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Glyphosate Tolerant Bacteria from Rhizosphere of Kangkong (*Ipomoea reptans* Poir.) and Soybean (*Glycine max* L.)

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Abstract: The use of organophosphate pesticides has some risks for human health and environment. One of the organophosphate pesticides is glyphosate. Various methods used to detoxify organophosphates including chemical methods, incineration, and landfills, produce acid and alkaline compounds, leaching pesticides around land and groundwater areas, as well as toxic emissions to the environment. The bacteria with this ability can be isolated from areas contaminated with glyphosate. Kangkong (*Ipomoea reptans*) and soybean (*Glycine max*) were chosen because of these plants are commonly found in rice fields which are areas that are frequent exposure to pesticide. The interaction between rhizosphere bacteria and plants as well as the composition of existing bacteria are closely related to the remediation occured. Kangkong and soybeans (2 weeks) were treated with glyphosate 377 mM. Soil pH was measured in third and seventh days after treat with glyphosate. The bacteria were isolated a week after treatment with glyphosate, and cultured in NA medium containing 5 mM and 10 mM glyphosate. The growing bacteria were selected and re-cultured in NA + glyphosate 10 mM medium. The selected isolates were tested for glyphosate degradation ability in Mineral Salt Media containing glyphosate 5 mM and glucose 50 mg/L. Eight isolates of bacteria grew in media containing glyphosate, i.e. Kd1, Kd2, Kd3, Kd4, Kd5 from soybeans rhizosphere, and K1, K3, K4 from spinach rhizosphere. The isolate Kd4 and K4 grew more abundantly compared the other isolates, exhibited good tolerant of glyphosate. From glyphosate degrading test, the isolate from soybean rhizosphere showed more tolerance than the isolate from kangkong rhizosphere. The molecular identification revealed that both isolates belong to species *Bacillus mycoides.*

Keywords: Glyphosate, organophosphate pesticide, rhizosphere bacteria.

Introduction

Glyphostae is the most widely used pesticide in the world today, both by farmers and urban communities (Soumis, 2018). Glyphosate is non-selective herbicide that inhibits the growth of most type of weeds, by inhibiting the synthesis of aromatic amino acids which are essential for the formation of protein in plants (Singh et al., 2020). Glyphosate is tightly bound to soil particles, which will inhibit glyphosate from being degraded by microbes. However, degradation of glyphosate by photodegradation was not significant in removing glyphosate in soil. Meanwhile, glyphosate in the aquatic environment will quickly settle in sediment, with a half-life of 12 days to 10 weeks (Tu et al., 2011).

The main product from glyphosate metabolism in microbes is AMPA (Aminomethyl Phosphonic Acid), which is still has potential to harm the human health (Leblanc et al., 2024). Glyphosate can dissolve in water, but its ability to bind soil particles is very strong, because the phosphonic acid part. Adsorption glyphosate in soil particles can cause the decrease of soil Ph

and increase soil phosphorus content (Sprankle et al., 1975b) and the glyphosate becomes immobile (Sprankle, Meggitt, & Penner, 1975a). Glyphosate also cannot be directly hydrolyzed and oxidized in the soil (Rueppel et al., 1977). Therefore, glyphosate degrading bacteria are important for biotechnological applications related to the further goal of the handling negative effects of glyphosate in the environment (Benslama & Bouhlarouf, 2013).

Some bacteria are known to have the ability to degrade glyphosate, including *Enterobacter cloacae* (Kryuchkova et al., 2014), *Sphingomonas paucimobilis* (Karpouzas et al., 2005), *Pseudomonas putida* (Kumari et al., 2012), and *Providencia alcalifaciens* (Nourouzi et al., 2011). The process of glyphosate degradation by microbes includes breaking the C-N bonds using the enzyme glyphosate oxidoreductase (GOX) which produces AMPA and glyoxylate, and then sarcosine (N-methyl-glycine) is degraded by the enzyme C-P lyase. Sarcosine is degraded into amino acids such as glycine, serine, cysteine, methionine, and histidine (Aparicio et al., 2013). More than 90% of the glyphosate residues are left in a depth of 15 cm to 35 cm from the soil surface due to the nature of the glyphosate which is strongly bound to the soil (Feng & Thompson, 1990).

Kangkong (*Ipomoea reptans* Poir.) is known as one of hyperaccumulator plants, and the main part of the plant playing the role of it is the root (Indrajati et al., 2005). The plant growth also mainly affects the abundancy of bacterial community in soil, especially in rhizosphere area. The abundance of bacteria in the rhizosphere area of soybean includes heat resistant bacteria (1011-1012 CFU/g soil), non-fluorescent bacteria (1011 CFU/g soil), chitinolytic bacteria (106-109 CFU/g soil), and fluorescent bacteria (103-108 CFU/g soil). The dominant heat resistant bacteria found was belong to *Bacillus* sp. which plays a role in inhibiting pathogens in soybean (Nawangsih et al., 2014). This research focus is on discovering the presence of organophosphate-degrading bacteria (glyphosate) in the rhizosphere area of kangkong and soybean, as well as identifying the glyphosate degrading bacterial isolates in the rhizosphere area of kangkong and soybean.

Materials and Methods

Plant Treatment

Kangkong and soybean plants are grown from seeds. Each polybag was planted with 3 test plants. On the 14th day after planting, the plants were treated with pesticides (glyphosate Monsanto Roundup) 377 mM (3 L / ha) (Nourouzi et al., 2011), 10 mL per poly bag (there are 10 polybags from each test plants with a diameter of 20 cm). For comparison, the plants without glyphosate treatment were also grown on different polybags. Furthermore, three days and one week after glyphosate administration, soil pH and moisture measurements were measured. The isolation of glyphosate degrading bacteria from the rhizosphere soil (of the plants treated with glyphosate as well as of the untreated plants) was carried out a week after treatment.

Bacterial Isolation

The isolation was performed based on the method by Kumari et al. (Kumari et al., 2012) with modifications, in which 10 g of soil samples were taken (15 cm depth), then were diluted in 90 ml of distilled water and suspended. After that, 0.1 ml of sample suspension (10-3 dilution) was inoculated by the spread plate method into NA media containing glyphosate (5 mM and 10 mM) (duplo) and incubated for 24 - 72 hours at room temperature (37^0C). The growing colonies were selected and purified into new media.

Selection of Glyphosate Degrading Bacteria

The glyphosate degradation test was performed with modifications of the method by Kryuchkova et al. (Kryuchkova et al., 2014), the bacteria isolated were selected based on their growth ability in NA media containing 10 mM glyphosate incubated for 24 hours. The growing isolates with the most abundancy were then sub-cultured in Mineral Salt Medium (MSM) containing 0.625 mM glyphosate as the source of C and P for bacterial growth. Furthermore, the inoculum was cultured in P-free MSM medium to make C and P-deficient bacteria (starved bacteria) for 20 hours. The bacterial suspensions were centrifuged and resuspended in MSM without a P source, then the results of the bacteria were resuspended in 100 mL of MSM medium containing 5mM glyphosate as the source of C and P. Furthermore, the bacteria were incubated

in shaker incubator at 140 rpm at 37oC for 28 days and their growth was observed every 7 days.

DNA Genomic Extraction and 16S rRNA Gene Amplification

The isolation of bacterial genomic DNA was done following the method of Moore et al. (2004) with modifications, using PCIA (25:24:1) method. For DNA extraction of isolate Kd4, 800 µL PVP 2% was added in lysis step. The 16S rRNA gene was amplified using 27F (5'-AGAGTTTAGTCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACT-3').

16S rRNA Gene Sequencing

The Sanger sequencing was done using dye terminator sequencing BigDye terminator V3.1. The cycle sequencing was performed using the PCR product of 16S rRNA gene as template. The sequence in ab1 file was then used to identify the species of isolates based on online gene databases.

Phylogenetic Tree Construction

The sequence of 16S rRNA gene (partial) was analyzed using BLASTn with the existing database in GenBank. Furthermore, the BLAST results were checked for the names of the valid isolates at www.ezbiocloud.net and the type of the bacterial strains were confirmed by using data from www.bacterio.net. The sequence alignment was done using Clustal W, and the phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis version 11 (MEGA 11) (Tamura, et al. 2021), using the Maximum Composite Likelihood substitution model with 1000X bootstrap replicates.

Results and Discussion

Glyphosate application causes the soil to become more acidic, and this can cause changes in plant morphology or the microorganism community in the rhizosphere. Soil pH in soybean rhizosphere was more stable than kangkong rhizosphere after glyphosate treatment (Table 1). The result from rhizosphere of kangkong is similar to the research by Zheng et al (Zheng et al., 2015). Three days after glyphosate application, the pH is quite low, and it was found that the glyphosate residue in the soil was still high, but it dropped drastically on the seventh day.

Table 1. Soil pH (3rd and 7th day after glyphosate treatment)

Treatment	Kangkong		Soybean	
	3rd	7th	3rd	7th
Control	6.8	7	6	6.2
Glyphosate 377 mM	4.71	6.94	5.78	6.16

Several bacterial isolates were obtained from the rhizosphere area of kangkong and soybean and only one type of fungus was found from the rhizosphere area of kangkong. The number of bacterial isolates from the rhizosphere area of soybean plants was more than from the rhizosphere area of kangkong (Table 2). The bacterial isolates were further selected using NA + glyphosate 10 mM medium to obtain tolerant isolates (Table 3).

Table 2. Isolation of glyphosate tolerant bacteria from the rhizosphere area of kangkong and soybean plants, growing in NA + glyphosate media at 37° C after 72 hours

Rhizosphere Area	NA + *Glyphosate* (mM)	Number of isolates	Microbial types	Isolate Code
Kangkong (untreated)	5	-	-	-
	10	-	-	-
Kangkong	5	2	Bacteria	K1, K3
		1	Fungi	K5
	10	1	Bacteria	K4
Soybean (untreated)	5	1	Bacteria	Kd5
	10	-	-	-
Soybean	5	6	Bacteria	Kd3, Kd7, Kd8, Kd9, Kd10, Kd11
	10	3	Bacteria	Kd1, Kd4, and Kd2

Table 3. Bacterial Isolates Grew in NA media with Glyphosate 10 mM

Rhizosphere Area	Isolates
Kangkong	K1, K3, and K4
Soybean	Kd1, Kd2, Kd3, Kd4, and Kd5

From morphological observation of the isolates, it was showed that all isolates have rod form (Bacilli). The colony of Kd4 isolate can make aggregate form and settled under NB media, while other isolates didn't form aggregate. Some isolates are Gram positive and some are Gram negative. All isolates have almost similar colony morphologies but show varying cell morphology. All isolates show negative result in indole and H_2S production. The isolate Kd4 and K4 were selected to glyphosate degradation test because of their abundant growth in NA media contain low glyphosate (0.625 mM), indicating the ability of the isolate to produce glyphosate degrading enzyme (Kryuchkova et al., 2014).

Degradation tests was performed using MSM plus 5 mM glyphosate and 50 mg/L glucose. The ability of isolates to degrade glyphosate can be seen from the ability of the isolates to grow in MSM containing 5 mM glyphosate (Fig. 2). Based on the results of the degradation test, Kd4 isolates from the rhizosphere area of soybean plants were better in adaptation to glyphosate. K4 isolates appeared to has slower growth (longer lag phase) than Kd4 isolates. The growth of K4 isolates only increased after 14 days of adapting to the presence of glyphosate, but decreased after 21 days. Therefore, it can be predicted that Kd4 and K4 isolates are only tolerant of glyphosate and are not able to degrade glyphosate because it is suspected that K4 isolate growth stops when the C source in the form of glucose has run out after 21 days. This is indicated by the undetectable growth of the Kd4 and K4 isolates measured by spectrophotometer (Fig 2).

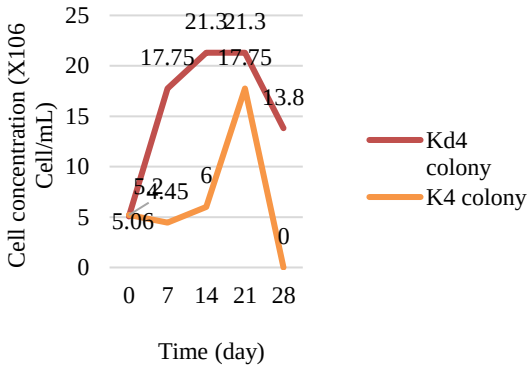

Fig 2. Growth curve of Kd4 and K4 in MSM media contain 5 mM glyphosate and 50 mg/L glucose

In this study, before the degradation test, the isolate Kd4 and K4 were stressed by C deficiency (starvation) by growing the colony in medium without C to accelerate the ability of colony to degrade the target component. Lack of C and lack of P as a stressor will accelerate the degradation process of target component by isolates later, because one of responses of C deficiency in bacteria is to increase the catabolic activity (Leung, Moore, Lee, & Trevors, 2005; Wang & Bakken, 1996).

Fig 3. Phylogram of Kd4 and K4 (based on 16S rRNA gene (partial) with maximum likelihood method

Based on 16S rRNA gene (partial), Kd4 and K4 were identified as *Bacillus mycoides* (Fig 3). Although Kd4 and K4 were identified as a same species, but Kd4 and K4 are different in their

growth rate and tolerant ability to glyphosate. Therefore, to confirm their variant will need further studies. To confirm the species *Bacillus mycoides* through phenotypic characters is also quite difficult, where we have to observe the ability of these bacteria to form rhizoidal colonies. However, the form of rhizoidal colonies cannot always be formed when the bacteria are grown in NA media (Gordon, Haynes, & Pang, 1973). *Bacillus mycoides* that are unable to form colony structures such as rhizoids (non-rhizoidal colonies) are also frequently found (Nakamura & Jackson, 1995).

Conclusion

This research concluded that Bacteria from rhizosphere of soybean exhibited tolerance of glyphosate rather than bacteria from rhizosphere of kangkong. The existence of the tolerant bacteria in the rhizosphere soil could affect the environmental surrounding the plants, produces soil conditions around the roots that are more conducive to plant growth, one of which is providing a buffering function for the soil so that the soil pH becomes more stable and can become a good place for plant growth. The isolate Kd4 and K4 were suspected being tolerant to glyphosate, but it is still need further studies to find out their ability in glyphosate degradation, to confirm the degradation ability through AMPA production, and the enzymes that playing role in degradation processes. The glyphosate-tolerant isolates Kd4 and K4 were both identified as *Bacillus mycoides.*

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