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THE APPLICATION OF *Trichoderma viride* STRAIN FRP 3 FOR BIODEGRADATION OF GLYPHOSATE HERBICIDE IN CONTAMINATED LAND

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ABSTRACT

In this current study, we observed *Trichoderma viride* strain FRP3 capability for biodegradation of glyphosate on contaminated land in Indonesia. There were two blank plots that have been involved as representatives of indigenous fungal, that prepared as control (non-contaminated soil) and P1 (GP-contaminated soil) while the treatments were represented by two plots. Plot 2 (P2) was introduced with conidia suspension of *Trichoderma viride* strain FRP3 one time application, and plot 3 (P3) was introduced with conidia suspension of *Trichoderma viride* FRP3 two time applications. At the end of observation, the CFU of two times application was the highest with CFU of 15.97 x 10^6 gr^{-1} soil. The CFU of P3 was corresponding to 45% higher than P2 (8.83 x 10^6 gr^{-1} soil). The CFU of GP-contaminated soil without conidia suspension application had 0.66 x 10^6 gr^{-1} soils, only 0.7% and 0.4% corresponding to P2 and P3, respectively. Direct indicator of glyphosate degradation was determined using GC analysis. Within 7 days after *Trichoderma viride* FRP3 was introduced, glyphosate content of treated soil decreased. This fungal strain provided 48% (P2) and 70% (P3) of glyphosate degradation higher than indigenous soil microbial community (P1) within 28 days of application.

Keywords: biodegradation; glyphosate herbicide; survival; *Trichoderma viride*

INTRODUCTION

Glyphosate (GP) is a broad-spectrum herbicide widely used in the world. It is applied to the leaves of plants to kill both broadleaf plants and grasses. The application of GP results in the leaves yellowing and decaying within 5–10 days (sometimes 30 days) caused by the breakdown of aromatic amino acids synthesis. The studies observed at the initial stage of Round-up production by Monsanto Chemical Co. (USA) showed a relatively high GP biodegradation in the contaminated soils with a half-life period of degradation about 20 days (Rueppel, Brightwell, Schaefer, & Marvel, 1977). However, the 30-year application of the glyphosate herbicide under different ecological conditions showed that GP insists in the contaminated soil for a longer time depending on type of soil, tillage, climatic conditions and other factors (Cox, 1998). According to Eberbach (1998), the application of glyphosate after 2 years of applications, remained its accumulation in soils and it could influence on human health and the environment.

Indonesia is the second producer of palm oil after Malaysia, together with a total production of 80% of the total world production of palm oil. In 2020, Indonesian government plan to develop oil palm plantations become 20 million hectares of which now have reached 6 million hectares. It means that large amounts of chemicals particularly glyphosate has been applied into the soil and continues to pollute the soil. In order to prevent the accumulation of glyphosate in soils and its effect on ground water, it is important to develop technology for its rapid elimination after the weed treatment.

In the environment, glyphosate's primary route of decomposition is through microbial degradation in soil. The available reference on the microbial degradation of glyphosate is mostly among bacteria (Hallas, Hahn, & Korndorfer, 1988; Krzysko-Lupicka & Orlik, 1997; Ermakova et al., 2010). However, the study on the biodegradation of glyphosate by fungal species is

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lacking. It has been known that the member of genus *Trichoderma* has a wide range of economically useful aspects that have applications for multiple biotechnological uses, mainly in agriculture (Shovan, Bhuiyan, Begum, & Pervez, 2008; Rajendiran, Jegadeeshkumar, Sureshkumar, & Nisha, 2010), industry and environmental biotechnology (Rincon, Benitez, Codon, & Moreno-Mateos, 2009). *Trichoderma* species show ecological capacity, due to its ability to produce a wide range of enzymes and the high ability of the enzyme to degrade substrates. Many strains of *Trichoderma* have the nature of the resistance to various toxic and xenobiotic compounds (Rincon, Benitez, Codon, & Moreno-Mateos, 2009). Currently, *Trichoderma viride* is commonly used for seed and soil treatment for suppression of various plant diseases caused by fungal pathogens. They are easily isolated, rapidly cultivated and very efficient to control a broad range of plant pathogens (Quiroz-Sarmiento, Ferrera-Cerrato, Alarcon, & Hernández, 2008). It also has been reported that *Trichoderma viride* could degrade herbicide such as trifluralin (Zayed et al., 1983) and bromoxynil (Askar, Ibrahim, & Osman, 2007). However, the report about the degradation of *Trichoderma viride* to glyphosate herbicide is lacking.

In previous study (Arfarita et al., 2011), it was selected *Trichoderma* sp. strain FRP3 because they had the highest ratio of growth diameter and this species has been known for agriculture application. This *Trichoderma* strain FRP3 has been identified and named as *Trichoderma viride* strain FRP3, based on morphology observation and also performed by 18S rRNA gene amplification (Arfarita et al., 2013). This present study observed the application of *Trichoderma viride* strain FRP3 for *in situ* bioremediation of glyphosate contaminated lands in Indonesia on application dose and more than 10 years of application history of Round-Up.

MATERIALS AND METHODS

Soil

The site intended for this bioremediation study was a part of agriculture land in Batu, East Jawa, Indonesia. The type of soil in experimental plots was inceptisol. This area has a history of more than 10 years using Round-Up with twice a year of glyphosate application and is located on 800 asl (above sea level). Climatology situation of Batu has minimum temperature of 18-24ºC and maximum temperature of 28-32ºC. Its air humidity is 75 - 98% and the rainfall average is 875 - 3,000 mm year^{-1}.

In Malang, the glyphosate content of soil in 2009 was 2.13 - 8.81 mg kg^{-1}. However, this data did not point specific areas Glyphosate content of soil treated in this study was 20.35 mg kg^{-1} 10 days after first herbicide spraying. The initial glyphosate content after 3 days second herbicide spraying was 67 - 73 mg kg^{1}.

Strain Preparation

Trichoderma viride strain FRP3 obtained from previous study that showed appreciable growth in culture medium containing glyphosate as the sole phosphorus source (Arfarita et al., 2013). *Trichoderma viride* strain FRP3 was purified, cultivated on Potato Dextrose Agar (PDA) and incubated for 5 days at 30ºC for further study. To be introduced to soil, *Trichoderma viride* strain FRP3 was then cultivated for mass production. For it, jam bottles (250 ml) containing 100 g of sterile rice husk was inoculated with five mycelial plugs (5 mm in diameter) of *Trichoderma viride* strain FRP3 taken from five days old cultures on PDA. Bottles were inoculated in aseptic condition and placed in incubator at 30ºC for 14 days. The conidia were harvested from 14 days old of cultivation media. The number of conidia per mg of cultivation media was determined by dilution method with the aid of an haemocytometer.

Field Application

The experiments were conducted on the size of plots 1 × 1 m. Seven days after spraying of glyphosate herbicide, the plots were extracted and loosened evenly to the depth of 30 cm; trash, residual roots, plant debris and other foreign inclusions were discarded. Herbicide glyphosate was applied in the amount equivalent to field application 3 days before microbial application. There were two blank plots that have been involved as representatives of indigenous fungal, that prepared as control (non-contaminated soil) and P1(GP-contaminated soil) while the treatments were represented by two plots. Plot 2 (P2) was introduced with conidia suspension of *Trichoderma viride* strain FRP3 one time application, and plot 3 (P3) was introduced with conidia suspension of *Trichoderma viride* strain FRP3 two time applications.

Conidia was harvested from two bottles of cultivation media (26 x 10^9 conidia g^{-1} media), suspended in a volume of 5 L water and applied to each plot by drip method. The experiment was carried out on May-June 2013 (dry season).

Soil samples were collected every week by observation for 28 days. Soil sampling techniques were started from 0-20 cm soil horizon by a core auger made of steel with an inner diameter of 2 cm. From each plot, soil samples were taken from five different points, then collected and mixed. Each sample was analyzed at least average in triplicate. At the end of the observation, the soil sample was also observed to a depth of 20-30 cm to estimate the mobility herbicide vertically through the soil profile.

Microbial Analysis

Soil samples were first processed through a series of dilutions, then inoculated on media RBAC with 500 mg L^{-1} of the GP using the spread plate method, to count viable cells of GP-degrading microorganisms in the soil population. The total numbers of GP-degrading cells were observed every week. CFU (Colony Forming Units) was determined after incubation of agar plates at 27°C for 48 hours. The CFU of GP-degrading microorganisms were calculated as the difference between the total amount GP-degrading cells in treatment plots and the amount of indigenous GP-degrading cells (Control); following this formula: P1-P3 = Total CFU – CFU Control. CFU is Colony Forming Units which perform on Media RBAC containing glyphosate herbicide from any soil samples. Control is came from soil samples without the application of *Trichoderma*.

Glyphosate Content in Soil Samples

Hexane**-**isopropanol extraction was used for the evaluation of the total GP content in soil samples. The soluble fraction of GP was determined after the aqueous extraction of soil samples. Ten grams of fresh soil were transferred to a 250 ml buffer bottle and 50 ml of a 3:1 (N-Hexane: Isopropanol) solvent was added. The flasks were put on a rotary shaker at 150 rpm and incubated in room temperature (± 25ºC) for 2 hours. After shaking, the flasks were allowed to stand for overnight. Ten ml of aqueous solutions were then transferred to 25 ml weighing bottle**,** adjusted to a volume of 25 ml with deionized water and then mix well. The upper solution (5 ml) was used for total GP content measurement.

GC Shimadzu 2010, equipped with a capillary columnRtx-1 (30 m × 0.25 mm ID, 0.25-µm-thick film) and a nitrogen-phosphorus detector was employed. The chromatographic conditions used for the analysis of glyphosate residues were as follows: detector temperature was 300ºC; injector temperature was 150ºC; oven temperature program was 1.0 min at 100º C, 20 K min^{-1} to 130°C, 1 K min^{-1} to 133ºC, hold for 10.5 min, 20 K min^{-1} to 150ºC, hold for 2.0 min. The total run time was 18.85 min. The injection volume was 5 µl. N_2 was used as the gas carrier, maintained at constant flow rate of 0.3 ml min^{-1}. The approximate retention time of the glyphosate was 14.2 min.

Soil pH Measurement

The pH was determined on a 1:5 (soil: deionized water) suspension as described by Hesse (1971). Measurement of pH involved detection of the charge in potential of a silver chloride combination electrode system using a millivolt meter standardized against known buffer solutions. A 1:5 (soil : water) suspension was prepared which 10 g air-dry soil (<2 mm) was put into an Erlenmeyer flask containing 50 ml deionized water and then shaking for 1 hour at 15 rpm. The pH meter was calibrated according to manufacturer's instructions using the buffer at pH 6.86 and either the 4.0 buffer depending on the expected values for the soils.

Data Analysis

Data was analyzed statistically using analysis of variance (ANOVA) and Duncan Multiple Range Tests to show the effects of *Trichoderma viride strain* FRP3 application on soil of fungal communities, Glyphosate content and soil pH.

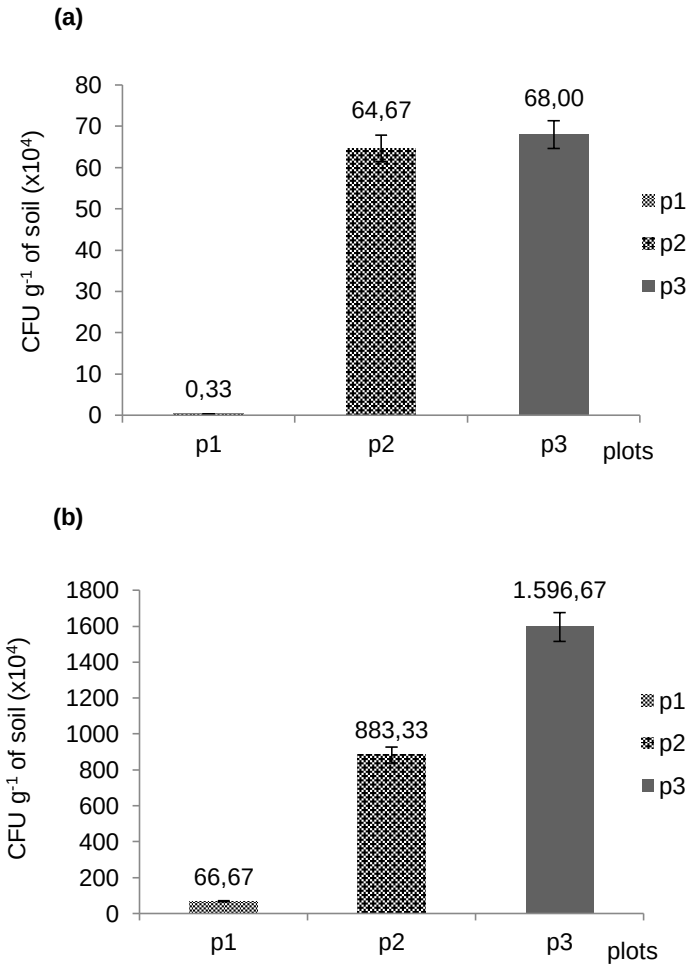
RESULTS AND DISCUSSION

Soil Fungal Communities under Field Conditions

Four plots were observed for the existence of soil fungal communities under field conditions. The existence was determined using Colony Forming Unit. As initiation (Figure 1a), where *Trichoderma* was applied, a large number of colonies of fungi were found. The CFU g^{-1} soil of indigenous microorganisms (P1), one time introducing of *Trichoderma viride* strain FRP3

(P2) and two times introducing (P3) were 0.33 x 10^4, 64.67 x 10^4 and 68.00 x 10^4 respectively. At the end of observation (Figure 1b), the CFU of P3 was the highest with CFU of 15.97 x 10^6 g^{-1} soil. The CFU of P3 was corresponding to 45% higher than P2 (8.83 x 10^6 g^{-1} soil). The CFU of P1 had 0.66 x 10^6 g^{-1} soil, only 0.7% and 0.4% corresponding to P2 and P3, respectively. It should be noted that the number of colonies present in units P2 and P3 was related to the amount of conidia suspension applied.

Figure 2 shows the significant increasing of soil fungal communities in every weeks of 28 days observation. The CFU of all plots were likely constant in first week and then increasing until the end of observation, except P1 which decreasing after 3 weeks. Perhaps, in the first of glyphosate application caused the inhibition and toxic to soil microorganisms. It also had the possibility of microbial degradation process during first week after *Trichoderma* application. The record on rain fall was two times during second weeks, 7.4 and 8.4 mm. The rain fall volume could increase the soil moisture to support the fungal grow.



Remarks: Blank soil plots consist of indigenous microorganisms: Control = non contaminated soil (No additional GP treatment), P1 = GP-contaminated soil (with additional GP treatment); Treatments plots were treated with glyphosate (GP-contaminated soil): P2 = *Trichoderma* conidia suspension 1 x application, P3 = *Trichoderma* conidia suspension 2 x application.

Figure 1. The CFU of soil fungal communities as initiation (a) and at 28 days of observation (b).

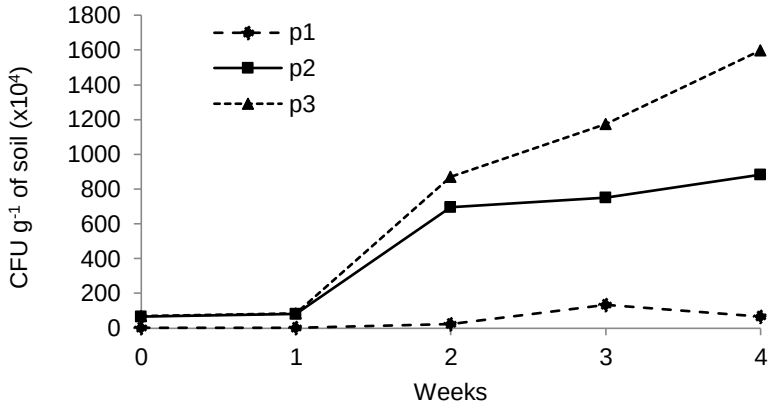


Figure 2. The existence and the increasing of soil fungal communities in plots P2 (■), plots P3 (▲) and indigenous microorganisms P1 (♦) in every weeks of 28 days observation.

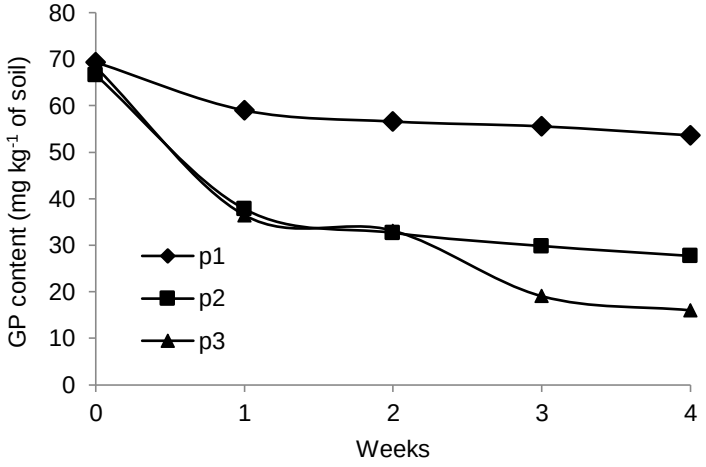


Figure 3. Glyposate contents during bioremediation process in 28 days of observation.

Glyphosate Content of Soil Samples

In situ bioremediation experiment results were expressed on GP content shown in Figure 3 which demonstrates that the GP biodegradation rate was maximal during the first week after introduction of *Trichoderma viride* strain FRP3. In the first week, GP content was decreasing significantly in the case of P2 and P3; by 37.8 mg kg^{-1} (56.8%) and by 36.5 mg kg^{-1} (53.4%), respectively. By the end of observation (28 days), the GP content of P3 decreased by 16 mg kg^{-1} (23.4%) and P3 decreased by 27.7 mg kg^{-1} (42.6%). From the third weeks, the GP content in the case of P2 higher than P3. This fungal strain provided 48% (P2) and 70% (P3) of glyphosate degradation higher than indigenous soil microbial community (P1) within 28 days of application. The GP content in this case was related to the amount of conidia suspension applied in the second week. Two times fungal introducing in the soil (P3) resulted the higher decreasing of GP content corresponding to P2. However, in the application we need to consider the labor efficiency and cost.

Soil pH

Table 1 shows soil pH values of all plots before glyphosate application, after glyphosate application and at 28 days of observation. Soil pH values of all plots before GP application were in the range of 5.3 to 5.5. According to pervious study, *Trichoderma viride* strains FRP3 were able to grow in a wide range of pH from 4.0 to 6.5 with the optimum pH being 5.5. This means that the

soil pH before glyphosate herbicide application is suitable for the growth of *Trichoderma*.

Table 1. Soil pH values

Plots	Soil pH values		
	Before GP application	After GP application	28 days observation
P1	5.52 a	6.56 a	6.28 a
P2	5.37 a	6.33 a	5.61 b
P3	5.45 a	6.44 a	5.42 ab

After glyphosate application, the soil pH of all plots increased, in the range of 6.33 to 6.56. Effect of glyphosate herbicide application thus increasing the pH of the soil has been reported by Adnan, Hasanuddin & Manfarizah (2012). All plots showed a decrease in pH at the end of observation, however a significant decrease shown by P2 and P3. Twice of *Trichoderma* application (P3) was able to restore the pH of the soil conditions such as pre-applied herbicide, slightly lower than P2, but this difference is not significant statistically. Although this property depends on many factors, it is worth mentioning that for this study, the application of *Trichoderma viride* strains FRP3 could decrease the soil pH as contrary of the glyphosate herbicide application.

CONCLUSION

In this study, we observed *Trichoderma viride* strain FRP3 capability for *in situ* bio-remediation of glyphosate- contaminated soils. *Trichoderma viride* strain FRP3 demonstrated high survival on field conditions with field application dose and more than 10 years of application history of glyphosate herbicide. At the end of observation, the CFU of two times application of conidia suspension (P3) was the highest with CFU of 15.97 x 10^6 g^{-1} soil. The CFU of P3 was corresponding to 45% higher than P2 (one time application of conidia suspension) (8.83 x 10^6 g^{-1} soil). The CFU of P1 (without conidia suspension application) had 0.66 x 10^6 g^{-1} soil, only 0.7% and 0.4% corresponding to P2 and P3, respectively. It should be noted that the number of colonies present in units P2 and P3 was related to the amount of conidia suspension applied. P2 was introduced one time with conidia suspension of *Trichoderma viride* strain FRP3 and P3 was intoduced twice during experimental period.

Direct indicator of glyphosate degradation was determined by using GC analysis. Within 7 days after *Trichoderma viride* strain FRP3 was introduced, glyphosate content of treated soil decreased. Within 7 days, GP content was decreasing significantly in the case of P2 and P3. By the end of observation (28 days), the GP content of one time application of conidia suspension P2) decreased by 16 mg kg^{-1} (23.4%) and two time application of conidia suspension (P3) decreased by 27.7 mg kg^{-1} (42.6%). This fungal strain for one time application provided 48% and two times application provided 70% in glyphosate degradation higher than indigenous soil microbial community (P1 is without conidia suspension application) within 28 days of application.

The application of this fungal strain could also decrease the increased soil pH as influence of treatment of glyphosate herbicide. *Trichoderma viride* strain FRP3 has been widely used as biological control agent in agricultural. The treatment of soil with this fungal strain seems also useful on the area where this herbicide is extensively used. Therefore, this fungus strain is very suitable for the remediation, because it is efficient, safe for the environment and quickly deal with soil contaminated by glyphosate.

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