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Isolation and screening of calcareous and non calcareous soil rhizobacteria producing osmoprotectant and indol acetic acid in Gunung Kidul, Yogyakarta, Indonesia

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Abstract

Maryani, Y., Sudadi, Dewi, W. S., & Yunus, A. (2019). Isolation and screening of calcareous and non calcareous soil rhizobacteria producing osmoprotectant and indol acetic acid in Gunung Kidul, Yogyakarta, Indonesia. *Bulgarian Journal of Agricultural Science*, *25*(1), 36–41

Gunung Kidul regency is geographically divided into calcareous and non calcareous soils. Calcareous soil has limitations including mostly hilly terrain, deep hardpan layer, calcareous stone and shallow cultivated layer. Furthermore, it has disadvantageous climate such as long dry season, short rainy season and high temperature. Therefore, this research aimed to isolate rhizobacteria. Sampling of soil was conducted in 6 types of soil on different sites in Gunung Kidul with calcareous and non calcareous soils, during the dry season. Rhizobacteria isolates were tested on media M63 added with 0.3M NaCl. Then, the rhizobacteria isolates were tested on resistance to osmotic stress on media M63 added with NaCl concentrations of 0.3M, 0.5M, 0.7M, 0.9M and 1M. There were 15 isolates obtained having resistance to M63 + 0.3M NaCl. Calcareous isolates having resistance to osmotic stress are from strains A124-k and Ver5-k. Isolate of strain Al24-k can produce glycine betaine of 9.7756 mg/g cell and isolate of Ver5-k can produce glycine betaine of 11.1221 mg/g cell. Non calcareous isolates having resistance to osmotic stress are from strains Ul24-a and Ul16-k. Isolate of strain Ul16-k can produce glycine betaine of 5,6616 mg/g cell and isolate of Ul24-a can produce glycine betaine of 7,9152 mg/g cell. Strain Al24-k isolate form IAA 7.089,3185 ppm/g cell, while strain Ver5-k forms 9.619,9582 ppm/g cell, Ull6-k forms 6.713,4572 ppm/g cell and Ul24-a forms 6.673,0378 ppm/g cell. Calcareous isolates strains A124-k and Ver5-k produce glycine betaine and IAA higher than non calcareous isolates strains Ul16-k and Ul24-a.

Keywords: rhizobacteria; osmotic stress; calcareous soils; glycine betaine; IAA
Abbreviations: ANOVA – analysis of variance;IAA – indol acetic acid; M – molar; NaCl – natrium chloride; PGPR – plant growth promoting rhizobacteria; Sp – species; SAS – statistical analysis system; Trp – triptophan

Introduction

Gunung Kidul regency is a calcareous soil district with limited availability of water supply for humans, animals, plants and microbes. Agricultural land in Gunung Kidul is rainfed agriculture. In the dry season, some parts of the farmland are left fallow due to water shortage but there are plants that can still live. They are cassava, corn, grass and reed.

Gunung Kidul regency is geographycally divided into two areas, namely areas of calcareous soil and non calcareous soil. Calcareous soil has limitations including mostly hilly terrain, deep hardpan layer, calcareous soil, and shallow cultivated layer. In addition, it has disadvantageous climate such as a long dry season, short rainy season and high temperature.

Rhizobacteria are microorganisms in Gunung Kidul regency, which is divided into two geographical features of calcareous soil and non calcareous soil. Therefore, calcareous soil in Gunung Kidul regency has the likelihood of isolating rhizobacteria in order to obtain rhizobacteria that help plants to grow well.

Rhizobacteria are microorganisms that have high survival and can survive in various stress conditions such as hot water, cold water, anaerobe, acid, base, dryness and so on. Therefore, calcareous soil area in Gunung Kidul has the possibility of isolating rhizobacteria in order to obtain rhizobacteria that can help plants to live well in osmotic stress condition. Rhizobacteria that are tolerant of osmotic stress have the capability of forming an osmoprotectant compound. Osmoprotectant compound synthesized by rhizobacteria is glycine betaine. Osmoprotectant rhizobacteria can help other organisms that cannot form glycine betaine by secreting glycine betaine into the environment. It is proved by Hartmann (1988) that *Azospirillum brasilense* absorbs glycine betaine produced by *Azospirillum halopreferens*.

Bacterium is a microbe that has an adaptation system on osmotic stress by osmolite annulation in cytoplasm. Le Rudulier and Bouillard (1983) reported that osmotic stress condition with 0,8M NaCl and 1mM glycine betaine can stimulate the growth of vertisol *E. coli*, *Salmonella typhimurium* and *Klebstella pneumonia*. Hutkins et al. (1987) reported that *Lactobacillus acidophilus* can grow at 1,8M NaCl. *Lactobacillus acidophilus* can accumulate osmoprotectant compound of glycine betaine. Glycine betaine is osmolite synthesized by rhizobacteria to protect it from osmotic stress such as high salt content and dry soil. Colony forming activity in osmotic stress in *E. coli* is indicated by glycine betaine formation (Yuwono et al., 1997).

Rhizobium meliloti, *Ectothiorhodospira halochloris* secrete glycine betaine when there occur media osmolarity decrease. The glycine betaine is only used as osmoprotectant and is regulated by medium osmolarity (Tschichholz and Truper, 1990). Yuwono et al. (2005) showed that isolate A82 can help *gogo* (dry field) paddy grow well at 40% moisture from field capacity. Isolate A82 in stress condition can produce glycine betaine, which is an osmoprotectant compound.

Several bacteria of the genus *Azospiriilum* spp*., Alcaligenes faecalis*, *Klebsiella* sp., *Enterobacter* sp., *Xanthomonas* sp., *Herbaspirillum seropediccae, Rhizobium* spp., *Bacillus* spp. and *Bralyrhizobium* spp. has been listed as Plant Growth Promoting Rhizbacteria (PGPR)(Chagas-Junior et al., 2015). The results obtained by Shohri and Giti (2010) show that some of microorganisms, including bacteria, produce auxin. Some of bacteria in rhizosfere produce Indol Acetic Acid (IAA) and release IAA as secondary metabolism.

The modes of action of PGPR are diverse; therefore, the growth may be favored by them both directly and indirectly. The promotion of growth is direct when the microorganisms facilitate the uptake of certain nutrients in the soil, solubilizing minerals such as phosphorus and making it available to the plant or when they produce phytohormones, such as indole-3-acetic acid (Chagas-Junior et al., 2015).

The auxin, IAA, is the most abundant phytohormone and also can be synthesized by various microorganisms. The IAA can synthesize both from tryptophan (Trp-dependent pathways) and from a Trp precursor but by passing Trp (Trp-indepent pathways). Despite progress in identifying enzymes in Trp-dependent IAA biosynthesis, no singgle IAA biosynthetic pathway is yet defined to the level that all of the relevant genes, enzymes, and intermidiates are indentified (Barrel et al., 2001).

Materials and Methods

Isolation and screening

Sampling of soil was conducted in 6 sites with different soil types from calcareous and non calcareous soils in Gunung Kidul regency, during the dry season. Microbes were resulted from isolation and screening from various soil types from calcareous and non calacareous soil. Soil types in the calcareous soil are alfisol, entisol, inseptisol and vertisol. Soil types in the non calcareous soil are ultisol and oxisol. Rhizobacteria isolates were tested on media M63 added with 0.3M NaCl.

Screening of osmotic stress test

Gathered microbes were grown in media M63 added with 0.3M NaCl and 7 microbial strains were selected. Then, the rhizobacteria isolates were tested on resistance to osmotic stress by growing the rhizobacteria isolates on media M63 added with NaCl at final concentrations of 0.3M, 0.5M, 0.7M, 0.9M and 1M. For resistance test on osmotic stress, two rhizobacteria isolates from calcareous soil and two isolates from non-calcareous soil having high resistance were selected.

Glycine betaine production

The capability of forming glycine betaine compound of isolate calcareous rhizobacteria and non calcareous rhizobacteria is tested. Procedure for determining glycine betaine adopts the periodide method developed by Barak and Tuna (1981) and Nicolaus et al. (1989) including cell production, extraction and glycine betaine determination. Analyses of variance (ANOVA) using statistical analyses system (SAS) were performed on the data (glycine betain) and the treatment means were compared using Duncan's test at $p \leq 0.05$.

Biochemistry tests

The biochemistry tests of rhizobacteria isolates Al24-k, Ver5-k, Ul16-k and Ul24-a show that they are grown pure. The biochemistry tests includes Gram characteristic, Nitrate reductation, Urea hidrolizise, Catalase production, Indol production.

Indole Acetic Acid (IAA) production

Yeast malt broth medium with tryptophan suplement was used on basis of IAA production and it was centrifuged. The supernatant was collected and mixed with ethyl acetate (1:2). Extract was evaporated and then it was dissolved in 5 ml metanol absolute, IAA was extract within solvent layer and repeated 3 to 4 times (Mohite, 2013). The extract sample and standart IAA were spotted on high-performance lyquid chromatography. Analyses of variance (ANOVA) using statistical analyses system (SAS) were performed on the data (IAA) and the treatment means were compared using Duncan's test at $p \leq 0.05$.

Results and Discussion

We obtained fifteen isolates having resistance to M63 + 0.3M NaCl from calcareous and non calcareous soil. There were eight isolates from calcareous soil and seven isolates from non calcareous soil.

The calcareous rhizobacteria isolates are Ver5-k and Al24-k strains. The non calcareous rhizobacteria isolates are Ul16-k and Ul24-a strains. Rhizobacteria isolates then were tested on the capability of forming glycine betaine. The results correspond to those obtained from Sharma et al. (2013) who conducted isolation and screening of the desert area in Rajasthan, India. Desert is an area with high osmotic stress condition. Isolation and screening in Rajasthan desert resulted in 24 rhizobacteria isolates that were tolerant of osmotic stress: *Bacillus* sp. (45.8%), *Corynebacterium* sp. (41.7%) and *Acinotobacter* sp., *Aeromonas* sp. and *Staphylococus* sp. group (2.5%). The research is the first report to describe cacti-associated bacteria from Brazillian semi-arid. From a total of forty eight bacteria, 65% were able to growth in medium with reduced water availilability, exopolysaccharida ride production was observed for 65% of strains (Kavamura et al., 2013).

Calcareous and non calcareous rhizobacteria have the capability of forming osmoprotectant compound in the form of glycine betaine. The capability reflects the rhizobacterial activity. Rhizobacterial activity in forming glycine betaine is presented in Fig. 1. Calcareous and non calcareous isolates rhizobacteria from strains Al24-k, Ver5-k, Ul16-k and Ul24-a have the capability of forming osmoprotectant compound in the form of glycine betaine. The capability of producing glycine betaine of strains Al24-k and Ver5-k is higher than that of strains Ul16-k and Ul44-k. Strain Al24-k isolate forms glycine betaine of 9.9251 mg g^{-1} cell, while strain Ver5-k forms 11.1218 mg g^{-1} cell, Ull6-k forms 5.6616 mg g^{-1} cell and Ul24- forms 7.9152 mg g^{-1} cell.

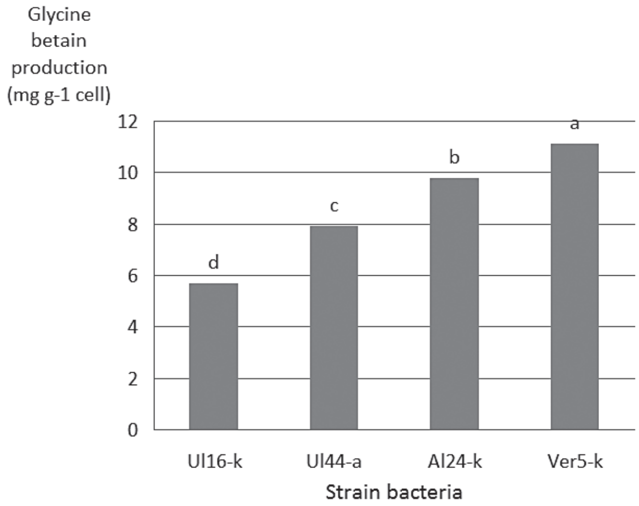

Fig. 1. Rhizobacterial activity in forming glycine betaine

Rhizobacteria isolates of strains Al24-k and Ver5-k are rhizobacteria found in calcareous soil. Rhizobacteria isolates of strains Ul24-a and Ul16-k are rhizobacteria found in non calcareous soil. Rhizobacteria isolates have the capability of being tolerant of high osmotic stress. Rhizobacteria are capable of adapting to high osmotic stress because they have the capability to form osmoprotectant compound. Rhizobacteria isolate of strains Al24-k, Ver5-k, Ul24-a and Ul16-k also have the capability of forming osmoprotectant compound of glycine betaine (Fig. 1). *Escherichia coli*, *Salmonella typimurin* and *Klebsiella pneumonia* are known to be capable of synthesizing glycine betaine (Le Rudulier and Bouillard, 1983, Landfald and Strom, 1986; Smith et al., 1988). Glycine betaine is one of the important osmoprotectants, accumulated by various organisms in osmotic stress condition (Perroud and Le Rudulier, 1985; Landfald and Strom, 1986). Hutkins et al. (1987) reported that *Lactobacillus acidophilus* can grow in 1.8M Nacl medium and osmoprotectant compound of glycine betaine is accumulated in their cell. The results accord with Gouffi and Carlos (2000). *Sinorhizobium meliloti* synthesize osmoprotectant of Glutamate and N-acetyl Glutaminyl Glutamine Amide. However, in this research the rhizobacteria isolates obtained have the capability of synthesizing glycine betaine.

This research corresponding to results obtained by Ohnishi and Murata (2006) in which *Cyanobacterium syncchoccus* PCC 7942 have the capability to be tolerant on salt stress and low temperature stress. The rhizobacteria have capability of synthesizing glycine betaine and choline. Klähn and Hagemann (2011) suggest that *Cyanorhizobacteria* have the capability of synthesizing glycine betaine and have tolerance for salt stress.

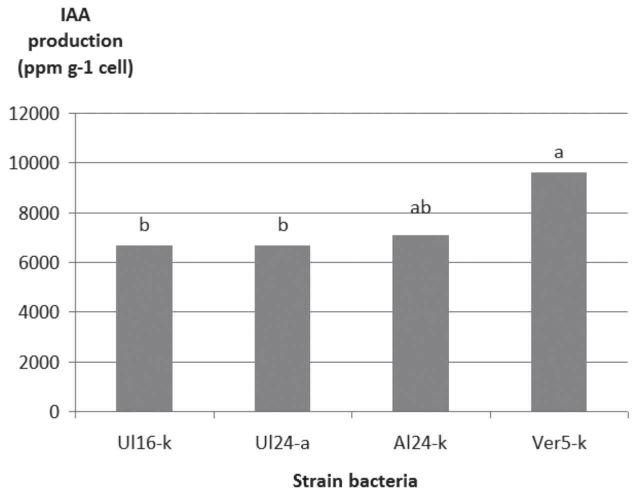

Fig. 2. Rhizobacterial activity in forming IAA

Calcareous rhizobacteria isolates have higher capability of producing glycine betaine than non calcareous rhizobacteria (Fig. 1). This indicates that calcareous rhizobacteria have better tolerance for osmotic stress than non calcareous rhizobacteria.

Calcareous and non calcareous rhizobacteria have the capability of forming IAA. The capability reflects the rhizobacterial activity. Rhizobacterial activity in forming IAA is presented in Fig. 2. Calcareous rhizobacteria and non calcareous isolates from strains Al24-k, Ver5-k, Ul16-k and Ul24-a have the capability of forming IAA.

Fig. 2 rhizobacteria isolates from calcareous soil Al24-k, Ver5-k and from non calcareous soil Ul16-k, Ul 24-a have the capability of forming growth hormone in the form of IAA. The capability of producing IAA of strains Al24-k and Ver5-k is higher than that of strains Ul16-k and Ul44-k. Isolate of strain Al24-k can produce IAA of 7.089,3185 ppm g^{-1} cell and isolate of Ver5-k can produce IAA of 9.619,9582 ppm g^{-1} cell. Isolate of strain Ul16-k can produce IAA of 6.713,4572 ppm g^{-1} cell and isolate of Ul24-a can produce IAA of 6.673,0378 ppm g^{-1} cell.

Table 1 indicated that isolation and screening of rhizobacteria from calcareous soil resulted in obtaining 15 isolates, which have resistance to medium M63+0.3M NaCl. The tolerance test on osmotic stress of 15 rhizobacteria isolates resulted in two isolates of calcareous and two isolates f calcareous rhizobacteria that have high osmotic stress tolerance.

Rhizobacteria isolates don’t have to capable catalase production and indol production (Table 2). This is indicate that the production IAA of rhizobacteria isolates is not direct. But, rhizobacteria isolates can produce phy-

Table 1. Tolerance of rhizobacteria isolate on agar M 63 medium at various NaCl concentrations

Soil	Microbia		Response				
	Strain	Type	0,3M	0,5M	0,7M	0,9M	1,0M
Calcareous	En2-a	bacteria	+++	++	+	+	–
	En4-j	actinomicetes	+++	++	+	–	–
	Al24-k	bacteria	+++	+++	++	+	+
	Ver5-k	bacteria	+++	+++	+++	+	–
	Ver41-k	bacteria	+++	++	–	–	–
	Ins11-a	actinomicetes	+++	++	+	–	–
	Ins27-k	bacteria	+++	++	+	–	–
	Ins36-j	bacteria	+++	+	–	–	–
Non calcareous	Ul16-k	bacteria	+++	+++	+	+	–
	Ul20-p	bacteria	+++	++	+	–	–
	Ul22-j	actinomicetes	+++	++	–	–	–
	Ul24-a	bacteria	+++	+++	++	+	+
	Ox2-j	actinomicetes	+++	+	–	–	–
	Ox9-j	bacteria	+++	++	+	–	–
	Ox68-a	actinomicetes	+++	++	+	+	–

Table 2. Biochemistry characteristic of rhizobacteria isolates from calcareous and non calcareous soil

No	Biochemistry characteristic	Calcareous soil		Non calcareous soil	
		Al24-k	Ver5-k	Ul16-k	Ul24-a
1.	Gram characteristic	Negative	Negative	Negative	Negative
2.	Nitrate reductation	+	++	++	+
3.	Urea hidrolizise	–	–	–	–
4.	Catalase production	+	+	+	+
5.	Indol production	–	–	–	–

tohormones, such as IAA, indirect. Isolate rhizobacteria strains Al 24-k, Ver5-k, Ul16-k and Ul24-a synthesized IAA and they need to suplement tryptophan. The modes of action of PGPR are diverse; therefore, the growth may be favored by them both directly and indirectly (Chagas Junior et al., 2015). The auxin, IAA, is the most abundant phytohormone and also can be synthesized by various microorganisms. The IAA can synthesize from tryptophan (Trp-dependent pathways) (Barrel et al., 2001).

According to Ikhwan (2011) rhizobacteria strains ICDT-IM are capable to produce IAA extracellular. Etesami et al. (2009) reported that rhizobacteria isolates from Iranian soil are capable to produce IAA. According to Idris et al. (2007) *Bacillus amyaquefaciens* needs tryptophan to produce and exude IAA. Egamberdieva (2008) reported that *Bacillus* sp. strain 41/1, *Cellulomonas* sp. strain 32 and bacteria strain In-7 can produce IAA. According to Kumar et al. (2014) *Azotobacter* sp. produce IAA 95,60-175,20 µg/ml higher than *Pseudomonas* sp. (IAA 44,40-95,60 µg/ml). According to Lestari et al. (2007) *Azospirillum* sp. strains AZ15 and AZ44 are capable to produce IAA (57,93 µg/ml at 12 days and 40,42 µg/ml at 7 days). Wedastri (2002) reported that *Azotobacter* isolates produce growth hormone (*Azotobacter* sp. 15b and *Azotobacter* sp. 23b) and nitrogen fixation (*Azotobacter* sp. 1a).

Conclusions

Isolates from calcareous soils having resistance to osmotic stress are from strains A124-k and Ver5-k, isolate of strain Al24-k can produce glycine betaine of 9.7756 mg g^{-1} cell and isolate of Ver5-k can produce glycine betaine of 11.1221 mg g^{-1} cell.

Isolates from non calcareous soils having resistance to osmotic stress are from strains Ul16-k and Ul24-a, isolate of strain Ul16-k can produce glycine betaine of 5, 6616 mg g^{-1} cell and isolate of Ul24-a can produce glycine betaine of 7,9152 mg g^{-1} cell.

Strain Al24-k isolate forms IAA 7.089,3185 ppm g^{-1} cell, while strain Ver5-k forms 9.619,9582 ppm g^{-1} cell, Ull6-k forms 6.713,4572 ppm g^{-1} cell and Ul24-a forms 6.673,0378 ppm g^{-1} cell.

Isolates from calcareous strains A124-k, Ver5-k produce glicine betain and IAA higher than isolates form non calcareous strain Ul16-k and Ul24-a.

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