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The Endophyte Bacteria Isolated from Java Coastal Plants Promote Plant Growth and Induce Salt Tolerance of Two Rice Varieties

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Abstract:
Bacteria associated with plants grown in saline natural habitats may be a key factor in the mechanisms responsible for some plants' tolerance of saline environments. To be able to utilize this plant-microbe interaction to alleviate the salt stress on plants growing under saline conditions would be of great benefit to crops like rice grown in coastal areas subject to salt water intrusion due to rising sea water levels. With the objective of finding the most promising bacteria that were able to induce salt tolerance in plants growing in tidal coastal areas of Indonesia, we evaluated 9 bacterial isolates that had previously been isolated from plants grown in the Java coastal area. A Greenhouse trial was carried out to evaluate the ability of these isolates to improve salt tolerance in two rice varieties (Ciherang and IF8) under different concentrations of NaCl. The results suggest that promising bacterial isolates E194-3, D205-1 and R146-6 might enhance salt tolerance in rice plants for root endophyte, leaf endophyte and rhizoplane respectively. These isolates gave the best effect on plants height, shoot dry weight, root length and root dry weight in both rice varieties tested Ciherang and IF8.

Keywords: *Salt tolerance, leaf endophyte bacteria, rhizoplane bacteria, root endophyte bacteria*

1. Introduction

Salt stress is becoming a significant factor constraining agricultural production in Indonesia especially in coastal areas, where rice is the crop most affected by salt stress. An increase of 1 m in average sea level would threaten the yield over approximately 1,600,000 ha of rice fields in Indonesia because of inundation and accumulation of salt in the soil (Nicholls and Mimura 1998). Rice is one of the most important food crops in Indonesia (Asian Development Bank 2009). Hence, it is becoming increasingly important to develop sustainable techniques to manage the threat. Inducing salt tolerance in rice so the crop is better adapted to salt stress is one strategy being considered.

The use of beneficial microbes such as plant growth promoting bacteria (PGPB) to improve salt tolerance in the plant is one of the prospective technologies that could be considered for rice cultivation under saline soil conditions (Mayak et al., 2004). Within the scope of PGPB, rhizosphere bacteria or endophytic bacteria might fill the role of beneficial microbes. Bacterial endophytes are bacteria that can be found on surface sterilized plant tissue and can be widespread within plants where they colonize the inner spaces of all plant compartments, but do not cause plant disease or significant morphological changes (Hallmann et al., 1997; Rosenblueth and Martínez-Romero, 2004). Tolerance to abiotic stress in plants can be induced by rhizobacteria through various mechanisms that include physical and chemical changes in the plant host which has been defined as an induced systemic tolerance (IST) by Yang et al. (2009). The effective and efficient of bacterial colonization of halotolerant bacterial inoculant into a broad range of agricultural crops is one of the key ways to determine inoculum efficacy for agricultural practices (Compant et al., 2005).

Microorganism associating with plants in this way grow in extreme habitats to contribute significantly to the plant's robustness and diversity among plant species. One group of microorganisms that behave in this way includes the bacterial endophytes, which are defined as bacteria that colonize healthy plant tissue without causing obvious symptoms or produce obvious injury to the host (Rosenblueth and Martınez-Romero, 2006). The major benefits for host plants partnering with endophytic microbes can include enhanced nutrition and improved tolerance to biotic and abiotic stresses (Hallmann, 1997). Bacterial endophytes found in association with plants grown under high soil salinity might have been adapted to those stressful conditions and may provide a significant benefit by inducing salt tolerance in the host plants they colonize.

A coastal area with high soil salinity is the natural habitat for halophilic/halotolerant bacteria (Lichfield, 2002). The Java coastal area is unique due to the presence of sea water that has intruded into the area where a significant number of diverse microorganisms can be found due to extreme conditions of soil salinity, swampy land, and high ultraviolet radiation. Therefore, isolating bacteria associating with plants growing in this ecosystem habitat might be of considerable benefit in alleviating salt stress in commercial crops growing in the coastal zone.

The unique properties of these bacteria could be a key factor in the mechanisms responsible for the plant microbe interactions that lead to salt tolerance in these saline environments. This ability for tolerance of stress and survival of plants induced by bacterial endophytes can be transferred to other plants. However, the efficiency of bacteria to enhance plant growth under stressed condition could be different among crops, varieties or species, plant growth stage, cultural conditions, inoculant strains and other environmental factors (Hallmann et al., 1997; Mayak et al., 2004; Andreote et al., 2010). In order to get the significant benefit of induced salt tolerance by bacterial endophytes and rhizoplanes, the most promising and effective salt tolerance inducing bacteria need to be selected as a mandatory, preliminary step. The aim of the research reported here was to find the most promising bacterial strains to be used for induction of salt tolerance in rice. In this study, we evaluated nine bacterial strains isolated from endemic plants from the Java coastal area that had been evaluated previously under gnotobiotic system of their ability to enhance rice growth under salinity stress. The green house trial reported here was carried out to characterize the ability of these isolates to induce salt tolerance in two varieties of rice i.e. Ciherang and IF8 under different levels of NaCl concentration.

2. Material and Methods

2.1. Preparation of Bacterial Cultures

Nine salt tolerant bacterial strains i.e. three rhizoplane bacteria (R146-6; R188-2; R55-11); three root endophyte bacteria (E194-3; E203-1; E196-1) and three leaf endophyte bacteria (D205-1; D150; D183-4) were tested in the experiment. These isolates were taken from plants growing in saline soil of the Java coastal area as part of a previous study (Table 1). These bacterial strains were routinely grown on tryptic soy agar (TSA) medium supplemented with 600 mM NaCl. For inoculation, each bacterial strain was grown in a 250ml flask containing 100 ml tryptic soy broth (TSB) for 48 h at 30^{o}C while continually shaken (150 rpm). Optical density was measured and a population of 10^{8} – 10^{9} colony forming units (CFUs) per mL of each different strain was maintained prior to seedling inoculation. In the previous study, different strains of bacteria associating with plant growing in the Java coastal area were found to differ greatly in their ability to promote seedling growth of rice when they were under salt stress. The screening of these bacteria was based on the relative growth rates of inoculated rice seedlings. The list of these isolates and their source/host is presented in Table 1.

Isolates	Source
R146-6	rhizoplane of Oryza sativa
R188-2	rhizoplane of Euphorbia vermiculata
E194-3	root endophyte of Spagneticola trilobata
E196-1	root endophyte of Rhizopora stylosa
D150	leaf endophyte of Rhizopora sp
R55-11	rhizoplane of Cactacea
E203-1	root endophyte of Ipomea pescaprae
D205-1	leaf endophyte of Cromolaena odorata
D183-4	leaf endophyte of Portulaca oleraceae

Table 1: List of Bacterial Isolates Used in This Study

2.2. Characterization of plant growth promoting (PGP) traits

2.2.1. Indole Acetic Acid Production

The nine selected bacterial isolates were characterized for their ability to produce indole acetic acid (IAA) using the method as described by Brick et al. (2004). 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 FeCl3 solution). The mixture was then incubated for 20 min at room temperature, followed by measurement of light absorbance 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).

2.2.2. Production of (ACC) deaminase

The ability of the isolates to produce ACC deaminase was measured on the media containing ACC as the sole nitrogen source following the method described by Penrose and Glick (2003) with little modification other than to reduce the ACC concentration. 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 ACC deaminase production.

2.2.3. Phosphate Solubilization

The bacterial isolates were evaluated for their ability to solubilize inorganic phosphate using Pikovskaya's agar medium containing calcium phosphate as the inorganic form of phosphate. A loopful of bacterial culture was streaked on the plates and kept for incubation at 28°C for 4-5 days. The appearance of a transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria (Pikovskaya, 1948).

2.2.4. Production of Hydrogen Cyanide

Production of hydrogen cyanide (HCN) was detected according to the method of Lorck (1948). The nutrient agar medium was amended with 4.4 g/L glycine and bacteria were streaked on this agar plate. A Whatman filter paper No.1 soaked in 2% sodium carbonate and 0.5% picric acid solution and placed in the lid of a petri plate was 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.

2.2.5. Production of Ammonia

Ammonia production was detected using the method described by Cappucino and Sherman (1992). Freshly prepared culture was inoculated into 10 ml peptone water in each tube and incubated for 48-72 h at 28-30°C. Each tube then had 0.5 ml of Nessler's reagent added. Development of brown to yellow colour was a positive sign of ammonia production.

2.2.6. Production of Exopolysaccharide

For evaluating the exopolysaccharide (EPS) activity quantitatively, bacterial cultures were grown on weaver mineral media that was enriched with glucose and production of EPS was assessed visually (Weaver et al, 1975). The EPS production was detected by the formation of fluffy material on the plates after 48 h of incubation at 28-30°C.

2.2.7. Ability to Fixed Nitrogen

Fresh bacterial culture from TSB medium was inoculated in test tube containing 4.5 ml of Nfb (Nitrogen free bromothymol) semisolid media (Döbereiner, 1995). The inoculated tubes then were incubated at 28-30°C for 48 h and observed the growth by the formation of pellicles. Appearance of pellicle formation on Nfb semi-solid medium indicated that bacterial isolate has ability to fixed nitrogen.

2.3. Greenhouse Test Assay

The greenhouse experiment was conducted in plastic pots containing 500 g of soil: sand (1:1 w/w). There was no hole in the bottom of the pots to stop leaching. The salinity treatments, i.e., 0, 25, 50, 75, 100, 150 and 200 mM NaCl were developed using the following formula to calculate the amount of NaCl salt in each pot as described by Shaw (1994):

EC_{Cl}= 6.64 x %Cl (per weight of soil)…… (1)

%Cl= ($mgCl^-$/kg soil)*10^{-4} …… (2)

1dS/m=10 mM NaCl…… (3)

Amount of NaCl added in each pot were 25 mM=0.78 g; 50 mM=1.55 g; 75 mM=2.17 g; 100 mM=3.10; 150 mM=4.65g dan 200 mM=6.20 g and mixing it with mechanical mixer. Control pots were maintained with no plants as reference pots to check the effect of irrigation water on salinity levels. EC was monitored regularly in the reference pots but only measured in the experimental pots at the end of the trial. There was no significant change in the salinity levels in any of the experimental pots at the end of the trial. Rice seedlings of Ciherang and IF8 varieties (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 9 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 sterile soil compost germination medium. After 14 days, seedling was transferred to pot containing salinity treatment medium as described above and keep for one plant per pot. Each treatment was made in 5 replicates. The pots for each treatment were irrigated as it need for rice plants growth and was monitor and maintained. Pots were were arranged in a greenhouse with ambient light and temperature. Plants were uprooted after 30 days after planting. The plant height, root length, were recorded.

2.4. 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 Duncan's Multiple Range Test (α=0.05) of the agricolae package from R (R Core Team, 2013).

3. Result and Discussion

3.1. Characterization of Plant Growth Promoting Traits

The result of plant growth promoting characterization is summarized in Table 1. Except isolate D150 and R55-11, all isolates had ACC deaminase activity under condition testing in this study. Bacteria that produce the enzyme ACC deaminase can cleave ACC to form αketobutyrate and ammonium and therefore reducing the level of ethylene of the plants

under salinity stress (Glick, 2005). Isolate D183-4 did not synthesize auxins, while other isolates synthesized auxin by more than 10 µg/ml IAA. Under conditions tested, isolate D150, E196-1 and E194-3 have the ability to solubilize phosphate. Isolate D150, D205, R55-11, R146-6, and E203-1 produce HCN. Isolate E194-3 and R188-2 produced exopolysaccharide. Only isolates D205-1 and R55-11 were not produced NH_3. No isolate has the ability to fixed nitrogen (N_2) under this condition tested. All isolates demonstrated multiple PGP traits. Bacteria that produced multiple PGP traits and able to colonize a broad range of hosts spectrum is one of the preferences in the selection of the most promising bacterial strain that could give a huge beneficial to increased plant growth under stress conditions (Bal et al, 2013).

No	Isolates	ACC	IAA (µg/ml)	PO_4	HCN	NH_3	EPS	N_2-fix
1	D150	-	19.442	+	+	+	-	-
2	D205-1	+	35.102	-	+	-	-	-
3	D183-4	+	0.945	-	-	+	-	-
4	R55-11	-	10.404	-	+	-	-	-
5	E196-1	+	40.692	+	-	+	-	-
6	E194-3	+	34.390	+	-	+	+	-
7	R146-6	+	11.173	-	+	+	-	-
8	R188-2	+	34.670	-	-	+	+	-
9	E203-1	+	18.068	-	+	+	-	-

Table 2: Characterization of PGP Traits and Hydrolyzing Enzyme Activity of Bacterial Isolates (ACC=ACC Deaminase, IAA; PO_4=Phosphate Solubilizing; HCN; NH_3= Ammonia; 1EPS= Exopolysaccharide; N_2-Fix= N_2 Fixing; Pec=Pectinase)

3.2. Effect Of Bacterial Inoculation under Greenhouse Test

The result showed that after 3 weeks of planting, no plant was surviving at pot with salinity concentration of NaCl 200 mM except the 13.3% of rice plants from Ciherang variety that inoculated with isolate D205-1. A control plants for both of rice variety were all died at the concentration of NaCl 75 mM after 30 days of planting, while rice plants inoculated with bacterial isolates were 40% to 100% surviving at concentration of NaCl 0-100 mM. Amount of 6.7-46.7% plants were surviving at 150 mM of NaCl concentration and 0-13% were surviving at 200 mM of NaCl concentration after 30 days of planting (figure 1 and 2). This result indicating that increased in NaCl concentration has significantly decreased plant growth and inoculation of bacterial isolate increased the plants survival rate. This result suggested that bacterial isolates could decrease the detrimental effects affected by salinity, accordingly increasing plants growth and survival under salinity stress.

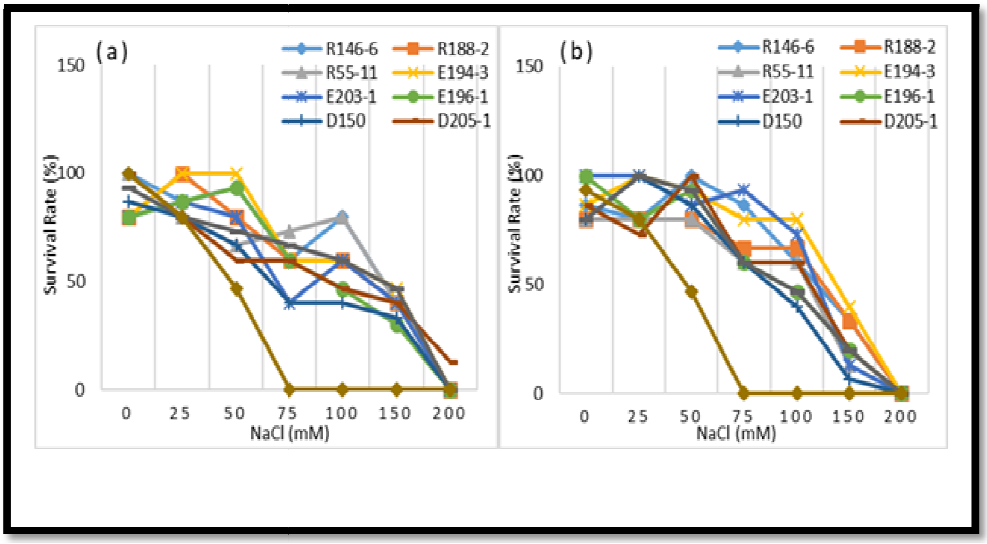

Figure 1: Effect of NACL Treatment and Inoculation of Bacterial Isolates on Survival Rate of (A) Ciherang and (B) IF8 Rice Plants

Salinity treatment significantly decrease plant height and inoculation of bacterial isolate positively affected plant growth of both rice variety at 30 days after planting. Isolate D205-1 gave the best effect on rice plants growth of Ciherang variety under saline, which increased plant height of 88.81% over uninoculated control. Isolate E203-1 and R55-1 were increased Ciherang rice plants height by 86.86% and 83.42% respectively over the control plants. Inoculation with isolate R55-11 gave the best result in height of 30 days old IF8 rice plants by 97.42% over the control, then follow by isolate R146-6 and E203-1 by 85.96% and 77.96% respectively. Isolate D205-1 is bacterial leaf endophyte that produce ACC

deaminase and IAA. ACC-deaminase containing bacteria can act as a sink for ACC when they colonize the plant tissues and keep ethylene levels below the point where root growth is obstructed. Decreasing levels of ethylene in plants tissue promoted growth and elongation of roots and shoots (Glick, 2005). The result also indicated that there was a systemic tolerance induction mechanism in which bacterial inoculation can help plant to growth under salinity stress.

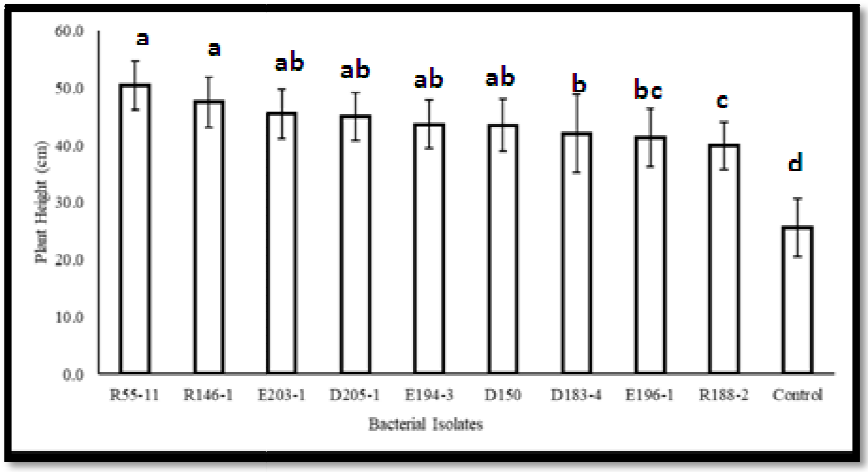

Figure 2: Effect of Bacterial Inoculation on Ciherang Rice Plants Height

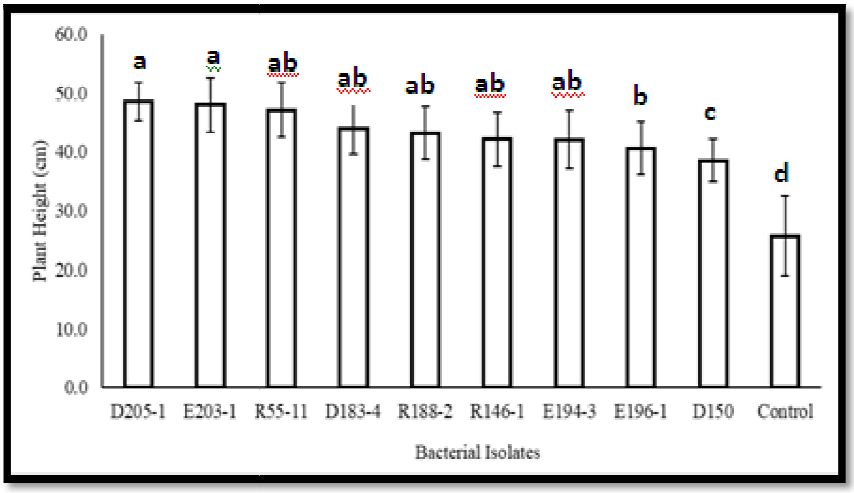

Figure 3: Effect of Bacterial Inoculation on IF8 Rice Plants Height

The rice plants were uprooted at 30 days after planting. Bacterial inoculation has significantly affected shoot dry weight, root length and root dry weight of 30 days old rice plants. Salinity has significantly decreased shoot dry weight, root length and root dry weight of both rice plants variety under conditions tested. This growth reduction caused by NaCl treatment was ameliorated by inoculation of bacterial isolates. Inoculation of Ciherang rice seedling with isolate D205-1 resulted in highest root length, root dry weight and shoot dry weight at concentration of NaCl 150 mM (Figure 3). Isolate D205-1 has also the highest positive effect on shoot dry weight of IF8 rice plants under NaCl treatment. Isolate D150 has significantly increased the root length of 30 days old IF8 rice plants, while isolate E196-1 and R55-11 were most effectively in increasing root dry weight. Greater shoot biomass production under salinity stress is an essential indicator to evaluate that bacterial inoculation caused better protection to photosynthesis apparatus from salinity injury (Gao et al, 2005). Salinity obstructs the photosynthetic ability by reducing the specific enzyme contribute to the synthesis of photosynthetic pigments. It leading to the reduction of chlorophyll content that resulted in lessening of plant biomass (Hidri et al, 2016).

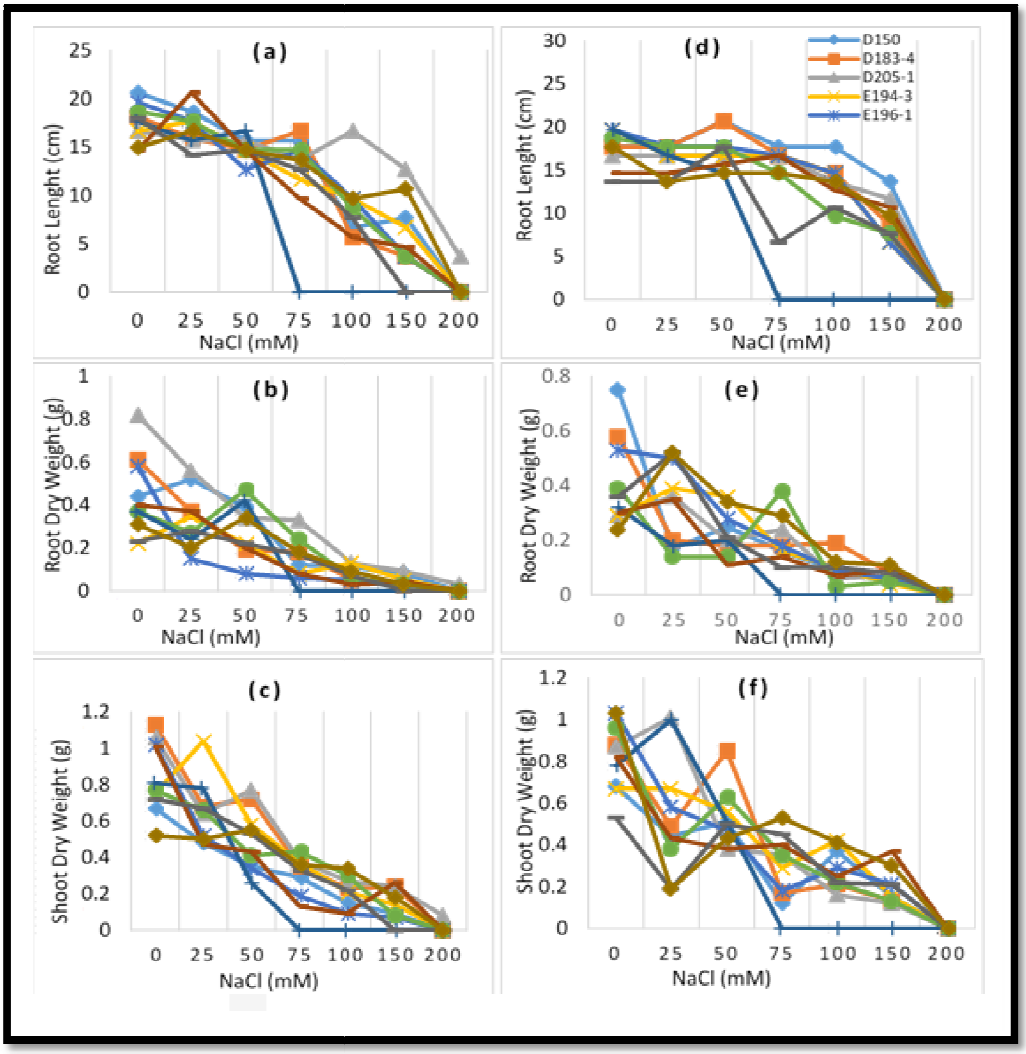

Figure 4: Effect of Nacl Treatment and Inoculation of Bacterial Isolates on Root Length, Root Dry Weight and Shoot Dry Weight of 30 Days Old Ciherang (A, B, C) and IF8 (C, D, E) Rice Plants

4. Conclusions

The most promising bacterial isolate is D205-1 that showed the highest plant growth parameters on both variety of rice plants under salinity condition in this study. On the basis of our finding we concluded that the most promising bacterial isolate in inducing systemic salt tolerance in plants are those that have high activity of multiple plant growth promoting. The bacterial leaf endophyte may be being the most effective in inducing salt tolerance since they are residing in plant tissue that close to the photosynthesis apparatus, thus better protected from photosynthesis apparatus damage. Nevertheless, extensive research is needed to clarify which is the key factor that bacteria could ameliorate salt stress in plant they colonized.

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