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To cite this article: Sulastri et al 2018 IOP Conf. Ser.: Earth Environ. Sci. 187 012024

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Induced systemic salt tolerance in rice by indigenous bacteria isolated from Java coastal plants under gnotobiotic system

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Abstract. Bacteria associated with plants grown in saline natural habitat provide an advantage to the host they colonize and their utilization would give a great benefit for alleviating the salt stress to the plants growing in such environment. With the objective to find the most promising bacterial strains to be used for induction of salt tolerance in agricultural crops, we evaluated 12 bacterial isolates that previously isolated from plants grown in the Java coastal area. Gnotobiotic system was performed to test the ability of isolates in enhancing salt tolerance in three different varieties of rice (Ciherang, IF8, Situbagendit) under different levels of NaCl. The result suggested that promising isolates for enhanced salt tolerance in rice seedling are E194-3, D150 and R146-6 for root endophyte, leaf endophyte and rhizoplane respectively. Isolate E194-3 (root endophyte) gave the best result at highest concentration of NaCl by increasing the root length, shoot length, fresh weight and dry weight 21%, 39%, 62% and 49% respectively (means from three rice varieties) in comparison to uninoculated control. This study suggested that gnotobiotic assay for evaluating rice seedling growth promotion by bacterial endophyte and rhizoplane under salt condition could be used for the selection of potential bacterial strains subjected to further testing of bacterial isolates to be used in inducing salt tolerance in rice under field conditions.

1. Introduction
Salt stress in coastal areas and irrigated land are one of the main factors decreasing agriculture production in Indonesia. The rice field is the most affecting by salt stress. An approximately 1,600,000 ha of rice harvesting area could be in danger due to a 1 m sea level rise in Indonesia because of inundation and increased soil salinity [1]. Rice is one of the most important food crop in Indonesia [2]. Therefore, it is becoming increasingly important to utilize sustainable techniques for inducing salt tolerance in rice for better adapted to salt stress.
Recent studies reported that many plant species have ability to adapt to salinity stress because of their association with certain microbes. Yang et al. 2009 [3] has projected the termed of this phenomenon as “induced systemic tolerance” (IST) for PGPR-induced physical and chemical changes in plants resulting in enhanced tolerance to abiotic stresses. The development of salt tolerant crops through molecular engineering and plant breeding is very important, but it is not an economical approach to sustainable agriculture, while bacterial inoculation to alleviate salt stress is an economical technology that minimizes budgets and environmental risk [4]. This biological approach of plant-microbe interaction to alleviate salt stress in agricultural crops has recently gained a great interest of researchers in the world [5,6,7,8,9].

Microorganism associating with plant growth in extreme habitats is very important to the plant fitness and plant species diversity. One group of microorganism includes the bacterial endophytes, which are defined as bacteria that colonize healthy plant tissue without causing obvious symptoms in or produce obvious injury to the host [10]. The major benefits for host plants partnering with endophytic microbes can include enhanced nutrition and improved tolerance to biotic and abiotic stress [11]. Bacterial endophyte that found in association with plants grown under high soil salinity might have been adapted to the stress conditions and could provide a significant benefit in inducing salt tolerance to the host they colonized over non-infected plants. A coastal area with high soil salinity are natural habitats of halophilic/halotolerant bacteria [12]. The Java coastal area is unique due to the presence of natural sea water that intruded into the area which embraces diverse microorganisms due to extreme conditions of soil salinity, swamped and ultraviolet irradiation. Therefore, isolation of bacteria associating with plants grown in this ecosystem habitat and their utilization would give a great benefit for alleviating salt stress. Their unique properties would be acted as key factors in the mechanisms responsible for plant microbe interaction in the saline environments. This mechanism of ability for stress tolerance and survival of plants induced by bacterial endophyte can be transferred to other plants. However, the degree of efficacy of bacteria to enhance plant growth under stress condition could be different within crops, varieties or species, plant growth stage, cultural conditions, inoculant strains and other environmental factors [11,5,13].

In order to get the significant benefit of salt tolerance induction by bacterial endophyte as well as rhizoplane, the selection of the most promising and effective salt tolerance inducing bacteria is a necessity. The in vitro screening procedure appears to be very effective and less time consuming. The in vitro screening under gnotobiotic system in the laboratory studies are usually performed using an enclosed tube culture system to exclude microbial contaminants from the roots under climate-controlled growth chamber conditions [14]. The use of gnotobiotic systems for selecting the best strain also would give an advantage to control the environment that reduced other microbial competence effects and minimized environmental fluctuations that affecting the population of the microorganism under study [15,16]. The aim of the research is to find the most promising bacterial strains to be used for induction of salt tolerance in agricultural crops. In this study, 12 bacterial isolates that previously isolated from endemic plants of the Java coastal area were studied. Gnotobiotic system was performed to test the ability of these isolates in enhancing salt tolerance in three different varieties of rice i.e. Ciherang, IF8, Situbagenditm under different levels of NaCl.

2. Materials and Methods

2.1. Preparation of bacterial Cultures

Twelve salt tolerant bacterial strains i.e. 4 rhizoplane bacteria (R146-6; R188-2; R146-3; R155-11); 4 root endophyte bacteria (E194-3; E102-1; E203-1; E196-1) and 4 leaf endophyte bacteria. These isolates were isolated from plants grown in saline soil of the Java coastal area from the previous study (Table 1). Bacterial strains were routinely grown on tryptic soy agar (TSA) medium supplemented with 1 M NaCl. For inoculation used, each bacterial strain was grown in 250 mL flask containing 100 mL tryptic soy broth (TSB) for 48 h at 30 °C under shaking (150 rpm) conditions.
Optical density was measured and a population of $10^8 - 10^9$ colony forming unit (CFU) per mL of different strains was maintained prior to seedling inoculation.

2.2. Gnotobiotic assay
The in vitro study of salt tolerance inducing bacteria in rice was done under gnotobiotic conditions using the method described by Simons et al. 1996 [11] with modification. The experiment was carried out in test tubes (25 mm in diameter, 200 mm in length) containing 10 g of a sterilized mixture of washed quartz sand and vermiculite (1:1) saturated with an appropriate of Yoshida nutrient solution [17]. Salinity conditions were established by adding 50; 100; 150 and 200 mM NaCl into the Yoshida nutrient solution. Rice seeds from three different rice variety, i.e. Ciherang, Situbagendit and IF8 (from AB2TI) were surface sterilized by treatment with 70 % ethanol for 1 min followed by 5 % sodium hypochlorite solution for 5 min and then washed for 5 times with sterile water. Surface-sterilized rice seeds were treated with pure cultures of 12 selected bacterial strains described above with the density of $10^8-10^9$ CFU mL$^{-1}$ in 0.85 % sterilized saline solution for 24 h. Control seeds were incubated in 0.85 % sterilized saline solution for 24 h. Inoculated seeds were transferred to test tubes as described above for each treatment and enclosed with plastic plugs. Tubes were arranged using completely randomized design with ten replications for each treatment. Plants were incubated in a growth room with temperatures maintained at ±25°C, with a cycle of 12 h dark/light. After 14 days, root length, shoot length, fresh weight and dry weight of rice seedlings were recorded.

2.3. Characterization of plant growth promoting (PGP) traits
All bacterial strains were tested for production of indole acetic acid (IAA). IAA production was detected as described by Brick et al. 2004 [18]. Bacterial cultures were grown for 72 h in nutrient broth media at 36-38°C. Fully grown cultures were centrifuged at 5000 rpm for 10 min. The supernatant (2 mL) was mixed with 2 drops of orthophosphoric acid and 4 mL of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl$_3$ solution). Tube then incubated for 20 min at room temperature before the absorbance was measured at 527 nm (Shimadzu UV Probe). The concentration of each sample was calculated from a standard plot ranging from 0.5-30 µg mL$^{-1}$ pure IAA (Sigma). The ability of the isolates to produce ACC deaminase was screened on minimal media containing ACC as their sole nitrogen source as described by Penrose and Glick 2003 [19]. Optical density (OD) was measured after 48 h at 540 nm by spectrophotometer and considered as an index for evaluating ACC deaminase producing isolates. Isolates with OD more than 0.6 indicated positive for ACC deaminase production. Phosphate solubilization of isolates was evaluated for the ability to solubilize inorganic phosphate. Pikovskaya’s agar medium containing calcium phosphate as the inorganic form of phosphate was used in the assay. A loopful of bacterial culture were streaked on the plates and kept for incubation at 28°C for 4-5 days. The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria [20]. Production of hydrogen cyanide (HCN) was detected according to the method of Lorck 1948 [21]. The nutrient agar medium was amended with 4.4 g glycine/L and bacteria were streaked on this agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate and 0.5% picric acid and placed in the lid of a petri plate inoculated with bacterial isolates. The plates were incubated at 28-30 °C for 5 days. HCN production was assessed by the color change of yellow filter paper to reddish brown. Ammonia production was detected using method describe by Cappucino and Sherman 1992 [22]. Freshly grown culture were inoculated in 10 mL peptone water in each tube and incubated for 48-72 h at 28-30 °C. Each tube then added with 0.5 mL of Nessler’s reagent. Development of brown to yellow color was a positive for ammonia production. For exopolysaccharide (EPS) activity (qualitative), strains were grown on Weaver mineral media enriched with glucose and production of EPS was assessed visually [23]. The EPS production was visually seen as the formation of fluffy material on the plates after 48 h of incubation at 28-30 °C.
2.4. Characterization of hydrolyzing enzyme activity

Amylase activity was determined on agar plates following the protocol of Männistö and Häggblom 2006 [24]. Formation of an opaque halo around colonies indicated amylase activity. Cellulase activity was assayed on plates containing 5 g L\(^{-1}\) of carboxymethyl cellulose, 1 g of peptone and 1 g of yeast extract. After incubating for 48 h at 28-30 °C the plates were overlayed with congo red (1 mg mL\(^{-1}\)) solution for 30 min. Congo red solution was then poured off, followed by washing the surface of the plate with 1 M NaCl solution [25]. Chitinase activity of the isolates was determined as zones of clearing around colonies following the method of Chernin et al. 1998 [26]. Protease activity was determined using 1 % skimmed milk agar plates, formation of halo zone around colonies was used as indication of activity [27]. Pectinase activity was determined using method described by Mateos et al. 1992 [28]. Bacterial isolates were spotted on nutrient agar supplemented with 0.5% pectin for the assay of pectinase. After incubating the plates at 30 °C for 5 days the surface of the medium was overlayed with 2% hexadecyl trimethyl ammonium bromide (CTAB) solution for 30 min. CTAB solution was then poured off and the surface of the plate was washed with 1 M NaCl to visualize the halo zone around the bacterial growth.

2.5. Statistical analyses

All results presented are the means of ten replicates. Data were subjected to statistical analysis. The mean difference comparison between the treatments was analyzed by analysis of variance (ANOVA) and subsequently by Tukey’s tHSD test function (α=0.05) of the agricolae package from R [29].

3. Results

3.1. Gnotobiotic assay

In the previous work, different strains of bacteria associating with plant grown in the Java coastal area were found to differ greatly in their ability to promote seedling growth of rice seedling under salt stress. The screening was performed based on the relative growth rate of rice seedling. Twelve strains that have selected to be used in this study had the potential to improve rice seedling growth under axenic salt stress conditions. The list of this twelve isolates were presented in Table 1.

Gnotobiotic assay of rice seedling under saline condition performed in a test-tube growth chamber using arsenic three rice seed varieties i.e. Ciherrang, Situbagendit and IF8 (AB2TI Gene Bank). For rice variety of Ciherrang, isolate D150 significantly increased shoot length, fresh weight and dry weight of seedling at a concentration of 50 mM (54%, 103% and 65% higher than control). Isolate D150 also significantly increase fresh weight of Ciherrang rice seedling at 100 mM by 67% and significantly increased fresh weight at 150 mM and 200 mM by 100% and 79% respectively over uninoculted control. Shoot length and dry weight of Situbagendit rice seedling increased significantly as inoculated by isolate D150 at 50 mM up to 25% and 9 % over the control. While isolate E194-3 significantly increased fresh weight at 50 mM and dry weight at 150 mM that was 30% and 40% greater than control. At concentration NaCl 100 and 150 mM, isolate D102-1 significantly increased fresh weight of Situbagendit rice seedling that was 49% and 41% over control. While at concentration NaCl 200 mM isolate E194-3 significantly increased shoot length, root length and dry weight 39%, 52% and 25%, respectively, and isolate D102-1 significantly increased fresh weight by 69% over control of Situbagendit rice seedling. Isolate E196-1 and R146-6 have 25% dry weight significantly higher than control for Situbagendit rice seedling at 200 mM NaCl.

Isolate R146-6 significantly increased root length at 50 mM and shoot length at 150 mM for IF8 rice seedling that was 62% and 67% respectively. Isolate E194-3 significantly increased dry weight at 50 mM that was 13% greater than control while isolate E203-1 significantly increased dry weight of IF8 rice seedling at 100 mM for up to 16% over the control. At a concentration of 150 mM, isolate D102 has significantly increased fresh weight of IF8 rice seedling by 96% and follow by isolate R146-6 that was 52% greater than control. Isolate R146-6 also significantly increased dry weight of IF8 rice seedling by 78% over control at 150 mM ACL. At concentration of 200 mM, isolate E194-3
significantly increased shoot length, root length, fresh weight and dry weight of IF8 rice seedling by 40%, 77%, 101% and 70% respectively. It seems that each isolate demonstrated specific effect at different level of salinity. Isolate E194-3 gave the best result at highest concentration NaCl of 200 mM in all varieties of rice by increasing the root length, shoot length, fresh weight and dry weight 21%, 39%, 62% and 49% respectively that calculated by means of three rice varieties in comparison to uninoculated control (Table 2).

Table 1. List of bacterial isolates used in this study

| Isolates  | Source |
|----------|--------|
| R146-6   | rhizoplane of Oryza sativa |
| R188-2   | rhizoplane of Euphorbia vermiculata |
| E194-3   | root endophyte of Spangnolica trilobata |
| E196-1   | root endophyte of Rhizopora stylosa |
| D102-1   | leaf endophyte of Cromolaena odorata |
| D150     | leaf endophyte of Rhizopora sp |
| R55-11   | rhizoplane of Cactacea family |
| E109-1   | root endophyte of Vigna radiate |
| R146-3   | rhizoplane of Oryza sativa |
| E203-1   | root endophyte of Ipomea pescaprae |
| D205-1   | leaf endophyte of Cromolaena odorata |
| D183-4   | leaf endophyte of Portulaca oleracea |

Table 2. Effect of bacterial inoculation on growth of rice seedling of three varieties (CHR=Ciherang; SBT=Situbagendit; IF8) at 200 mM NaCl

| Isolates  | CHR Root length | SBT Root length | IF8 Root length | CHR Shoot length | SBT Shoot length | IF8 Shoot length | Fresh weight | Dry Weight |
|-----------|-----------------|-----------------|-----------------|------------------|-----------------|-----------------|--------------|-----------|
| CHR       | SBT             | IF8             | CHR             | SBT             | IF8             | CHR             | SBT          | IF8       |
| R146-6    | 5.8a            | 6.65ab          | 6.4ab           | 10.3bcd          | 11.55ab         | 11.3ab          | 0.013ab      | 0.021ab    | 0.0176defg  |
| R188-2    | 6.25a           | 5.8abc          | 6.6ab           | 10.3bcd          | 8.1ab           | 8.86cde         | 0.017abc     | 0.019bc    | 0.018cefg   |
| E194-3    | 6.25a           | 7.25a           | 7.2a            | 14.8a            | 12.3a           | 12.6a           | 0.019ab      | 0.018bc    | 0.028a      |
| E196-1    | 6.63a           | 5.6abc          | 6.6ab           | 10.9ab           | 11.75a          | 10.54cde        | 0.014bcde    | 0.017cd    | 0.021bcde   |
| D102-1    | 6.22a           | 5.7abc          | 6.2ab           | 10.6bc           | 10.6ab          | 12ab            | 0.016abcde   | 0.022a     | 0.0086cde   |
| D150      | 4.6a            | 7ab             | 6.3ab           | 12.05ab          | 9.85ab          | 9.2cde           | 0.02a        | 0.019bc    | 0.025ab     |
| R55-11    | 5.75a           | 5.8abc          | 5.4ab           | 6.25d            | 9.7ab           | 7.4cde           | 0.00306      | 0.014defg  | 0.020bcdef  |
| E109-1    | 5.7a            | 5.5abc          | 5.5ab           | 7.3cd            | 7.5b            | 6.4e             | 0.0105de     | 0.012        | 0.023bcde   |
| R146-3    | 6.9a            | 6.2abc          | 5.5ab           | 6.2cd            | 7.2b            | 6.5e             | 0.0101de     | 0.0095f    | 0.019bcde   |
| E203-1    | 6a              | 6.4ab           | 6.5ab           | 7.9cd            | 8.5ab           | 8.6bcde          | 0.012cde     | 0.011f     | 0.024abcde  |
| D205-1    | 6.29a           | 6.6ab           | 5.3ab           | 8.1cd            | 11.67ab         | 6.2e             | 0.011de      | 0.016cde   | 0.015fg     |
| D183-4    | 5.2a            | 4.1c            | 6.1ab           | 8.9cd            | 9.7ab           | 8.8cde           | 0.011de      | 0.014defg  | 0.0068cde   |
| Control   | 5.8a            | 5.3bc           | 5.3b            | 6.7cd            | 8.2ab           | 7.3de            | 0.013de      | 0.013fg    | 0.0061d     |

Mean values sharing the same letter (s) in the column do not differ significantly according Tukey’s HSD test α=0.05

3.2. Characterization of PGP traits and hydrolyzing enzyme activity

All isolates were characterized for all PGP traits and hydrolyzing enzyme activity, the result is summarized in Table 3. Seven isolates had ACC deaminase activity, 12 isolates produced more than 10 µg/mL IAA, 6 isolates have the ability to solubilize phosphate and produce HCN respectively. Isolate E109-4 and R188-2 produced exopolysaccharide. All isolates produced NH₃ except isolates D205-1 and R55-11 were not produced NH₃. Isolate E109-2 was identified an ability to fix nitrogen.
Figure 1. Effect of bacterial inoculation in Ciherang rice seedling at different salt concentration applied (0; 50; 100; 150 and 200 mM)
Figure 2. Effect of bacterial inoculation in Situbagendit rice seedling at different salt concentration applied (0; 50; 100; 150 and 200 mM)
Figure 3. Effect of bacterial inoculation in IF8 rice seedling at different salt concentration applied (0; 50; 100; 150 and 200 mM)
All isolates were able to degrade pectin, 9 isolates produced amylase, 8 isolates produced protease, 4 isolates were able to degrade cellulose and 7 isolates were able to degrade chitin. All isolates were demonstrated multiple PGP traits.

**Table 3.** Characterization of PGP traits and hydrolyzing enzyme activity of bacterial isolates (ACC=ACC deaminase, IAA; PO₄=phosphate solubilizing; HCN; NH₃= ammonia; Pro=protease; Amy=amylase; Chit=chitinase, Cell= cellulase; EPS= exopolysaccharide; N₂-Fix= N₂ fixing; Pec=pectinase)

| No | Isolates | AC C | IAA (μg/ml) | PO₄ | HCN | NH₃ | EPS | N₂-fix | Amy | Chit | Cell | Pro | Pec |
|----|----------|------|-------------|-----|-----|-----|-----|--------|-----|------|------|-----|-----|
| 1  | D150     | -1   | 19.442      | +   | +   | -   | -   | +      | +   | +    | +    | +   | +   |
| 2  | R146-3   | -1   | 16.773      | +   | +   | -   | -   | +      | +   | +    | -    | +   | +   |
| 3  | E109-2   | -1   | 17.738      | +   | -   | -   | -   | +      | +   | +    | +    | -   | +   |
| 4  | D205-1   | +    | 35.102      | -   | +   | -   | -   | -      | +   | +    | +    | +   | +   |
| 5  | D183-4   | +    | 0.945       | -   | -   | +   | -   | -      | -   | -    | +    | +   | +   |
| 6  | R55-11   | -1   | 10.404      | +   | -   | -   | -   | -      | +   | -    | +    | +   | +   |
| 7  | D102-1   | -1   | 4.077       | -   | +   | -   | -   | -      | -   | +    | +    | +   | +   |
| 8  | E196-1   | +    | 40.692      | -   | +   | -   | -   | +      | +   | -    | +    | +   | +   |
| 9  | E194-3   | +    | 34.390      | -   | -   | +   | -   | -      | +   | -    | -    | +   | +   |
| 10 | R146-6   | +    | 11.173      | -   | +   | -   | -   | -      | -   | +    | +    | +   | +   |
| 11 | R188-2   | +    | 34.670      | -   | -   | +   | -   | -      | +   | -    | -    | +   | +   |
| 12 | E203-1   | +    | 18.068      | -   | +   | -   | -   | -      | -   | -    | +    | +   | +   |

**Figure 4.** Gnotobiotic system used for assay of rice seedling growth under saline

### 4. Discussion

Inoculations of indigenous bacterial isolates from Java coastal plants into three rice varieties significantly improved rice seedling growth under saline condition. This improvement may be due to the production of PGP traits by the isolates. Each isolate has demonstrated a specific effect at different rice varieties and levels of salinity. Andreote et al. 2010 [13] reported that different cultivars of the same plant species and plant growth stage affect the composition of associated bacterial communities because of differences in plant metabolism that result in different growth stimulate. In other hand culture condition and environmental factor have also affected to the bacterial community in the plant-
bacterial interaction. For three rice varieties, isolate E194-3 performed the best in regard to rice growth seedling parameters. This result may be in correlated with our finding that isolate E194-3 showed the ability to produce ACC deaminase, IAA and ability to solubilize phosphate that are known as a mechanism in induced systemic salt tolerance in plant [5,30,31,7]. Isolate E194-3 also produced exopolysaccharides (EPS). Exopolysaccharides has identified protects microorganisms from osmotic stress and fluctuations in water potential by enhancing water retention and regulating the diffusion of carbon sources in the microbial environment [32]. Isolate D183-4 demonstrated a poorest seedling growth in three of rice varieties. This result correlated with the lowest of IAA production of isolate D183-4 as well it potential PGP traits that has been characterized. It is better to screen bacteria for the most promising isolates having suitable colonization and PGP traits when selecting bacteria as bioagent to promote plant growth [33]. PGP traits that assessed under gnotobiotic system would be beneficial for selecting the most promising bacterial strains that could lead to increased plant growth [34].

Based on the most promising in promoting rice seedling growth and the ability to produce multiple PGT traits, we suggested that promising isolates for enhanced salt tolerance in rice seedling are E194-3, D150 and R146-6 for root endophyte, leaf endophyte and rhizoplane respectively. In general, isolate E194-3 is the best isolate in enhancing salt tolerance in rice seedling. Efficient establishment of induced systemic salt tolerance in rice by endophytic and rhizoplane isolates was demonstrated with a gnotobiotic system using axenic rice seedling. Therefore a gnotobiotic system under controlled condition complementary with identification of PGP potential suggested as a powerful tool for efficient screening of potential bacterial strains subjected to further testing of bacterial isolates to be used for inducing salt tolerant in rice under field conditions.

Acknowledgments
This research was supported by the Indonesia Endowment Fund for Education (LPDP), Indonesia Ministry of Finance. The authors wish to thank the Director of Centre for Plant Production Technology (PTPP), Agency for The Assessment and Application of Technology (BPPT) for providing the necessary facilities for this study. In addition, we are thankful to the Chairman of Indonesian Association of Seed Banks and Farming Technology (AB2TI), Indonesia for providing the rice varieties.

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