Growth of paddy inoculated by *Azotobacter* spp. in tetracycline contaminated culture

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Abstract. Excess tetracycline consumed by the cattle is excreted in the form of urine or feces commonly used as organic matter for rice production. The plant growth promoting *Azotobacter* that used as biofertilizer is usually carried by manure as the source of organic matter. Tetracycline residues in the manure might interfere the plant growth. The objective of this preliminary study was to verify the influence of two *Azotobacter* isolates on early vegetative growth of rice grown in media contaminated tetracycline. Bioassay was performed by using filter paper moistened with liquid growth media media with some level of tetracycline. Paddy seed was grown for 12 days on the paper and inoculated with *Azotobacter c2a9* from Maluku soil and *Azotobacter K4* from West Java soil. In general, 25-75 mg/L of tetracycline significantly decreased shoot height and root length of paddy seedlings inoculated with *Azotobacter c2a9*. Lower level tetracycline, 15 mg/L, increased shoot heights and root lengths of paddy seedlings inoculated with *Azotobacter K4*, a saline resistant isolate. However 30 mg/L of tetracycline reduce all parameters.

1. Introduction
Rice (*Oryza sativa* L.), an important staple food, is cultivated on 8 million hectares of area in Indonesia [1]. Nutrient source in rice cultivation is mainly inorganic fertilizer which shows harmful effect on soil and water when farmers apply it too much and in inappropriate application method. In sustainable agriculture, the use of environmental-friendly nutrient source is a way to reduce fertilizer use. Plant growth promoting rhizobacteria (PGPR) can be included in fertilizing routine due to its prosperity in increasing the nutrient availability and producing plant growth substances[2]. Microbial inoculation is reported elsewhere to maintain and increase the quantity and quality of paddy [3].

*Azotobacter* is a PGPR widely used as biofertilizer; aerobic and heterotrophic *Azotobacter* fixes N₂ to NH₃ which hydrolyzed and nitrified into NH₄⁺dan NO₃⁻, the ionic nitrogen that are available for plant [4]. Most of *Azotobacter* isolates are able to produce growth substance indole acetic acid (IAA), gibberellin and cytokines as plant growth regulators [3]. Extrapolisaccharide (EPS) was produced by *Azotobacter* to protect the oxygen-sensitive nitrogenase in aerobic fixation process [5]. *Azotobacter* also protects the plants from unfavorable conditions, such as salinity and drought [6]. Recently, *Azotobacter vinelandii* (SRIAz3) was discovered from rice rhizosphere that increase the tolerance of var. IR64 rice to salinity [6].
The inoculation of Azotobacter to paddy field should be followed by organic matter amendment since the heterotroph bacteria depend on organic carbon as energy and carbon source. Most of rice fields in Indonesia use manure as organic matter to improve plant growth and yield. Manure might be contaminated by antibiotics because animal production always use antibiotic to control animal health [7]. 40-90% of unabsorbed antibiotics are found in the excreted manure and urine [8]. Another study mentioned that around 58% of veterinary antibiotics that consumed by the livestock are excreted to terrestrial area [9].

Tetracycline is one of veterinary antibiotics that used in the livestock farming. This antibiotic can easily binds with di- or trivaleces metal ions [10]. This veterinary input is soluble in non polar solvent like chloroform, asetic ethyl, and dichloromethane [11]. The inhibition way of tetracycline forms the bond with 50S subunit ribosome in bacteria and prevent the T-RNA bond with ribosome thus there is no protein synthesis [12]. The rest of antibiotics that can not be absorbed by the livestock are carried in the manure. Some samples of manure and green house soil contains 156.2-5001.4 μgkg⁻¹ of antibiotics and <1000 μg kg⁻¹ of tetracycline [13]. In the soil samples received manure intensively, the concentration of tetracycline was 86.2 (0–10 cm), 198.7 (10–20 cm), and 171.7 μg kg⁻¹ (20–30 cm) and an average of 4.6–7.3 μgkg⁻¹ chlortetracycline was found [14].

The increase concentration of antibiotics in soil might be harmful either for plant and beneficial microbes. It is needed to develop the resistance ability of rhizobacteria, especially Azotobacter, thus it would has dual functions as nutrient server and bio protector. Some novel research showed that certain Azotobacter isolates were resistant to tetracycline [15,16]. Azotobacter demonstrated resistance ability to veterinary tetracycline excreted to the cultivation land [17]. Screening of tetracycline-resistance Azotobacter against some tetracycline concentration is important to ensure Azotobacter’s activity to increase rice growth. The objective of this preliminary study was to verify the ability of two Azotobacter isolates to improve and protect the early vegetative phase of rice in media with several tetracycline concentrations.

2. Materials and methods

2.1 Biological Material
Azotobacter c2a9 was isolated at 2014 from chili rhizosphere grown in Entisols of Ambon, Maluku Province. Saline-resistant Azotobacter K4 was isolated at 2015 from soil around roots of paddy grown in Ultisols of Karawang, West Java Province. One g of soil sample was grown in free-nitrogen Ashby broth until the pellicle was appeared. The pellicle was streaked on the Ashby plate in the petri dish. The separated colony was purified on another plate and stored in the test tube that contain Ashby Agar. The cultures of both isolates were maintained in nitrogen-free Ashby media. A total of 200 mL of broth was autoclaved at 121°C for 15 minutes and left out one night at room temperature. One slant of Azotobacter pure culture was mixed thoroughly with 10 mL of NaCl 0.85% for 5 minutes. As much as 5% of liquid pure culture was inoculated to 200 mL sterilized nitrogen-free Ashby broth in a 500 mL Erlenmeyer flask. The culture was incubated for 72 hours before the plant test.

2.2 Bioassay
The experiment used non-soil media to support the plant growth. Growth medium for the bioassay was Jensen’s broth (20 g sucrose; 1 g dipotassium phosphate; 0.5 g sodium chloride; 0.1 ferrous sulphate; 0.005 g sodium molybdate; 2 g calcium carbonate, pH 7). Bioassay was performed in transparent plastic bag of 11 cm height, 9 cm wide and arranged in a rack inside the growth chamber with 12 hours of irradiation time using 8400 lm of lamp. Filter paper were filled with 30 ml of sterilized Jensen’s broth contaminated with tetracycline. The tetracycline concentration was 25-75 mg L⁻¹ for paddy seed inoculated with Azotobacter c2a9 and 15 and 30 mg L⁻¹ for paddy seed inoculated with Azotobacter K4. Difference in tetracycline level was due to different IC₅₀ of the two bacteria. IC₅₀ of c2a9 and K4 were 90 mg L⁻¹ and 32 mg L⁻¹, respectively. All c2a9 and K4 inoculations were six time replications.
The seeds were sterilized using sodium hypochlorite then washed by sterile aquadest three times and incubated for three days on the sterile paper in the Petri dish. After three days, the seeds were inoculated with c2a9 or K4 broth and grown on the upper tip of filter paper. Paddy seedlings were maintained for 12 days in growth chamber in room temperature. Shoot height, root length, sum of primary roots and secondary roots were measured in the last day of plant test. These parameters were statistically analyzed by ANOVA and 5% Duncan multiple range test.

3. Result and Discussion

Plant test of c2a9 isolate in various tetracycline contamination showed the significant results in each treatment. The 25-75 mg L⁻¹ treatment gave the growth barriers to plants (Table 1; Figure 1). The inhibition could be seen from the low canopy height, root length, and the number of primary and secondary roots. The ratio of canopy height and root length showed that treatment with 50 mg L⁻¹ tetracycline had the highest ratio of 5.83 and showed an inhibition of root growth. The plant height without tetracycline and inoculated by c2a9 isolate was 11.13 cm followed by 25-50 mg L⁻¹ treatment with significantly decreased height of 2.51 - 2.68 cm. The best root length was found in the treatment without tetracycline, which was 10.01 cm and the root length with tetracycline treatment was significantly different from non-tetracycline treatment which was between 0.41 to 1.98 cm. The highest number of primary and secondary roots was also found in treatments without tetracycline. The sum of primary and secondary root in the plants with c2a9 and tetracycline had non-significant result, but they had instable trend. The sum was lowering in the concentration of 62.5 mg L⁻¹ and increased a bit in the concentration of 75 mg L⁻¹.

| Tetracycline (TC) concentrations | Parameter       | Shoot Height (cm) | Root Length (cm) | Shoot : Root Ratio | Sum of Primary Roots | Sum of Secondary Roots (n cm⁻¹) |
|---------------------------------|-----------------|-------------------|------------------|--------------------|---------------------|----------------------------------|
| Without TC                      |                 | 11.13c            | 10.01a           | 1.13a              | 3.83c               | 9.53c                            |
| 25.0 mg L⁻¹                     |                 | 2.51b             | 1.98b            | 1.21a              | 1.50ab              | 2.58ab                           |
| 50.0 mg L⁻¹                     |                 | 2.68b             | 0.63b            | 5.83b              | 2.33b               | 7.68bc                           |
| 62.5 mg L⁻¹                     |                 | 0.78a             | 0.41b            | 2.12a              | 0.50a               | 0.33a                            |
| 75.0 mg L⁻¹                     |                 | 1.10ab            | 0.68b            | 1.42a              | 1.66ab              | 1.26ab                           |

Treatments with superscripted alphabet showed the significant value (P<0.05)
Figure 1. Paddy seedling at day 12 in several level of tetracycline inoculated with Isolates c2a9. a) without tetracycline; b, c, d, e is with 25, 50, 62.5 and 75 mg L\(^{-1}\) tetracycline, respectively.

The plant growth using K4 isolates with a range of tetracycline concentrations of 0-15-30 mg L\(^{-1}\) showed that plants were still able to grow well with the given range of tetracycline concentrations (Figure 2). The height of the plant and number of secondary roots in the concentration range of 0-15 mg L\(^{-1}\) of tetracycline ranged between 7.38 - 9.11 cm and 77 - 92.15 which were significantly higher than the plants contaminated by 30 mg L\(^{-1}\) of tetracycline. Plant root length was significantly higher in treatment with 15 mg L\(^{-1}\) tetracycline which was 8.73 cm. The number of primary and secondary roots in all treatments showed the non-significantly difference from the treatment without tetracycline. In the ratio of plant height to root length, 15 mg L\(^{-1}\) of tetracycline treatment showed the non-significantly difference result from the treatment without tetracycline and treatment without tetracycline was not significantly different from the treatment of 30 mg L\(^{-1}\) tetracycline. This result expressed that all treatments gave the tolerance to tetracycline.

Table 2. Growth of paddy seedling inoculated with k4 isolates in several tetracycline level

| Tetracycline concentrations | Shoot Height (cm) | Root Length (cm) | Shoot : Root Ratio | Sum of Primary Roots | Sum of Secondary Roots (n cm\(^{-1}\)) |
|-----------------------------|-------------------|------------------|-------------------|----------------------|-------------------------------------|
| 0 mg L\(^{-1}\)            | 7.38\(^{b}\)      | 5.53\(^{b}\)     | 1.49\(^{ab}\)     | 5.66                 | 13.73                               |
| 15 mg L\(^{-1}\)          | 9.11\(^{b}\)      | 8.73\(^{c}\)     | 1.14\(^{a}\)      | 6.5                  | 12.20                               |
| 30 mg L\(^{-1}\)          | 3.06\(^{a}\)      | 1.31\(^{a}\)     | 2.24\(^{b}\)      | 4.33                 | 11.44                               |

Treatments with superscripted alphabet showed the significant value (P<0.05)
Figure 2. Paddy growth at day 12 with two concentration of tetracycline inoculated with K4. a) without tetracycline; b) with 15 mg L\(^{-1}\)tetracycline, c) with 30 mg L\(^{-1}\) tetracycline contamination

The inhibition of plant growth by tetracycline was visually and statistically apparent in the plant test using c2a9 with a range of tetracycline of 0-75 mg L\(^{-1}\) and K4 with a range of tetracycline from 0 to 30 mg L\(^{-1}\). The plants inoculated by K4 isolates with a concentration of 15 and 30 mg L\(^{-1}\) still grew well. By observing the visual appearance, there was not much differences compared to inoculation treatment without tetracycline. This indicated that plants inoculated with K4 isolates were relatively resistant to tetracycline up to 30 mg L\(^{-1}\). Different things happened to plants inoculated by c2a9 isolates which gave an inhibitory response at a concentration of 25 mg L\(^{-1}\) tetracycline at the end of the experiment. This indicated that the combination of plant and Azotobacter was able to survive until 30 mg L\(^{-1}\) of tetracycline. Some in vitro experiment without plant had discovered that Azotobacter was resistant to tetracycline in various concentration. Azotobacter that was isolated from non-legume rhizosphere was resistant to 20 mg L\(^{-1}\) of tetracycline [18]. Some Azotobacter isolated from domestic and industrial wastewater-irrigated rhizosphere were more resistant to 30 mg L\(^{-1}\) of tetracycline [19].

The inhibition of plant growth by tetracycline also occurred in rai grass (Loliumperenne L.) with a biomass reduction of 40% in 0.1 mg L\(^{-1}\) of tetracycline [20]. The decrease in root length was discovered on wheat (Triticum aestivum L.) along with an increase in tetracycline concentrations of up to 10,000 mg L\(^{-1}\)[21]. In addition, there was fungal contamination in the treatment with 50 mg L\(^{-1}\) tetracycline concentration with the inoculation of c2a9 isolates (Figure 1c). The suppression of the bacterial community in the solution increased the population of other microbes, such as fungi. This condition also found in all treatments with tetracycline with the highest concentration of 4.27 x 10\(^8\) CFU mL\(^{-1}\) at 60 mg L\(^{-1}\) tetracycline after 15 days of planting of wheat (Triticum aestivum L.) in hydroponic experiments [22]. This experiment indicated that the inoculation of Azotobacter was potentially able to protect the plant from certain tetracycline antibiotics. This protection might depend on the compatibility of isolates and the plant used.
4. Conclusion
The response of plants inoculated with c2a9 isolates with a range of tetracycline concentrations until 75mg L\(^{-1}\) and K4 isolates with a range of tetracycline concentrations until 30 mg L\(^{-1}\) showed a decrease in growth. However, there was no difference in the number of roots in the treatment of K4 isolates with and without tetracycline.

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