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Plant Growth Promoting Effects of Nepalese Sweet Potato Endophytes

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Abstract: Endophytic bacteria form a symbiotic relation with plants and generally cause no harmful effects to the host plants. In a previous study, we isolated eight bacterial endophytes from sweet potato plants harvested in Salyan, Nepal. These endophytes showed plant growth-promoting properties as a mixed culture. In this study, we evaluated the ability of these strains to produce indole-3-acetic acid (IAA) and to fix nitrogen. Based on these results, we selected two strains, *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3, and evaluated their ability to promote plant growth. IAA production activity peaked at 15–60 mg NH_4NO_3/L in plant-free medium. Similarly, acetylene reduction activity peaked at 0–6.25 mg NH_4NO_3/L. Both strains successfully colonized plants, promoted the growth of tomatoes, and induced phenotypes in plants consistent with IAA exposure. This suggests that these strains promote plant growth by producing IAA inside the plant, where nitrogen levels are expected to be low.

Keywords: endophyte; indole-3-acetic acid (IAA); sweet potato; tomato; nitrogen fixation; colonization

1. Introduction

Endophytic bacteria colonize the internal tissue of the host plant, forming a symbiotic relationship without detrimental effects to the tissue of most plants [1,2]. Many plant growth-promoting endophytes can fix nitrogen, produce phytohormones, and express 1-aminocyclopropane-1-carboxylate (ACC) deaminase [3–5].

Sweet potato (*Ipomoea batatas*, L.) is cultivated in several countries in Asia, Africa, Europe, and America. This plant can be cultivated with little fertilization [6,7], probably due to its association with endophytes [8] and a general behavior/response of sweet potato as a plant species. For example, sweet potato endophytes *Bacillus cereus*, *Achromobacter xylosoxidans*, and *Rahnella aquatilis* showed superior indole-3-acetic acid (IAA) production and phosphate solubilizing abilities, which may improve the nutrient uptake, root growth, and overall plant growth [9,10].

However several plants and soil types may lack the efficient endophytes that have superior plant growth-promoting activities. Therefore, the isolation and inoculation of plant growth-promoting endophytes can contribute to economically efficient crop production systems by reducing the use of chemical fertilizers or pesticides [11].

Nepal is a small Himalayan country with a high degree of biodiversity. The diverse climates and soils of Nepal produce favorable conditions for high microbial diversity, including microbes such as legume-nodulating bacteria [12,13]. However, there are limited reports on endophytic bacteria in

Nepal. Venkatachalam et al. [14] reported a diverse bacterial community in temperate soils of Nepal with different enzymatic activities. In our previous study, we reported diverse genotypes of sweet potato bacterial endophytes in 12 different locations in Nepal, and the inoculation of mixed cultures of the strains from each location improved the fresh weight and vine length of sweet potato in growth chambers [15]. In this study, the endophytes from the 'Salyan' location were selected based on their physiological and plant growth-promoting properties to identify endophytes that can promote plant growth. As the effects of nitrogen levels on plant growth-promoting properties of endophytes have not been extensively examined yet, IAA production and nitrogen fixation activities were examined at different levels of nitrogen in this study. Plant growth promotion by the endophytes was also examined using sweet potato as a host plant. Tomatoes were also used due to their sensitive response to inorganic nitrogen levels and preparation of uniform seedlings from seeds.

2. Materials and Methods

2.1. Bacterial Strains

Strains used in this study were isolated from Nepalese sweet potato tubers [15]. Eight sweet potato endophytes from the Salyan location were used in this study (Table 1).

Table 1. Endophytes used in this study isolated from sweet potato cultivated in Salyan, Nepal.

Strain	* Most Similar Genus	Class	Accession Number
Sal 1	*Klebsiella* sp.	Gammaproteobacteria	LC389410
Sal 2	*Flavobacterium* sp.	Flavobacteriia	LC389415
Sal 3	*Enterobacter* sp.	Gammaproteobacteria	LC389433
Sal 4	*Rhizobium* sp.	Alphaproteobacteria	LC389434
Sal 5	*Stenotrophomonas* sp.	Gammaproteobacteria	LC389439
Sal 6	*Herbasprillum* sp.	Betaproteobacteria	LC389442
Sal 7	*Agrobacterium* sp.	Alphaproteobacteria	LC389443
Sal 8	*Microbacterium* sp.	Actinobacteria	LC389445

* Most similar genus in 16SrRNA gene sequence data base.

2.2. Evaluation of Plant Growth Promoting Properties

2.2.1. IAA Production

The ability of the selected eight endophytes to produce IAA was determined following the Salkowski assay [16]. Strains were grown in Modified Rannie (MR) [17] liquid medium amended with NH_4NO_3 at 0.1 g/L (N + MR) and 200 µg/mL tryptophan, and incubated at 26 °C at 150 rpm. Samples without inoculation were set as the control. After 3 days of incubation, an aliquot of the supernatant was taken after centrifugation at 10,000× *g* for 10 min at 4 °C. Then, a double volume of Salkowski reagent was added, and the absorbance was measured at 530 nm using a UV-VIS spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) after 30 min in darkness.

The potential of *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3, which showed higher activity, was also examined in $\frac{1}{2}$ MS plant growth medium [18] (in which the amount of macroelement was adjusted to 1/2 strength) with sucrose and tryptophan levels at 0.87 g/L and 200 µg/mL, respectively. IAA production was also measured at ammonium nitrate levels of 0, 0.015, 0.03, 0.24, 0.48, and 1.2 g/L.

2.2.2. Nitrogen Fixation Activity

For *Klebsiella* sp. Sal 1 and *Herbaspirillum* sp. Sal 6, which were reported to have the *nif*H gene [15], the acetylene reduction assay (ARA) was conducted. Cell suspensions of the isolates were prepared at 10^9 CFU/mL after 2 days of culture in liquid MR medium and then washed twice with autoclaved distilled water by centrifugation (at 10,000 rpm at 4 °C for 10 min). A 50 µL aliquot of the cell suspension was poured over a slant of MR agar (1.1%) medium in a 121 mL glass bottle containing

different levels of nitrogen (NH_4NO_3; 0, 6.25, 12.5, 25, 50, 75, 100 mg/L). The bottle was capped, and 10% of the air inside the bottle was replaced by acetylene gas and then incubated in darkness at approximately 30 °C for 4 days. After incubation, the concentration of ethylene in the bottle was measured by a gas chromatograph (GC-14B, Shimadzu) equipped with a flame ionization detector and Porapak N (50/80 mesh; GL Sciences, Tokyo, Japan). The activity was also measured in $\frac{1}{2}$ MS plant growth medium with sucrose at 0.87 g/L at different levels of nitrogen as in the MR medium.

2.3. Effect of Inoculation on Sweet Potato

The experiment was conducted in a phytotron (LH-240, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) with 14 h light and 28/25 °C day/night temperature with 6000 to 7000 lux light intensity in white florescent light conditions. Each strain was prepared at 10^9 CFU/mL in the same way as in ARA and inoculated to sweet potato (variety 'Kokei') tissue culture cuttings. The inoculation experiments were repeated in two different conditions: vermiculite pots and agar tubes.

In the vermiculite pots, two Leonard jars were overlaid, and the top pot was filled with vermiculite and the bottom with liquid $\frac{1}{2}$ MS medium which was connected by a cotton wick to supply the liquid nutrient medium to the top pot. The pot was autoclaved before use. The cut part of the saplings was inoculated by dipping it in the cell suspension, and 1 mL of suspension was poured on the vermiculite around the plant after transplantation. The experiment was conducted in triplicate and the top of the pots were covered by ventilated (<0.2 mm pore size), transparent plastic bags (Sunbag, transparent, Sigma-Aldrich, Tokyo, Japan).

In the agar tube conditions, sweet potato cuttings were inoculated by dipping them in the cell suspension and planted in 1/2 MS agar (1.1%) medium in the capped glass tube (12 cm × 2.4 cm). Each experiment was set at least in triplicate.

Growth parameters were recorded after around 1 month. Based on the results of the repeated inoculation experiments, in both the vermiculite pot and agar tube conditions, two of the most potent bacterial strains, *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3, were selected for further studies in nitrogen-limiting conditions (NH_4NO_3 at 0.12 g/L).

2.4. Effect of Inoculation on Tomato

Tomatoes were selected for further tests because they were the most responsive to different levels of inorganic nitrogen when compared to spinach and carrot (data not presented). In addition, a large number of uniform seedlings could be produced from seeds as compared to sweet potato. Tomato seeds ('*Chika*' F_1 hybrid, Taki Company, Kyoto, Japan) were surface-sterilized by dipping them in 70% ethanol for 1 min, followed by 1% NaOCl for 15 min, then washed 7–8 times with sterilized distilled water. The inoculation experiments were conducted in two different culture conditions, liquid media tubes and Gelrite petri dishes, and the culture conditions were the same as those for the sweet potato experiment.

In the liquid media tubes, one inoculated seed was sown on a piece of single-ply wipe (Kimwipe, wipers S-200) in a capped glass tube (12 cm × 2.4 cm) containing 6 mL liquid medium. Growth parameters were recorded after 15 days, and then the concentration of IAA in the culture solution was determined by Prominence Ultrafast Liquid Chromatography (UFLC) System (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector (SPD-M20A) and 100 L × 3.0 column. The solvent system, 0.5% formic acid and acetonitrile (75/25; *v*/*v*), was used, and IAA was detected at 278 nm.

In the Gelrite petri dishes, three inoculated seeds were sown on Gelrite (0.27%) $\frac{1}{2}$ MS medium in a plastic petri dish (90 × 15 mm). Data were recorded 24 days after the seed sowing.

After recording the plant growth parameters, one plant from each treatment was used to check the colonization of the inoculants. The root part was dipped in 50 mL of sterilized distilled water and gently shaken to suspend the inoculants in the rhizosphere. Then, the root and the stem parts were separated by cutting around 1.5 cm below the cotyledon, washed in sterilized distilled water to remove most of the surface-attached bacteria, and macerated with 1 mL of sterilized distilled water using a

disposable homogenizer (BioMasher, Nippi, Tokyo, Japan). An aliquot of the diluted samples was plated on N^+MR agar medium, and the appeared colonies were counted after 2 days of incubation at 26 °C. The remaining plants in the petri dish were put in a 30 mL glass bottle, and ARA was measured as described above.

2.5. Statistical Analysis

Statistical analysis was conducted using William's test after MANOVA or Tukey's test after ANOVA.

3. Results

3.1. IAA Production

Five strains produced IAA in tryptophan-containing, liquid N^+MR medium (Figure 1). Among them, *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 produced IAA at the highest levels. These strains produced less IAA in the plant growth medium, and production decreased as the nitrogen levels increased (Figure 2). *Klebsiella* sp. Sal 1 produced higher IAA at NH_4NO_3 levels from 0 to 0.06 g/L, whereas *Enterobacter* sp. Sal 3 showed a similar response at a narrower range from 0.015 to 0.03 g/L.

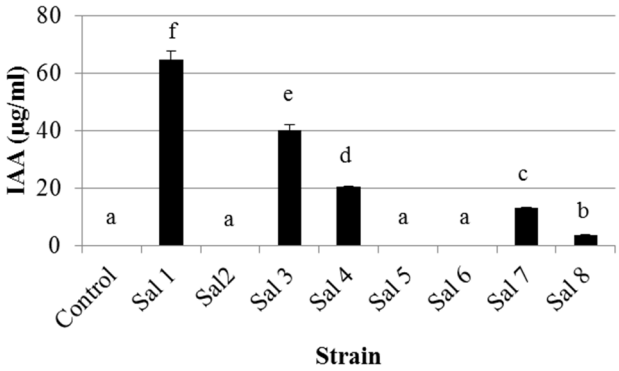

Figure 1. Indole-3-acetic acid (IAA) production by sweet potato endophytes in N^+MR medium. The bars represent standard deviation (n = 3) and different letters indicate significant differences at $P < 0.01$ by Tukey's test.

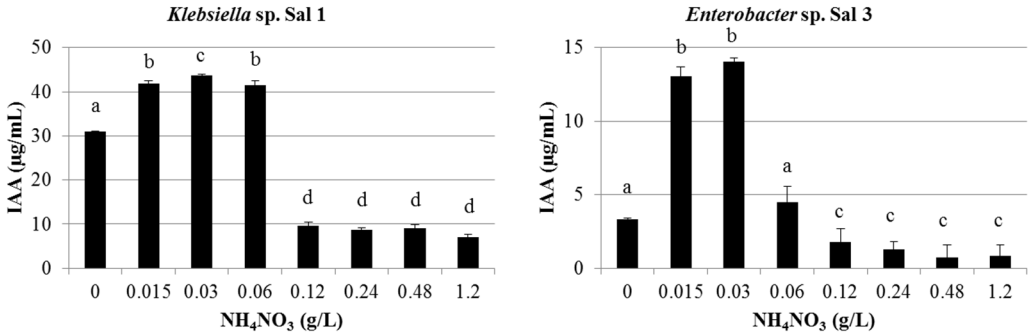

Figure 2. IAA production at different levels of nitrogen in $\frac{1}{2}$ MS liquid medium. The bars represent standard deviation (n = 3) and different letters indicate significant differences at $P < 0.01$ by Tukey's test.

3.2. Nitrogen Fixation Activity

Klebsiella sp. Sal 1 showed higher nitrogen fixation activity than *Herbaspirillum* sp. Sal 6 in the MR medium, and in both strains, the activity decreased with an increase in the level of nitrogen in the medium (Figure 3A,B). The activity was lower in the plant growth ($\frac{1}{2}$ MS) medium, and a similar trend was observed for the nitrogen level (Figure 3C). No activity was detected for *Herbaspirillum* sp. Sal 6 in the $\frac{1}{2}$ MS medium at all levels of nitrogen tested.

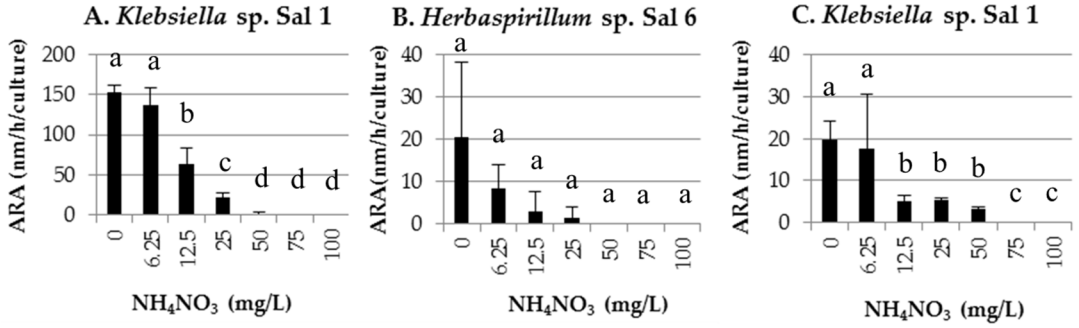

Figure 3. ARA in MR (**A**,**B**) and 1/2MS (**C**) agar medium at different levels of nitrogen. The bars represent standard deviation (n = 3) and different letters indicate significant differences at $P < 0.01$ by Tukey's test.

3.3. Effect of Inoculation on Sweet Potato

Inoculation showed a positive effect on the growth of sweet potato in three different experiments in non-limiting nitrogen conditions (Figure 4). The effects varied some within replications in most of the experiments. In experiment 1 (conducted in the vermiculite pots), inoculation of *Enterobacter* sp. Sal 3, *Stenotrophomonas* sp. Sal 5, and *Agrobacterium* sp. Sal 7 showed a tendency for greater total weight accumulation. Root weights were higher in all of the inoculated plants, and it was highest in *Enterobacter* sp. Sal 3. The number of roots in the pot experiment showed no clear effects (data not shown). Vine lengths were longer in *Enterobacter* sp. Sal 3 and *Rhizobium* sp. Sal 4.

Experiments 2 and 3 were conducted in the agar tube. Because the initial plant size in the agar tubes was smaller than in the vermiculite pots, the root weight and vine length were evaluated and the final fresh weight was used in the case of agar tubes instead of recording the increase in the weight. In both experiments, inoculation showed a positive effect on the increase in total fresh weight, whereas root elongation was not affected (experiment 2) or retarded (experiment 3) by most of the strains. Inoculation of *Flavobacterium* sp. Sal 2 and *Enterobacter* sp. Sal 3 in experiment 2 and *Enterobacter* sp. Sal 3, *Herbaspirillum* sp. Sal 6, and *Agrobacterium* sp. Sal 7 in experiment 3 induced larger numbers of roots. Overall, *Enterobacter* sp. Sal 3 repeatedly stimulated the growth of sweet potato.

In nitrogen-limiting conditions, inoculation with the two selected strains showed positive effects on the root number but not on the root weight of sweet potato cultivated in the agar tube (Figure 5).

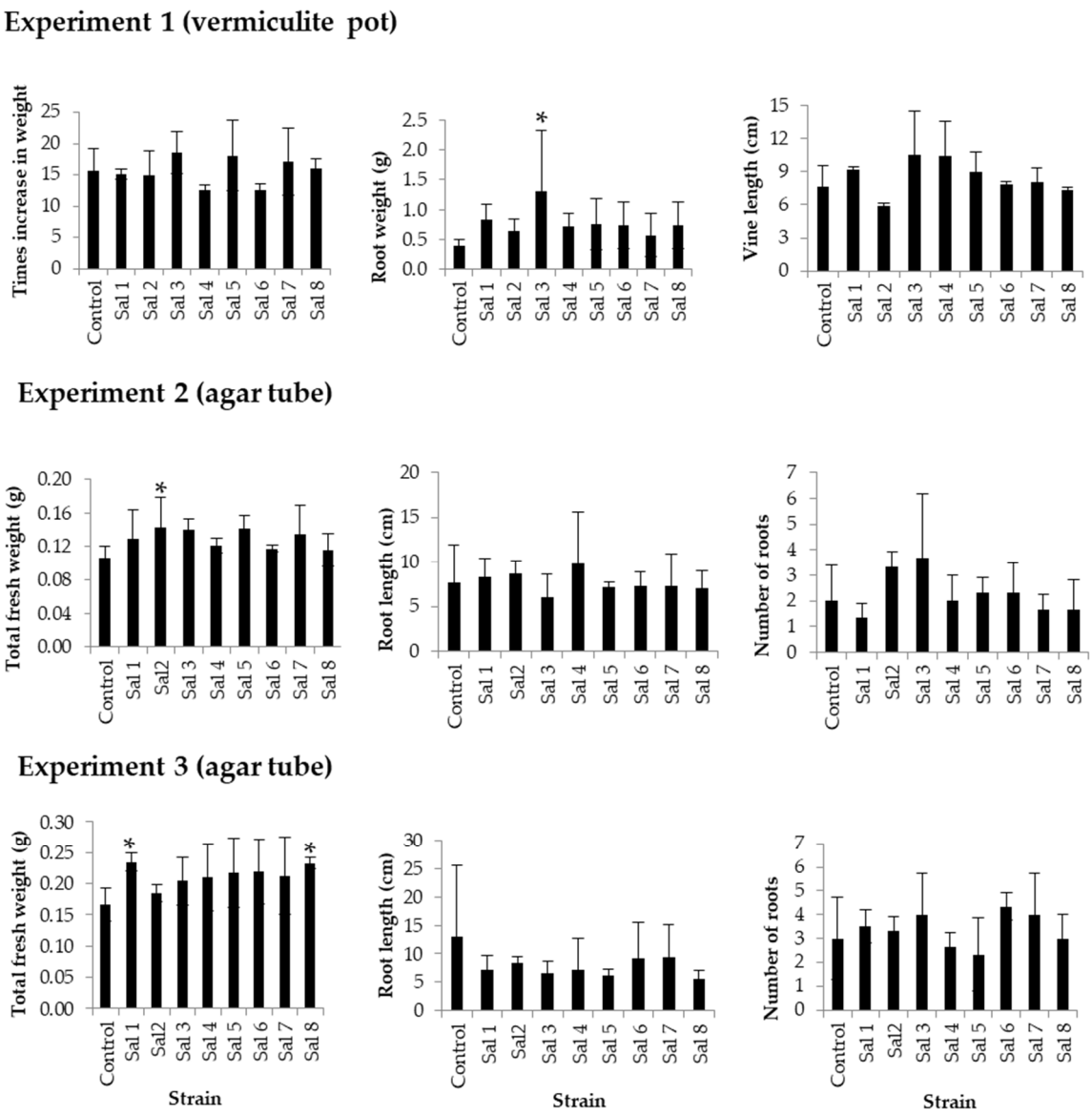

Figure 4. The effect of the inoculation with sweet potato endophytes on sweet potato plant growth in nitrogen non-limiting conditions. The bars represent standard deviation (n = 3) and asterisks indicate a significant difference from control at $P < 0.05$ by William's test.

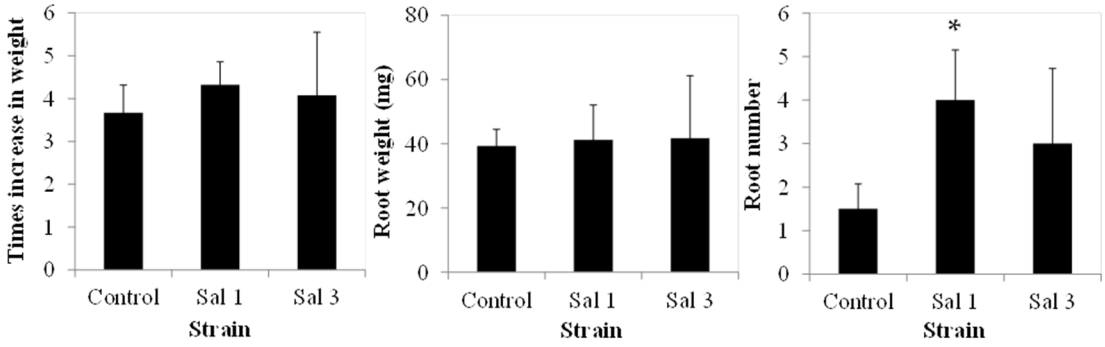

Figure 5. The effect of inoculation with sweet potato endophytes on sweet potato plant growth in nitrogen-limiting conditions in agar tubes. The bars represent standard deviation ($n \geq 3$) and asterisks indicate a significant difference at $P < 0.05$ by Tukey's test.

3.4. Effect of Inoculation on Tomatoes

In vitro tests suggested that the response of tomato seedlings to inoculation with endophytes was related to nitrogen availability. In N-limiting conditions, strains Sal 1 and Sal 3 significantly increased shoot length (Figure 6). In N-depleted conditions, Sal 3 increased root biomass. After the cultivation, IAA was not detected (<0.1 μg/mL) in any of the culture solutions when examined by UFLC.

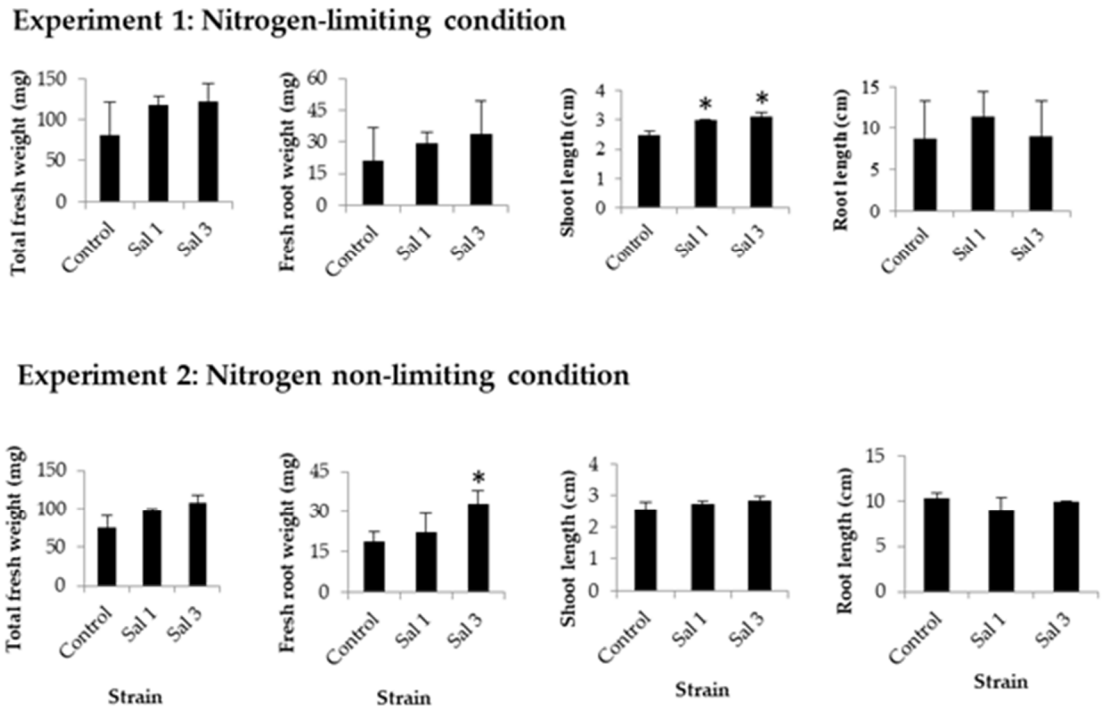

Figure 6. The effect of inoculation of sweet potato endophytes on the growth of tomato plants in nitrogen-limiting (Experiment 1) and non-limiting (Experiment 2) conditions in liquid media tubes. The bars represent standard deviation ($n \geq 3$) and asterisks indicate a significant difference at $P < 0.05$ by Tukey's test.

In nitrogen non-limiting conditions in a petri dish, the effects of the inoculation were apparent in all of the growth parameters (Figure 7). None of the samples showed ARA activity at the end of the cultivation.

Both *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 colonized tomato plants in large populations (Figure 8). In the liquid test tube condition, internal root colonization rate was higher ($2.5 - 2.6 \times 10^8$ and $1.6 - 2.7 \times 10^9$ CFU/g fresh weights by *Enterobacter* sp. Sal 3 and *Klebsiella* sp. Sal 1, respectively). In the other parts, lower populations at $1.5 - 3.4 \times 10^7$ CFU/g and $0.19 - 6.3 \times 10^7$ CFU/g fresh weight of stem and leaf were detected, respectively, in both strains. In the rhizosphere, the colonization rate was $0.22 - 1.3 \times 10^8$ CFU/g fresh weight.

Under nitrogen non-limiting conditions in a petri dish, colonization in the rhizosphere was higher at 9.5×10^9 and 1.9×10^{11} CFU/g fresh weight by *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3, respectively. After the rhizosphere, the colonization rate was higher in the root at $3.8 - 7.5 \times 10^8$ CFU/g fresh weight, $0.56 - 1.5 \times 10^8$ CFU/g in the stem, and $0.16 - 2.8 \times 10^8$ CFU/g in the leaf. In both systems, the population of *Enterobacter* sp. Sal 3 was higher than *Klebsiella* sp. Sal 1 in many samples, and no bacterial colony was observed in the control plants.

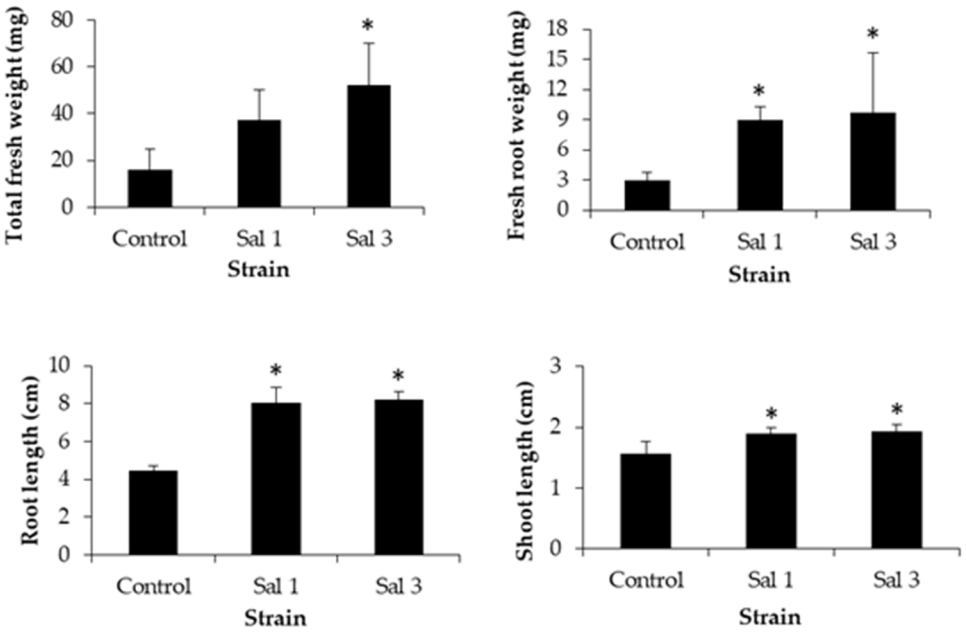

Figure 7. Effect of inoculation of sweet potato endophytes on the growth of tomato in non-limiting nitrogen conditions in Gelrite petri dishes. The bars represent standard deviation (n = 3) and asterisks indicate a significant difference at $P < 0.05$ by Tukey's test.

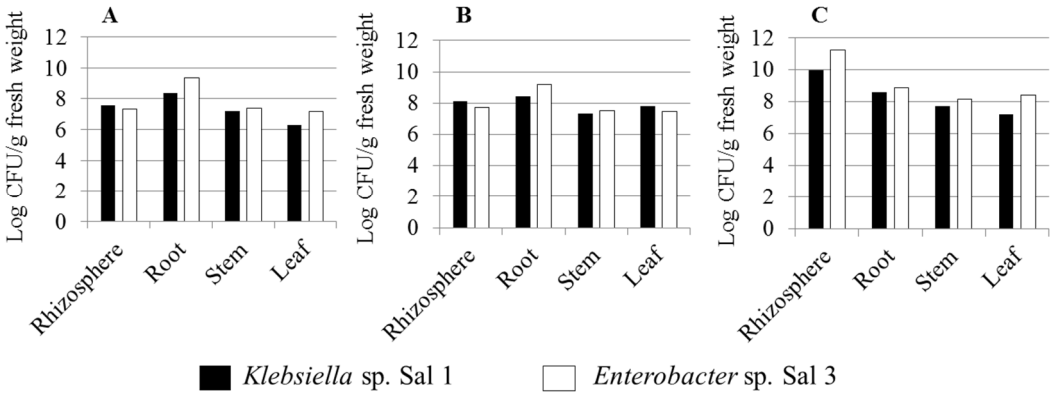

Figure 8. Colonization of the inoculants in tomato plant parts in nitrogen limiting (**A**) and non-limiting (**B**) conditions in test tubes and nitrogen non-limiting conditions in petri dishes (**C**).

4. Discussion

In this study, eight endophytic bacterial strains isolated from sweet potato were examined for their growth-promoting activities on inoculated plants. A similar nitrogen-dependent reduction of IAA production was reported in *Stenotrophomonas maltophilia* [19]. In the nitrogen-free medium, *Klebsiella* sp. Sal 1 produced a greater amount of IAA compared to Sal 3. Since the strain Sal 1 showed ARA activity, IAA-producing activity seemed to be associated with nitrogen fixation.

The *nif*H gene containing strains *Klebsiella* sp. Sal 1 and *Herbaspirillum* sp. Sal 6 showed a similar trend of ARA activity in the MR medium, where the activity decreased with a sufficient amount of nitrogen. This property was similar to the response of *Herbaspirillum seropedicae* Z78 to nitrogen [20].

Earlier reports have shown that plant-associated bacteria can improve plant growth by IAA production [10,21–23] and nitrogen fixation [8]. In this study, bacterial inoculation also showed positive effects on the growth of sweet potato. However, inoculation effects on growth parameters varied among replications due to the difficulty in preparing similar sizes of sweet potato cuttings in the experiments. Even under such varying conditions, most of the inoculants promoted lateral root growth, resulting in greater total root weight. The production of IAA by bacteria in the rhizosphere

was reported as an important plant growth-promoting factor that stimulates lateral root growth and absorption of nutrients [24]. In this study, the positive effects of the strains *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 might be the result of their IAA production abilities because a similar change in root morphology was observed after inoculation [25,26].

Sweet potatoes and tomatoes were tested under nitrogen limiting and non-limiting conditions at 0.12 and 1.2 g NH_4NO_3/L, respectively. IAA production peaked at relatively low nutrient levels. Although the nitrogen levels set in this study were within the inhibitory range for IAA production, it was expected that the high nitrogen levels might decrease the optimum level in the plants and microbial consumption. Because a similar morphological change to that caused by IAA was observed (unpublished data) by the inoculation, and IAA was not detected ($\leq$0.1 μg/mL) in the culture solution, it was hypothesized that IAA was produced in the plant at lower nitrogen levels where the high populations of the endophytes colonized. In addition, other mechanisms of plant growth promotion by the endophytes may have occurred including phosphate solubilization [23], 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity [27], siderophore production [28], and production of other plant hormones like gibberellic acid (GA3) [29] and cytokinins [30].

The effect of the inoculants was more apparent in the plants grown on Gelrite petri dishes. Higher colonization of the inoculated strains in the petri dish conditions also suggested microbial participation in plant growth promotion.

Both *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 colonized the rhizosphere and tissues of tomato when seed was inoculated at higher levels. Their cellullase- and pectinase-producing properties [15] might help them enter into the plant tissue. Hydrolytic enzymes at the infection site leads to cell wall degradation and entry of bacteria such as *Rhizobium* and *Azospirillum* strains in white clover [31].

High-colonizing endophytes could be applied as biofertilizers or biocontrol agents [32]. The effects of the sweet potato endophytes on tomato plants in this study suggested that they have the potential to colonize the rhizosphere and plant tissue, and to establish a symbiotic relationship with plants besides sweet potato. Further studies are necessary to confirm their endophytic establishment and plant growth promotion under field conditions where diverse microorganisms already exist. To protect the inoculated strains from competition against indigenous microorganisms, the establishment of the useful endophytes on seeds or seedlings before their planting in field environments is proposed.

Author Contributions: S.A.D. and K.I. conceived and designed the experiments; S.A.D. performed the experiments; R.R.P. isolated and sequenced the bacterial strains used in this study; S.H. helped to conduct the experiment, data recording and analysis; F.A. performed the statistical analysis; S.A.D. wrote the paper with significant contributions from K.I.

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