Synergism bioassay of selected indigenous halotolerant phosphorizobacteria from rice rhizosphere on saline soil ecosystem

F N F Athallah1*, U Dinar2, B N Fitriatin1**, T Simarmata1**
1*Student of Soil Science Magister, Faculty of Agriculture, Universitas Padjadjaran, Indonesia
1**Department of Soil Science Faculty of Agriculture, Universitas Padjajaran, Indonesia
2Department of Agronomy and Bioproduct Technology, Institut Teknologi Bandung, Indonesia.
*E-mail: farisnurfauzi@gmail.com

Abstract. The research was conducted to study the activity and synergism of indigenous halotolerant phosphorizobacteria. Three selected isolates (K5, TR-C2, CLR-A) isolated from different saline soil ecosystem were tested to find out the synergism of each isolate and its ability in P solubilizing. The experiment was arranged as randomize complete design, consisted of seven treatments and provided with four replications. Synergism test was carried out using filter paper disc method. The filter paper disc (d= 5 mm) were soaked in each suspension of selected isolates and stick onto NA plate media directly for single isolate treatments (B, C, D), while combined isolate treatments (E, F, G) were spread first against another isolate according to its treatments. Each treatments were incubated for 72 h at 30°C then inhibition zone was observed. The solubilizing index was tested by sticking soaked filter paper disc on supplemented Pikovskaya agar plate and incubated for 72 h at 30°C. Liquid inoculants were prepared by inoculating of each selected bacteria into 40 ml modified Pikovskaya media supplemented with 0,09 g.L⁻¹ NaCl (EC= 4 dS.m⁻¹), shaken at 100 rpm for five days. Inoculants were centrifuge at 7500 rpm for 10 minutes, 2.5 ml of supernatant were taken and P-available were observed using Bray II method. The experiment result showed that K5, TR-C2 and CLR-A isolates were compatible to grow in consortium condition. K5 isolate showed the highest P solubilizing activity but not significantly different when combined with TR-C2 isolate.

Keyword : synergism, bioassay, rice rhizosphere, saline soil ecosystem

1. Introduction
Around 407.4 Ha (5.03%) of the area of paddy fields in Indonesia is on tidal land [1]. The land was left by many farmers due to decreased productivity caused by soil fertility degradation [2]. One factor that causes a decrease in soil fertility in tidal land is a high level of salinity due to seawater intrusion [3]. Saline soil are usually found in arid and semi arid areas where irrigation system was performed...
Moreover, in 2050 it was expected that about 50% of arable land would be salinized [5]. Saline soil with electrical conductivity (EC) values of above 4 dS.m\(^{-1}\) has the potential to lose rice yields of 10-20%, moreover if the EC value above 6 dS.m\(^{-1}\) has the potential to lose 50% of rice yield [6].

Saline soils have the potential to exhibit toxic ions, osmotic stress, salt stress, deficiency and imbalance of nutrients availability (N, Ca, K, P, Fe, Zn) and inhibit the absorption of water from the soil [7-8]. Phosphorus element deficiency is one of the constraints on saline soil in tropics ecosystem [9]. Saline soil conditions also have been reported to cause decreased of phosphorus uptake by plant roots and decreased phosphorus availability due to low of soluble P by Ca-P fixation [10]. Phosphate is one of essential nutrient that plays important role in root, stalk and stem development, flower and seed formation, maturity and crop resistance [11].

Inoculation of saline soil indigenous bacteria is expected to be able to obtain superior bacteria that are able to maintain the saline soil fertility. Bacteria in the Plant Growth Promoting Rhizobacteria (PGPR) group isolated from the saline soil were reported to have saline tolerant characteristic or known as halotolerant bacteria [12]. One organism that plays role in supporting P nutrients supply on saline soils can be categorized as halotolerant phosphorizobacteria. Genera of *Pseudomonas, Bacillus, Rhizobium, Penicillium* and *Aspergillus* are known as superior P solubilizer [13]. These microbes are able to provide some essential nutrients, especially phosphorus element, which have an important role in maintaining plant resistance [14-15]. These P solubilizing microbes provide available P by transforming insoluble phosphate like Ca\(_3\)(PO\(_4\))\(_2\) to soluble phosphate (HPO\(_4^{2-}\) and H\(_2\)PO\(_4\)) with diverse mechanism, consist of acidification, chelation and exchange reactions [16]. Therefore, it is necessary to investigate and develop the halotolerant phosphorizobacteria isolates that isolated from saline soil so that they have the potential to be used as biofertilizer inoculants on saline soil conditions.

2. Materials and methods

2.1. Preparation of selected halotolerant phosphorizobacteria

Halotolerant phosphorizobacteria were isolated from rice rhizosphere on saline soil ecosystem in Karawang, West Java, Indonesia. Three selected isolates consist of K5, TR-C2 and CLR-A (Table 1.) were observed to find out its ability on phosphorus solubilizing and its compatibility to create an optimal consortium. Those selected isolates were refreshed on Pikovskaya agar media and incubated for 72 h at 30°C. The isolates were moved into the refrigerator for mother stock.

Table 1. Characteristic of selected halotolerant phosphorizobacteria

| No | Isolate | Colony Form | Cell Form | Gram | Fosfatase Activity (mg/g/h) |
|----|---------|-------------|-----------|------|-----------------------------|
| 1  | K5      | Round       | Basil     | Negative | 6.02                         |
| 2  | TR-C2   | Round       | Basil     | Negative | 21.11                        |
| 3  | CLR-A   | Round       | Basil     | Negative | 27.80                        |

2.2. Compatibility test of halotolerant phosphorizobacteria isolates

The isolate compatibility test was carried out qualitatively using filter paper disc method [17]. Filter paper disc with 5 mm of diameter was prepared then soaked into each suspension of selected isolate. The paper discs on each selected isolates were stuck directly on the center of nutrient agar (NA) media for single isolate treatment as positive control. The media on combined treatments were spread by selected isolate first against another selected isolate. K5 isolates were spread on the surface of NA media then TR-C2 and CLR-A discs were stuck on the media to obtain combination of K5+TR-C2 and K5+CLR-A treatment. The TR-C2 isolate were spread on the surface of NA media then CLR-A disc was stick on the media to obtain combination of TR-C2+CLR-A treatment. Each treatment were repeated three times and incubated for 72 h at 30°C. Inhibition zone were observed by observing the gap on isolate intersection.

2.3. Phosphorus solubilizing activity test

The phosphorus solubilizing test was carried out qualitatively by measuring the solubilizing index (SI) on Pikovskaya agar media [18] and quantitatively by measuring the available P in Pikovskaya broth.
Modified Pikovskaya media were prepared, containing 10 g.L\(^{-1}\) glucose, 5 g.L\(^{-1}\) Ca\(_2\)(PO\(_4\))\(_2\), 0.5 g.L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 0.2 g.L\(^{-1}\) KCl, 0.1 g.L\(^{-1}\) MgSO\(_4\), 0.1 g.L\(^{-1}\) MnSO\(_4\), 0.4 g.L\(^{-1}\) yeast extract in 1 liter of distilled water \[19\] and supplemented by 0.09 g.L\(^{-1}\) NaCl to adjust the EC value to 4 dS.m\(^{-1}\). Modified Pikovskaya agar was prepared about 15 ml on each petri dish. Isolate suspensions were prepared by adding 8 mL of selected inoculant into each petri dish for single isolate treatment as positive control. About 4 mL of each selected inoculant was mixed against other selected inoculant to obtain combination of K5+TR-C2, K5+CLR-A and TR-C2+CLR-A treatments. Filter paper discs were soaked into each isolate suspension and sticked onto centre of Pikovskaya media and incubated for 72 h at 30\(^\circ\)C. The growth of colony and halozone were observed by measuring solubilizing index with the formula:

\[
SI = \frac{\text{halozone diameter (mm) - colony diameter (mm)}}{\text{colony diameter (mm)}}
\]

Modified Pikovskaya broth were prepared by pouring 40 ml of media into a 100 ml erlenmeyer flask. About 4 mL of each selected inoculant was added into Pikovskaya broth (10% v/v) for single isolate treatment as positive control. About 2 mL of each selected inoculant was mixed against 2 mL of other selected inoculant to obtain combination of K5+TR-C2, K5+CLR-A and TR-C2+CLR-A treatments. The inoculants were shaken at 100 rpm for 5 d at room temperature and final media pH values were measured. After incubation, the inoculants were centrifuged at 7500 rpm for 15 minutes and 2,5 mL supernatant were taken. The P-availability of supernatant was analyzed by Bray II method (pH supernatant < 5.5) with observations at 889 nm wavelength \[20\].

2.4. Statistical analysis
Compatability test was not carried out with statistical analysis. The data of phosphorus solubilizing test were analysed by One Way ANOVA and the significances were analysed by Duncan Multiple Range Test (\(\alpha = 5\%\)) and supported by SPSS 21 software.

3. Results and discussion
3.1. Compatibility of selected halotolerant phosphorizobacteria isolate
The analysis result showed that both of the three selected isolates were compatible (Table 2). Those three selected isolates had the ability to grow simultaneously without exhibited the inhibition zone or gap on isolate intersection area (Figure 1). The selected halotolerant phosphorizobacteria were expected not to secrete an antibiotic compound that could inhibit other selected bacteria. The microbial incompatibility may occur if the microbial secrete some element that contains toxin compounds produced by these microbes \[21\]. The compatibility of \textit{Azospirillum brasilense} with \textit{Pseudomonas fluorescens} occurs because it has a similar life mechanism and support plant growth \[22\]. Based on the compatibility results it can be concluded that the three isolates were able to be combined without inhibiting the growth of these bacteria.

| Table 2. Compatibility of combination selected halotolerant phosphorizobacteris isolate |
|-----------------------------------------------|
| Isolate Combination       | Compatibility |
| K5                           | ✓              |
| TR-C2                        | ✓              |
| CLR-A                        | ✓              |
| K5 + TR-C2                   | +              |
| K5 + CLR-A                   | +              |
| TR-C2 + CLR-A                | +              |

(✓)Grow
(+ Compatible


3.2. Phosphorus solubilizing activity by selected halotolerant phosphorizobacteria isolate

The results of phosphorus solubilizing activity showed that there were differences in the ability of these bacteria on solubilizing index value and P-available by single isolate treatments compared with combine isolate treatments (Table 3).

**Table 3.** Phosphorus solubilizing activity by selected halotolerant phosphorizobacteria isolates

| Isolate Combination | Solubilizing Index Value | P-available (ppm P$_2$O$_5$) |
|---------------------|--------------------------|-------------------------------|
| Control             | -                        | 62.45 a                       |
| K5                  | 0.405                    | 292.40 d                      |
| TR-C2               | 0.219                    | 281.93 cd                     |
| CLR-A               | 0.214                    | 272.39 bcd                    |
| K5 + TR-C2          | 0.318                    | 284.25 cd                     |
| K5 + CLR-A          | 0.268                    | 253.29 B                      |
| TR-C2 + CLR-A       | 0.196                    | 264.69 bc                     |

The value followed by different letters were significantly different based on DMRT $\alpha = 5\%$

The highest solubilizing index value was found in single isolate K5 followed by combined isolates K5 + TR-C2 which were not significantly different compared with combined isolates K5 + CLR-A as described in Figure 2. Single isolates of K5 has a superior ability to solubilize P compared to other single isolates and able to increase the solubility of P when combined with other selected isolates (CLR-A and TR-C2). Combining of K5 isolates with other selected isolates were expected to be able to support in phosphate solubilizing with the addition of organic acids produced by these microbes. Organic acids produced by beneficial microbes have an important role in solubilizing phosphate elements [23]. The qualitative of P solubilizing ability was cannot fully interpret the ability of the bacteria to provide available P, therefore it is necessary to observe the P solubilizing ability of selected isolate quantitatively on Pikovskaya broth [24]. The results of P-available analysis showed that single K5 isolate still had the best P solubilizing ability (292.40 ppm) compared to the combined isolate treatment of K5 + CLR-A (253.29 ppm) and TR-C2 + CLR-A (264.69 ppm). Single isolate treatments showed the P solubilizing ability were not significantly different from each other.
The best isolate combination was found in the combination of K5 + TR-C2 isolates while other isolates combinations tended to reduce the ability P solubilizing as described in Figure 3.

Combining the selected halotolerant phosphorizobacteria isolates could affect the P solubilizing activity. Combining the bacterial isolates would have an impact on increasing or decreasing the activity of these bacteria. Factors that affect the activity of microbial consortium include secretion of toxin metabolites and depletion of nutrient sources such as nitrogen and phosphate sources [25]. Carbon sources that are less diverse in the media can also affect the performance of microbial consortium [26].
4. Conclusions
The selected halotolerant phosphorizobacteria (K5, TR-C2 and CLR-A) were compatible to grow in consortium condition but had a diverse impact on phosphate solubilizing activity. The best isolate combination was obtained on K5 isolate combined with TR-C2 isolate. The other combination could lead to decreasing of phosphate solubilizing activity compared with single isolate treatments. This conclusion leads to the importance of synergism and inoculant consortia compatibility to ensure optimal biofertilizer.

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