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Research Article

Role of soil bacterial consortia on glyphosate degradation and growth of maize seedlings

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Abstract: Pre-growing weed control by glyphosate herbicides is effective for increasing yield, but glyphosate residues in the soil might reduce soil quality and can accumulate in agricultural products. Naturally, microbes are able to breakdown glyphosate into nontoxic substances orthophosphate and glycine. Glyphosate degradation in soil by single soil microbes are reported elsewhere, but the information about glyphosate removal by soil bacterial consortia was limited. The objective of this research was to determine the effect of carbon (C), nitrogen (N), and phosphorus (P) composition in liquid media to increase glyphosate degradation and its degradation product by soil bacterial consortia and 2) verify the effect of bacterial consortia on maize seedlings growth, their N and P uptake, as well as total and soluble P in soil. Glyphosate degradation test was set up by incubating bacterial consortia in a different composition of C-N-P liquid basal media. Greenhouse experiment has been performed in a randomized block design to treat maize grown in Inceptisols with bacterial and glyphosate application. The results showed that C-N-P composition of liquid media affected the concentration of glyphosate, as well as orthophosphate and glycine as by-products. In-planta experiment verified that inoculation of glyphosate-degrading bacterial to maize seedling grown in glyphosate-contaminated soil enabled to enhance shoot dry weight of maize seedling and N and P uptake at 4 weeks after inoculation.

Keywords*: bacterial consortia, glycine, maize, nitrogen, orthophosphate, phosphorus*

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Introduction

The massive use of glyphosate-based herbicide may result in significant residues in soil. Glyphosate (N-phosphonomethyl-glycine) prohibits 3-enolpy-ruvylshikimate-5-phosphate synthase to catalyse the synthesis of amino acids tyrosine, tryptophan and phenylalanine of the shikimic pathway in all plants (Vivancos et al., 2011; Helander et al., 2019). Inhibition of Amino acids synthesis induce chlorosis mainly in the young leaves due to nitrogen mobilization toward old leaves and hence limit weed growth. The application of glyphosate in large quantities induce glyphosate and aminomethyl-phosphonic acid (AMPA) contamination; an intermediate product of glyphosate degradation in crop yields and groundwater bacteria. In Iowa, the United States, level of glyphosate residues and AMPA in transgenic soybean seeds were 0.4-8.8 mg/kg and 0.7-10 mg/kg, respectively (Bohn et al., 2014). The 30-year application showed that glyphosate was detectable in contaminated soil for a longer time (Cox, 1998). Glyphosate contamination in

Indonesian has not been yet reported. The use of glyphosate in the Hative Besar Village, Ambon City was 2-3 times a year and caused the low accumulation of glyphosate in soil, 0.004 mg/kg. Nonetheless, increased yield due to glyphosate application related to the increase in glyphosate accumulation in the edible part of crops (Faqihhudin et al., 2014; Kesuma et al., 2015). Application of 6 L/ha glyphosate resulted in residue up to 0.169 mg/kg in maize cobs (Faqihhudin et al., 2014); slightly higher than the maximum concentration of glyphosate residue in food crops (0.1 mg/kg) according to the Indonesian regulation.

Glyphosate is glycine derivative with molecular formula $C_3H_8NO_5$ which is readily removed from soil by microbial enzymatic metabolism. In soil, glyphosate will be degraded enzymatically to phosphorus-, carbon-, or nitrogen-containing compounds which partly uptaken by microbes (Hove-Jensen et al., 2014). Glyphosate can be removed through AMPA and Sarcosine pathways. The first pathway produces AMPA which is more toxic than glyphosate (Landry et al., 2005). In the Sarcosine pathway, the enzymatic reaction produced free phosphate ions and sarcosine which is further broken down into formaldehyde and glycine (Duke and Powles, 2008; Rodríguez et al., 2019).

Impact of high rate glyphosate application was reported to increase P content in agricultural soil since glyphosate acid consists of 18.3% P and become a significant input of P (Hébert et al. 2019). The increase of P up to 9.4 kg/km^2 was recorded across the United States in 2014 (Hébert et al., 2019). Phosphate is the main source of phosphorus for plants, while glycine is metabolized by most of soil microbes and plants (Duke et al., 2012; Ma et al., 2017). Keeping glyphosate residues in edible part below the minimum concentration reduce the harmful of glyphosate to animal and human (Cattani et al., 2014; Myers et al. 2016). Lowering glyphosate residue in the soil might be carried out by glyphosate-degrading bacteria.

Glyphosate biodegradation was demonstrated by single soil microbes such as *Pseudomonas putid*a and *Serratia marcescens* (Benslama and Boulahrouf, 2013), and *Trichoderma viride* fungi (Arfarita et al., 2016). The biodegradation and mineralization of organic substances generally require a synergism between microbes prior to transforming organic substances effectively (Song et al., 2018). A Fungal consortium was reported to proliferate in glyhosate-enriched plate agar and increase their growth during 24-day incubation in broth contaminated with glyphosate (Arfarita et al., 2014). However, glyphosate degradation by bacterial consortia has not been studied intensively. A bacterial consortium isolated from slightly glyphosate-contaminated agricultural areas in Ambon showed the glyphosate IC50 2.04 mg/L (Condrosari et al., 2018). The degradation capacity of this bacterial consortium related to growth media has not been studied. The C:N:P of the substrate has a prominent role in nutrient cycling since organic substances degradation is often restricted due to adverse macronutrient availability for the bacteria. The C:N:P ratio of glyphosate is 3:1:1 the C:N:P for glyphosate degradation by E. coli was around 17:5:1, according to Stanier et al. (Doran, 1995). Changes in the composition of C, N, P might affect glyphosate degradation by bacterial consortia.

Glyphosate degradation contributes to essential nutrient content in the soil, mainly N and P. Both macronutrients are limiting factor of crop production in the tropics. Contamination of glyphosate in the soil might provide the nutrient mainly N and P for plant uptake when the phosphate-degrading microbes are available. The objectives of this experiments were to 1) determine the effect C, N and P composition in the liquid media to increase glyphosate degradation by a bacterial consortium; and 2) evaluate the effect of bacterial consortia on the growth, and N and P in the soil as well as their uptake by maize grown in glyphosate-contaminated soil.

Materials and Methods

The study consisted of laboratory and greenhouse experiment during August 2017 to May 2018. A laboratory experiment was performed in liquid basal media with different composition of C, N and P, and was conducted at the Research Laboratory of PT Pupuk Kujang, Karawang Regency, West Java, Indonesia. The second experiment was carried out at the Faculty of Agriculture, Universitas Padjadjaran, Jatinangor Campus. The greenhouse was located in an altitude of 735 m above sea level. The temperature during the experiment was 19.5 ^{0}C-36 ^{0}C, and mean daily temperature was 25.3 ^{0}C.

Consortia of glyphosate-degrading bacteria

Bacterial consortia consisted of *Stenotrophomonas maltophilia* MHFENV 20, *Bacillus subtilis* FX4, *B. subtilis* IP18, *Lysinibacillu*s sp. BNPK-15, *Staphylococcus* sp. InS-021-1, *Stenotrophomonas* sp. DIB76BC2 and some unculturable bacteria. The consortia belong to PT Pupuk Kujang fertilizer company.

Laboratory experiment establishment

The experimental design was a completely randomized block design to test five different ratio

of C, N and P of liquid media (Table 1) with five replication. All liquid medium contaminated by glyphosate of 499.92 mg/L from a commercial herbicide that contained 41% of glyphosate. The concentrations of the C, N and P in all media were categorized as excessive, sufficient and less according to the C:N:P for glyphosate degradation by *E. coli* (17:5:1) and the C:N:P of glyphosate molecules (3:1:1). A total of 200 mL liquid media was sterilized at 121 ^{0}C for 20 minutes and inoculated with 1.5% consortia liquid culture with the cell density of 10^7 CFU/mL

Table 1. Ratio of carbon to nitrogen to phosphorus in liquid basal media enriched with glyphosate.

C:N:P[1]/ sufficiency category (Trial code)	Growth media composition[2]
17:5:1; Sufficient in C, N and P (A)	Na-acetate 1.69 g/L, $MgSO_4.7H_2O$ 12 mg/L, $CaCl_2$ 14 mg/L, $FeCl_3.6H_2O$ 125 mg/L, NH_4Cl 316.52 mg/L
3:1:1; less in C and N, sufficient in P (B)	Without Na-acetate and NH_4Cl
3:5:1/ less in C, sufficient in N and P (C)	Without Na-acetate
17:1:1; Sufficient in C and P, less in N (D)	Without NH_4Cl
34:10:1; excessive in C and N, sufficient in P (E)	With Na-acetate 3.76 g/L

[1]C:N:P, Carbon:Nitrogen: Phosphorus ratio; [2]Basal media in control was according to Alsop et al. (1980).

The culture was incubated on a 120-rpm orbital shaker at 23-25 ^{0}C for 30 days. Glyphosate, orthophosphate, glycine, and total bacterial populations were analyzed at day 30. Glyphosate concentrations were analyzed by UV/Vis spectrophotometer at a wavelength of 260 nm (Catrinck et al., 2014) while glycine concentration was determined at 470 nm (Shah et al., 2007). The orthophosphate concentration was determined by UV/vis spectrophotometer at 885 nm wavelength (Paytan and McLaughlin, 2007). Bacterial population count was carried out by the serial dilution plate method (Sanders, 2012). According to this laboratory trial, the media composition that supports glyphosate degradation was chosen for the pot experiment.

Greenhouse experiment establishment

The soil was silty clayed Inceptisols which is neutral in pH (6.86); very low in organic-C, N total, and available P_2O_5; and low in K_2O and average in cation exchange capacity. The experimental design of pot trial with maize as a test plant was a completely randomized block design with six treatments and four replications. The treatments were inoculation of bacterial consortia without and with 50 mg/L and 100 mg/L glyphosate rates in the soil; the control soil received neither consortia nor glyphosate. The liquid inoculant (10^9 CFU/mL) was scaled up in the liquid media with C:N:P of 3:1:1 based on the first trial. The media contained $MgSO_4.7H_2O$ 12 mg/L, $CaCl_2$ 14 mg/L, $FeCl_3.6H_2O$ 125 mg/L, and glyphosate 499.92 mg/L.

A total of 1.5 kg Inceptisols was put into black polyethylene bags and incorporated with 20 g cow manure. At the same time, 15 mL or 35 mL of glyphosate solution (5 g/L) was added to soil in order to have a final concentration of 50 mg/kg and 100 mg/kg. The soil was incubated for 2 days in the greenhouse before inoculation. The soil surface in an individual bag was sprayed with 5 mL bacterial consortia mixed with 85 mL sterilized distilled water and incubated for 7 days. Single maize seed was sown in each polybag which then placed in the greenhouse for 4 weeks. The half dose of inorganic fertilizers (100 kg Urea/ha, 50 kg SP 36/ha, and 50 kg KCl /ha) were applied at one week after sowing.

At 4 weeks after sowing, shoots and roots dry weight and the leaf number have been measured. The ratio of root to shoot dry weight were calculated. N and P in shoots as well as total N and soluble P_2O_5 in soil have been analyzed. The plant dry weight was measured by weighing the shoot after heating at 70 ^{0}C for 48 h. The analysis N and P in shoots by using the Kjeldahl method and UV-Vis spectrophotometer consecutively. N uptake and P uptake were calculated by multiplying N or P content in the shoot by shoot dry weight.

Statistical analysis

All data were subjected to analysis of variance ($p<0.05$). If the mean square of treatments were significant, then Duncan Multiple Range Test at $p<0.05$ were performed.

Results and Discussion

Glyphosate degradation in the liquid culture

Before inoculation, glyphosate in all liquid media was 499.92 mg/L. At day 30, C:N:P ratio of media determined glyphosate level in the culture in which the glyphosate content was reduced (Table 2). The lowest decrease in glyphosate level was

demonstrated by the culture with C:N:P ratio of 3:1:1 (B), but it did not significantly differ with the culture of 17:1:1 (D). The ratio of C:N:P of growth media significantly affected both metabolites content at day 30 (Table 2). The higher recovery of orthophosphate was found in the media with C:N:P of 3:1:1 (B) and 34:10:1 (E). However, glycine levels were significantly higher in the growth media with C:N:P of 34:10:1 (E) although the decrease of glyphosate in the said ratio was significantly lower than B and C treatments.

Analysis of variance showed that the mean square of treatments was significant at $p<0.05$ for the total bacterial population in the culture. The count of total bacteria verified that the medium with C:N:P of 17:5:1 and 3:1:1 supported bacterial proliferation more than other media (Table 2). Nonetheless, lower bacterial population in E media resulted in high orthophosphate and glycine

Table 2. Glyphosate, orthophosphate, glycine content, and total bacteria count in the media with different C:N:P ratio at 30 days after bacterial inoculation.

C:N:P ratio	GH (%)	OP (µM)	Gly (g/L)	Total bacteria
17:5:1 (A)	21^{a}	14.0^{b}	1.23^{bc}	6.10^{a}
3:1:1 (B)	7^{c}	34.9^{a}	1.20^{bc}	6.09^{a}
3:5:1 (C)	13^{b}	16.9^{b}	1.25^{b}	5.90^{c}
17:1:1 (D)	10^{b}	17.1^{b}	1.16^{c}	5.98^{b}
34:10:1 (E)	28^{a}	30.4^{a}	1.42^{a}	5.93^{c}

Numbers followed by the same letters were not significantly different based on Duncan's Multiple Range test at $p<0.05$.

The decline of glyphosate content at day 30 indicated that bacterial consortia enable to degrade glyphosate as a source of C, N and mainly P in the presence of other macro- and micronutrients. The experiment proved that consortia effectively catabolized glyphosate mainly in the absence of inorganic N. The absence of NH_4Cl induce the bacteria to metabolize glyphosate to fulfil their need for N. Researchers stated that single culture of similar bacterial species found in the consortia was resistant and able to degrade glyphosate (Shehata et al., 2012; Yu et al., 2015; Iyer et al., 2018). However, organic matter biodegradation should be accomplished through multiple enzymatic proceeds by various microbes that play a role in the certain reaction of biodegradation pathways. Our result verified that consortia were able to degrade glyphosate effectively up to 93%. The ability of consortia to the glyphosate was likely to be more effective compared with single bacteria currently reported. The bacteria was still viable at the end of the experiment in the culture with 499.92 mg/L glyphosate, although we didn't reisolate single bacterial species. Single culture of *Bulkholderia vietnamiensis* AQ5-12 has glyphosate-degrading ability for 100 mg/L in the presence of fructose as C source and ammonium sulphate as N source (Manogaran et al., 2018). This suggested that bacterial consortia resisted to a higher level of glyphosate.

The microbial consortia inoculated to liquid media might produce other intermediate besides orthophosphate and glycine, but in this study, we had only orthophospate and glycine profile. Glyphosate removal produced C-, P-, and N-containing intermediate include mainly sarcosine (N-methylglycine), glycine, AMPA, formal-dehyde, and orthophosphate (Kryuchkova et al, 2014). Microbes can utilize some of the said substance for their metabolism. In the absence of inorganic N (NH_4Cl), high glyphosate degradation was in line with orthophosphate level at 30 days showed that the consortia catabolized glyphosate for N. Media composition with excessive in C and N but sufficient in P produced highest orthophosphate and glycine due to higher glyphosate degradation. Glycine synthesized in glyphosate degradation pathway is catalized by C-P lyase enzyme which is induced by the presence of glyphosate (Hove-Jensen et al., 2014). they also stated that oxidase enzyme activity in the pathway resulted in the formation of AMPA and glycosylates; the AMPA is hence catabolized by among other C-P lyase to produce orthophosphate.

Plant parameters

The shoot dry weight of seedling was clearly higher when soil received consortia and glyphosate (Table 3) compared to control. While the effect of bacteria consortia-glyphosate to increase root dry weight was only significant when 100 mg/kg glyphosate was added along with the consortia.

The increase in dry weight resulted in the change of R/S. Significantly decreased in R/S was shown by a plant grown in 50 mg/kg glyphosate with inoculation. Irrespective of glyphosate levels in soil, inoculation reduced root to shoot ratio, but the R/S was <1 which indicated that the growth of shoots was more intensive than roots. This demonstrated that all treatments play a significant role to support the shoot growth. The combination of microbial consortia and glyphosate had a significant effect on N and P content of shoots as well as their uptake (Table 4). Comparing to the control treatment, application of 50 mg/kg and 100 mg/kg glyphosate on soil with and without inoculation resulted in the increase of P content, as well as N and P uptakes. However, only the application of 100 mg/kg glyphosate increased N content. All inoculated maize had a higher N and P

status at 4 weeks. Overall the best treatment to increase all N and P status of maize seedling was bacterial inoculation combined with higher rate glyphosate addition before sowing. The results verified that glyphosate contributed to N and P uptake due to bacterial consortia degradation. Glyphosate degradation to glycine as well as orthophosphate will increase N and P contents in soil and hence N and P uptake. In this experiment P uptake by roots might be restricted due to lower soil pH (5.3-5.5) so that some portion of orthophosphate produced during glyphosate degradation might be washed out of the root zone Increased in N and P uptake caused the plant to contain sufficient N and P for cell synthesis and subsequent plant metabolisms.

Table 3. Root and shoot dry weight and root to shoot ratio of 4 week-old maize seedling grown in soil with glyphosate and bacterial consortia.

Bacterial and glyphosate treatments	Roots dry weight (g)	Shoot dry weight (g)	R/S
A: Control	0.2[a]	2.70[a]	0.09[b]
B: no bacteria with 50 mg/kg GH	0.3[a]	5.30[c]	0.06[ab]
C: no bacteria with 100 mg/kg GH	0.4[a]	4.42[b]	0.08[b]
D: with bacteria, no GH	0.3[a]	5.44[c]	0.06[ab]
E: with bacteria and 50 mg/kg GH	0.3[a]	6.54[d]	0.04[a]
F: with bacteria and 100 mg/kg GH	0.5[b]	7.19[e]	0.07[b]

Numbers followed by the same letter were not significantly different based on Duncan's Multiple Range test at $p<0.05$, GH = glyphosate herbicide.

Table 4. Nitrogen and phosphorus profile in shoot of 4 week-old maize seedling grown in soil with glyphosate and bacterial consortia.

Bacterial and glyphosate treatments	N (%)	P (%)	N uptake (g/plant)	P uptake (mg/plant)
A: Control	2.41[a]	0.008[a]	0.07[a]	0.222[a]
B: without bacteria with 50 mg/kg GH	5.06[c]	0.025[c]	0.27[d]	1.333[c]
C: without bacteria with 100 mg/kg GH	2.32[a]	0.014[b]	0.10[b]	0.631[b]
D: with bacteria, no GH	2.90[a]	0.018[b]	0.16[c]	0.924[b]
E: with bacteria and 50 mg/kg GH	3.71[b]	0.025[c]	0.24[d]	1.604[c]
F: with bacteria and 100 mg/kg GH	3.61[b]	0.027[c]	0.26[d]	1.950[d]

Numbers followed by the same letter were not significantly different based on Duncan's Multiple Range test at $p<0.05$, GH = glyphosate herbicide.

Consortia of microbes are generally able to carry out a series of organic substances biotransformation that cannot be proceeded by single species or strain. Consortia also accelerate the rate of reaction compared to a single species. The consortia also verified to promote early vegetative growth of maize seedling besides metabolize the glyphosate. Bioremediation process by using bacterial consortia also played a role in promoting plant growth were recommended elsewhere. The *B. subtilis* and *S. maltophilia* are well known Plant Growth Promoting Rhizobacteria which demonstrated some promising plant growth-promoting effect on plant growth (Hashem et al., 2019; Alexander et al., 2019).

Conclusion

Consortia of soil bacteria degraded glyphosate and produce orthophosphate and glycine as well at 30 days after inoculation, but the percentage of glyphosate degradation depended on the sufficiency of carbon, nitrogen and phosphorous. Higher glyphosate degradation and orthophosphate content in the media was induced by less carbon and nitrogen but sufficient phosphorous (C:N:P, 3:1:1). In contrast, higher glycine content was detected in media contained excessive carbon and nitrogen but sufficient phosphorous (C:N:P, 34:10:1). Nonetheless, the content of glycine in liquid media with C:N:P of 3:1:1 was as much as that in media with the C:N:P ratio of 17:5:1. in general, the glycine contents were not too varied compared to orthophosphate.

Consortia of glyphosate-degraded bacteria combined with glyphosate increased shoot dry weight of 4 week-old maize seedling as well as N and P content in the shoots and their uptake. The experiment verified that the bacterial consortia have an important role in maintaining maize

growth in soil with a high content of glyphosate. The results suggested that the bacterial consortia have a promising function for remediating glyphosate-contaminated soil.

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