Isolation and Identification of Bacterium Resistant to Glyphosate and Paraquat Herbicide from Rice Fields

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Abstract. Herbicides are chemicals that commonly used to control weeds in rice fields. The aim of this study was to isolate and identify bacteria that were resistant to glyphosate and paraquat herbicide. The soil sample was collected from two locations in rice fields at Desa Mulyajaya and Kutalanggeng, Karawang. Thirteen bacterial isolates were isolated from rice fields and screened for their resistance to glyphosate and paraquat. One isolate was resistant to 3,500 ppm of glyphosate and 1,400 ppm of paraquat. Based on Biolog omniLog system, the isolate was identified as Ensifer meliloti.

1. Introduction

Glyphosate and paraquat are herbicides that commonly used in agricultural to help minimum/zero tillage, providing productivity and soil conservation benefits. The agricultural extensification and intensification is dependent on the use herbicides to control weeds that are persistent threat to crop productivity. Glyphosate is a non-selective herbicide with broad spectrum used to control or kill annual grasses including rooted perennial weeds, herbaceous plant and some shrubs. It can be used in no-till agriculture to prepare fields before planting, during crop development and after crop harvest. Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), resulting in shikimate accumulation and reduced production of aromatic amino acids [1].

Paraquat is also a non-selective herbicide with a broad spectrum and rapid action. Paraquat is one of the most common herbicides used in agriculture including rice production. Paraquat is toxic because it diverts photosynthetic electron transport to oxygen to produce free radicals that cause lipid peroxidation and membrane damage [2]. It has inhibitory and repressing effect, reduces enzymes activity and growth on microorganisms. Resistance or sensitivity of microbes on paraquat toxicity depend on their capability to neutralized radical superoxide

The use of chemical herbicides can alter other environmental components, including microbial community. These chemicals can harm non-target organisms which may reduce biodiversity and ecosystem that support food security and on-farm profitability [3]. Herbicides were able to degraded by some soil bacteria and inhibit the growth of bacteria. Different effects of herbicides on the growth of microbes could be ranging from inhibition to tolerance.

The development of a cheap and environmentally friendly bioremediation method using glyphosate and paraquat-degrading bacteria is a promising approach to cleansing and restoring soils contaminated...
with those herbicides. The aim of this study is to isolate and identify bacteria from rice fields which resistant to glyphosate and paraquat herbicides.

2. Materials and Methods

2.1. Collection of soil sample

Soil samples were obtained from two locations namely Desa Mulyajaya and Kutalanggeng at Karawang, West Java. These locations are known as one of the rice cultivation area in Karawang. Soil samples were collected from depth of 0 – 15 cm from five different sites in each of the two locations. Soil samples from each site were thoroughly mixed and stored at 4°C. Soil samples from Desa Mulyajaya were numbered 1, while samples from Desa Kutalanggeng were numbered 2.

2.2. Isolation of bacteria

Minimum Salts Medium (MSM) was used for bacteria isolation. The composition of MSM (gram per liter) are 0.2 MgSO_4\cdot7H_2O, 1 KH_2PO_4, 1 K_2HPO_4, 0.5 (NH_4)2SO_4, 0.01 CaCl_2, 0.001 FeSO_4 and 0.5 NaNO_3 [4]. Ten gram of each soil sample was dissolved in 250 ml Erlenmeyer flask containing a mixture of 90 ml of MSM and supplemented with 1 ppm of glyphosate and 0.4 ppm of paraquat. The addition of herbicides in the media was 2X doses of herbicide use in the field. The flasks were incubated on rotary shaker at 120 rpm for 7 days. Ten ml of the broth culture was transferred into 90 ml of fresh MSM containing 1 ppm of glyphosate and 0.4 ppm of paraquat then incubated for 7 days. This treatment was repeated for 5 times. The culture was serial diluted (10^{-3} to 10^{-6}) and spread on the MSM agar. The separated single colonies were purified and sub-cultured for further experiment.

2.3. Selection of bacteria resistant to glyphosate and paraquat

Thirteen isolates were grown on Nutrient Broth (NB) medium and then shaken incubated for 24 h at room temperature. Twenty μl of isolate was subculture on NA medium supplemented glyphosate with concentration 0 – 4.000 ppm and paraquat 0 – 1.600 ppm. All treatments were incubated at room temperature for 48 h and repeated 2 replicates. Isolates which grow on medium with the highest herbicide concentration used as a bioremediation agent candidate.

2.4. Identification of bacteria resistant to glyphosate and paraquat

Isolate that are resistant to the highest glyphosate and paraquat concentration was identified chemically using Biolog microsation system models ELX808BLG series 1306184 (Biolog Hayward, CA, USA). This system used Buffer IF-A protocol A to determine of utilization of carbon source. Isolate was grown on NA medium for 24 h. Furthermore, 150 µl of bacterial suspension was inoculated into micro plate well and incubated for 24 hours in a Biolog incubator machine [5]. The micro plates are read with the Biolog microstation system compared to database and result is determined.

Biology system is used as an easier of phenotypic identification. The Biolog technique of microbial identification is based on carbohydrate utilization by microorganisms. The Biolog micro plates consists various media of specific carbohydrates and a redox indicator. The redox dye, tetrazolium changes into purple if microbial growth occurs in particular well representing catabolism of the substrate [6].

2.5. The growth of bacteria resistant to glyphosate and paraquat

The potential isolate was grown on NB medium added glyphosate or paraquat with a concentration 1X, 3X and 5X doses of herbicide usage in the field. The dose of glyphosate usage is 0.5 ppm, while the paraquat dose is 0.2 ppm. This treatment was conducted to determine the growth of isolate after added herbicide. NB medium without herbicide and inoculated with potential bacteria is used as a control. Sampling was done every day for 7 days. The growth rate of isolate was measured using spectrophotometer at λ 620 nm.
3. Results and Discussion

3.1. Isolation and screening bacteria resistant glyphosate and paraquat

In this study, thirteen bacterial isolates were obtained from 2 rice fields. Seven bacterial isoleates were obtained from Desa Mulyajaya and 6 bacterial isolated from Desa Kutalanggeng. These isolates have shown an ability to grow in culture medium containing glyphosate and paraquat. Rice fields of Desa Mulyajaya are known have high herbicide residues.

Herbicide residues of rice fields at Desa Mulyajaya are 0.202 ppm of glyphosate and 0.114 ppm of paraquat, while at Desa Kutalanggeng are lower 0.098 ppm of glyphosate and 0.024 ppm of paraguay [7].

Selection of bacteria resistant glyphosate showed that all isolates were able to grow on NA medium added with glyphosate concentration from 0–1000 ppm (Table 1). Only isolate 1.2 isolate that can survive up to glyphosate concentration 3500 ppm.

| Code | Concentration of glyphosate (ppm) |
|------|----------------------------------|
|      | 0      | 50     | 100    | 200    | 500    | 1000   | 1500   | 2000   | 2500   | 3000   | 3500   | 4000   |
| 1.1  | +      | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      |
| 1.2  | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | -      | -      |
| 1.3  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 1.4  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 1.5  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 1.6  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 1.7  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 2.1  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 2.2  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 2.3  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 2.4  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 2.5  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |
| 2.6  | +      | +      | +      | +      | +      | -      | -      | -      | -      | -      | -      | -      |

+: grow, -: not grow

The data showed that higher concentration of glyphosate treatment resulted in fewer isolates are able to grow on the medium. Moneke et al. [8] reported that the number of bacteria or fungus that grows decreased on solid media added by glyphosate. The breakdown of glyphosate through two pathways, the first is cleavage of the C-N bond by the enzyme known as glyphosate oxidoreductase with formation of glyoxylate and aminomethylphosphonic acid (AMPA). The second pathway is cleavage of the C-P bond to give sarcosine, glycine and formaldehyde by a C-P lyase activity [4].

The selection of bacteria resistant paraquat showed that all isolates were able to grow on NA medium added with paraquat concentration from 0–200 ppm. Isolate 1.2 was also able to survive up to paraquat concentration 1400 ppm (Table 2).
Table 2. Selection of bacteria resistant to paraquat

| Code | Concentration of paraquat (ppm) |
|------|---------------------------------|
| 1.1  | 0 50 100 200 400 600 800 1000 1200 1400 1600 |
| 1.2  | + + + + + + + + + + + |
| 1.3  | + + + + + + + + + + + |
| 1.4  | + + + + + + + + + + + |
| 1.5  | + + + + + + + + + + + |
| 1.6  | + + + + + + + + + + + |
| 1.7  | + + + + + + + + + + + |
| 2.1  | + + + + + + + + + + + |
| 2.2  | + + + + + + + + + + + |
| 2.3  | + + + + + + + + + + + |
| 2.4  | + + + + + + + + + + + |
| 2.5  | + + + + + + + + + + + |
| 2.6  | + + + + + + + + + + + |

+: grow, -: not grow

The higher concentration glyphosate and paraquat will be more toxic and inhibit the growth of bacteria. The number of isolates tolerant to paraquat decreased when paraquat concentration increased [9]. Paraquat has been found to induce oxidative stress by production of superoxide anion and cause toxicity to aerobic cells [10].

3.2. Identification of bacteria resistant glyphosate and paraquat

Based on biochemical test using Biolog system, potential isolate 1.2 can use various source of carbohydrate, amino acid and reduce violet and blue tetrazolium (Table 3). Positive (+) or negative (-) reaction indicated capability or disability of bacteria use substrate for metabolism process.

Table 3. Metabolite activity of isolate 1.2 in Biolog microstation system

| Substrate       | Activity          |
|-----------------|-------------------|
| α-D-glucose     | +                 |
| D-glucose-6-phosphate | +/-            |
| D-sorbitol      | +                 |
| Dextrin         | +                 |
| D-fructose-6-phosphate | -              |
| Myo-inosytol    | +                 |
| D-fructose      | +                 |
| Glycl-L-proline | +                 |
| Tetrazolium violet | +             |
| D-fucose        | +                 |
| L-arginine      | +                 |
| Tetrazolium blue | +             |
| L-fucose        | +                 |
| L-aspartic acid | +                 |
| Lyncomycin      | +                 |
| Maltose         | +                 |
| L-glutamic acid | +                 |
| Guanidine hydrochloride | +/-          |
| D-trehalose     | +/-               |
| L-Histidine     | +/-               |
| Niaproof 4      | +/-               |
| Gentiobiose     | +                 |
| L-alanine       | +/-               |
| D-galacturonic acid | +/-         |
| Sucrose         | +                 |
| L-serine        | +/-               |
| L-galactonic acid-g-lactone | +/-   |
| D-turanose      | +                 |
| Gelatin         | -                 |
| Glucuronamide   | +/-               |
D-raffinose, + L-Pyroglutamic acid - D-glucoronic acid +/- N-acetyl-D-Glucosamine + D-Lactic acid methyl ester - Acetic acid +/- D-mannose + a-ketoglutaric acid - Formic acid +/- L-rhamnose + D-mannitol + D-gluconic acid - D-galactose + L-arabitol + Mucic acid - N-acetyl-D-Mannosamine + Glycerol + Citric acid - a-D-Laktose +/- Quinic acid +/- Pectin + D-salicin +/- D-malic acid +/- L-Lactic acid + N-acetyl-D-Galactosamine +/- Acetoacetic acid +/- L-malic acid + 3-methyl-D-Glukoside +/- Tween 40 +/- Bromosuccinic acid + D-melibiose + D-aspartic acid +/- Potassium tellurite + D-cellobiose +/- Propionic acid +/- Sodium lactate 1% +/- 3-methyl- Glucose +/- g-amino-N-butyric acid -

Each microorganisms use different carbon sources depending on their nutritional requirement. Based on the positive and negative reaction, the specific characters of species can be determined. Identification of isolate 1.2 using Biolog system obtained result as a *Ensifer meliloti* with a probability of 0.992.

*Ensifer meliloti* (formerly *Sinorhizobium meliloti*) is an aerobic, motile, Gram negative, non-spore-forming rod bacterium. *Ensifer* has a facultative symbiotic of legumes, primarily Medicago, Melilotus and Trigonella genera. *E. Meliloti* converts atmospheric N\textsubscript{2} into a plant usable for m, thereby providing plants with an essential nutrient and contributing to plant growth and productivity [11]. Mhamdi et al. [12] reported that *E. meliloti* can exist as a soil saprophyte or as a legume microsymbiont of common bean (*Phaseolus vulgaris*).

de la Pena et al. [13] reported the symbiotic fixing-nitrogen bacterium *E. meliloti* tolerant to oxidative stress was tested in solid medium supplemented with hydrogen peroxide, paraquat and atrazine. The Rhizobia isolates of *Vicia faba* L. showed reduction of cell population in liquid medium treated with different concentration of glyphosate [14]. The other research stated that some Rhizobia genera tolerant to glyphosate and paraquat [15].

3.3. The growth of bacteria resistant glyphosate and paraquat
The growth of isolate 1.2 at different glyphosate concentration showed different growth rate (Fig. 1). The optimum growth of isolate 1.2 at different glyphosate concentration is at 96 h, with different optical density. The growth of isolate 1.2 decreased at concentration 0 and 1X doses of glyphosate at 120 h. Decreasing growth of isolate 1.2 at concentration 3X and 5X doses of glyphosate occurred at 144 h.
Isolate *Ensifer meliloti* 1.2 used in this study showed growth in the medium culture containing various concentration of glyphosate. The rapid growth of isolate *E. meliloti* was from 0 to 48 h, showed effective utilization of glyphosate. The growth of this isolate increased slowly at 48 h and reached maximum growth at 96 h. The growth of isolate 1.2 tends to decreased and stagnant after 120 h. This indicated that *E. meliloti* 1.2 could utilize glyphosate as a carbon or phosphorus source to support its growth. Many bacterial isolates have been reported to utilize glyphosate as a carbon [4] and phosphorus source [8]. Several research reported that the ability of microorganisms utilize glyphosate by naturally synthesizing appropriate enzymes or result of genetic mutation [8]. The high capacity of this isolate to utilize this herbicide in vitro could be attributed to their previous contact with the herbicide in the rice fields from where they were isolated.

The growth of isolate 1.2 at concentration 0, 3X and 5X doses of paraquat increased until 96 h (Fig. 2). The peak growth of isolate 1.2 at 1X doses of paraquat occurred at 120 h then decreased at next hour. Decreasing growth of isolate 1.2 at concentration 0, 3X and 5X doses of paraquat occurred at 120 h then increased and decreased again at next hour.

The isolate *E. meliloti* 1.2 could growth in the medium containing different doses of paraquat. The growth of isolate 1.2 increased rapidly from 0-48 h. This is indicated that utilization of paraquat by
isolate 1.2 was effective. After 48 h, the growth of *E. meliloti* increased slowly and reached maximum growth at 96 h. The stationary phase of isolate 1.2 occurred after 96 h. The growth rates of *E. meliloti* 1.2 at different paraquat concentration showed capability this isolate utilize paraquat as energy or nutrient sources to support its growth. Maldani et al. [16] reported that several Rhizobium strains were tolerant to paraquat. The tolerance of some bacteria to paraquat is due to their ability to synthesize paraquat neutralizing enzymes, namely catalase and superoxide dismutase.

4. Conclusion
Thirteen bacterial were isolated from rice fields and screened for their resistance to glyphosate and paraquat herbicide. One isolate was resistant to 3.500 ppm of glyphosate and 1.400 ppm of paraquat. Based on Biolog omnilog identification system, isolate 1.2 was identified as *Ensifer meliloti*. Isolate *Ensifer meliloti* 1.2 could growth at different concentration of glyphosate or paraquat.

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6. References
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