

Isolation and Selection of *Rhizobium* Tolerant to Pesticides and Aluminum from Acid Soils in Indonesia

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ABSTRACT

Application of *Rhizobium* as inoculum in acid soil requires specific characters, namely high tolerance to pesticide residues, soil acidity, and high concentration of Aluminum. This study was conducted to isolate *Rhizobium* having these characters. In spite of acid soils from Kalimantan, Sumatra, Sulawesi and Java; root nodules of legumes planted in those regions were used as source of isolates. Rhizobial isolation was done using direct isolation and enrichment technique. A paper disc diffusion technique was used in selecting tolerance to pesticides. The selected isolates were examined the tolerance to pH, Al, and ability to form root nodule with soybean. From soil analysis, it could be seen the correlation between pH value and Al concentration. It means that the lower pH value the higher Al concentration. The number of *Rhizobium* isolates and its tolerance to paraquat was depended on soil type. From 173 strains of isolated *Rhizobium*, 24 strains were tolerance to pesticides and Aluminum. They were able to grow in wide range of pH, namely 3 – 8, or some of them in 5 - 8. Around 92% of the selected bacteria could form root nodules with soybean plant in different number and size. Hopefully, these isolates can be applied in the pesticide polluted agricultural lands, especially in acid soils with high concentration of Al, and it can also increase soybean production.

Keywords: Acid soils, aluminium, *Rhizobium*, pesticides

INTRODUCTION

Rhizobium is important in nitrogen fixation and induction of resistance on pest and phyto-pathogen (Karban and Kue 1999). In Indonesia *Rhizobium* inoculation has been done long ago, but the result was not maximal, causing soybean import increases every year. The failure of inoculation might be caused by low survival of *Rhizobium* in environments, in which soil acidity and toxic materials, such as Aluminum and pesticide residue, limited their growth and activity (Foth 1990; Paudyal *et al.* 2007).

Application of pesticides in agricultural land for years caused accumulation of their residues. This residue is a general limiting factor to the failure of *Rhizobium* inoculation. According to Naylor (1996), herbicide is the most frequent pesticide applied in agricultural lands, followed by many kinds of insecticides. Herbicides with paraquat active agent are applied routinely in Kalimantan and Sumatra's

peat lands since more than 10 years. Unfortunately, this chemical is stable in acid solution; and in routine application, it was accumulated in peat soil (Martani *et al.* 2002). In spite of these herbicides, carbamate insecticides is also applied routinely since 1980's in agricultural lands. Therefore, this active agent might be accumulated in soils. Recently, another insecticide with deltamethrin active agent is widely applied to substitute the application of banned DDT.

Agricultural land expansion was done especially in acid soils located in Kalimantan and Sumatra. However, there are environmental constraints should be faced, *i.e.*, soil acidity, Aluminum toxicity and mineral deficiency. The low pH in acid soil limited microbial diversity and activities (Margino *et al.* 2007; Martani 2005). As neutrophilic bacteria, *Rhizobium* can not grow optimally in acid soils. Therefore it may cause the failure of *Rhizobium* to function (Hartel and Bouton 1991). Fortunately, some of these bacteria have broad range of pH values for their growth, and it is a benefit character if they are introduced in acid soils.

Aluminum is a normal soil component bound to organic substances or silicate clay, and in low concentration it is required for plant growth. However, in acid soils Al is released and cause instability and toxicity of this metal. Aluminum toxicity is detected in histosol due to organic decomposition that release Al into soil solution. In this solution, Al^{3+} is higher than 1 mg kg^{-1} and cause thickness and hardness of root epidermis, inhibit cell metabolism, and destroys root system (Rout *et al.* 2001). These conditions may decrease excretion of root substances which are important in early steps of nodulation process and may inhibit *Rhizobium* penetration into root tissues (Foth 1990; Keyser *et al.* 1993; Hanum *et al.* 2007). Aluminum at 0.5ppm is also toxic to *Rhizobium* and delays the first step of root nodulation (Richardson *et al.* 1988). Aluminum is also affected the cell size and intracellular water content of *Arthrobacter* sp. PI/I-95, which then inhibit the growth of this bacteria (Illmer and Erlebach 2008).

For a development and successful inoculation, it requires *Rhizobium* strains with high survival and function in many environmental constrains, including pesticides residue, soil acidity, and high concentration of Al. In this study, *Rhizobium* sp. was isolated from several Indonesian acid soils. Following some examinations, it were selected some strains with high tolerance to pesticides, Aluminum, and pH values. Hopefully, they can be used as *Rhizobium* inoculum with specific benefits and can be also successful in many environmental soil conditions.

MATERIALS AND METHODS

Pesticide Chemicals

In this study, a Gramoxone herbicide (Zeneca Ltd. with $200 \text{ mg paraquat ion L}^{-1}$) was used as paraquat source. Two kinds of insecticides were also used as Carbofuran (with active agent Carbamate) and Decis (Deltamethrine with active agent). These pesticides were used as representing widely applied agricultural chemicals.

Acid Soil Samples

The sample of acid soils used as source of *Rhizobium* isolate were (1) Ultisol from South East Sulawesi, Lampung, and West Java; (2) Acid Sulfate Soil from South Kalimantan; and (3) Peat Soils from Central Kalimantan, South Kalimantan, Riau and Central Java. In spite of acid soils, soybean root nodules planted in some acid soils were also used as *Rhizobium* sources.

Rhizobium Isolation

Soil samples were suspended in sterilized distilled-water, diluted, and were surface plated on Yeast-Extract Mannitol Agar (YMA) added with 1% congo-red. After 2 days, colorless and slimy colonies of *Rhizobium* were isolated and grown in the same medium. If there was no *Rhizobium* colony found by direct isolation, enrichment technique was used by growing the soil sample in broth medium and then surface plated in YMA (Yanti *et al.* 2003).

Selection of *Rhizobium* Isolates

The isolates were examined their tolerance to three kinds of pesticides (paraquat, deltamethrine, and carbofuran) and Aluminum, and also the optimal pH for the rhizobial growth. Three Aluminum sulfate [$\{\text{Al}(\text{SO}_4)_3\}$] was used in Al tolerance selection. Tolerance to pesticides and Al were conducted using a *paper disc diffusion technique*. Growth inhibition was shown by the appearance of clear zone around the *paper disc* after 2-3 day incubation time. The range of pH value for growth was detected based on turbidity of the Yeast Extract Mannitol Broth medium. Bacterial growth was indicated by the maximum optical density at 525 nm.

The ability to form symbiotic association with legume of selected *Rhizobium* strains were examined.

Symbiotic Association with Legume Plant

Successful symbiotic association between *Rhizobium* and soybean plant was indicated by effective nodule formation. Selected *Rhizobium* was inoculated to the soybean (3-4 days) planted on sterilized sand medium. During planting period of 3 weeks, the plant was watering by mineral broth medium (Subba Rao 1994). The numbers, size, and distribution of nodule, as well as the color of inner nodules were used as parameters for selecting the best *Rhizobium* isolates.

RESULTS AND DISCUSSION

Soil Samples Analysis

The results of soil samples analysis are shown in Table 1. There was a correlation between soil acidity and Aluminum concentration in which soil with lower pH value has higher concentration of Al. In acid soils, Al was released from clay minerals and organic substances forming soluble Al, which was hydrolyzed and resulted Al^{3+} and ionic H^+ (Foth 1990). Therefore, exchangeable Al could be

Table 1. Characters of acid soil samples.

No	Soil type	Soil Code	Location	pH value	Al ¹ (mg kg ⁻¹)	Organic C (%)
1	Acid SO ₄ soil	ASS	Banjar, South Kalimantan	2.4-3.0	2.246	8.40
2	Saphric peat soil	SPS-1	Pangkoh, Central Kalimantan	3.7	0.297	63.00
3	Saphric peat soil	SPS-2	South Kalimantan	3.0-3.5	0.07	NA ³
4	Saphric peat soil	SPS-3	Riau Province	5.2	0.36	NA ³
5	Hemic peat soil	HPS-1	Pangkoh, Central Kalimantan	3.7	0.165	67.49
6	Hemic peat soil	HPS-2	Riau Province	5.1	0.44	NA ³
7	Fibric peat soil	FPS-1	Pangkoh, Central Kalimantan	4.0	0.067	68.53
8	Fibric peat soil	FPS-2	Riau Province	3.6	0.52	NA ³
9	Peat soil	PS	Rawa Pening, Central Java	4.0-5.0	ND ²	NA ³
10	Latosol	Lat	Gunung Kidul, Yogyakarta	5.2-5.8	ND ²	NA ³
11	Ultisol	RYP-1	Kendari, South East Sulawesi	4.3-4.6	0.028	NA ³
12	Ultisol	RYP-2	Jasinga, West Java	6.2	ND ²⁾	NA ³
13	Ultisol	RYP-3	Jasinga, West Java	4.3	0.13	NA ³
14	Ultisol	RYP-4	Pekalongan, Lampung	5.0	0.03	NA ³
15	Ultisol	RYP-5	Tigeneneng, Lampung	4.5	0.03	NA ³

Notes: ¹Exchangeable aluminum; ²ND = Not detected; ³NA = Not analyzed.

detected in soils with pH value below 5, and no Al was detected in RYP-2 soil with pH value above 6 (Table 1). Aluminum was released from soil particles if soil acidity was below 5, and in pH 5.5 this metal would be in particulate form. In this case, it would not be toxic to plant and microorganisms. Maximal detected exchangeable Al concentration was 2.246 mg kg⁻¹, which was found in Kalimantan's acid sulfate soil (ASS). In this study the Al concentration used for examining its tolerance was 3 mg kg⁻¹.

Organic carbon concentration in soil was depended on the soil type. The Peat soils contained organic Carbon higher than 60%, while the ASS and RYP contained only 8.4 and 14.4%, respectively. The organic Carbon in soil is important because paraquat herbicide which has two positive charges will be adsorbed by organic substances and/or clay minerals. In this condition, paraquat will be persistence and non-biodegradable. Persistence of paraquat in peat soil will be higher due to the soil acidity, and could be accumulated in repeated and routine application of the herbicide (Martani *et al.* 2002).

It is hope that from these acid soil samples many strains of *Rhizobium* which were able to grow in acid condition and tolerant to Aluminum toxicity could be isolated .

Isolation of *Rhizobium* sp. from Soils and Root Nodules

Rhizobium was isolated using direct isolation and enrichment culture techniques. The second

method was conducted if direct isolation was fail to get a *Rhizobium* isolate. The failure of direct isolation might be due to very low soil pH that limited the survival of *Rhizobium*. In this study, direct isolation from Kendari's RYP (1) was fail; suggesting that soil acidity (Table 1) and type of soil (forest soil, not agriculture) were responsible to the failure. Enrichment technique was applied to this soil by addition of paraquat into YM broth medium (Yanti *et al.* 2003) and also by planting with soybean for a month; hoping those conditions

Table 2. *Rhizobium* isolated from acid soils.

No	Soil Type	Number of sub-sample	Number of isolates
1	ASS	7	69
2	SPS-1	1	1
3	SPS-2	1	3
4	SPS-3	1	1
5	HPS-1	1	2
6	HPS-2	1	2
7	FPS-1	1	2
8	FPS-2	1	0
9	PS	1	5
10	Latosol	1	6
11	RYP - 1	2	19
12	RYP - 2	1	4
13	RYP - 3	2	9
14	RYP - 4	1	4
15	RYP - 5	1	5
Total <i>Rhizobium</i> isolates			132

would induce survival and growth of the intended bacteria. After incubated for several weeks, some rhizobial colonies were grow on YMA-CR medium. The results were shown in Table 2.

The highest number of strain was isolated from Kalimantan's ASS, and the lowest were from PS (Table 2). Kalimantan's ASS was agricultural land planted with soybean or rice; and the PS sample was usually planted with corn. Soil treatments and kind of crops usually planted affected the number and characteristics of *Rhizobium* isolated from those soils. In case of RYP (2) of West Java, although the soils were from field and garden, some rhizobial strains could be isolated.

Although in general *Rhizobium* grew optimally in neutral pH, some of them able to grow in low pH, and have a wide pH range (Long 2007). Martani et al. (2001) succeeded to isolate some *Rhizobium* from root nodules of soybean planted in peat soil of Kalimantan. They usually known as "acid soil *Rhizobium*" (Appunu and Dhar 2006) These results show a good chance to get specific *Rhizobium* inoculum for acid soils which widely spread in Indonesia, especially in Sumatra and Kalimantan.

In-spite of soil isolation, *Rhizobium* bacteria were also isolated from legume's root nodules planted in the acid soils. At the sampling time, in some locations, soybean and/or ground nut were planted; and some of these crops were able to form nodules. Several *Rhizobium* strains could be isolated from these nodules (Table 3).

More than 40 strains of *Rhizobium* could be isolated from root nodules (Table 3). Keyser et al. (1993) and Subba Rao (1994) reported that some strains of *Rhizobium* in rhyzosphere which match in symbiotic association with legume species planted in this area will infected root hair and enter some microbiological and biochemical phases in root. At final phase, some of these bacteria will grow faster resulted in the formation of root nodules.

Table 3. *Rhizobium* isolated from root nodules.

No.	Legume sample	Sample location	Number of isolates
1	Soybean	Central Kalimantan	9
2	Soybean	South Kalimantan	11
3	Soybean	South East Sulawesi	4
4	Soybean	Lampung	15
5	Ground nut	Lampung	2
Total <i>Rhizobium</i> isolates			41

Here, the effective *Rhizobium* fixed air nitrogen and formed ammonium as nitrogen source for the legume plant. Hopefully, in this study some of the *Rhizobium* isolated from these nodules and the above acid soils are effective strains in nitrogen fixation and also adapted with acid soil environment containing high concentration of aluminum and accumulated pesticides residues.

Tolerance of *Rhizobium* Isolates on Pesticide and Aluminum

Paper disc diffusion technique was done because it could show maximum tolerance of rhizobial strain to specific concentration of pesticides or Aluminum based on the formation of growth inhibition zone. The data are shown in Table 4.

Soil type affected tolerance of *Rhizobium* on paraquat herbicide (Table 4). Generally, the higher concentration of paraquat has higher toxicity to the bacteria. Therefore, the number of isolates tolerant to paraquat decreased when paraquat concentration increased. About 6% of isolates were tolerant to 1000mg.kg⁻¹ of paraquat. Enrichment technique for RYP-1 soil resulted in the higher numbers of *Rhizobium* tolerant to paraquat compared to direct isolation. The possibility of intended bacteria to grow was higher in enrichment culture with specific conditions (Yanti et al. 2003). Conversely, there was no rhizobial strain isolated from RYP – 2 tolerant to paraquat even at 20 mg kg⁻¹.

These results were supported by Katayama and Kuwatsuka (1992) who reported that there were some species of soil microorganisms tolerant to paraquat until 1,000 mg kg⁻¹. Yanti et al. (2003) also isolated paraquat degrading *Micrococcus* sp S-2 and *Achromobacter* sp. SM-1, with high tolerance to this chemical. These two bacteria were isolated from Kalimantan peat soil and acid sulphate soil, respectively. The tolerance of some bacteria to paraquat is due to their ability to synthesize paraquat neutralizing enzymes, namely catalase and superoxide dismutase (Margino et al. 2007). As widely known that photolysis of paraquat resulted in some toxic radicals, such as super-oxides (O₂⁻) and hydrogen peroxide. These free radicals will induce lipid, depolymerize polysaccharide and degrade protein in cell (Donely and Robinson 1991).

There are some specific characters that have to be shown by rhizobial strains to success to be applied on many soil types, that is high survivability and activity where ever they introduced. These selected strains should be able in adaptation to

Table 4. Tolerance of *Rhizobium* isolates to pesticides, aluminum and soil pH range.

No	Code of isolate	Maximum tolerance to pesticide (mg kg ⁻¹)			Tolerance to Al (mg kg ⁻¹)	Soil pH range
		Paraquat	Carbofuran	Deltamethrin		
1	PMKP 1.2	2,000	2,000	15,000	0.1	3 - 8
2	PMKH 1.2	100	4,000	15,000	3.0	3 - 8
3	PMKP 2.2	> 2,000	1,000	4,000	1.5	4 - 8
4	PMKP 5.2	> 2,000	1,000	5,000	2.5	4 - 8
5	TRP 1-3	300	5,000	1,000	0.1	5 - 8
6	TP 2-3	300	5,000	1,000	0.5	5 - 8
7	TL 5	300	5,000	1,000	1.0	5 - 8
8	SJ-6	750	1,000	750	--	5 - 7
9	SJ-11	750	5,000	750	--	5 - 8
10	TSMK-2	500	1,000	1,000	2.0	4 - 8
11	TSMK-3	750	1,000	1,000	1.0	4 - 8
12	TSMK-4	500	500	1,000	1.0	4 - 8
13	AnSM-3	500	1,000	1,000	--	5 - 8
14	AnSM-6	300	1,000	750	1.5	4 - 7
15	AnSM-8	300	1,000	1,000	2.5	3 - 7
16	TnLmKp-1	750	5,000	1,000	1.5	3 - 8
17	TnLmKp-2.1	1,000	1,000	2,000	1.5	4 - 8
18	TnLmKp-2.2	1,000	1,000	300	0.1	3 - 8
19	PnKb-2	1,000	5,000	1,000	0.5	5 - 8
20	Hemik-2	500	1,000	1,000	0.5	5 - 7
21	SR-1	300	5,000	2,000	--	5 - 8
22	SK-2	300	500	500	1.5	3 - 8
23	IK-2	750	100	1,000	2.5	3 - 8
24	W-2	300	1,000	750	0.5	4 - 8

limiting factors found in natural environments. In this study some specific characters were analyzed, namely the tolerance to soil acidity, high Aluminum concentration, and pesticides residue. Table 4 shows the rhizobial isolates and their benefit characteristics.

The paraquat application of 1,000 mg kg⁻¹ is much higher than dosage application of 20 mg kg⁻¹. This high concentration is used to anticipate if there is paraquat accumulation in soil due to stability of this chemical in acid soil (Martani *et al.* 2003). Carbamate insecticide that has been applied for many years also might be accumulated in some soils. In case of deltamethrin, this insecticide is applied very recently, especially in developed countries to substitute the banded DDT (Xia *et al.* 2004). Therefore, this new insecticide has not been accumulated in soil, but might be widely used in Indonesia in the coming few years.

These selected strains could grow in wide range of pH values (4 – 8) (Table 4). The pH value of soils in Indonesia usually is ranged from 4 (for acid soils, such as acid sulphate soil, peat soil and ultisols) to 7.3 (for some relatively high pH, *i.e.* vertisol). Therefore, the soil pH range from 3 – 8 is

save enough to guarantee the success of *Rhizobium* application in acid soils and also in neutral soils. These strains usually known as “acid soil *Rhizobium*” or “acidotolerant bacteria” (Appunu and Dhar 2006; Long 2007), namely neutrophilic bacteria but still able grow in relatively low pH value.

Although the Aluminum toxicity mechanism toward *Rhizobium* is not clarified yet, Illmer and Erlebach (2008) found that Al affected the cell size and intracellular water content of *Arthrobacter* sp. PI/I-95, and inhibit the growth of this bacteria. The tolerance of these selected *Rhizobium* are higher than 1 mg kg⁻¹ of Aluminum. It is good enough since the concentration of Al in some Indonesian acid soils is below 1 mg kg⁻¹ except the acid sulphate from Kalimantan, that reach 2.246 mg kg⁻¹ (Table 1).

Rhizobial inoculum that could be survived and was able to make adaptation on some environmental limiting factors were found in many soil types. Due to benefit characteristics of these *Rhizobium* strains isolated from this study (Table 4), it can be hoped that application of this new inoculum can be a solution for probability of the failure of inoculation,

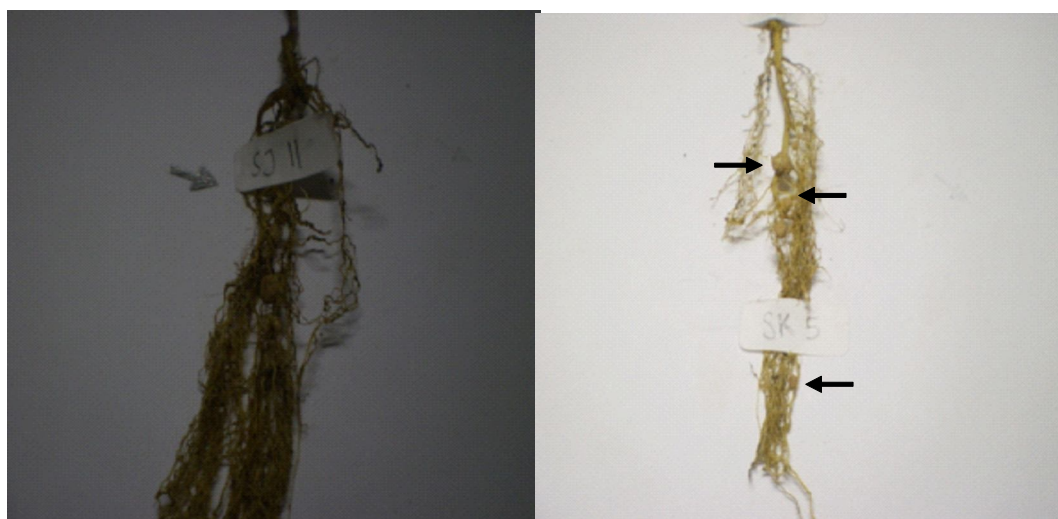


Figure 1. Soybean root containing nodules.

Table 5. Characteristic of root nodules.

No.	Isolate	Number	Diameter of Root nodules (mm)	Distribution
1	PMKP 1.2	7	2-5	main root
2	PMKP 5.2	5	1-2	distributed
3	TRP 1-3	20	0.3-2	main root
4	TP 2-3	13	0.5-5	main root
5	SJ-6	25	0.5-3	distributed
6	SJ-11	16	0.5-5	distributed
7	TSMK-2	20	1-4	main root
8	TSMK-3	18	1-4	main root
9	TSMK-4	14	2-5	distributed
10	AnSM-3	16	1-5	main root
11	AnSM-6	17	1-3	distributed
12	AnSM-8	16	2-5	main root
13	TnLmKp-1	17	1-5	distributed
14	TnLmKp-2.1	15	1-5	main root
15	TnLmKp-2.2	17	1-5	distributed
16	PnKb-2	14	1-5	main root
17	Hemik-2	27	2-5	main root
18	SR-1	18	2-4	main root
19	SK-2	16	1-4	distributed
20	IK-2	16	1-4	distributed
21	W-2	14	1-4	main root

especially if it is applied in acid soils. The 24 isolates are very potential, because they can be used as *Rhizobium* inoculum which have some benefits compared to other usual *Rhizobium* inoculum which were widely found in the market for legumes. The benefits of these *Rhizobium* strains are: (1) high tolerance to some kinds of pesticides (paraquat herbicide, deltamethrin and carbamate insecticides);

(2) have wide range of soil pH value for their growth (pH 4 to 8); and (3) high tolerance to Aluminum.

In spite of the specific benefit adaptation to ecological limiting factors, the ability to form symbiotic association with legume is necessary. Therefore, these 24 rhizobial strains were examined their ability to form effective root nodules with soybean plant.

Formation of Root Nodule by the Selected *Rhizobium*

Sand medium was used to substitute liquid medium because of the better air circulation. Additionally, the sand was chosen based on consideration that plant harvesting is easier than soil medium to avoid root damage. After 3 weeks incubation period, the soybean plants were harvested carefully. The results show that almost all of the 24 strains were able to form nodule; and the nodule characters were depended on the *Rhizobium* strain. Some soybean root containing nodules could be seen in Figure 1; and the detail data of the characteristic of nodulation are shown in Table 5.

Table 5 shows that the number, diameter and distribution of the nodules were strain dependent. Namely, it depended on the ability of each strain in symbiotic association with soybean and the *Rhizobium* growth and activity in soybean root. When crushing the nodule, it showed that almost all of the nodules had a red-pinky color, indicated that they were effective nodules in nitrogen fixation (Subba Rao 1994).

Up to now, farmers in acid land usually applied soil amendments to increase pH value and to reduce Aluminum toxicity (Ano and Ubochi 2007), which increased the production cost. This study resulted in selected *Rhizobium* strains tolerant to acid environment, high concentration of pesticides and Aluminum (Table 4), and also their ability to form effective nodules with soybean plant (Figure 1, Table 5). Hopefully, application of these strains as *Rhizobium* inoculum in acid soils will improve soybean growth and increase the cost product (without or at least minimize production cost for increasing the soil pH).

In another study, we also tried to apply these selected *Rhizobium* strains as soybean inoculum planted in acid and neutral soil, and it was reported in another publication.

CONCLUSIONS

The number of *Rhizobium* isolates and its tolerance to paraquat was depended on soil type. From 173 strains of isolated *Rhizobium*, 24 strains were tolerance to pesticides, and Aluminum. They were able to grow in wide range of pH, namely 3 – 8, or some of them in 5 - 8. Around 92% of the selected bacteria could form root nodules with soybean plant in different number and size. Hopefully, these isolates can be applied in pesticide polluted agricultural lands, especially in acid soils

with high concentration of Al, and it also increase soybean production.

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