10^{-1}-10^{-7}). One hundred µl of each diluted solution (10^{-3}-10^{-7}) were plated into Starkey agar media with three replications. The bacteria obtained were analyzed for their density using Total Plate Count (TPC) and then purified. The bacteria were stored in Starkey agar media and broth.

16S rRNA gene sequence analysis

Phylogeny of the isolated bacterial strains was analyzed based on 16S rRNA gene sequences. General steps for 16S rRNA gene sequence analysis included DNA extraction, amplification of 16S rRNA gene sequences, purification of amplicons, and cycle sequencing. DNA extraction and PCR amplification were performed according to the manufacturer's protocol (KOD FX Neo, Toyobo). The genomic DNA isolated from SOB isolates were used to amplify the 16S rRNA gene by PCR using universal primers F27 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-TACCTTGTTACGACTT-3' forward and reverse primer, respectively (Amutha and Kokila 2012). The amplified 16S rRNA gene products were sequenced, analyzed, and compared with 16S rRNA gene sequences from the GeneBank database of NCBI (http://blast.ncbi.nlm.nih.gov/). Phylogenetic relationships of the isolated bacterial strains were determined using MEGA 7.0 software (Kumar et al. 2016).

Data analysis

Data obtained were analyzed using descriptive statistics.

RESULTS AND DISCUSSION

Soil properties

The physical and chemical properties of soil samples from three different planting patterns of shallot are shown in Table 1. The highest sulfate (41.31 ppm), organic C (0.957%), organic matter (1.650%) contents, C/N ratio (9.57), and bulk density (1.357 g/cm^3) were obtained in the soils of PP3. Whereas, highest particle density (2.495 g/cm3), cation exchange capacity (CEC) (41.913 me%), and porosity (46.012%) were observed in the soils of PP1. The results indicated that varying planting patterns affect soil properties.

Identification of bacteria

Three SOB isolates were successfully isolated from soil samples collected from different planting patterns of shallot (Figure 1). Isolate A-3245D was isolated from soil samples of PP3; isolate B-3246F was isolated from all planting patterns, and isolate C-3247 was isolated from soil samples of PP2 and PP3. The three isolates were identified as *Burkholderia cepacia* (isolate A-3245D), *Klebsiella variicola* (isolate B-3246F), and *Klebsiella aerogenes* (isolate C-3247C) (Table 2).

Diversity of sulfur-oxidizing bacteria

SOB diversity in different planting patterns of shallot is presented in Figure 2. *K. variicola* was identified as a bacterial species that were able to survive in the soil of three different planting patterns of shallot. The amplification of the 16S rRNA gene for all tested isolates produced fragments at 750 bp (A-3425D), 1000 bp (B-3246F), and 1500 bp (C-3247), respectively (Figure 3). Phylogenetic analysis showed three bacterial strains isolated i.e. A-3245D, B-3246F, and C-3247C were closely related to *Burkholderia cepacia, Klebsiella variicola,* and *Klebsiella aerogenes*, respectively (Figures 4-6).

Table 1. Physical and chemical properties of soil at three different planting patterns of shallot

Parameter	Planting pattern		
	PP1	**PP2**	**PP3**
Organic C	0.859%	0.886 %	0.957 %
Total N	0.132%	0.101 %	0.100%
C/N ratio	6.51	8.77	9.57
Organic matter	1.481 %	1.528 %	1.650 %
pH H_2O	7.49	7.33	7.2
Total P_2O_5	0.182 %	0.067 %	0.062 %
Total K_2O	0.230 %	0.093 %	0.128 %
Total S	138.06 ppm	181.96 ppm	178.74 ppm
Sulfate $(SO_4)^{2-}$	15.67ppm	4.79 ppm	41.31 ppm
Bulk density	1.347 g/cm^3	1.216 g/cm^3	1.357 g/cm^3
Particle density	2.495 g/cm^3	2.637 g/cm^3	2.555 g/cm^3
Porosity	46.012 %	53.887 %	46.888 %
CEC	41.913 me%	50.586 me%	43.089 me%

Note PP1 (Planting Pattern 1): shallot-dry season-shallot-shallot, PP2 (Planting Pattern 2): shallot-dry season-shallot-rice, and PP3 (Planting Pattern 3): shallot-pulses-shallot-rice, CEC: cation exchange capacity

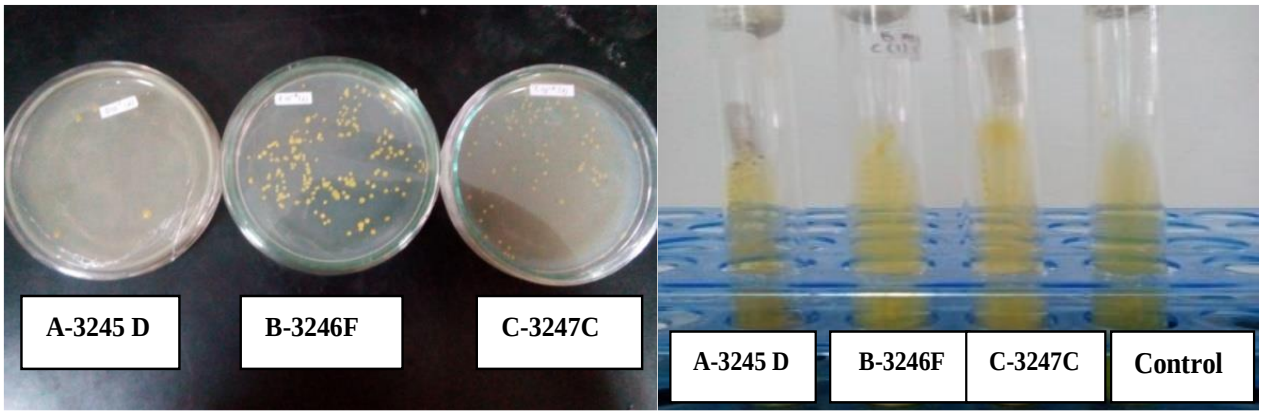

Figure 1. Sulfur-oxidizing bacteria (A-3245D, B-3246F and C-3247C) from soil samples of shallot rhizosphere

Table 2. Sulfur-oxidizing bacteria isolated from shallot rhizosphere

Isolate	Bacterial isolate		
	PP1	PP2	PP3
A-3245D	-	-	*Burkholderia cepacia*
B-3246F	*Klebsiella variicola*	*Klebsiella variicola*	*Klebsiella variicola*
C-3247C	-	*Klebsiella aerogenes*	*Klebsiella aerogenes*

Note: PP1 (Planting Pattern 1): shallot-dry season-shallot-shallot, PP2 (Planting Pattern 2): shallot-dry season-shallot-rice, and PP3 (Planting Pattern 3): shallot-pulses-shallot-rice

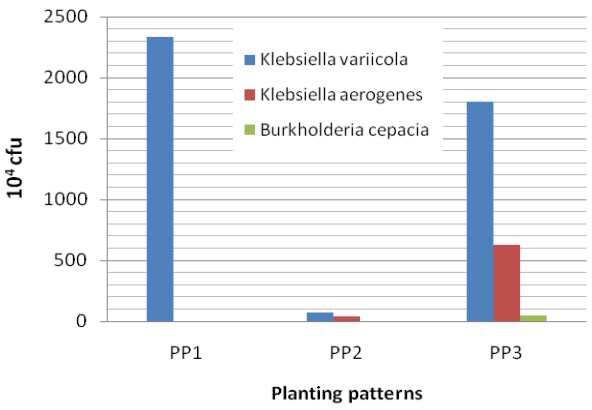

Figure 2. Diversity of Sulfur-oxidizing bacteria in three different planting patterns of shallot

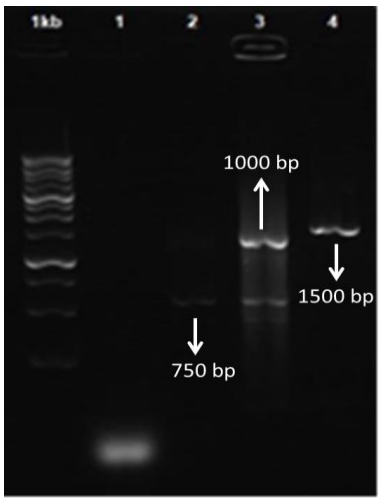

Figure 3. 16S rRNA gene amplification profile of SOB isolates. Lane 1kb: DNA marker, Lane 2: isolate A-3425D (750 bp), Lane 3: isolate B-3246F (1000 bp), Lane 4: C-3247C (1500 bp)

Discussion

The growing bacteria change the color of the growth medium from greenish-yellow to brownish-yellow. The color change from greenish-yellow to brownish-yellow can be used as an indicator that SOB can grow well and signs of oxidation reaction that converts sulfur to sulfate (Puspitasari et al. 2014; Vidyalakshmi and Sridar 2007). The SOB had round, smooth, and yellow colonies (Figure 1).

Soil samples in PP1 have the lowest total S, organic C, and organic matter contents. It might be associated with continuous shallot cultivation throughout the year so that the return of organic material was lower than organic matter from plant residues. After harvesting, the straw and pulses residues are left on the field to decompose and used by plants as sources of organic matter. The long-term addition of crop straw on soil increases organic C content in the soil, which is a key determinant of soil fertility (Li et al. 2015).

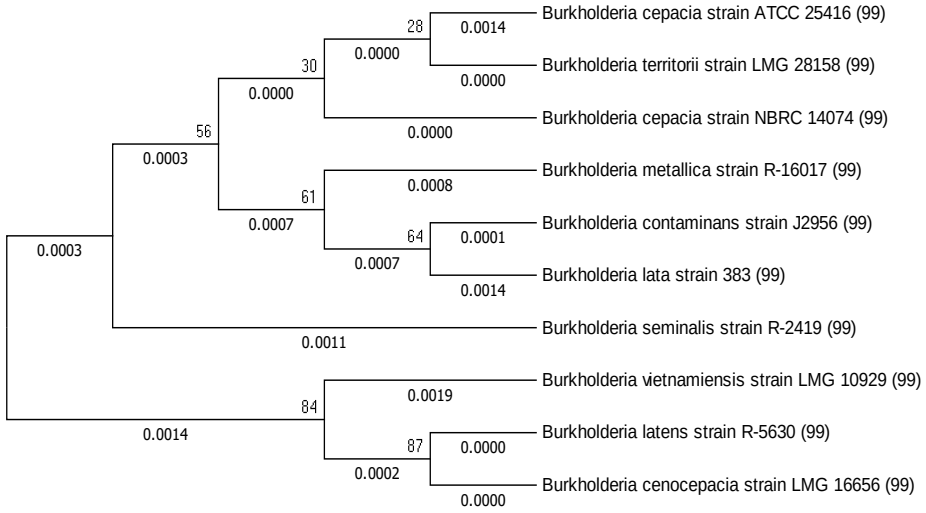

Figure 4. Phylogenetic tree of bacterial isolate A-3245D based on 16S rRNA gene identified **as** *Burkholderia cepacia*

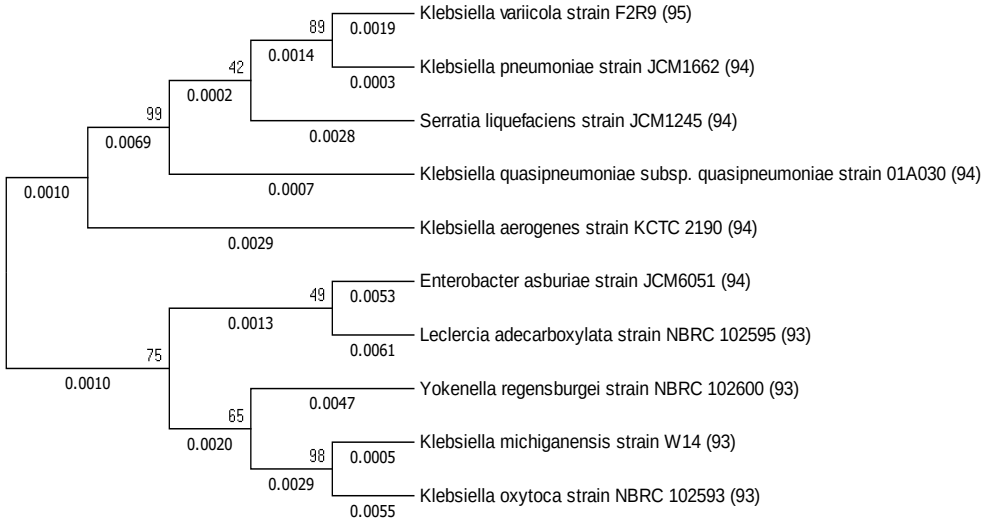

Figure 5. Phylogenetic tree of bacterial isolate B-3246F based on 16S rRNA gene identified **as** *Klebsiella variicola*

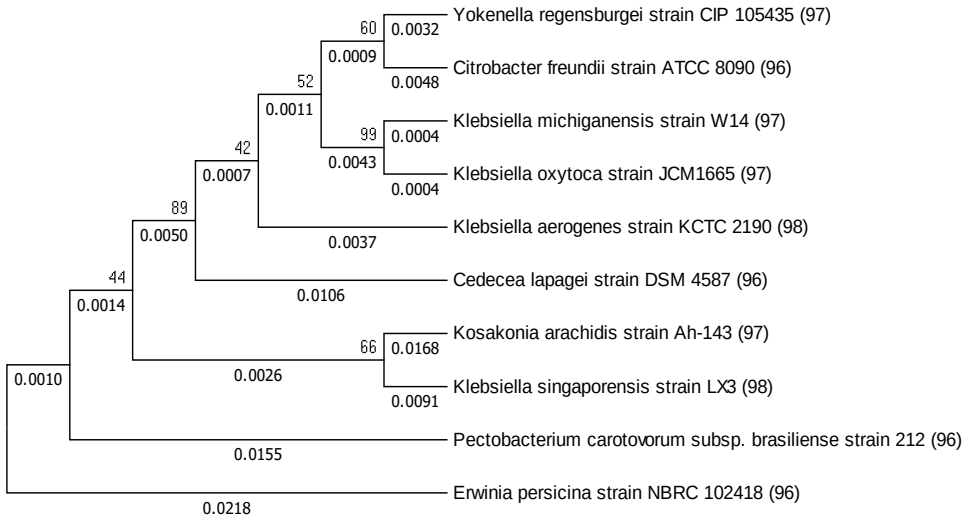

Figure 6. Phylogenetic tree of bacterial isolate C-3247C based on 16S rRNA gene identified **as** *Klebsiella aerogenes*

According to Gaofei et al. (2010), the content of soil organic matter correlates with the organic carbon content in the soil, but increasing soil organic matter content results in a decrease soil pH. The leaf straw of shallot has organic carbon content (35.2%) which is lower than rice straw (36.8%) and soybean straw (51%) (Pandey et al. 2008). Congreves et al. (2017) reported that plant cultivation without a rotation system results in low soil organic carbon content. Liu et al. (2015) reported that planting similar plant species without crop rotation systems may have a negative effect called "secondary soil salinization" due to excessive use of chemical fertilizer which can also influence soil fertility.

It is known that sulfur is an essential nutrient for shallot growth. In PP1, shallot cultivation throughout the year results in a decrease of total sulfur in soil caused by high absorption of sulfur by plant roots. Soil samples in PP1 had the highest pH (7.49) and the lowest CEC (41.913 me%) which may be due to low organic matter. Soil organic matter increases in line with increasing levels of organic C, sulfur, K_2O, CEC, and porosity of the soil, but an increase in soil organic matter decreases soil pH (Lasmini et al. 2015).

Organic matter in the soil undergoes decomposition by soil microorganisms resulting in the formation of a more complex organic matter called humus or humic acid (Schaeffer et al., 2015). The availability of humus increases soil CEC and improves nutrients absorption by the plant roots. Therefore, lower organic C and organic matter contents in PP1 might result in lower CEC. Tambone et al. (2007) reported that the addition of organic matter to the soil can increase cation exchange capacity.

In the present study, three isolates, namely A-3425D, B-3246F, and C-3247C, were successfully isolated from rhizosphere soils from three different planting patterns (Table 2). The genomic DNA of these isolates was subjected to amplification of the 16S rRNA gene (Figure 3). As a result, the amplified fragments obtained from all tested isolates were at 750 bp (A-3425D), 1000 bp (B-3246F), and 1500 bp (C-3247), respectively. Gel electrophoresis showed that the primers used were successfully amplified 16S rRNA gene from the tested samples, although DNA quality varied (Figure 3). It was shown that some samples produced PCR product fragments with a smear on the gel as observed in isolate A-3245D/*Burkholderia cepacia*. The smear of DNA along with the amplified product may be associated with the quality of DNA and possible contamination of the genomic DNA with other products such as protein. However, the PCR products of those samples still work and 16S rRNA sequences were successfully amplified. Based on the phylogenetic analysis, the isolate A-3425D was identified as *Burkholderia cepacia* (99% homologous sequence and 100% query coverage) (Figure 4), the isolate B-3246F was identified as *Klebsiella variicola* (95% homologous sequence 95% and 99% query coverage) (Figure 5), and the isolate C-3247C was identified as *Klebsiella aerogenes* (98% homologous sequence and 99% query coverage) (Figure 6).

Ammonium sulfate $(NH_4)_2SO_4$ and sulfur powder are the most widely used chemical fertilizers by shallot farmers in Brebes Regency, with a dose of 120.77 kg S/ha (Juwanda 2011) that results in a high level of total sulfur in the soil. Moreover, the presence of SOB such as *K. aerogenes, K. variicola,* and *B. cepacia* in rhizosphere soil of shallot can increase sulfate content in the soil. Therefore, SOB can be used as natural agents for reducing the use of chemical fertilizers.

Klebsiella sp. is a bacterial species that can oxidize sulfur to sulfate (Behera et al. 2014). Lin et al. (2015) reported that *K. variicola* has potential as a biofertilizer agent, while *K. aerogenes* can oxidize sulfur to sulfate. Sulfate in the soil is taken up by roots and used for plant growth and development (Mason and Kelly 1988). Adequate sulfur content in the soil affects the levels of N, P, and K taken up by plant roots (Pradhan et al. 2015). According to Behera et al. (2014), *Klebsiella* sp. has a better ability to oxidize sulfur to sulfate than *Bacillus* sp. and plays an important role in the degradation of chlorpyrifos pesticides. The presence of *Klebsiella* sp. is important in the cultivation of shallot with high use of pesticides (Farhan et al. 2013).

Burkholderia sp. is another bacterial species that can oxidize sulfur to sulfate (Sridar et al. 2015). Belonging to the genus *Burkholderia*, *B. cepacia* is very useful for bioremediation of endosulfan-contaminated soil (Kumar et al. 2008) and usually used as a bioinoculant in agriculture (Khambalkar and Sridar 2015). *Klebsiella* and *Burkholderia* can grow well at soil pH of 7 (Lin et al. 2015; Manogaran et al. 2018; Satapute and Kaliwal 2016), while maximum oxidation of sulfur to sulfate by *Klebsiella* sp. reaches on a medium with a pH of 7 (Behera et al. 2016). Based on the results of SOB isolation, it showed that the diversity and population density of bacteria in soil samples of PP3 were higher than those in PP1 and PP2, i.e. *K. aerogenes* ($3.9x10^6$ CFU/ml), *K. variicola* ($1.79x10^7$ CFU/ml) and *B.cepacia* ($7.45x10^5$ CFU/ml). As a result, there is the possibility of higher sulfur oxidation to sulfate in PP3. Soil samples in PP3 had an average pH of 7.2, which approaches neutral pH (7) as optimal pH for bacterial growth, but this pH was lower than other groups (Table 1). Increasing the level of soil sulfate results in decrease soil pH (Gevenez de Souza et al. 2015). The adequate availability of sulfate in the soil can increase the yield and quality of shallot bulbs (Pradhan et al. 2015). Soil samples from PP2 had the lowest sulfate content (4.79 ppm) that might be associated with lower SOB diversity, consequently, there was lower oxidation of sulfur to sulfate (Table 1 and Figure 2). Another study by Pivovarova (2012) showed that increasing soil sulfate results in increasing SOB diversity and decreasing soil pH.

The results of the study showed that soil samples in PP1 had the highest nitrogen content. It might due to the higher population density of *K. variicola* (Figure 2). *K. variicola* can bind nitrogen (Lin et al. 2015), as one of the primary macronutrients required by plants at high concentration. Nitrogen content in the soil affects the absorption of macro-and micro-nutrients (phosphorus, potassium, and sulfur) taken up by the root plants and a determinant for the

production of shallot bulbs (Hilman et al. 2014). Soil samples from PP1 had the highest nitrogen content and soil pH, but the lowest sulfate content (138.06 ppm).

In conclusion, soil properties and SOB diversity depend on the shallot planting patterns. It is recommended that shallots should use PP3 planting patterns to obtain the highest sulfate, organic matter contents, and SOB diversity (*Burkholderia cepacia*, *Klebsiella variicola,* and *Klebsiella aerogenes*) in the soil.

ACKNOWLEDGEMENTS

The authors thank the Ministry of Research, Technology, and Higher Education of Indonesia for providing financial support. The authors also thank the University of Jenderal Soedirman, Purwokerto, Banyumas, Indonesia for providing research facilities.

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