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Halotolerant Plant Growth-Promoting Bacteria Colonization of Agronomic Crops Under Saline Stress

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Abstract.
The ability of a plant growth-promoting bacteria (PGPB) strain to colonize the roots and tissues of inoculated plants is important for their successful use in agricultural practices. The purpose of this study was to determine how effective 15 indigenous halotolerant PGPB were at colonizing three different agronomic crops via seed inoculation. Using standard Hoagland's media and Hoagland's media amended with 100 mM NaCl, we tested 15 gfp-tagged halotolerant bacterial isolates for their ability to colonize rice, maize, and soybean seedlings. The quantitative dilution plating method and fluorescent microscopy were used to determine the colonization degree of gfp-tagged halotolerant PGPB isolates in the rhizoplane zone and in the inner tissue of the seedlings at 21 days after germination. All halotolerant PGPB isolates colonized the rhizoplane zone of all seedlings. In both standard and 100 mM NaCl amended Hoagland's media, isolates E194-3, D183-4, and E101-1 showed the highest colonization in rice, maize, and soybean seedlings, respectively. The ability of halotolerant PGPB isolates to colonize agronomic crops was found to vary depending on bacterial isolates, plant species, plant tissues, and NaCl concentration.

Keywords: *s: inoculation, colonization, halotolerant, endophyte, rhizoplane*

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1. Introduction

Global climate change and land use conversion are on the rise and are expected to increase environmental stresses that exacerbate crop stresses. One of the serious climate change impact is salinization of agricultural land. Salinization of agricultural land have significantly come across in decreasing it productivity. Therefore, it is urgently needed in deploying a sustainable scheme to overcome this concern. An eco-friendly technic by inducing salt tolerance in rice for better adapted to salt stress would be very promising strategy to generate climate change-resilient plants[1]–[3]. The use of beneficial microbe to mitigate salt stress in agronomic crops based on their plant growth-promoting capability has been proven by many researchers [4]–[12]. The PGPB can be a

rhizosphere bacteria or endophytic bacteria. Bacterial endophytes are bacteria that can be found in the surface-sterilized plant tissue and widespread within plants that colonize inner spaces of all plant compartments but do not cause plant disease or significant morphological changes [13, 14]. Diverse soil-grown plant species and all of their parts compartments have been identified as a host of diverse bacterial endophytes [15]. It is believed that the application of endophytic bacteria in agricultural practices may become more prospecting in the future than rhizosphere bacteria. Endophytic bacteria are located in the inner tissue of the plant, hence it does not have competition with other microorganisms in the rhizosphere. Furthermore, endophytic bacteria are better at protecting the plant host from environmental stresses, since they are more intimately interconnected with the plant host than those rhizospheric bacteria[14, 16–18]. .

Application of bacterial endophyte to induce abiotic stress tolerance in plants are extensively determined as a promising strategy to control plant stress. Induced Systemic Tolerance (IST) is the term being used for microbe-mediated induction of abiotic stress responses bacteria[19–20]. The next important step after a new promising inoculants for IST have been identified is how to effectively deliver these inoculant into specific plant tissues[21–22]. The effective and efficient colonization of halotolerant bacterial inoculant into an extensive range of crops play a role in determining inoculum efficacy[23–24]. It has been reported that after the inoculation step, bacteria will colonize and grow on or around the plant roots for the establishment of effective plant-bacterial interaction. That was a very crucial step for the bacterial inoculant to regulate their colonization performance, where some of them are capable to get into the internal plant tissues and exists as the colonization of bacterial endophyte[13, 25–26].

The successful formation of plant-bacteria association requires specific bacterial functions and also a specific feature from the plant host. It involves the reciprocal response and a substantial harmonization of the reactions between the bacterial inoculant and the plant[27]. The establishment and colonization of endophytic bacteria in the internal plant tissues are multifaceted and comprise plant host recognition, pre-colonization stage, penetration, multiplication, localization, and colonization[28]. Bacteria that can be found in the rhizosphere as the same bacterial colonies with those within all plant compartments indicated these bacteria may have the distinctive capability to produce a beneficial effect to the plant host. Seeds can act as a bio-agent for transferring the endophytic bacteria from one to the next generation of plants by infecting the plant and spread within the plant together with the plant growth or move inside the plant tissue[13–14, 29]. Plant seed has a unique structure, attractive characteristics, and they have a specific mechanism of selection of liable transmission from one to the next generation

that enables it to transmit the specific endophytic bacteria[30]. Bacterial strain that can be transmitted through seed inoculation would get an excellent systematic colonizer and capable to grow internally within the plant[14]. Even though seed inoculation has been widely used to deliver bacterial inoculant into agronomic crops, the efficacy of bacterial strain in colonizing plant roots and inner tissues is not always clearly recognized. Major factors contributing to the interactions of most bacterial endophytes with agronomic crops are also needed to be studied. The main objective of this study was to evaluate the ability of 15 indigenous halotolerant PGPB to colonize three agronomic crops (rice, maize, and soybean) under saline treatment.

2. Materials and Methods

2.1. Isolates of halotolerant plant growth-promoting bacteria

Total of 15 halotolerant plant growth-promoting bacteria (PGPB) previously isolated from Java coastal plants, Indonesia were used in this study. Those halotolerant PGPB isolates have been previously identified to be able to grow on tryptic soy agar (TSA) media containing 1000 mM NaCl and able to efficiently promote rice seedling growth under saline condition up to 200 mM of NaCl concentration (data not shown). The plant growth-promoting capabilities of halotolerant PGPB isolates used in this study were previously studied (the data are shown in Table 1). Before being used for colonization assay, the bacterial isolates were maintained on 1/10 strength of TSA media supplemented with 600 mM NaCl.

2.2. Gfp-tagged bacterial isolates

The HEB used in this study were tagged with green fluorescent protein (gfp) tagging. Plasmids EGFP-pBAD that harboring the enhanced green fluorescent EGFP-pBAD (Addgene plasmid # 54762, CITY of origin) was used. The plasmid was purified from *E. coli* DH5α host cells using the ATMTM Plasmid Mini Kit (ATP Biotech Inc., CITY of origin) in accordance to the instructional procedure given by the manufacturer. The electrocompetent cells of bacterial isolates preparation have followed the method described by Sambrook et al.[31]. Aliquots 100 µl of the electrocompetent cells of all bacterial isolates from the frozen stock were thawed and mixed with purified 5 µl gfp plasmid DNA in 1.5 ml microfuge tubes. The Bio-Rad Gene Pulser XcellTM Electroporation System was used to electroporate the plasmids into the bacterial cells

Table 1: Plant growth-promoting features of bacterial isolates.

No	Isolates	ACC	IAA (µg/ml)	PO_4	HCN	NH_3	EPS	N_2-fix	Amy	Chit	Cell	Pro	Pec
1	D150	-	19.442	+	+	+	-	-	+	+	+	+	+
2	R146-3	-	16.773	+	+	+	-	-	+	+	+	-	+
3	E109-2	-	17.738	+	-	+	-	+	+	+	+	-	+
4	D205-1	+	35.102	-	+	-	-	-	+	+	+	+	+
5	D183-4	+	0.945	-	-	+	-	-	+	-	-	+	+
6	R55-11	-	10.404	-	+	-	-	-	+	-	-	+	+
7	D102-1	-	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	-	+	+	-	-	-	-	-	+	+
13	E101-1	+	10.288	+	-	+	+	+	+	+	-	-	+
14	D102-1	-	4.077	+	+	+	+	+	+	-	-	-	+
15	D183-4	+	0.945	-	-	+	+	+	-	-	-	-	+

ACC=ACC deaminase, IAA; PO_4=phosphate solubilizing; HCN; NH_3= ammonia; Pro=protease; Amy=amylase; Chit=chitinase, Cell= cellulase; EPS= exopolysaccharide; N_2-Fix= N_2 fixing; Pec=pectinase

by a program default of “Bacterial2” with the electroporation unit 2.5kV; 25µF; 200 ohms; exponential decay pulse type. Directly, an aliquot of 900 µl of tryptic soy broth (TSB) was filled out into the electroporation cuvette after the pulse. Afterward, the bacterial cells were resuspended by pipetting up and down, then transferred to 1.5 ml tubes and shaken at 150 rpm at 37oC for 3 hours. After that, an amount of 100 ml of bacterial cell suspensions was spread gently onto the 1/10 strength of TSA agar plates containing 600 mM NaCl and ampicillin 100 µg/ml, then incubated at 30oC. After 24 to 48 h of incubation, the bacterial colonies were observed using a fluorescence stereo zoom microscope (Olympus SZX12, CITY of the producer). Colonies that appear with bright fluorescent were picked and stored in 15-10% glycerol stock on -80oC until further use.

2.3. Seed inoculation

Seeds of rice, maize, and soybean were inoculated with 15 selected HEB isolates that have been previously tagged with gfp plasmid. In detail, each gfp-tagged bacterial isolate was grown in 100 ml TSB media supplemented with 100 µg/ml ampicillin in a 250 ml flask for 48 hours at 30oC under shaking at 150 rpm and centrifuged (6000

rpm for 10 min). The bacterial pellet was resuspended in 0.85% saline solution. Optical density was measured and a population of 108-109 colony-forming unit (CFU) per ml of bacterial isolates was used for seed inoculation. Seeds of rice, maize, and soybean ware surface sterilized using 70 % ethanol for 1 min, then dipped in 5.25 % sodium hypochlorite solution for 5 min and finally washed five times with sterilized distilled water. The colonization ability of bacterial isolates was investigated as described by Yanni et al. [32] with minor modification. Surface-sterilized seeds were immersed in pure cultures of 15 selected HEB isolates in sterilized 0.85 % saline solution up to 24 h for rice, and 30 min for maize and soybean seeds. Seeds were also immersed in sterilized 0.85 % saline (no bacterial cells) as a control. Treated seeds were transferred to culture tubes with a diameter 25 mm and a length 200 mm. The tube was filled up with either 15 ml of normal Hoagland's nutrient medium, or 15 ml of Hoagland's nutrient medium supplemented with 100 mM NaCl for saline stress treatment. An amount of 1% purified agar was added to solidify all Hoagland's media. These tubes were upper-covered with ca. 3 g of sterilized quartz sand, each saturated with appropriate sterile normal Hoagland's liquid medium or Hoagland's liquid medium containing 100 mM NaCl Each treatment was made for three replications. Tubes were incubated under a maintained temperature of ±25°C, with 12 h dark/ light cycles in the culture's growth room.

2.4. Re-isolation, quantification, and visualization of the gfp-tagged HEB isolates

All seedlings were gently uprooted from the tube cultures at 21 days after inoculation and the gfp-tagged bacterial population numbers in and on the shoot and root tissue were determined. Roots were washed with sterilized water to clean the agar and sand that attached to it. For counting the gfp-tagged rhizoplane bacteria, washed roots were rinsed four times with sterilized distilled water and then dipped in 0.85 % saline solution in a 25 ml test tube, and vortex for 10 min in room temperature. The saline solution was serially diluted (up tp 10-4) with saline solution and spread on selective media consisting of 1/10 strength TSA agar plates containing 600 mM NaCl and 100 µg/ml ampicillin. The bacterial colonies apparent on agar plates after 3-5 days of incubation at 30oC were considered as rhizoplane populations.

For calculating the gfp-tagged endophyte bacteria, washed roots and shoots were separately surface-sterilized with 70 % ethanol for 1 min, dipped in 1 % NaOCl for 3 min, and rinsed four times with sterilized distilled water. Surface-sterilized seedling tissues

were aseptically macerated. For surface sterility check, sterile plant tissues were rolled over plates of TSA. The diluted seedling tissues macerates were plated on the selective media as described above. After 3-5 day incubation at 300C, the bacterial colonies apparent on the agar plate media were considered as a bacterial endophyte.

Bacterial colonies were counted, picked, and re-streaked on selective media, and stocked in pure culture. In addition, the shoot and root of freshly uprooted and washed seedlings were also observed directly under a fluorescence stereo zoom microscope (Olympus SZX12, CITY of the producer) to detect the presence of gfp-tagged bacterial cells in and on the seedling tissues.

3. Result and Discussion

3.1. Quantification of gfp-tagged halotolerant PGPB isolates on inoculated seedling

The total population of gfp-tagged halotolerant PGPB isolates colonizing seedling tissues were quantified using dilution plating methods. The results showed great variability of recovered bacterial cells among the gfp-tagged halotolerant PGPB isolates tested, crops, and seedling tissues. It was observed diverse population density up to 10^5 CFU g^{-1} fresh weight on root tissue and rhizoplane of rice, maize, and soybean seedlings for all isolates tested at normal and salt stress treatment media. Results showed that concentration NaCl of 100 mM decreased the colonization degree of bacterial inoculant in rice, maize, and soybean seedling in this study. Isolates D205-1, D183-4, and E194-3 were recovered from the shoot, root, and rhizoplane of rice seedling at both normal and salt stress treatment media. Isolates D217-2, E101-1, D205-1, D183-4, E193-4, and D102-1 were recovered from the shoot of rice seedling grown at normal media, but no bacterial isolates of E101-1 and D102-1 were recovered from the shoot of rice seedling at media containing 100 mM NaCl. The notable exception was the isolate D150 which was not recovered from the shoot of rice seedling at normal media, but it was detected at the shoot of rice seedling from media containing 100 mM NaCl (Table 2). The inoculation method may also influence the colonization degree of bacterial inoculant into the treated plant. Seed inoculation considers as an efficient, reliable, and farmer-friendly method to deliver microbial inoculants into agronomic crops[33–34]. Nevertheless, it may not be effective in the colonization process for some bacterial strains.

The population density of bacterial isolates on maize seedling displayed a similar pattern as observed in rice seedling. Most of the bacterial isolates in this study showed

good colonization on the rhizoplane and root interior tissues of rice, maize, and soybean seedling but decreased significantly on the shoot interior tissues. This finding following the previous study that most endophytes are usually observed high density in underground part (roots) compared with above-ground part (shoots) [26, 28, 35]. As described by Hallmann and Berg [36], the root system act as buffered habitat and provide root exudates that providing habitat niches for endophyte colonization. That is why the rhizosphere is a primary source for endophytic colonization[37]. As another speculation, it could be because the observation of bacterial colonization was at the seedling stage that might be the bacterial isolates have not yet reached above ground (shoot). This reason is supported by Kandel et al. [38], who observed the colonization of poplar endophyte (WP5gfp) in the root, leaf, and steam of Maize. They concluded that the rhizosphere is the start point for the bacterial endophyte to access the inside of the plant tissues. Isolate E101-1; D205-1; D183-4; E194-3; R55-11; E109-2 showed good colonization for maize seedling at both colonization media with and without NaCl and the highest population recovered from maize seedling was isolate D183-4 (Table 3). Isolate D183-4 also recovered from all observed tissues (shoot, root, and rhizoplane) of maize seedling at both normal and salt stress treatment media. Isolate D205-1 and E194-3 showed colonization ability on all observed seedling tissues.

Table 2: Number of bacterial isolates recovered from rice seedling after 21 daysgermination at normal and saline stress treatment media (containing 100 mM NaCl).

	CFU/gram fresh weight					
	Normal media			Media containing 100 mM NaCl		
Isolates	Shoot	Root	Rhizoplane	Shoot	Root	Rhizoplane
D217-2	5×10^2	5.1×10^4	3×10^5	-	14	30
R146-6	-	2×10^5	4.33×10^5	-	2.73×10^2	8.33×10^3
D150	-	1.33×10^5	1.67×10^5	3.33×10^2	4×10^2	2.33×10^3
E193-2	-	3.67×10^4	4×10^4	-	8.33×10^2	4×10^2
E101-1	2.67×10^2	3.33×10^3	9.67×10^5	-	57	7.33×10^2
D205-1	2×10^3	7×10^4	9×10^5	6×10^2	3.33×10^2	1.9×10^3
D183-4	5×10^3	1.67×10^4	1.67×10^5	5.67×10^2	81	9×10^3
E194-3	1.33×10^2	8.67×10^5	2×10^5	4×10^2	6.67×10^2	2.33×10^3
E196-1	-	1.33×10^4	7×10^4	-	3.21×10^2	1.67×10^2
R55-11	-	5×10^3	3×10^4	-	3×10^3	8×10^3
E109-2	-	1.33×10^2	2×10^3	-	68	2×10^2
R188-2	-	6×10^4	3×10^5	-	6.55×10^2	7×10^2
D102-1	3×10^2	2.67×10^3	2×10^5	-	17	88
E203-1	-	3×10^3	2×10^4	-	9.33×10^2	8.73×10^3
R146-3	-	7.67×10^2	2.67×10^3	-	11	77.33
Control	-	-	-	-	-	-

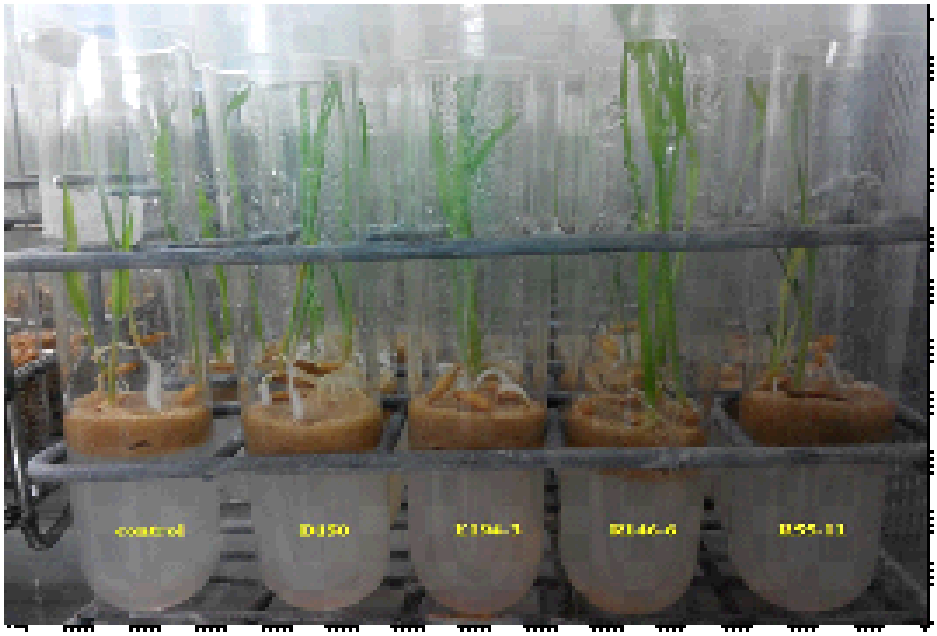

Figure 1: Colonization test performance of rice seedling at media containing 100 mM NaCl.

The isolates E101-1, D205-1, E194-3, and D183-4 were recovered from soybean seedling at both normal media and media with 100 mM NaCl (Table 4). Isolate of D217-1, R146-6, E193-2, E109-2, E203-1, and R146-3 were not recovered from soybean seedling at media containing 100 mM NaCl. Those indicated that 100 mM NaCl concentration is a limiting factor for effective colonization of those isolates in soybean seedling and it is crucial to achieving successful induction of salt-tolerant in crops. The numbers of halotolerant bacterial isolates recovered from the inoculated seedlings at 21 days after germination in this study were varied within bacterial isolates, plant species, plant tissues, and NaCl concentration. The best isolate would have better colonization under high salinity conditions and colonize a wide range spectrum of crops. But it also takes into consideration from the plant side that plant species also have different abilities to be colonized endophytically by the same bacterial isolate. The plant that has an attractive character in the colonization process is desired[39]. Isolate E101-1 showed the highest population of bacterial cells recovered from shoot, root, and rhizoplane of soybean seedling compare to other isolates at both normal media and media containing 100 mM NaCl.

3.2. Visualization of gfp-tagged bacterial isolates

Throughout this study, no gfp-tagged fluorescent bacterial cells were observed on the root and shoot of all the uninoculated control seedlings. . This result was correlated with

TABLE 3: Number of bacterial isolates recovered from maize seedling after 21 days germination at normal media and saline stress treatment media (containing 100 mM NaCl).

	CFU/gram fresh weight					
	Normal media			Media containing 100 mM NaCl		
Isolates	Shoot	Root	Rhizoplane	Shoot	Root	Rhizoplane
D217-2	1.12×10^2	$2{,}36 \times 10^2$	21	-	-	37
R146-6	-	-	1×10^4	-	1.04×10^2	2.67×10^2
D150		34.33	1.67×10^5	-	-	12
E193-2	-	4×10^2	2.2×10^4	-	9×10^2	2.9×10^2
E101-1	3×10^2	2×10^2	1×10^2	22.33	20	4.3×10^2
D205-1	7×10^2	2.33×10^3	1×10^4	5×10^2	23	8.8×10^2
D183-4	1.58×10^3	1×10^4	3×10^5	18	1.67×10^2	1.5×10^3
E194-3	1.67×10^2	9×10^3	2.33×10^4	43	1.13×10^2	5×10^2
E196-1	-	3.67×10^3	4.33×10^4	-	-	2.7×10^3
R55-11	-	4×10^4	9.67×10^4	-	-	6.3×10^3
E109-2	-	3.33×10^2	5×10^3	-	5.67×10^2	4×10^2
R188-2	-	1×10^3	9.67×10^3	-	-	2×10^2
D102-1	-	-	2.67×10^2	-	-	7×10^2
E203-1	-	-	5×10^3	-	7	3.33×10^2
R146-3	-	-	7×10^2	-	-	11
Control	-	-	9	-	-	-

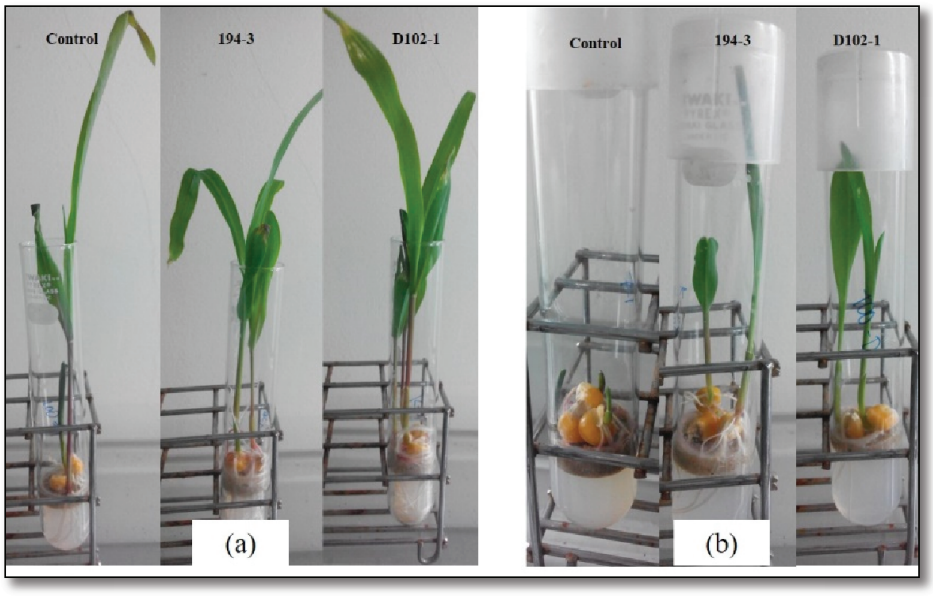

Figure 2: Colonization test performance of maize seedling at (a) normal media and (b) media containing 100 mM NaCl.

the growth of the seedling, which uninoculated seedlings showed a stunted growth at salt stress treatment media (Figure 1, 2 and 3). Microscopic observation of roots seedling at 21 days after inoculation of gfp-tagged HEB isolates showed that most isolates were

TABLE 4: Number of bacterial isolates recovered from soybean seedling after 21 days germination at normal media and saline stress treatment media (containing 100 mM NaCl).

	CFU/gram fresh weight					
	Normal media			Media containing 100 mM NaCl		
Isolates	Shoot	Root	Rhizoplane	Shoot	Root	Rhizoplane
D217-2	-	1.24×10^2	3×10^4	-	-	-
R146-6	-	-	19	-	-	-
D150	-	5×10^4	$2{,}67 \times 10^4$	-	48	1.2×10^2
E193-2	-		33	-	-	-
E101-1	7.33×10^3	2×10^2	6.33×10^4	2.6×10^2	53	1.33×10^3
D205-1	6.67×10^2	1×10^2	1×10^2	17	1.5×10^2	2.6×10^2
D183-4	3.33×10^3	2×10^3	2.67×10^4	92	2×10^2	8.8×10^2
E194-3	-	-	1×10^4	-	-	1.2×10^3
E196-1	-	1×10^4	6.33×10^4	11	24	2.5×10^2
R55-11	-	-	1×10^4	-	-	57
E109-2	-	-	1.9×10^2	-	-	-
R188-2	-	-	7.67×10^4	-	-	3.2×10^2
D102-1	2×10^2	3×10^3	2×10^4	44	93	2.7×10^2
E203-1	-	-	2.67×10^2	-	-	-
R146-3	-	-	9×10^2	-	-	-
Control	-	-	23	-	-	-

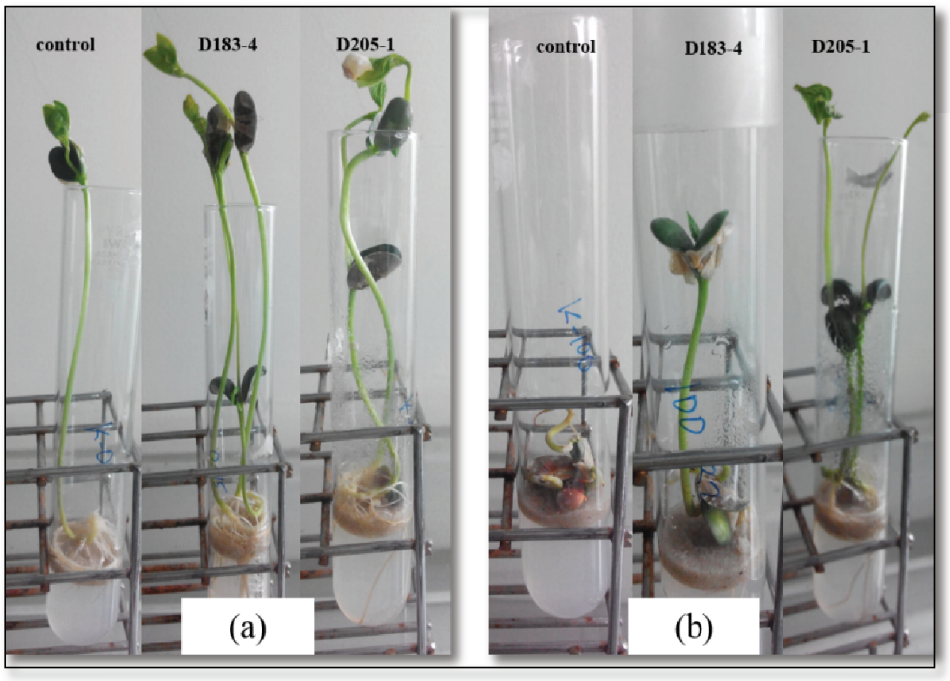

Figure 3: Colonization test performance of soybean seedling at (a) normal media and (b) media containing 100 mM NaCl.

found predominantly in rhizoplane zone and the high cell population was emitted from the root surface (Figure 4) and it was also detected on around root hairs in some isolates.

This result was in accordance with the result from the dilution plating method which also resulted in a high population of bacterial isolates recovered from rhizoplane and root of the observed seedling. In rice, maize as well as soybean seedling in this study, leaf surface colonization by isolate R146-6, R146-3, R188-2, R55-11, E203-1, and isolate E109-2, were not observed in both dilution plating and fluorescence microscopy methods, but they have observed the high cell density at the root surface. This result indicated that these bacterial isolates were noticeably confined predominantly in the rhizoplane zone. Hansen et al.[40] explained a colonization pattern for *Pseudomonas fluorescens* DF57 on barley roots and Compant et al.[39] a colonization pattern for *Burkholderia* sp. strain PsJN on the grapevine that in line with this study result. Moreover, Soldan et al.[41]. reported the same result which found that the surface of the root hairs in the developing zone of barley seedling was the most extensive colonization of endophyte inoculant. Some researcher has been reported that it was a common colonization behavior for many bacteria. Only some samples were observed the high cell density on the leaf surface, this was due to the lower degree of shoot colonization by gfp-tagged halotolerant PGPB cells as observed on the dilution plating method. Gfp-tagged halotolerant PGPB cells that appear on the leaf surface did not spread evenly, but they were apparent as a small spotted cell colony (Figure 5).

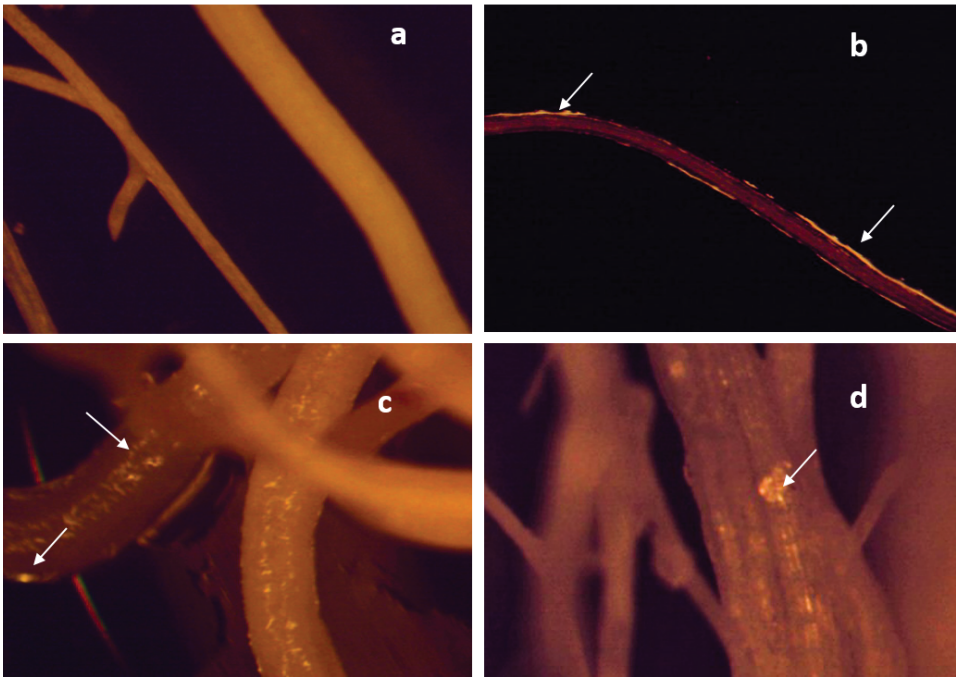

Figure 4: Colonization pattern of spot inoculated bacteria on the root surface at: (a) control root; (b) rhizoplane of rice seed; (c) internal root tissue of maize; (d) internal root tissues of rice) under 120x magnification Olympus SZX12 stereo zoom fluorescent microscope .

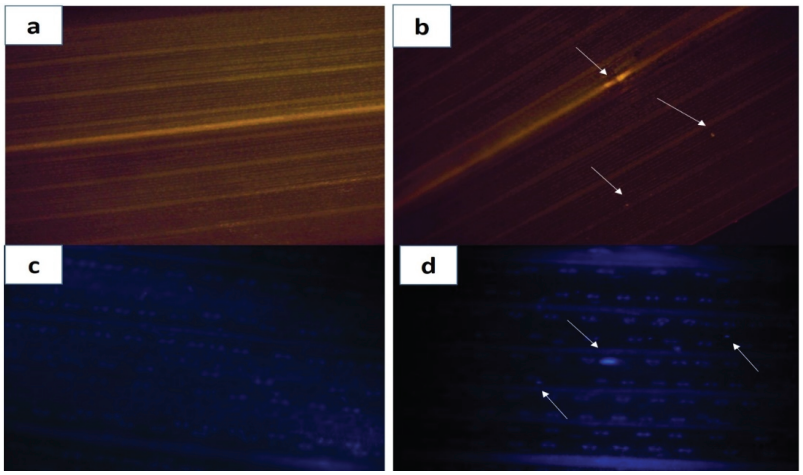

Figure 5: The gfp-tagged bacterial cells apparent as a small spotted colony at the leaf surface of (a) maize control (b) maize leaf with gfp tagged-bacterial cells (c) rice control (d) rice leaf with gfp tagged-bacterial cells. under 120x magnification Olympus SZX12 stereo zoom fluorescent microscope.

4. Conclusion

The result showed that the number of bacterial cells recovers from the plant tissues of rice, maize, and soybean seedlings was decreased in a growing media containing100 mM NaCl. Most bacterial isolates were recovered a higher number of colonies at the rhizoplane than at the shoot and internal root. Furthermore, under the condition of this study, the competence of bacterial isolates to colonize the inoculated plants (rice, maize, and soybean) were likely to be a key factor in figuring the colonization pattern of these bacterial isolates. We suggested that to select the most superior inoculant in agricultural application, selected inoculant is those that having a high competence to colonize targeted host and has relatively stable colonization ability in a wide range of environmental condition. Isolate E194-3, D183-4 and E101-1 consider as a promising isolates for further evaluation. Further study should be completed with the mechanism of interaction and other positive efficacy to select the best inoculant for agricultural cultivation practice.

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