JOURNAL OF DEGRADED AND MINING LANDS MANAGEMENT
ISSN: 2339-076X (p); 2502-2458 (e), Volume 5, Number 1 (October 2017): 995-1000
DOI:10.15243/jdmlm.2017.051.995

Research Article

Glyphosate biodegradation by plant growth promoting bacteria and their effect to paddy germination in glyphosate contaminated soil

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Received 23 August 2017, Accepted 18 September 2017

Abstract: Glyphosate is the most widely used herbicide in Indonesia. Glyphosate persistence between 55 days to 3 years. Widespread and uncontrolled use can cause weeds to become resistant and residue contaminates the soil and water environment. Due to the residual impact of glyphosate, it is necessary to identify a method that can increase the degradation of glyphosate. Several studies have shown that glyphosate can be degraded by microorganisms (fungi, rhizosphere and endophytic bacteria), some of which are members of plant growth-promoting bacteria. This study used the bacteria *Enterobacter cloacae, Enterobacter* sp and *Pseudomonas fluorescens.* These three types of bacteria have growth-promoting properties and potentially increase glyphosate degradation. Results of chromatogram on the residual test of glyphosate in liquid medium and soil containing glyphosate showed that glyphosate residue decreased with the addition of bacterial treatment when compared to control. The percentage of degradation in liquid medium are 96.06% by *Enterobacter cloacae,* 57% by *Enterobacter* spand 93.45% by *Pseudomonas fluorescens.*The percentage of degradation in soil medium are 4.32% by *Enterobacter cloacae,* 23.49% by *Enterobacter* spand 12.19% by *Pseudomonas fluorescens.*A positive result indicates that bacterial growth boosters from the plant (endophyte) as well as the area of rooting (rhizosphere) have additional potential as biofertilizer, bio stimulant, bio protectant but also as bio degradator pollutants such as the herbicide glyphosate.

Keywords*: bacteria, degradation, endophyte, glyphosate, rhizosphere*

To cite this article: Andriani, L.T., Aini, L.Q. and Hadiastono, T. 2017. Glyphosate biodegradation by plant growth promoting bacteria and their affect to paddy germination in glyphosate contaminated soil. J. Degrade. Min. Land Manage. 5(1): 995-1000, DOI: 10.15243/jdmlm.2017.051.995

Introduction

In 2015, Indonesian agrochemical market includes 350 brands of fungicides, 600 brands of herbicides and 800 brands of insecticides registered with the Indonesian authorities, according to Ministry of Agriculture's Pesticide Commission numbers. By product type, the pesticides market of Indonesia is segmented into herbicides, insecticides, fungicides and other pesticides. From the perspective of product category the most utilized products were herbicides (40%). Glyphosate and paraquat are the most utilized chemicals in herbicides category (Mordor Intelegence, 2015). The widespread use of glyphosate may result in weeds resistant and the residual causing environmental pollution. The persistence of glyphosate ranges from 55 days to 3 years. Because of the impact of glyphosate, it is important to identify methods for enhancing glyphosate degradation. Many studies have shown that glyphosate can be degraded by microorganisms (rhizospheric and endophytic bacteria); some of them are member of plant growth promoting bacteria (Grosbard and Atkinson, 1985; Yu et al., 2015). Glyphosate biodegradation in soil by *Pseudomonas* sp and *E. Coli* have shown high efficiency compare with it naturally biodegradation (Permanasari et al.,

2005). *Bacillus subtilis* strain Bs-15 can significantly promote glyphosate degradation in soil and play an important role in the bioremediation of glyphosate-contaminated soils (Yu et al., 2015). *Azospirillum* sp. and *Pseudomonas* sp. can degrade glyphosate residues both in vitro and in vivo in maize plants (*Zea mays* L.) (Travaglia et al., 2015). This study aimed to investigate glyphosate biodegradation by plant growth promoting bacteria and their effects on seedling growth in glyphosate soil contaminated.

Materials and Methods

Chemicals and reagents

Chemical reagents used for this study were commercial herbicides (Round up), Nutrient agar (Na). Pikovskaya agar, 0.01% tryptophan, HPLC grade acetonitrile., N-hexane HPLC grade, Na_2SO_4 HPLC grade.

Microorganisms and preparation of inoculum

The bacteria used were *Enterobacter cloacae, Enterobacter* sp. and *Pseudomonas fluorescens*. *Enterobacter cloacae* and *Enterobacter* sp. *Pseudomonas fluorescens* obtained from the collection of Bacteriology Laboratory Department of Plant Pests and Diseases, Faculty of Agriculture, Brawijaya University. The bacteria was cultured on NA medium and incubated for 24 hours.

Growth test of bacterial cells on medium containing glyphosate

Tests were performed by growing selected bacteria having potential to degrade glyphosate herbicides on 500 mL nutrient broth (NB) medium which had been added with 250 µL glyphosate herbicide. The control medium (without bacteria) and treatment medium (with bacteria) were incubated at room temperature (for 9 days) and shaked at 150 rpm. Cell density (optical density/OD) was measured using a spectrophotometer at 590 nm wave length (Travaglia et al., 2015).

Characterization of growing bacterial growth

Phosphate solubilization test

Selection of potentialbacteria in dissolving phosphate was done by using Pikovskaya selective media refers to Pant et al. (2016). The bacteria grown on NA medium was taken 1 loop of ose needle and scraped on picovskaya medium then incubated for 4-5 days at room temperature. The ability of dissolving phosphate was observed based on the formation of clear zones around bacterial colonies

Indole acetic acid (IAA) production test

The selection of potential IAA-producing bacteria was based on Loper and Scroth (1986). Bacterial suspension was cultured on NA medium plus 0.01% tryptophan. The tube containing the suspension was incubated at 27 ° C for 120 hours. After that it was harvested and centrifuged at 8000 rpm for 15 minutes. The supernatant was transferred to a sterile reaction tube and treated with Salkowski reagent (7.5 mL $FECl_3.7H_2O$ 0.5M; 150 mL concentrated H_2SO_4; 250 mL sterile Aquades). The solution was incubated for 1 hour and then absorbance value was measured at 530 nm wavelength. The absorbance value then added to the IAA standard curve equation to determine the concentration of IAA produced (Khairani, 2009).

Bacterial ability in degrading herbicide glyphosate

Biodegradation of herbicides glyphosate in soil medium

The test was used surface soil (depth 0-15 cm) field with a history no herbicide application. The soil was sterilized by autoclave for three consecutive days at 121^0C for 25 minutes each. The glyphosate herbicide was mixed with 1 kg of soil by adding 250 mL of sterile aquades which had added 125 µL of glyphosate herbicide and bacterial suspension of 1 mL on bacterial treatment and no bacteria addition to bacterial treatment. The herbicide residue was measured 7 days after incubation.

Biodegradation of Pesticides in Liquid Medium

Testing was done by adding glyphosate herbicide as much as 250 µL / 500 mL of liquid medium then suspension of bacteria was added as much as 1 mL on bacterial treatment and no addition of bacteria in treatment without bacteria (control). All erlenmeyers were incubated in dark conditions at room temperature and 150 rpm in a rotary shaker for 7 days. After that, the residual herbicide in the liquid medium was calculated.

Assesment of degradation

Percent degradation was calculated by the formula of Sawhney et al. (2015) and Yu et al. (2015) as follows: Degradation rate (%) = (a-b)/ a x 100, where a is the quantity of glyphosate in the treated sample (glyphosate + bacteria), and b is the quantity of glyphosate in control (only glyphosate)

The effect of growth-promoting bacteria in paddy seedlings on medium containingglyphosate

The experiment was compiled based on a complete non factorial randomized design with 6 replications. The combination of treatments used was as follows; A0: control (without bacteria and without glyphosate), A1: *Enterobacter cloacae* bacteria, without glyphosate, A2: *Enterobacter* sp bacteria, without glyphosate, A3: *Pseudomonas fluorescens* bacteria without glyphosate, A4: *Enterobacter cloacae* + glyphosate bacteria, A5: Bacteria *Enterobacter* sp. + Glyphosate, A6: Bacteria *Pseudomonas fluorescens* + glyphosate, and A7: Glyphosate. Healthy seeds that having similiar size were selected and soaked in water for 24 hours. The seed surface was sterilized using 0.2% NaOCl and 70% alcohol and washed with sterile water 4-5 times. The seeds were then soaked with 10 mL / 100 mL of plant growth suspension which had been selected previously. The seed immersion treatment was repeated three times. The seeds were grown on germicidal glyphosate added media of 250 µL. Seeds were incubated at room temperature for 10 days for germination. Observation parameters were sprout length (cm), root length (cm), seed vigor index and germination percentage (IP)

Results and Discussion

Characterization of plant growth promoting bacteria

Phosphate solubilization

Based on phosphate solubilization ability test, *Enterobacter cloacae* and *Pseudomonas fluorescens* were able to solubilize phosphate as evidenced by the formation of clear zone around the bacteria. While the *Enterobacter* sp. can not solubilize phosphate as evidenced by no clear zone around the bacteria (Figure 1).

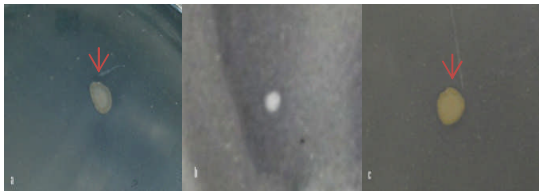

Figure 1. Phosphate Solubilization Test. a: *Enterobacter cloacae*, b: *Enterobacter* sp., c: *Pseudomonas fluorescens*

Based on IAA hormone production test, three bacteria (*Enterobacter* sp. *E. Cloacae* and *Pseudomonas fluorescens*) may produce IAA hormones as evidenced by the discoloration of redness in bacteria grown on tryptophan medium and reacted with Salkowski reagents. Differences in the thickness of red, indicate the different levels of IAA (Figure 2). Based on the calculation of IAA levels, *Enterobacter* sp has the highest IAA level of 12.443 ppm, followed by *Pseudomonas fluorescens* with a level of 6.871ppm and *Enterobacter cloacae*witha level of 2.443 ppm (Table 1).

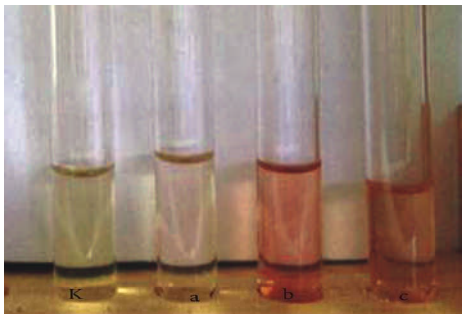

Figure 2. IAA Hormone Production Test. k: Control a: *Enterobacter cloacae*, b: *Enterobacter* sp., c: *Pseudomonas fluorescens*

Table 1. Concentrations of IAA Hormone

Isolate	Absorbance Value	IAA Hormone Concentration (ppm)
Enterobacter cloacae	0.039	2.443
Enterobacter sp.	0.240	12.443
Pseudomonas fluorescens	0.128	6.871

Result of bacterial growth test on medium containing glyphosate showed that *Enterobacter cloacae*, *Enterobacter sp* and *Pseudomonas fluorescens* could adapt well on medium containing glyphosate as indicated by significant difference between bacterial growth in medium containing glyphosate and non glyphosate. The results of chromatogram on the residual test of glyphosate in liquid medium and soil containing glyphosate showed that glyphosate residue decreased with the addition of bacterial treatment when compared to control (Figure 4) The percentage of degradation can be seen in the Table 2. The effectiveness of plant growth promoting bacteria (PGPB) who capable degrading glyphosate herbicides to paddy germination was visible on the Table 3. On the length of the root there was a marked difference between bacteria-treated and non-bacterial on the glyphosate

medium. In germinationon medium containing glyphosate the addition of effective bacteria increases root length. At shoot length, there was no significant difference between treatments. In the seed vigor index, the same as the root length in which the bacterial addition treatment significantly increased the seed vigor index compared with the bacteria-free treatmenton medium containing glyphosate

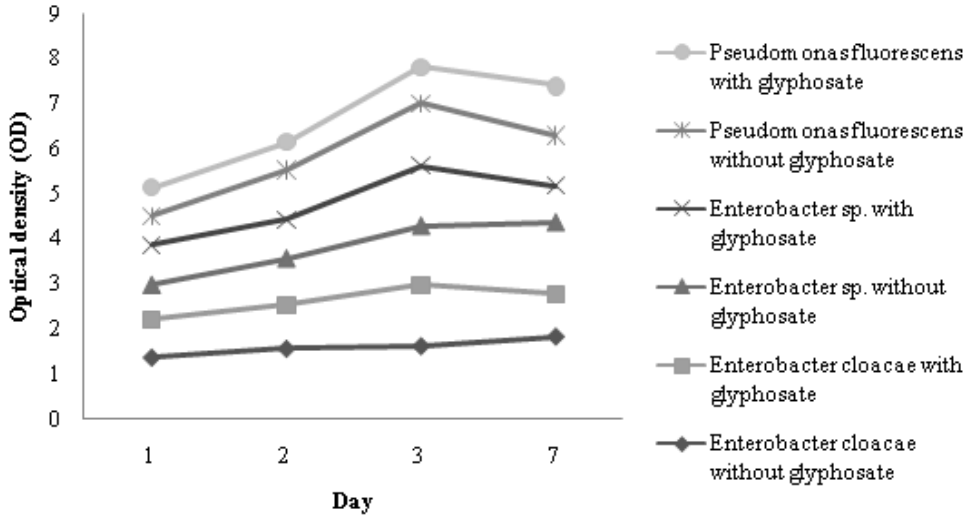

Figure 3. The Growth of Bacteria

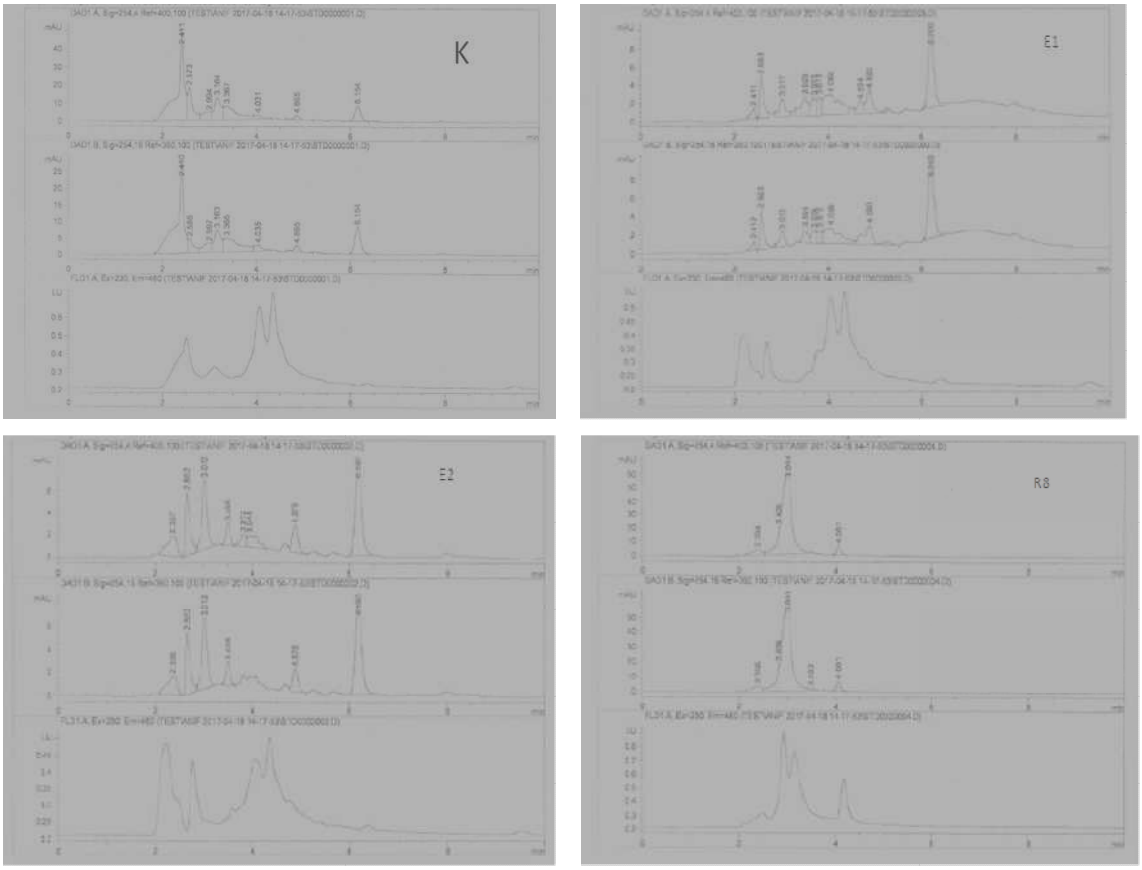

Figure 4. Chromatogram of Glyphosate Herbicide Residues. K: Control, E1: *Enterobacter cloacae*, E2: *Enterobacter*sp., R8: *Pseudomonas fluorescens*

Table 2. Percentage of degradation in liquid and soil medium

Isolate Name	Liquid Medium		Soil Medium	
	Glyphosate (ppm)	Percentage of Degradation (%)	Glyphosate (ppm)	Percentage of Degradation (%)
Control	0.484		0.1505	
Enterobacter cloacae	0.019	96.060	0.1440	4.32
Enterobacter sp.	0.203	57.990	0.1152	23.49
Pseudomonas fluorescens	0.032	93.453	0.1322	12.19

Table 3. Effects of Bacteria on Paddy Germination

Treatment	Root Length (cm)	Shoot Length (cm)	Seed Vigor Index
Without bacteria, without glyphosate	8.23 a	13.95 ab	22.18 b
Enterobacter cloacae without glyphosate	8.45 a	14.00 ab	22.45 b
Enterobacter sp.without glyphosate	8.70 ab	14.93 b	23.63 b
Pseudomonas fluorescens without glyphosate	10.32 b	14.07 ab	24.38 b
Enterobacter cloacae with glyphosate	8.65 ab	14.18 b	22.83 b
Enterobacter sp.with glyphosate	7.87 a	13.85 ab	21.72 ab
Pseudomonas fluorescens with glyphosate	8.80 ab	13.28 ab	22.08 b
Glyphosate without bacteria	7.28 a	11.92 a	19.20 a
CV	16.82	13.71	10.88
LSD 5%	1.68	2.20	2.83

Means within a row followed by the same lower case letter are not significantly different at p 0:05 LSD level.

Discussion

Bacteria used in biodegradation of herbicides glyphosate were *Enterobacter*sp., *Enterobacter cloacae* and *Pseudomonas fluorescens*. These bacteria show that there are resistant to glyphosate and further tested both of bacteria in degrading glyphosate. Pseudomonas fluorescens bacteria is a rhizospheric bacteria that also resistant to glyphosate. This test begins with characterization of growth-promoting bacteria where the results are not all bacteria capable of solubilized phosphate, but the are three types of bacteria that capable to produce IAA hormone which is a growth hormone.In bacterial growth test on medium containing glyphosate showed that bacteria were able to live on glyphosate medium and utilize the nutrients in medium to survive. The growth phase of bacteria in static cultures is experiencing early growth phase and lateral phase (lag phase), exponential stage (logarithmic), stationary stage and stage toward death (Schlegel, 1994). Usually, in artificial medium the bacteria begin to death phase at 3 days, but inthis test, the bacteria begin to death phase at 9 days after inoculation. According to Travaglia et al. (2015), he suggested that *Pseudomonas* and *Azospirillum* bacteria are capable in detoxifying glyphosate undergo longer stationary phases and delayed phases of death. The percentage of degradation by *Enterobacter* sp., *Enterobactercloacae* and *Pseudomonas fluorescens* were high enough about *Enterobacter* sp (57,99%)., *Enterobacter cloacae* (96.06%) and *Pseudomonas fluorescence* (93,45%) in liquid medium. This suggests that bacteria have two function as plant growth promoting bacteria and to degrade glyphosate herbicide. These characters are quite beneficial for humans where bacteria can help to reduce levels of glyphosate that has high persistence and poison for plants, besides it has growth promoting properties that can increase the production of cultivated crops. Microorganisms can metabolize glyphosate in two pathways: 1) Carbon-Phosphorus Chain (C-P) pathways produce phosphate and sarcosin (C-P) lyase pathways eg *Pseudomonas sp.* 2) Oxidative cleavage of the C-N chain on the carboxyl side catalyzed with glyphosate oxidoreductase (GOX), resulting in aminomethylphosphonic acid (AMPA) and glyoxilate (AMPA Pathway) (Pollegioni et al., 2011). Glyphosate degradation rates depend on the adaptation of bacteria to herbicides, phosphate status in bacterial cells, and bacterial culture growth phases (Kryuchkova et al., 2014. In test of growth-promoting plant bacteria effect that capable at degrading glyphosate to germination of

paddy plants shows more advantageous to use this bacteria on medium containing glyphosate, because beside itcan degrade glyphosate, the bacteria can also produce IAA hormone with certain levels that can help increase growth. The results of germination test showed that the addition of bacteria can increase roots length, shoots length and seed vigor indexes. The herbicide glyphosate is known are phytotoxic against crop cultivation, with the addition of glyphosate, the herbicide bacteria containing of glyphosate in the medium can be decreased so that increased plant growth. A positive result indicates that plant growth promoting bacteria (PGPB) from the plant (endophyte) or rhizosphere have additional potential as biofertilizer, bio stimulant, bioprotectan but also as biodegradator pollutants such as the herbicide glyphosate.

Conclusion

Enterobacter sp., *Enterobacter cloacae* and *Pseudomonas fluorescens* are plant growth promoting bacteria that can produce IAA hormones. *Enterobacter cloacae* and *Pseudomonas fluorescens* bacteria can solubilized phosphate while *Enterobacter* sp. cannot. *Enterobacter* sp., *Enterobacter cloacae* and *Pseudomonas fluorescens* have the ability to degrade glyphosate herbicides which will be useful in environmental improvement. Enterobacter sp., *Enterobacter cloacae* and *Pseudomonas fluorescens* may help increase germination of paddy in medium containing glyphosate.

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