Study on plant growth promoting properties of endophytic bacteria affected by their abiotic and biotic environments

（非生物的および生物的環境条件に影響される細菌エンドファイトの
植物成長促進特性に関する研究）

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APPROVAL SHEET

This thesis entitled **'Study on plant growth promoting properties of endophytic bacteria affected by their abiotic and biotic environments'** prepared and submitted by **Ms. Sabitri Adhikari Dhungana** in partial fulfillment of the requirements for the degree of Doctor of Philosophy has been examined and hereby recommended for approval and acceptance**.**

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CHAPTER 1

General Introduction

1.1. Endophytic bacteria

'Endophyte' is derived from the two Greek words 'endon' (within) and 'phyte' (plant). The bacteria, which live inside the plant tissues and generally cause no harmful effects to the plants, are called endophytic bacteria. Hallmann et al. (1997) defined endophytic bacteria as; isolates from the surface sterilized plant parts or from the extracts of inner tissues which generally cause no damage to the host plant. It is well known that many of them have the growth promoting abilities for plants directly by producing plant hormones and fixing dinitrogen or indirectly by reducing damage caused by pathogens or environmental stresses (Etminani and Harighi, 2018; Khan and Doty, 2009). Since living within plant's tissues on the other hand, bacterial endophytes obtain a reliable source of nutrients from the host plant (Yadav and Yadav, 2017). In addition of the plant-microbe interaction, it is supposed that endophytic bacteria interact positively or negatively within their community in plant.

1.2. Direct roles of endophytic bacteria to plant growth

1.2.1. Production of phytohormones

Phytohormones are the class of organic substances, acting as chemical messengers that can control plant growth and development at very low concentrations. They are synthesized in defined organs of the plant that can be translocated to other sites, where it triggers specific biochemical, physiological and morphological responses. Not only plants, microorganisms including bacteria and fungi are also the other sources of phytohormones (Costacurta and Vanderleyden, 1995; Patten and Glick, 1996; Botini et al., 2004; Egamberdieva et al., 2017). Many endophytic bacteria isolated from tropical legumes (UmaMaheswari et al., 2013), rice (Shylla et al., 2016) and millets (Raveendra Reddy et al., 2018) were reported to produce phytohormones such as auxin (indole-3-acetic-acid (IAA)), gibberellic acid (GA) and cytokinin (CK).

Auxin

Indole-3-acetic acid (IAA) is the most studied auxin and frequently auxin and IAA are considered as interchangeable terms (Spaepen, 2007). IAA controls many physiological processes including cell enlargement, division and differentiation (Haber, 1962; Abidin and Metali, 2015), phototropism, geotropism and various other developmental changes (Zhao, 2010). Plant response to IAA varies from plant to plant. Suitable concentration of IAA in one plant might be detrimental or less sensitive to another plant. For example, IAA at higher than 10^{-8} M inhibited the root growth in lettuce (Tanimoto and Watanabe, 1986), while concentrations below 10^{-5} M was not effective in *Dillenia* plant (Abidin and Metali, 2015). Many endophytic bacterial isolates has been reported with high IAA producing ability as follows; *Pseudomonas*, *Bacillus*, *Enterobacter* and *Micrococcus* in rice (Mbai et al., 2013); *Burkholderia*, *Enterobacter*, *Pseudomonas* and *Acinetobacter* in soybean (Kuklinsky-Sobral et al., 2004) *Rahnella*, *Pseudomonas* and *Enterobacter* in sweet potato (Khan and Dotty, 2009); *Klebsiella* and *Enterobacter* in sugarcane, rice and *Piper nigrum* (de Santi Ferrara et al., 2011; Ji et al., 2013; Jasim et al., 2013a, 2013b).

Gibberellin

Gibberellin (GA) or gibberellic acid is plant hormone, involved in a number of developmental and physiological processes such as stem elongation, leaf growth, stem branching, female flower induction (Dalai et al., 2015), dormancy breaking (Debeaujon and Koornneef, 2000; Patel and Mankad 2014) and root elongation (Inada and Shimmen, 2000). It was discovered during a study of rice seedling disease '*bakanae'* in Japan in 1930s. The soil-borne fungus, *Gibberella fujikuroi* secreted GA that made the plant taller with no seed production. GA is produced not only by the fungus and plants but also by bacteria. In bacteria, gibberellin is a secondary metabolite with unknown role for themselves but believed to be used as a signaling factor to make association with plants (Bottini et al., 2004). Atzorn et al. (1988) reported the presence of GA1, GA4, GA9 and GA20 along with IAA in the culture of *Rhizobium phaseoli*. Many studies have shown that *Azospirillum* have ability to produce this hormone. Inoculation of GAs-producing endophytic strains of *Azospirillum brasilense* Cd and A. *lipoferum* USA 5b improved the sheath elongation of GA-deficient dwarf rice mutants (Cassan et al., 2001). In another study, GA3 producing

Azospirillum brasilense improved seed germination in soybean (Cassan et al., 2009). *Arthrobacter koreensis*, an endophyte of halophyte *Prosopisstrom bulifera* was also able to produce GA1 and GA3 along with IAA, abscissic acid (ABA) and jasmonic acid (Piccoli et al., 2011).

Cytokine

Cytokine (CK) is an important group of plant hormones involved in cell division, growth and differentiation, and prevention of senescence (Schmulling, 2002). Balance of the auxin and cytokine plays an essential role in plant morphogenesis with a strong impact on the root formation and relative growth of roots and shoots. Endophytes *Psuedomonas resinovorans*, *Paenibacillu spolymaxa* and *Acenitobacter calcoaceticus* are known as CK producers (Bhore et al., 2010).

1.2.2. Nitrogen fixation

Nitrogen (N) occupies a conspicuous place in all living cells. It is an essential component of many biomolecules including amino acids, proteins, enzymes, DNA and chlorophyll. Although it is highly abundant in atmosphere as dinitrogen gas (N_2), plants cannot use it as nitrogen source. Certain bacteria and archaea can convert atmospheric nitrogen into ammonia by an enzyme nitrogenase which is called biological nitrogen fixation (BNF). The bacteria having an ability of BNF are called diazotrophs. Presence of several diazotrophic endophytes in sweet potato tuber and stems as genus *Sinorhizobium*, *Rhizobium*, *Klebsiella*, *Paenibacillus* (Reiter et al., 2003) and *Bradyrhizobium*, *Pelomonas*, *Bacillus* (Terakado-Tonooka, 2008) suggested the associative N_2 fixation and contribution to the N uptake in sweet potato. Diazotrophic endophytes *Herbaspirillum*, *Ideonella*, *Enterobacter* and *Azospirillum* were also isolated from rice leaves (Elbeltagy et al., 2001).

1.2.3. Stress induced aminocyclopropane-1-carboxylate (ACC) deamination

Growth of plant is affected by numbers of biotic and abiotic stresses. Stress induces ACC production (Morghan and Drew, 1997) and act as a precursor of ethylene synthesis in plants (Adams and Yang, 1979). As a part of stress response, it inhibits root elongation, speeds aging

and promotes senescence and abscission. Some bacteria that can degrade ACC to ammonia and alpha-ketobutyrate by ACC deaminase (ACCD) can help plant growth by inhibiting ethylene production (Hao et al., 2007). The bacteria use ACCD to utilize ACC as the source of carbon and nitrogen. Consequently, the growth of the microorganisms is accelerated and the level of ACC decreases in plants. Such bacteria can protect the plants from ethylene induced by stresses such as flood, drought, high salt and pathogen attacks (Glick, 2014). Inoculation of bacterial endophytes such as *Ralstonia* sp., *Pantoea agglomerans* and *Pseudomonas thivervalensis* from the cupper tolerant plants with ACCD producing ability improved the biomass of *Brassica napus* (Zhang et al., 2011).

1.3. Indirect roles of endophytes to plants

1.3.1. Production of antibiotics and lytic enzymes

Prevention of plant disease is one of the biggest challenges for food production as well as ecosystem stability. Intensive farming system is increasing the dependency on agrochemicals and consequently increasing the pesticide resistance (Christina et al., 2013). Some microbes can produce a range of bioactive secondary metabolites which could control the plant pathogens. Such microbes could indirectly promote the plant growth by suppressing the diseases.

Several studies have shown control of plant diseases by endophytic bacteria. Chilli root endophytic bacteria *Klebsiella oxytoca* AVSCE5 showed an antimicrobial activity against the pathogens *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* sp., *Streptococcus sp.* and *Colletotrichum* sp., while, did not against the beneficial rhizospheric bacteria *Pseudomonas aureginosa* (Syed et al., 2017).

Production of the lytic enzymes such as chitinases, glucanases, lipase and proteases can lyse the cell components of several pathogenic fungi (Glick, 2012). In another study, maize seed bacterial endophytes *Bacillus* synthesized the lytic enzymes such as amylase, esterase, lipase and protease, and exhibited antagonistic effects against the rotting pathogenic fungi *Rhizoctonia solani*, *Sclerotia rolfsii* and *Macrophomina phaseolina* (Bodhankar et al., 2017).

1.3.2. Induction of systemic resistance against pests and pathogens

Some plant growth promoting bacteria can trigger a phenomenon in plants known as induced systemic resistance (ISR), in which defense mechanisms of the plants are activated when pathogens or insects attack. The significant reductions in juvenile penetration rate and number of root-knot galls of the tomato plants were observed when the tomato was treated by the endophytic bacteria *Pantoea agglomerans* MK-29, *Cedecadavisae* MK-30, *Enterobacter* spp. MK-42 and *Pseudomonas putida* MT-19 (Munif et al., 2001).

1. 4. Factors affecting functions of bacterial endophytes

Endophytic functions were influenced by culture conditions *in vitro*. Such as, IAA production by *Bacillus subtilis* was influenced by the temperature, inoculums size and the incubation period (Hussein et al., 2016). Source of carbon and nitrogen, and pH have also affected the IAA production in *Bacillus* and *Paenibacillus* (Acuna et al., 2011; Mohite, 2013). A diazotrophic bacteria *Herbaspirillum seropedicae* Z78 showed the highest nitrogen fixation activity and the lowest IAA production activity in the absence of nitrogen in the medium, whereas, the highest external nitrogen caused a significant decrease in nitrogen fixation activity and an increased production of IAA (Yin et al., 2015). Inoculation of a rice endophytic diazotrophic bacterium *Pantoea agglomerans* YS19, having IAA, ABA, GAs and CK producing ability, improved the growth of rice seedlings under nitrogen and carbon free gnotobiotic conditions compared with the nitrogen supplemented condition (Feng et al., 2006).

1.5. Effect of host environment on endophytic community

Plants are colonized by a diverse group of endophytic bacteria (Sturz and Nowak, 2000). Endophytic bacteria appear to originate from seeds, vegetative planting material, rhizosphere soil and the phylloplane (Hallmann et al., 1997). Bacterial endophytes enter through stomata, hydathodes, lenticels germinating radicles or the tissue wounds, then move from external to internal layers of the tissue cells and finally into the xylem, and could distribute into the whole plant system including flowers, fruits and seeds (Sturz et al., 2000). Endophytic colonization would depend on the host plants conditions influenced by soil types, moisture content, temperature, agriculture inputs and management practices. It is not clear how the communities

interact with the host plants but it is supposed that their interaction with the host plant effects on plant growth.

1.6. Use of endophytes as biofertilizers

Biofertilizers consistes of living beneficial microorganisms as discussed in previous section in case of endophytic bacteria applied to seed/seedling, soil and composting process to promote plant growth.

The endophytic bacteria, which can effectively colonize the plants and promote plant growth, are considered as potential biofertilizers. Several endophytic nitrogen fixing bacteria such as *Burkholderia*, *Rhizobium*, *Bradyrhizobium*, *Gluconacetobacter*, *Herbaspirillum*, *Serratia* and *Pantoea* isolated from rice; *Burkholderia*, *Rhizobium*, *Sinorhibium*, *Herbaspirillum*, *Pseudomonas* and *Azospirillum* in maize; *Gluconacetobacter*, *Herbaspirillum*, *Bradyrhizobium*, *Enterobacter* and *Klebsiella* in sugarcane and *Rhizobium*, *Cellulomonas* and *Herbaspirillum* in wheat are the good candidates of biofertilizers (Bhattacharjee et al., 2008). IAA producing endophytic bacteria having plant growth promoting abilities, for example; sweet potato endophytes, *Bacillus* sp. P42 and *Bacillus* cereus P31 (Dawwam et al., 2013), berseem clover endophytes *Pseudomonas putida* (Etesami et al., 2015) and strawberry endophytes *Bacillus* spp. and *Sphingopyxis* sp. (Dias et al., 2009) are also the potential candidates for biofertilizers.

It is well known that all plants are inhabited internally by diverse microbial communities (Hardoim et al., 2015). In this context, the important factors that must be considered while using the plant growth promoting endophytic bacteria as a biofertilizers are probable competition among microbial communities.

Some bacterial endophytes promote the plant growth individually or through the synergistic interaction with other bacteria and fungi. Co-inoculation of endophytic *Pseudomonas fluorescens* and rhizospheric *Pseudomonas putida*, bacteria saved the nitrogen fertilizer upto 25% in rice cultivation than single inoculation (Etesami and Alikhani, 2016). Co-inoculation of bacterial endophytes *Bacillus* with *Mesorhizobium* improved the plant growth, nodulation and nitrogen fixation than single inoculation of *Mesorhizobium* in chickpea (Saini et al., 2015).

The effect of co-inoculation is usually beneficial for plant growth (Andrews et al., 2010), but a negative effects of co-inoculation were observed in endophytic *Bacillus megaterium* with rhizospheric *Pseudomonas putida* or *Pseudomonas penetrans* bacteria in tomato (Flor-Peregrín et al., 2014).

1.7. Rationale of this study

Nepal is a small Himalayan country with a high degree of biodiversity. High microbial diversity such as legume nodulating bacteria (Adhikari et al., 2012, 2013) and sweet potato bacterial endophytes (Puri et al., 2018) were reported in Nepal. In our previous study, inoculation of mixed cultures of sweet potato endophytes from each location improved the fresh weight and vine length of sweet potato in growth chamber (Puri et al., 2018). However, responsible strains have remained unclear.

In this study, based on the physiological and plant growth promoting properties, endophytes from 'Salyan', a warm temperate location in Nepal, were selected to further characterize their plant growth promoting activities. Effects of nitrogen level on production of IAA and nitrogen fixation activity have been examined.

There are a lot of studies on plant growth promotion by bacterial IAA producers but its degradation is a less study topic. Considering endophytic community, the plant growth promotion by IAA would be reduced when IAA degrader could degrade IAA in the community. In this study, IAA degrading ability of the selected strains were examined and the interaction of the IAA producer and degrader were elucidated by examining the effect on plant growth.

CHAPTER 2

Plant growth promoting effects of Nepalese sweet potato endophytes

2.1. Introduction

Endophytic bacteria colonize the internal tissue of the host plant, forming a symbiotic relationship without detrimental effects to the tissue of most plants (Rosenblueth and Martínez-Romero, 2006; Ryan et al., 2008). Many plant growth-promoting endophytes can fix nitrogen, produce phytohormones, and express 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Gamalero and Glick, 2011; Gamalero and Glick 2015; Kandel et al., 2017).

Sweet potato (*Ipomoea batatas*, L.) is cultivated in several countries in Asia, Africa, Europe, and America. This plant can be cultivated with little fertilization (Hill et al., 1990; Santoyo et al., 2016), probably due to its association with endophytes (Yonebayashi et al., 2014) 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 (Khan and Doty, 2009; Dawwam et al., 2013).

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 (Souza et al., 2015).

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 (Adhikari et al., 2012; 2013). However, there are limited reports on endophytic bacteria in Nepal. Venkatachalam et al. (2015) 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 (Puri et al., 2018). 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. Tomato and strawberry were also used due to their sensitive response to inorganic nitrogen levels and preparation of uniform seedlings from seeds.

2.2. Materials and Methods

2.2.1. Bacterial strains

Strains used in this study were isolated from Nepalese sweet potato tubers (Puri et al., 2018). Eight sweet potato endophytes from the Salyan location were used in this study (Table 2.1).

2.2.2. Evaluation of plant growth promoting properties

2.2.2.1. IAA production

The ability of the selected eight endophytes to produce IAA was determined following Salkowski assay (Gordon and Weber, 1951). Strains were grown in MR (Modified Rannie) (Elbeltagy et al., 2001) (Table 2.2) liquid medium amended with NH_4NO_3 at 0.1 g/L (N+MR) and 200 µg/mL tryptophan, and incubated at 26°C in 150 rpm. Samples without inoculation were set as control. After 3 days of incubation, an aliquot of the supernatant was taken after centrifugation at 10,000xg for 10 minutes at 4°C. Then, 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 minutes in dark.

The potential of *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3, which showed higher activity, was also examined in 1/2MS plant growth medium (Murashige and Skoog, 1962) (Table 2.3) (in which macro element was adjusted at 1/2 strength) amended with sucrose and tryptophan at 0.87 g/L and 200 µg/ml, respectively. IAA production was also measured at different levels of ammonium nitrate at 0, 0.015, 0.03, 0.24, 0.48, and 1.2 g/L.

2.2.2.2. Nitrogen fixation activity

For *Klebsiella* sp. Sal 1 and *Herbaspirillum* sp. Sal 6, which were reported to have *nif*H gene (Puri et al., 2018), the 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 ×g at 4°C for 10 minutes). A 50 µL aliquot of the cell suspension was poured over slant of MR agar (1.1 %) medium in 121 mL glass bottle containing different levels of nitrogen (NH_4NO_3; 0, 6.25, 12.5, 25, 50, 75 and 100 mg/L). The bottle was

capped, 10% of the air inside the bottle was replaced by acetylene gas, and then incubated in dark at around 30°C for 4 days. After incubation, 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 1/2MS plant growth medium with sucrose at 0.87 g/L at different levels of nitrogen as in MR medium.

2.2.3. Effect of inoculation on sweet potato

The experiment was conducted in a phytotron (LH-240, Nippon Medical & Chemical Instruments Co., Ltd.) with 14 h light and 28/25°C day/night temperature with 6000 to 7000 lux light intensity in white florescent light condition. 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 pot condition, two Leonard jars were overlaid, and the top pot was filled with vermiculite and bottom with liquid 1/2MS medium which was connected by 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 in the cell suspension and 1 mL of suspension was poured on the vermiculite around the plant after transplanting. The experiment was conducted in triplicate and top of the pots were covered by ventilated (< 0.2 mm pore sized) transparent plastic bag (Sunbag, transparent, Sigma-Aldrich, Tokyo, Japan).

In the agar tube condition, sweet potato cuttings were inoculated by dipping in the cell suspension and planted in 1/2MS 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 vermiculite pot and agar tube conditions, two of the most potential bacterial strains; *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 were selected for the further studies at nitrogen-limiting condition (NH_4NO_3 at 0.12 g/L).

2.2.4. Effect of inoculation on tomato

Tomato was selected for further tests because it was most responsive to different levels of inorganic nitrogen than spinach and carrot (data not presented). And it was easy to produce large number of uniform seedlings from seeds as compared to sweet potato. Tomato seeds ('*Chika*' F_1 hybrid, Taki company, Japan) were surface sterilized by dipping in 70 % ethanol for 1 min followed by 1% NaOCl for 15 minutes, then washed 7-8 times with sterilized distilled water. The inoculation experiments were conducted in 2 different culture conditions; liquid media tubes and gelrite petridishes, and the culture conditions were the same as for the sweet potato experiment.

In the liquid media tube condition, 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 concentration of IAA in the culture solution was determined by Prominence Ultrafast Liquid Chromatography System (Shimadzu, Kyoto, Japan) equipped with photodiode array detector (SPD-M20A) and 100L × 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 petridish condition, three inoculated seeds were sown on gelrite (0.27%) solidified 1/2MS medium in a plastic petridish (90 mm × 15 mm). Data were recorded after 24 days of seed sowing.

After recording the plant growth parameters, one plant from each treatment was used for checking colonization by the inoculants. The root part was dipped in 50 mL of sterilized distilled water and gently shaken for suspending the inoculants in the rhizosphere. Then, the root and the stem parts were separated by cutting around 1.5 cm below the cotyledon leaf, 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, 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 petridish were put in a 30 mL glass bottle and ARA was measured as described above.

2.2.5. Effect of inoculation on strawberry

Strawberry was inoculated by one of the potential strain, *Klebsiella* sp. Sal 1 under nitrogen non-limiting condition. This crop was selected as an additional commercial crop other than tomato and sweet potato. Wild strawberry seeds ('Shikinari', Sakata no Tane company, Japan) were stored in -20°C for 5 weeks to break the seed dormancy and surface sterilized by dipping in 70 % ethanol for 1 min followed by 1% NaOCl for 5 minutes, then washed 7-8 times with sterilized distilled water. Sterilized seeds were placed on gelrite (0.27%) solidified 1/2MS medium. At three weeks after germination, similar sized seedlings were selected and used for the inoculation experiment. The inoculant was prepared as in the sweet potato and tomato inoculation experiments. Controlled plants were inoculated by autoclaved cell suspension. Roots of the small seedlings were dipped in the cell suspension for around 30 seconds and then transplanted to gelrite (0.27%) solidified 1/2MS medium in petridish (90 mm × 15 mm). Sealed petridish was cultured under the same conditions as the sweet potato experiment. Experiment was conducted in triplicate and total weight was measured after 16 days of inoculation.

After recording the growth parameter, one plant was used for checking colonization by the inoculant. The procedures were the same as the tomato experiment. In the case of strawberry, only root and leaf parts were used for checking the internal colonization.

2.2.6. Statistical analysis

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

2.3. Results

2.3.1. IAA production

Five strains produced IAA in tryptophan containing liquid N^+MR medium (Figure 2.1). Among them, *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 produced the IAA at high level. These strains produced less IAA in plant growth medium and the production was decreased as the nitrogen levels increased (Figure 2.2). *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 the similar response at a narrower range from 0.015 to 0.03 g/L.

2.3.2. Nitrogen fixation activity

Klebsiella sp. Sal 1 showed higher nitrogen fixation activity than *Herbaspirillum* sp. Sal 6 in MR medium, and in both strains, the activity was decreased with increasing level of nitrogen in the medium (Figure 2.3A and B). The activity was lower in the plant growth (1/2MS) medium and the similar trend was observed for the nitrogen level (Figure 2.3C). No activity was detected for *Herbaspirillum* sp. Sal 6 in 1/2MS medium at any levels of nitrogen tested.

2.3.3. Effect of inoculation on sweet potato

Inoculation showed the positive effect on growth of sweet potato in 3 different experiments in nitrogen non-limiting condition (Figure 2.4). The effects were rather deviated 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 Sal 7 showed a tendency of total weight increment. 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 was recorded. 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 agar tubes was too small as compared to that in the vermiculite pot, root weight and vine length were evaluated and final fresh weight was used in the case of agar tubes instead of recording times increase in the weight. In both experiments, the inoculation showed positive effect on the

increment of total fresh weight, whereas the 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. In overall observation, *Enterobacter* sp. Sal 3 repeatedly stimulated the growth of sweet potato.

In the nitrogen-limiting condition, inoculation of the selected 2 strains showed positive effects on the root number but not on the root weight of sweet potato cultivated in the agar tube (Figure 2.5).

2.3.4. Effect of inoculation on tomato

In vitro tests suggested that the response of tomato seedlings to inoculation with endophytes was related to the nitrogen availability. In N-limiting conditions, strains Sal1 and Sal3 significantly increased shoot length (Figure 2.6). In N-depleted conditions, Sal3 increased the root biomass but the overall effects were not significant. After the cultivation, IAA was not detected (<0.1 µg/ml) in all of the culture solutions when examined by UFLC.

In nitrogen non-limiting condition in petridish, effects of the inoculation were apparent in all of the growth parameters (Figure 2.7). None of the sample showed ARA activity at the end of the cultivation.

Both *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 colonized into tomato plants in large populations (Figure 2.8). In the liquid test tube condition, internal root colonization rate was higher (2.5 - 2.6 × 10^8 and 1.6 - 2.7 × 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 × 10^7 CFU/g and 0.19 - 6.3 × 10^7 CFU/g fresh weight in stem and leaf were detected, respectively for both strains. In rhizosphere, the colonization rate was 0.22 - 1.3 × 10^8 CFU/g fresh weight.

Under nitrogen non-limiting condition in petridish, colonization in 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 rhizosphere, the colonization rate was higher in root at 3.8 – 7.5 × 10^8 CFU/g fresh weight, followed by 0.56 – 1.5 × 10^8 CFU/g in stem and 0.16– 2.8 × 10^8 CFU/gin leaf. In

both systems, population of *Enterobacter* sp. Sal 3 was higher than *Klebsiella* sp. Sal 1 in many samples and no bacterial colony was observed in control plants.

2.3.5. Effect of inoculation on strawberry

The inoculation of *Klebsiella* sp. Sal 1 increased the growth of the strawberry seedlings (Figure 2.9). But due to the large deviation in the inoculated replicates, the effect was not significant.

The inoculated strain Sal 1 colonized the strawberry plant parts in high population (Figure 2.10). As in the petridish grown tomato (Figure 2.8C), colonization in rhizosphere was the highest and followed by roots and leaves. Colonization in rhizosphere was 1.2 x 10^{10} CFU/g fresh weight and internal colonization in root and leaf was 1.6 x 10^{8} and 1.5 x 10^{7} CFU/g fresh weight, respectively. No colony was observed in the control plants.

2.4. Discussion

In this study, eight endophytic bacterial strains isolated from sweet potato were examined for their growth promoting activities on the inoculated plants. In the nitrogen free medium, *Klebsiella* sp. Sal 1produced a greater amount of IAA compared to Sal 3. Similar nitrogen-dependent reduction of IAA production was reported in *Stenotrophomonas maltophilia* (Othman et al., 2013). 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 MR medium in which the activity was decreased with sufficient amount of nitrogen. This property was similar to the response of *Herbaspirillum seropedicae* Z78 to nitrogen (Yin et al., 2015).

Earlier reports have shown that plant associated bacteria can improve the plant growth by IAA production (Dawwam et al., 2015; Raut et al., 2017; Ali et al., 2009; Dias et al., 2009) and nitrogen fixation (Yonebayashi et al., 2014). In this study, the bacterial inoculation also showed the positive effects on the growth of sweet potato but the effects were not apparent. The growth parameters were deviated within the replications due to the difficulty of preparing similar size of sweet potato cuttings in the experiments. Even under such deviated conditions, most of the strains promoted the lateral root growth resulting intothe total root weight increment. Production of IAA by bacteria in rhizosphere was reported as an important plant growth promoting factor that stimulates lateral root growth and absorption of nutrients (Egamberdieva, 2012). 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 the similar change in root morphology was observed by the inoculation (Haahtela et al., 1988; Lin et al., 2012).

Sweet potato and tomato were tested under nitrogen limiting and non-limiting conditions at 0.12 and 1.2 g NH_4NO_3/L, respectively. IAA production was peaked at relatively low nutrient levels. Although the nitrogen levels set in this study were the inhibitory range for IAA production, it was expected that the high nitrogen levels might decrease to the optimum level through the plant and microbial consumption. Because the similar morphological change as

caused by IAA was observed (unpublished data) by the inoculation, and IAA was not detected (≤ 0.1 µg/ml) in the culture solution, it was supposed that IAA was produced in plant at lower nitrogen levels where the high populations of the endophytes colonized. In addition, other mechanisms of plant growth promotion by the endophytes include phosphate solubilization (Dias et al., 2009), 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Gravel et al., 2007) siderophore production (Sharma and Johri, 2003) and production of other plant hormones like gibberellic acid (GA3) (Brown and Burlingham, 1968) and cytokinins (Akiyoshi et al., 1987).

The effect of the inoculants was more apparent in tomato grown on gelrite petridishes. Higher colonization of the inoculated strains in the petridish conditions also suggested the microbial participation in the plant growth promotion.

Both *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 colonized the rhizosphere and tissues of tomato when inoculated at higher levels by the seed inoculation. Similar result was observed in strawberry seedlings when root was inoculated by *Klebsiella* sp. Sal 1. Their cellullase and pectinase producing properties might help them to enter into the plant tissue (Puri et al., 2018). The hydrolytic enzymes at the infection site leads to the cell wall degradation and entry of bacteria such as *Rhizobium* and *Azospirillum* strains in white clover (Plazinski and Rolfe, 1985).

2.5. Conclusions

High colonizing endophytes could be applied as biofertilizers or biocontrol agents (Compant et al., 2010). The effects of the sweet potato endophytes on tomato and strawberry plants, in this study, suggested that they have potential to colonize at rhizosphere and in plant tissues, and to establish symbiotic relationship with the 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, establishment of the useful endophytes in seeds or seedlings before planting in field environments is proposed.

Table 2.1. Endophytes used in this study isolated from sweet potato cultivated in Salyan, Nepal.

Strain	***Most similar genus**	**Class**	**Accession number**
Sal 1	*Klebsiella* sp.	Gammaproteobateria	LC389410
Sal 2	*Flavobacterium* sp.	Flavobacteria	LC389415
Sal 3	*Enterobacter* sp.	Gammaproteobacteria	LC389433
Sal 4	*Rhizobium* sp.	Alphaproteobacteria	LC389434
Sal 5	*Stenotrophomonas* sp.	Gammaproteobacteria	LC389439
Sal 6	*Herbaspirillum* sp.	Betaproteobacteria	LC389442
Sal 7	*Agrobacterium*sp.	Alphaproteobacteria	LC389443
Sal 8	*Microbacterium* sp.	Actinobacteria	LC389445

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

Table 2.2. Nutrient composition of Modified Rannie (MR) medium

Constituent	**Final concentration (g/L)**
K_2HPO_4	0.8
KH_2PO_4	0.2
NaCl	0.1
Na_2MoO_4-$2H_2O$	0.025
Fe（III）-EDTA	0.012
Yeast extract	0.1
Sucrose	0.5
Mannitol	0.3
DL-Malic acid	0.13
$MgSO_4$-$7H_2O$	0.2
$CaCl_2$-$2H_2O$	0.044
p-aminobenzoic acid	10 μg
Biotin	5 μg

Adjusted pH at 6.8

Source: Elbeltagy et al., 2001

Table 2.3. Nutrient composition of Murashige and Skoog (MS) medium

Ingredient	**Constituent**	**Final concentration (mg/L)**
Macro nutrient	$NH_4 NO_3$	2400
	KCl	1400
	$CaCl_2.7H_2O$	440
	$MgSO_4. 7H_2O$	370
	KH_2PO_4	170
Micronutrients	KI	0.83
	H_3BO_3	6.2
	$MnSO_4.4H_2O$	22.3
	$ZnSO_4.7H_2O$	8.6
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
	Na_2.EDTA	37.3
	$FeSO_4.7H_2O$	27.8
Vitamins and organics	Pyridoxine (HCL)	0.5
	Thiamine (HCL)	0.1
	Myoinositol	100
	Glycine	2
	Nicotinic acid	0.5

Adjusted pH at 5.78

Source: Murashige and Skoog, 1962

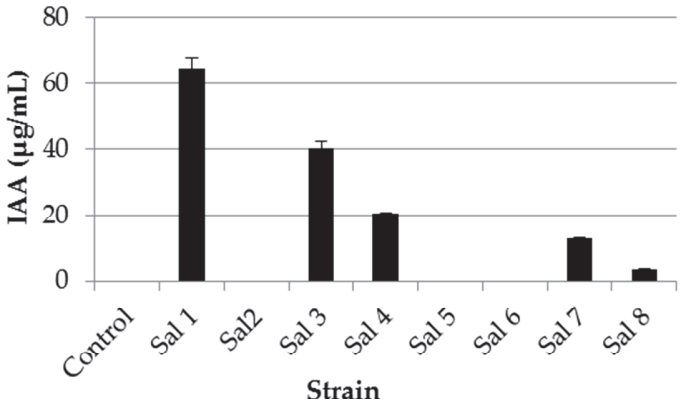

Figure 2.1. IAA production by sweet potato endophytes in N^{+}MR medium. The bars represent standard deviation (n=3).

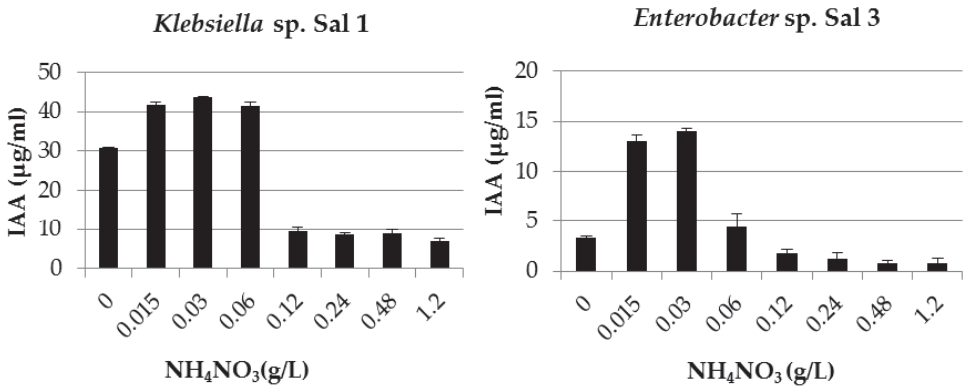

Figure 2.2. IAA production at different levels of nitrogen in 1/2MS liquid medium. The bars represent standard deviation (n=3).

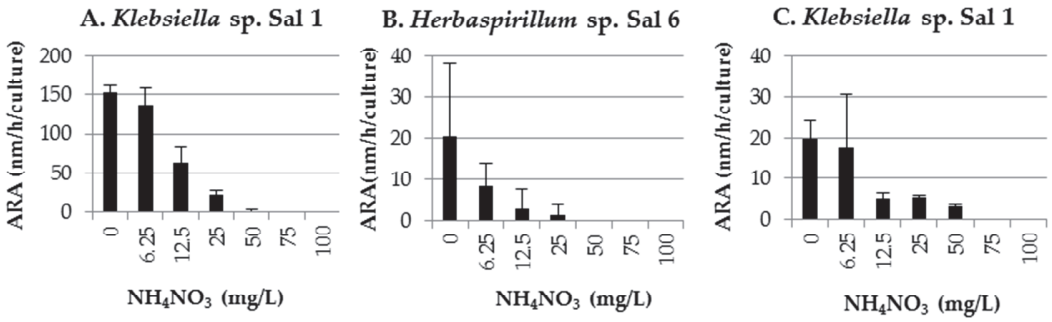

Figure 2.3. ARA in MR (A and B) and 1/2MS (C) agar medium at different levels of nitrogen. The bars represent standard deviation (n=3).

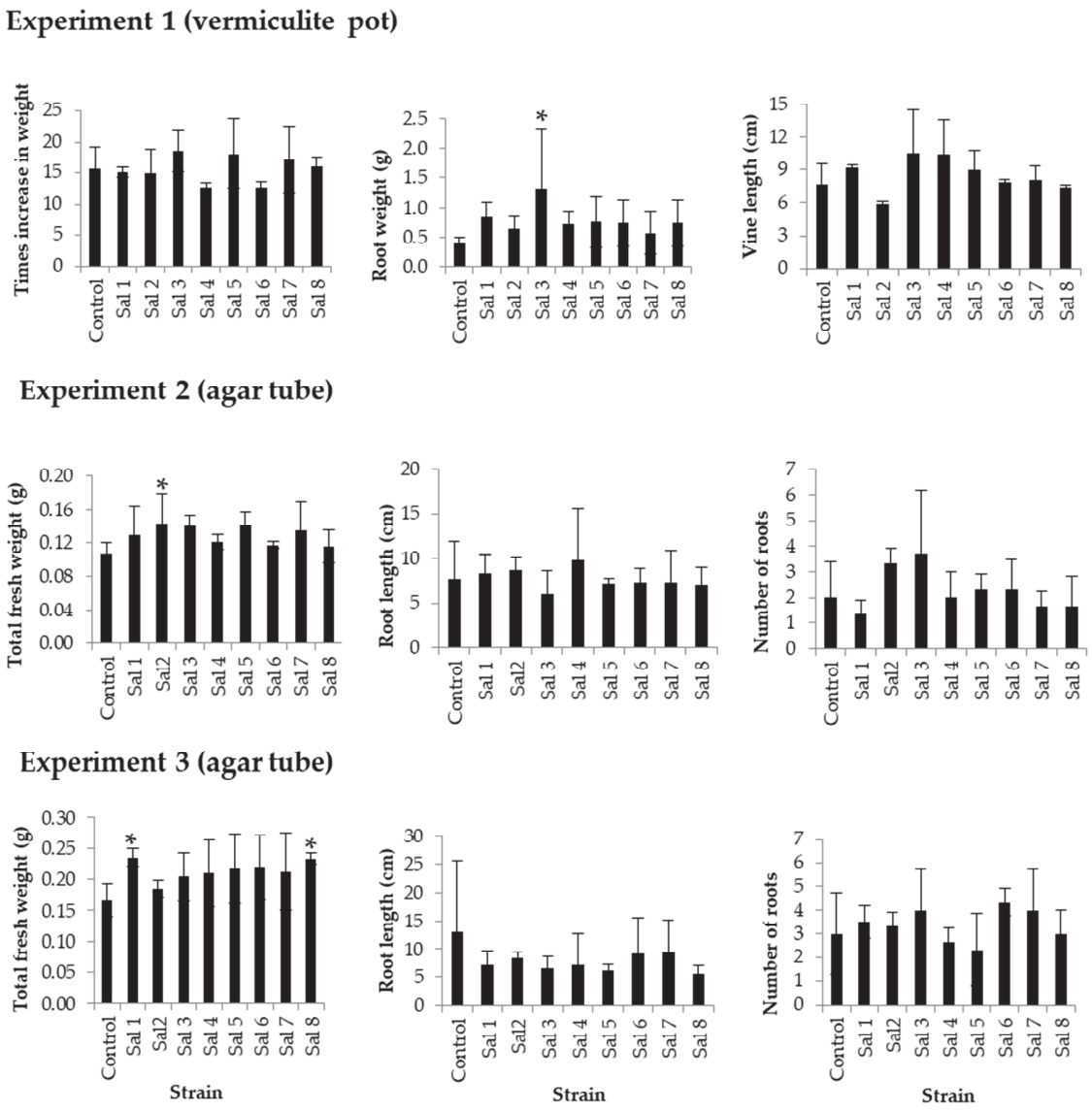

Figure 2.4. Effect of inoculation of sweet potato endophytes in nitrogen non-limiting condition. The bars represent standard deviation (n=3) and asterisks indicate significant difference at P<0.05 by William's test.

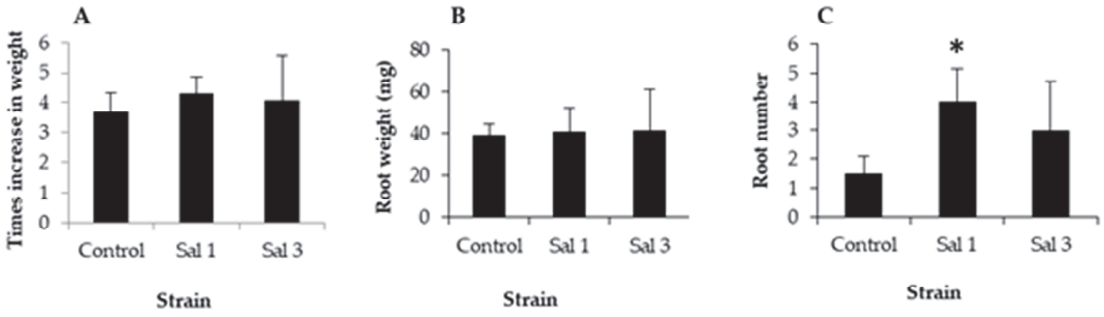

Figure 2.5. Effect of inoculation of sweet potato endophytes on nitrogen-limiting condition in agar tube. The bars represent standard deviation ($n \geq 3$) and asterisks indicate significant difference at $P<0.05$ by Tukey's test.

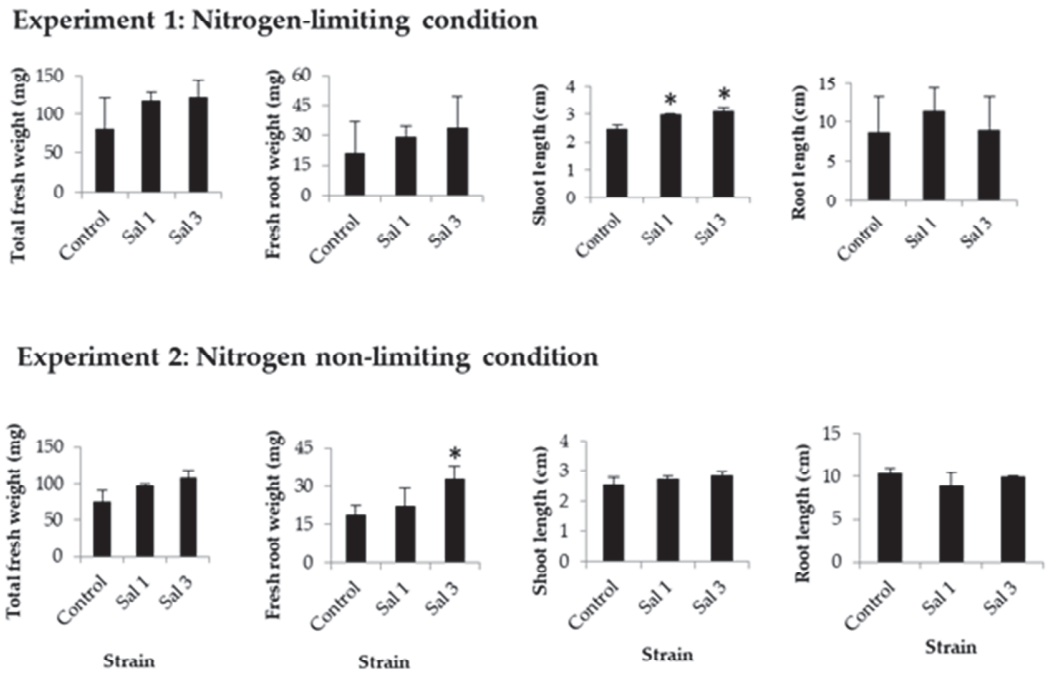

Figure 2.6. Effect of inoculation of sweet potato endophytes on the growth of tomato in nitrogen-limiting (Experiment 1) and non-limiting (Experiment 2) conditions in liquid media tube. The bars represent standard deviation ($n \geq 3$) and asterisks indicate significant difference at $P<0.05$ by Tukey's test.

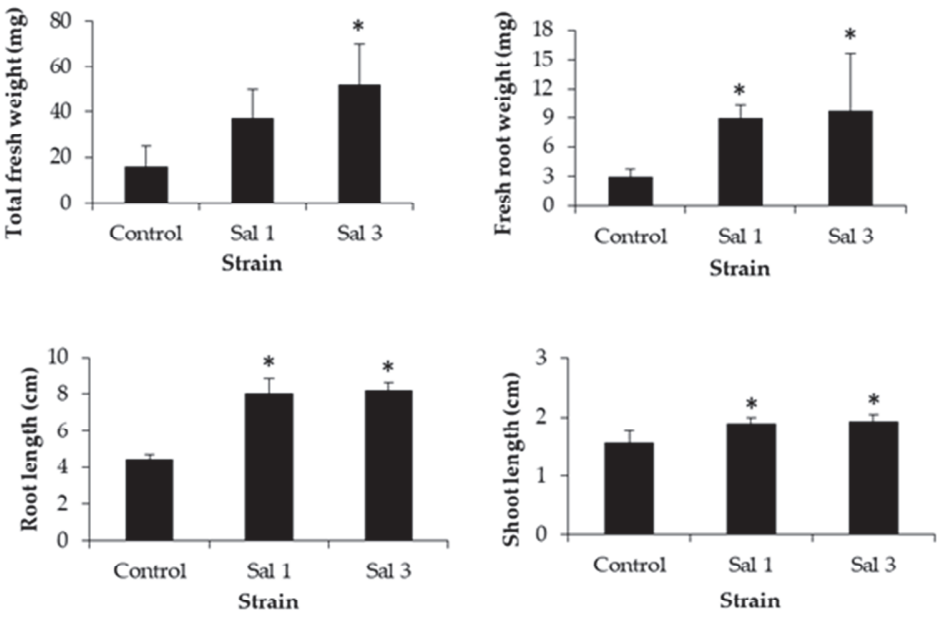

Figure 2.7. Effect of inoculation of sweet potato endophytes on the growth of tomato in nitrogen non-limiting condition in gelrite petridish. The bars represent standard deviation (n=3) and asterisks indicate significant difference at $P<0.05$ by Tukey's test.

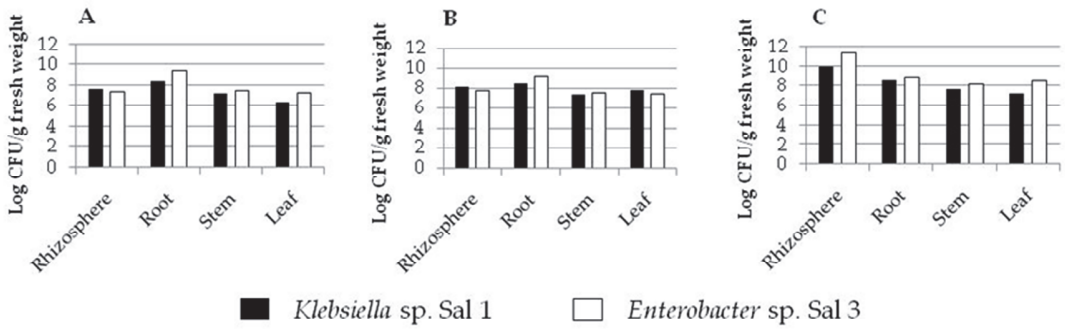

Figure 2.8. Colonization of the inoculants in tomato plant parts in nitrogen limiting (A) and non-limiting (B) conditions in test tube and nitrogen non-limiting condition in petridish (C).

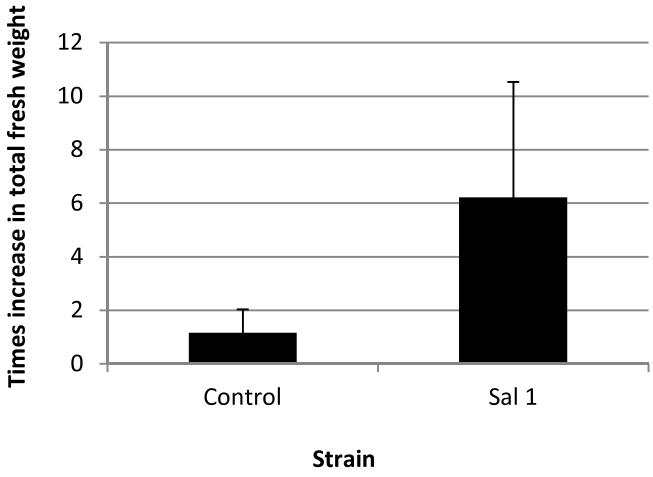

Figure 2.9. Effect of inoculation of sweet potato endophyte Sal 1 on the growth of strawberry in nitrogen non-limiting conditions. The bars represent standard deviation (n=3).

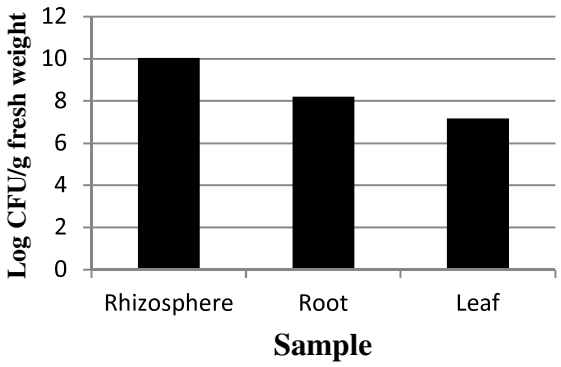

Figure 2.10. Colonization of the inoculants Sal 1 in strawberry plant parts.

CHAPTER 3

Effects of co-inoculation of indole-3-acetic acid (IAA) producing and degrading bacterial endophytes on plant growth

3.1. Introduction

Indole-3-acetic acid (IAA) is an important substance that regulates different developmental processes in plants such as cell division, elongation, differentiation, and response to gravity and light. Concentration of IAA in plant is crucial for controlling the growth (Tanimoto, 2005), and controlled through biosynthesis, conjugation, degradation and intercellular transport (Tromas and Perrot-Rechenmann 2010). The IAA action on plant growth is considered to be concentration dependent, and externally applied IAA showed optimal concentration around 10^{-9} M for roots and 10^{-5} M for stems (Tanimoto, 2005). On the other hand, application of IAA biosynthesis inhibitors resulted in reduction of endogenous IAA contents followed by suppression of elongation and growth of tomato seedlings (Higashide et al., 2014).

In addition to the endogenous IAA in plants, there have been a plethora of studies describing the IAA-producing bacteria including endophytes (Khan and Doty, 2009; Mohite, 2013; Dawwam et al., 2013; Etesami et al., 2015). Application of IAA producing bacteria has shown significant increase in plant growth and yield as follows; *Pseudomonas fluorescens* and *Bacillus subtilis*in onion (Reetha et al., 2014), *Rahnella aquatilis*in hybrid poplar (Khan and Dotty, 2009), *Enterobacter ludwigii* in rice (Susilowati et al., 2018) and *Klebsiella pneumonia*in wheat and moth bean (Sachdev, 2009), however, most of them were conducted under controlled conditions and single strain inoculation. It is well known that all plants are inhabited internally by diverse microbial communities (Hardoim et al., 2015), therefore, interactions in both positive and negative aspects are supposed among them.

Not only IAA producing bacteria, IAA degrading bacteria have been reported as a member of epiphytic community in pea plants (Libbert and Risch, 1969) and in rhizospheres of pine tree (Raczkowska- Błach et al., 1995) and tomato (Gravel et al., 2005; Gravel et al., 2007). So, in this context, when IAA producing bacteria are present as plant growth promoting endophytes or used as biofertilizer, presence of IAA degrading bacteria in the endophytic community could

eliminate or reduce the effects by decreasing concentration of IAA in plant. A number of studies have been carried out on IAA producers and their role in plant growth promotion, but the endophytic IAA degraders have been a less study topic regardless their importance considering their interaction with IAA producers. In our previous work, eight endophytic bacterial strains isolated from the same sweet potato sample were studied for their IAA producing and nitrogen fixing abilities, and their potentials of plant growth promotion (Dhungana et al., 2018). The aim of this study was to examine the IAA degrading ability of these strains, and to elucidate the effects on the plant growth of co-inoculation of the IAA producers with the degrader.

3.2. Materials and methods

3.2.1. Bacterial strains

Eight sweet potato bacterial endophytes used in previous section for plant growth promotion study were used in this study (Table 3.1).

3.2.2. IAA degrading ability of the bacterial strains

To determine the IAA degrading ability, the strains were cultivated in Modified Rannie (MR) (Elbeltagy et al., 2001) (Table 2.2) liquid medium amended with NH_4NO_3 at 0.1 g/L (N+MR) and 50 µg/mL IAA (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan), and incubated at 26°Cat 150 rotation per minute (rpm) for 6 days. Control was set under the same conditions without inoculation. During the cultivation, OD_{660} was monitored every day, and at 3 and 6 days, 200 □l aliquot of the bacterial culture was taken and centrifuged at 10,000 ×g for 10 min at 4°C. The supernatant was mixed with a double volume of Salkowski reagent (Gordon and Weber, 1951), and kept for 30 min in darkness, and then the absorbance was measured at 530 nm using a UV-VIS spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan).

3.2.3. Fate of IAA under co-cultivation of IAA producing and degrading strains

The IAA producing strains *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 were co-cultivated with IAA degrading strain *Herbasprillum* sp. Sal 6, and fate of IAA was examined. Each strain was cultured in N^+MR liquid medium for 2 days, washed twice with sterilized distilled water after centrifugation at 10,000 ×g at 4°C for 10 min., and then suspended to obtain equal population at10^9colony forming unit (CFU)/mL.A9 µL aliquot of the cell suspension was added to 3 mL of N^+MR liquid medium amended with tryptophan at 200 µg/mL and incubated as mentioned above. OD at 660 nm, concentrations of IAA and tryptophan were measured at 12, 24, 36, 48, and 72 hours after inoculation. Each strain was individually cultured under the same conditions. IAA and tryptophan in the culture were quantified by Prominence Ultrafast Liquid Chromatography (UFLC) System (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector (SPD-M20A) and 100Lx3.0 column. The solvent system, 0.5% formic acid and

acetonitrile (75/25; V/V) was used, and IAA and tryptophan were detected at 278 nm. The experiment was done in triplicate.

3.2.4. Effect of co-inoculation of IAA producing and degrading strains on plants

The IAA producing (Sal 1) and degrading (Sal 6) strains were individually inoculated and co-inoculated to tomato and radish plants, and their effects on the plants were examined. Seeds of tomato (Momotaro F_1 hybrid) and radish (Taibyousoubutori 2gou) were purchased from Takii & Co., Ltd.(Kyoto, Japan), and surface sterilized by dipping in 70% ethanol for 1 min followed by 1% NaOCl for 13 and 18 min for tomato and radish, respectively, and washed 7-8 times with sterilized distilled water. The seeds were inoculated by dipping them overnight in the bacterial cell suspensions prepared as mentioned above. Control was prepared by dipping the seeds in sterilized cell suspensions by autoclaving at 121°C for 20 min. One inoculated ungerminated seed was sown in a glass tube (1.5 cm id × 10 cm) containing 1.5 g of sterilized vermiculite and 1 mL of liquid 1/2MS medium (Murashige and Skoog, 1962) (Table 2.3) in which the amount of macro element was adjusted to 1/2 strength, and capped with a silicon plug. Each treatment was conducted in 7-12 replications. Growth parameters were recorded after growing for 6 days in a phytotron (LH-240, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) with 14 h light and 28/25°C day/night temperature and 6000 to 7000 lux light intensity in white fluorescent light conditions.

After recording the plant growth parameters, colonization of the inoculated strains was examined using one plant from each treatment. Rhizosphere colonization was examined by dipping and gently shaking the roots in sterilized distilled water. Colonization in the root and leaf was examined by macerating the separated parts in sterilized distilled water using a disposable homogenizer (BioMasher, Nippi, Tokyo, Japan) after surface washing with sterilized distilled water. 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. Morphologies of the colonies of the strains were clearly different on the plate for counting separately.

3.2.5. Effect of exogenous IAA on tomato

The tomato seeds were sterilized in the same way as described above. Then, the seeds were soaked overnight in sterilized distilled water and sown in glass tube (1.5 cm id x 10 cm) containing 1.5 g of sterilized vermiculite supplied with 1 mL of 1/2MS liquid medium containing IAA at 0, 0.005, 0.01, 0.1, 0.5 and 1 µg/mL. The tubes were capped with silicon plug and grown in the phytotron same as in the inoculation experiment. The experiment was conducted in 10 replications. Root length, fresh root and total weight were recorded after 6 days of the seed sowing.

3.2.6. Statistical analysis

Statistical analysis was conducted by student's t test or Tukey's test after one-way ANOVA using MINITAB ver. 14 (MINITAB Inc., USA).

3.3. Results

3.3.1. IAA degrading activity of the bacterial strains

Herbaspirillum sp. Sal 6 degraded all the IAA within 3 days of cultivation (data not shown). *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3, IAA producing strains, significantly degraded IAA by ca. 40%, and *Rhizobium* sp. Sal 4, *Agrobacterium* sp. Sal 7 and *Microbacterium* sp. Sal 8 degraded IAA by ca. 20% at 6 days (Figure 3.1).

The IAA degrading abilities of the strains were reflected to their growth differences in the media with and without IAA. The difference was the largest in the IAA most degrading strain Sal 6, and it was larger in the IAA moderately degrading strain Sal 3. Instead, the difference in the other IAA degrading strain Sal 1 was as same as the IAA little degrading strain Sal 2 (Figure 3.2).

3.3.2. Fate of IAA under co-cultivation of the IAA producing and degrading strains

Fates of tryptophan and IAA are shown in Figure 3.3. Tryptophan was individually degraded by all strains, and IAA was produced by Sal 1 and Sal 3. When IAA-producing Sal 1 and Sal 3 were co-cultivated with IAA degrading Sal 6, lower levels of IAA were detected in the culture, especially in Sal 1 + Sal 6.

3.3.3. Effect of inoculation of IAA-producing and IAA-degrading strains on plants

Inoculation of IAA producing strain Sal 1 caused significantly higher fresh root weight than control, but the effect was reduced by co-inoculation of IAA degrading strain Sal 6 (Figure 3.4). The reduced level was as same as the individual inoculation of Sal 6. Root length and fresh plant weight were not affected by the any inoculations.

In radish plants, the similar tendency was observed in fresh root weight but the difference was not significant due to the large deviations (Figure 3.4). In addition, fresh plant weight was improved by the inoculation of IAA producing strain Sal 1, and the effect was reduced by co-inoculation of IAA degrading strain Sal 6 lower than individual inoculation of Sal 6 (Figure 3.4).

In root length, inoculation of Sal 6 showed negative effects, but it was improved by co-inoculation of Sal 1.

In the individual inoculation, both *Klebsiella* sp. Sal 1 and *Herbaspirillum* sp. Sal 6 colonized tomato and radish plants in high populations (Figure 3.5A). Colonization in rhizosphere was 2-3 order higher than root and leaf in both plants. Population of Sal 1 in tomato was 13 times higher in root than leaf, whereas Sal 6 was 7 times higher in leaf than root. In case of radish, the populations in root and leaf were almost same in both strains.

In the co-inoculation, the rhizosphere, root and leaf were colonized by the bacteria in the similar way as in the individual inoculation (Figure 3.5B). Relative percentage of the population of Sal 6 was higher in all plant parts (75-95%) than Sal 1 except for the root of tomato (33%). Root colonization of Sal 1 was 23 and 8 times higher than leaf in tomato and radish, respectively. Higher root colonization was also observed in Sal 6, but the differences were less (1.4 and 1.3 times in tomato and radish, respectively). No colony was observed in control plants.

3.3.4. Effect of exogenous IAA on tomato

Application of IAA showed the apparent effect on root parameters (Figure 3.6). IAA at 0.01 µg/mL significantly improved the root weight over control. Both root length and total fresh weight significantly decreased at 0.5 and 1 µg/mL of IAA, while the root fresh weight showed the tendency of increase with IAA, the roots were distorted and looked like knot (picture not presented). At the high concentration of IAA, the root fresh weight was not decreased as it might be the reason why the weight was not affected by IAA.

3.4. Discussion

All of the endophytic bacterial strains used in this study presented some ability to degrade IAA in which the IAA non-producing *Herbaspirillum* sp. Sal 6showed the highest activity. The high IAA producing strains *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 also moderately degraded IAA. The growth difference of Sal 3 and Sal 6 with and without IAA in the medium suggested that the IAA degraders utilized IAA as a source of energy. Usage of IAA by the degraders as carbon and energy sources could be related to their colonization potential in the plants. Utilization of IAA as a sole source of carbon, nitrogen and energy for growth was also reported in *Pseudomonas putida* (Leveau and Lindow, 2005). In our study, both IAA producing and degrading endophytes were found in the same sweet potato sample, suggesting that concentration of IAA in plant would be affected by balance of their activity.

Under *in vitro* conditions, co-cultivation of IAA degrading *Herbaspirillum* sp. Sal 6 decreased the concentration of IAA, which was produced by the co-cultured IAA producers. In addition, Sal 6 degraded tryptophan as well as IAA with the highest activity. Therefore, it was suggested that the level of IAA was reduced in two ways; one by degrading the produced IAA and another by degrading tryptophan, a precursor of IAA, in the medium. There have been several examples of tryptophan catabolizing bacteria utilizing it as a sole source of carbon and nitrogen, like *Bacillus cereus*, *Pseudomonas aeruginosa*, *Ralstonia metallidurans* (Kurnasov et al., 2003), *Pseudomonas aureofaciens* (Salcher and Lingens, 1980) and *Bacillus megaterium* (Bouknight and Sadoff, 1975). In the culture amended with tryptophan, the degradation rate of tryptophan by Sal 6 was reduced in the presence of Sal 3, suggesting negative interaction between the two strains. There is also an example of positive interaction such as *Sphingomonas* sp. SRS2 significantly enhanced the metabolism of phenylurea herbicide isoproturon by utilizing the methionine released by co-cultured an unidentified bacterial strain SRS1 (Sorensan et al., 2002). Nutritional conditions have also been reported to affect the bacterial IAA metabolism. The IAA production decreased as the nitrogen levels increased in sweet potato endophytic strains *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 (Dhungana et al., 2018). Degradation of IAA by epiphytic *Alcaligenes* and *Pseudomonas* strains was inhibited in the presence of glucose (Libbert

and Risch, 1969). These results also suggested that levels of IAA in plant would be determined by the results of complex interactions among host plants, microbes and environmental conditions.

Inoculation of *Klebsiella* sp. Sal 1, having the highest IAA producing activity, improved lateral root growth resulted in the increase of fresh root weight of tomato and radish, suggesting that the promoted plant growths were caused by IAA produced by the strain Sal 1. The similar morphological changes observed in exogenous IAA treatment within the tomato. IAA regulates the promotion of lateral root growth (Bao et al., 2004), and inoculation of the other IAA producing plant associated bacteria showed similar effects on the inoculated strawberry (Dias et al., 2009) and mung bean (Patten and Glick, 2002). Decreasing of the effects by co-inoculation of IAA degrading *Herbaspirillum* sp. Sal 6 also suggested that bacterial IAA improved the plant growth. Since both IAA producing and degrading bacteria were found in any parts of the inoculated plants, the bacterial IAA produced seemed to be readily available to the co-existing IAA degrading bacteria.

3.5. Conclusions

When IAA producing strains are used as biofertilizer in agriculture, their interactions with indigenous endophytic communities, especially with IAA degrading endophytes, should be considered. They would interact with the inoculant positively and/or negatively, and the potential of the inoculants might be reduced when the degraders are active in the plant. One of the examples is presented in this study, and this is the first report to the best of our knowledge. Potential of an individual endophyte should be considered as a result of interaction with its community. Therefore, IAA production and its plant growth promotion observed in laboratory experiments would not act in the same way under the actual conditions. These factors should be considered and the mechanisms of the microbial interactions should be further studied.

Table 3.1. Sweet potato endophytic bacterial strains used in this study

Strain	*Most similar genus	Class	Accession number	IAA producing ability (µg/mL)
Sal 1	*Klebsiella* sp.	Gammaproteobacteria	LC389410	65
Sal 2	*Flavobacterium* sp.	Flavobacteria	LC389415	0
Sal 3	*Enterobacter* sp.	Gammaproteobacteria	LC389433	40
Sal 4	*Rhizobium* sp.	Alphaproteobacteria	LC389434	20
Sal 5	*Stenotrophomonas* sp.	Gammaproteobacteria	LC389439	0
Sal 6	*Herbaspirillum* sp.	Betaproteobacteria	LC389442	0
Sal 7	*Agrobacterium*sp.	Alphaproteobacteria	LC389443	13
Sal 8	*Microbacterium* sp.	Actinobacteria	LC389445	4

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

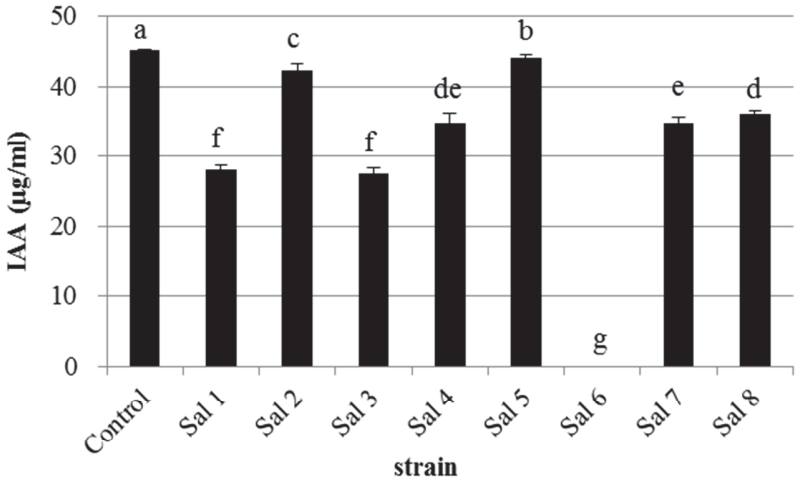

Figure 3.1. IAA degradation by sweet potato N^{+}MR medium. The bars represent standard deviation (n=3) and different letters indicate significant differences at $P < 0.01$ by Tukey's test.

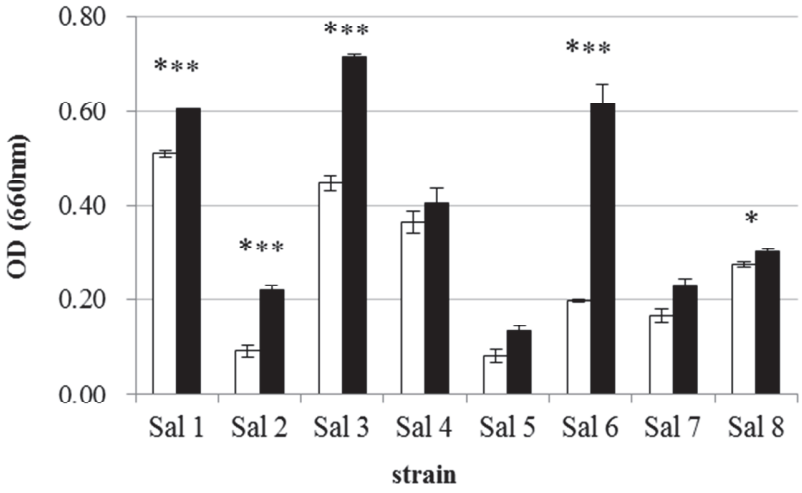

Figure 3.2. Growth of the endophytic bacterial strainsin N^{+}MR media at 6 days with (closed box) and without (open box) IAA. The bars represent standard deviation (n=3) and asterisks indicate significant difference (***P<0.001 and *P<0.05) by student's t test.

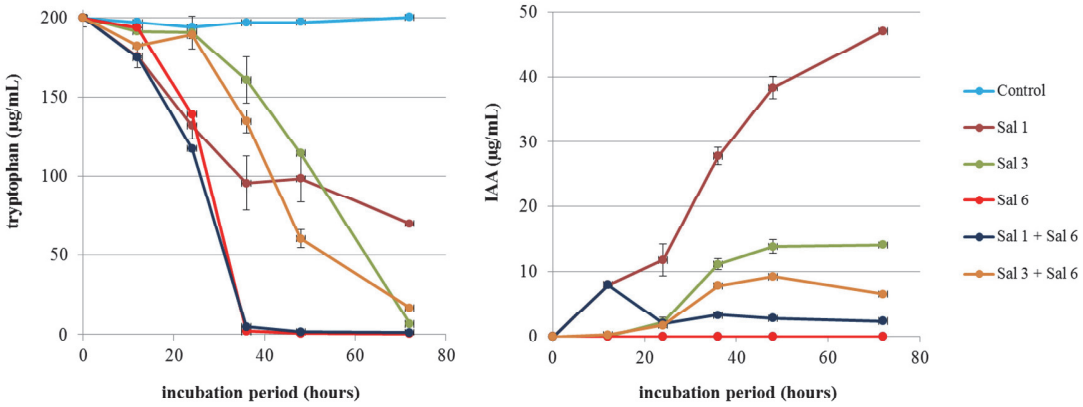

Figure 3.3. Fate of tryptophan and IAA under co-cultivation of the IAA producing (Sal 1 and Sal 3) and degrading (Sal 6) strains of sweet potato endophyte in tryptophan amended medium. The bars represent the standard deviation (n=3).

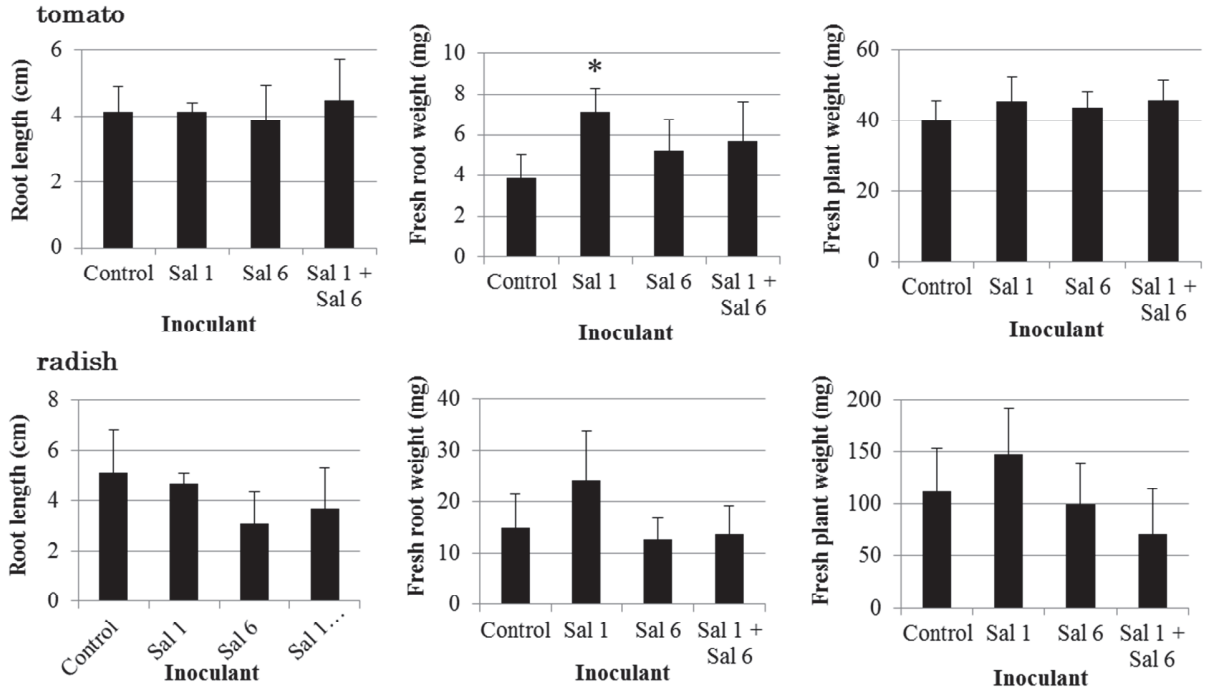

Figure 3.4. Effect of inoculation of IAA producing (Sal 1) and degrading (Sal 6) strains on growth of tomato and radish plants. The bars represent the standard deviation (n=7-12). Asterisk indicates a significant difference at P <0.001 by Tukey's test.

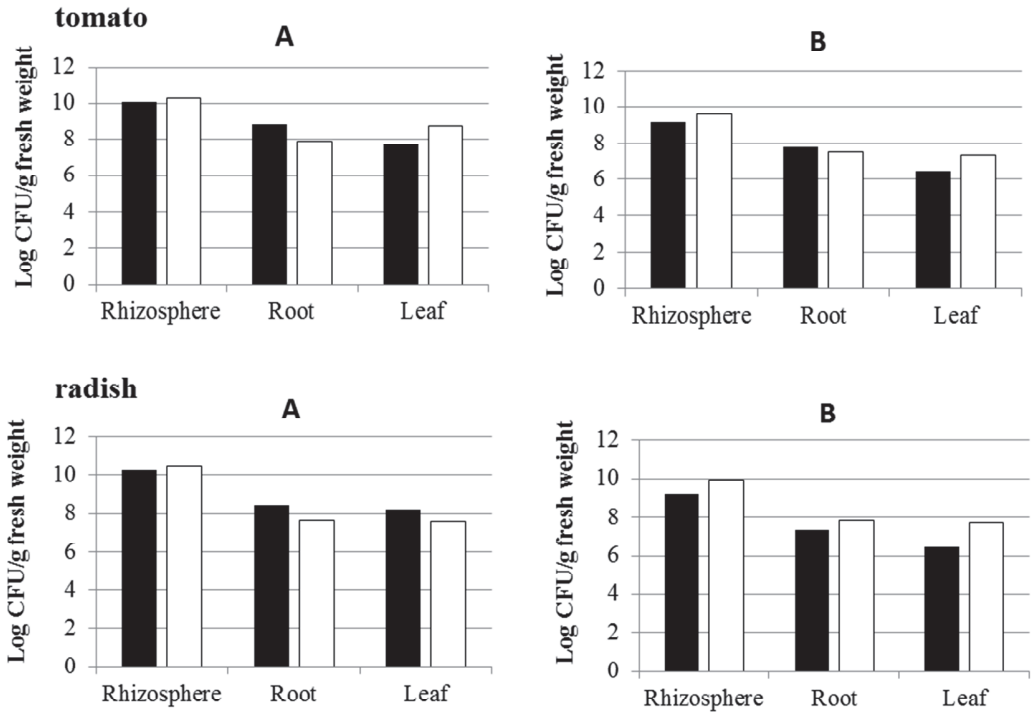

Figure 3.5. Colonization in plant parts of *Klebsiella* sp. Sal 1 (closed box) and *Herbaspirillum* sp. Sal 6 (open box) individually (A) and co-inoculated (B) to tomato and radish seeds.

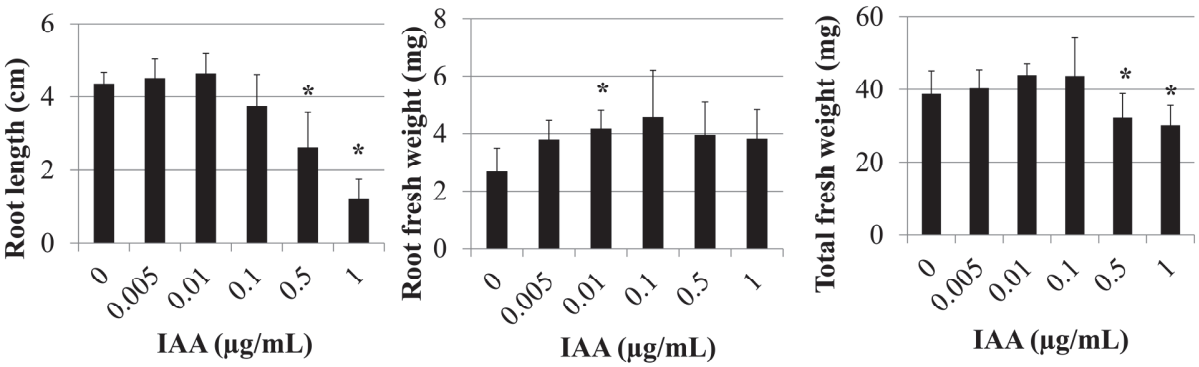

Figure 3.6. Effect of exogenous IAA on growth of tomato plants. The bars represent the standard deviation (n=10). Asterisks indicate significant difference at P <0.05 by Tukey's test.

Summary

Endophytic bacteria inhabit the internal tissue of the plants and generally cause no harmful effects to the host plants. Many of them promote the growth of the host plant through biological nitrogen fixation, synthesis of plant hormones, and so on.Such functions seem to be affected positively or negatively by the host environment and interaction within microbial community. Many researchers have studied the beneficial endophytic properties and their effect on plant growth, but it has not been extensively examined how the endophytic environments and their interaction affect the plant growth.

In our previous study, diverse endophytic bacterial strains were isolated from sweet potatoes cultivated in Nepal, and inoculation of a mixed culture of the isolates improved fresh weight and vine length of sweet potato in a growth chamber, however, responsible strains have remained unclear. In this study, we selected the isolated eight bacterial endophytes from Salyan location, and examined their plant growth promoting properties in relation to effects of nitrogen level in the culture and interaction in the endophytic community in the plants.

The first purpose of this study was to specify the responsible strains among the selected strains, and examine the effects of nitrogen level on their production of indole-3-acetic acid (IAA) and nitrogen fixation activity and on the plant growth promotion by the endophytes. Among the selected eight strains, *Klebsiella* sp. Sal 1, *Enterobacter* sp. Sal 3, *Rhizobium* sp. Sal 4, *Agrobacterium* Sal 7 and *Microbacterium* sp. Sal 4 produced IAA at 65, 40, 20, 13 and 4 μg/mL, respectively, in 0.1g/L NH_4NO_3 amended Modified Rannie (MR) medium. In the two *nif*H gene containing strains, *Klebsiella* sp. Sal 1 showed higher acetylene reduction activity than *Herbaspirillum* sp. Sal 6in MR medium.Inoculation of the strains showed positive effects on the growth of sweet potato in three different experiments in nitrogen non-limiting (1/2MS) conditions cultivated in vermiculite pot and agar tube. Based on the plant growth promoting properties, *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 were selected for further study. The high IAA producers, *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3, showed optimum concentrations at 15–60 mg NH_4NO_3/L for IAA production in plant growth basal medium. For the acetylene reduction activity, *Klebsiella* sp. Sal 1 showed the higher activity at 0–6.25 mg NH_4NO_3/L in MS medium, while no activity was observed in *Herbaspirillum* sp. Sal 6. Under

the nitrogen-limiting conditions (1/2MS with NH_4NO_3 at 120 mg/L), inoculation of the two selected strains showed positive effects on the root number of the sweet potato cuttings. In the tomato seedlings grown on Kimwipes in the liquid medium test tube cultivation conditions, the inoculation of the endophytes showed the tendency to increase total fresh weight and root fresh weight but not shoot and root lengths under both nitrogen-limiting and non-limiting conditions. While in the gelritepetri dish cultivation conditions, the effects of the inoculation were apparent in all of the growth parameters under nitrogen non-limiting conditions. As both the inoculants colonized rhizosphere, root and shoot part of inoculated tomato seedlings, it was supposed that the endophytes produced IAA in plant where lower nitrogen levels were expected. *Klebsiella* sp. Sal 1 also improved the growth of strawberry seedlings under the nitrogen non-limiting gelrite petridish conditions with high colonization of the inoculant in rhizosphere, root and leaf parts.

Bacterial production of IAA and its effects on plant growth have been much studied but less is about the ecology of IAA degrading bacteria. Some studies have shown that plants harbor not only IAA producing bacteria, but also IAA degraders as member of epiphytic and rhizospheric bacterial community.

The second purpose of this study was to examinethe IAA degrading ability of the selected eight endophytic isolates and to elucidate the interaction betweenthe IAA producer and degraderby their co-inoculation.All of the strains including five IAA producers showed the IAA degrading ability, among which *Herbaspirillum* sp. Sal 6 had the highest activity. Large difference in growth of Sal 6 in the media with and without IAA suggested that the IAA degrader utilized IAA as a source of energy. When IAA-producing *Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3 were co-cultivated with IAA degrading *Herbaspirillum* sp. Sal 6 in tryptophan amended medium, IAA concentrations detected were decreased compared with the single inoculation of the IAA producers. As *Herbaspirillum* sp. Sal 6 showed high activity for degrading tryptophan, it was suggested that the co-inoculation of the strain Sal 6 decreased the concentration of IAA by degrading IAA produced by the IAA producers, and/or by degrading tryptophan, a precursor of IAA for the IAA producers. When the IAA producer and degrader were individually or co-inoculated to tomato plantto examine the interactive effects on the plant growth, inoculation of the IAA producing strain *Klebsiella*sp. Sal 1 increased the root fresh weight, but the effect was

reduced by co-inoculation of the IAA degrading strain *Herbaspirillum* sp. Sal 6. Root length and fresh plant weight were not affected by the any inoculations. In radish plant, inoculation of the IAA producer increased the root fresh weight and total fresh weight, and the effect was reduced by co-inoculation of the IAA degrader as the tomato experiment. In the individual inoculation, both *Klebsiella* sp. Sal 1 and *Herbaspirillum* sp. Sal 6 colonized tomato and radish plants in high populations. Colonization in rhizosphere was 2-3 order higher than root and leaf in both plants. Population of Sal 1 in tomato was 13 times higher in root than leaf, whereas Sal 6 was 7 times higher in leaf than root. In case of radish, the populations in root and leaf were almost same in both strains. In the co-inoculation, the rhizosphere, root and leaf were colonized by the bacteria in the similar way as in the individual inoculation. Relative percentage of the population of Sal 6 was higher in all plant parts (75-95%) than Sal 1 except for the root of tomato (33%). High colonization of the inoculated strains suggested that the IAA degrader decreased IAA levels in the plants by degrading IAA and/or its precursor tryptophan. Similar response of tomato to exogenous IAA with that caused by the IAA producing *Klebsiella* sp. Sal 1 suggesting that the root growthpromotion observed in the inoculation of *Klebsiella* sp. Sal 1 was due to IAA produced by the strain.

In this study, colonization of the plant tissue by the endophytesat high population suggested their potential to establish symbiotic relationship with the host plants. Considering their usage as biofertilizer for actual agriculture, their probable positive and negative interactions with existing indigenous endophytic microbial community should be considered. Therefore, the observed effects of plant growth promotion by the inoculants in laboratory experiments would not act in the same way under the field conditions. Further study on understanding the mechanisms of the interactions with several biotic and abiotic environmental factors could help to achieve the positive potential of the efficient endophytes in agriculture.

要約

エンドファイト（植物内生菌）は植物の内部組織に生息しており、一般的には宿主植物に有益である。それらの多くは、生物学的窒素固定、植物ホルモンの合成などを介して宿主植物の成長を促進する。それらの機能は、宿主内環境および内生菌の相互作用によって影響を受けると考えられている。しかし、植物内環境や内生菌の相互作用が植物生長にどのように影響するかについてはまだ明らかになっていないところが多い。

本研究の最初の目的は、ネパール、Salyan から単離した 8 菌株について、インドール酢酸（IAA）の生産、窒素固定活性および植物成長促進における窒素レベルの影響を調べることであった。使用した 8 菌株のうち、*Klebsiell*a Sal1、*Enterobacter*Sal3、*Rhizobium*Sal4、*Agrobacterium*Sal7 および *Microbacterium*Sal4 は、0.1g/L の NH_4NO_3 添加 MR 培地中で、それぞれ 65、40、20、13 および 4μg/mL の IAA を生成した。2 株の *nifH* 遺伝子保有株において、Sal1 は、*Herbaspirillum*Sal 6 より高いアセチレン還元活性を示した。また、使用した菌株はバーミキュライトポットおよび寒天試験管を用いた窒素非制限（1/2 MS）下でのサツマイモへの 3 回の接種実験で、成長促進効果を示した。接種実験の結果に基づいて、Sal1 と Sal3 をこれ以降の実験に用いた。高い IAA 活性を示した Sal1 と Sal3 は、植物栽培用基本培地での IAA 生産に 15～60mg NH_4NO_3/L で至適濃度を示した。アセチレン還元活性については、Sal1 は 0～6.25 mgNH_4NO_3/L 添加 MS 培地でより高い活性を示したが、Sal 6 では活性は観察されなかった。窒素制限条件下（120 mg NH_4NO_3/L の 1/2 MS）で 2 菌株を接種した結果、サツマイモの根数が増加した。試験管を用いた液体培地中のキムワイプの上で成長したトマトの実生に対する内生菌の接種実験においては、窒素制限および非制限条件の両方で、総生体重および根生重を増加させるが茎長および根長には影響しない傾向を示した。シャーレを用いたゲルライト培地条件では、窒素非制限条件下で、総生体重、根生重、茎長および根長すべてにおいて成長促進が認められた。接種した両菌株は、トマト実生の根圏、根および茎での生息が認められたため、内生菌は植物中で IAA を生産したこと、また、そこでの窒素濃度は低いことが推定された。さらに、Sal1 はシャーレを用いた窒素非制限下のゲルラ

イト培地条件におけるイチゴ実生への接種実験で、根圏、根および葉で高い菌密度で検出され、イチゴ実生の成長を促進した。

本研究の第二の目的は、選択された 8 菌株の内生菌の IAA 分解能力を調べ、IAA 生産菌との植物への共接種により、それらの間の相互作用を明らかにすることであった。5 株の IAA 生産菌を含むすべての供試菌株は IAA 分解能力を示し、中でも Sal 6 が最も高い分解活性を示した。Sal 6 の増殖は、IAA を含む培地と含まない培地で大きく異なっていたため、この IAA 分解菌が IAA をエネルギー源として利用していることが示唆された。IAA 生産菌の Sal1 と Sal3 を IAA 分解菌の Sal 6 をトリプトファン(TRP)含有培地で共培養すると、IAA 生産菌の単独培養での場合と比較して IAA 濃度が大きく減少した。Sal 6 は TRP に対しても高い分解活性を示したので、共培養における Sal 6 は、IAA 生産菌によって生産された IAA を分解することによって、また、IAA の前駆体である TRP を分解することによって IAA の濃度を低下させたことが示唆された。トマトにこれらの IAA 生産菌および分解菌をそれぞれ単独で、または混合接種すると、IAA 生産菌の Sal1 は根の生重を増加させたが、その効果は IAA 分解菌の Sal 6 との混合接種によって減少した。根長と植物体の生重はいすれの接種によっても影響されなかった。ダイコンでは、IAA 生産菌の接種は根と植物体の生重を増加させたが、トマトの場合と同様に、IAA 分解菌との混合接種によってその効果は減少した。Sal1 と Sal 6、それぞれの単独接種では、トマトおよびダイコン、いずれも高い菌密度での生息が認められた。いずれの植物においても根圏における菌密度は根および葉より 2〜3 桁高かった。トマトにおける Sal1 の菌密度は、葉よりも根の方が 13 倍高かったのに対し、Sal 6 では根よりも葉の方が 7 倍高かった。ダイコンの場合、根と葉における菌密度は両菌株ともに同程度であった。混合接種の場合、いずれの植物でも、根圏、根および葉における菌密度は、単独接種の場合と同様であった。Sal 6 の菌密度の相対割合は、トマトの根（33%）を除いて、すべての植物の部分で Sal1 より高かった（75–95%）。接種された菌株の植物中での高い菌密度は、IAA 分解菌が IAA やその前駆体 TRP を分解することによって植物中の IAA レベルを減少させたことを示唆した。IAA の添加によるトマトの反応は、

IAA 生産菌 Sal1 の接種によるものと同様であったことから、Sal1 による根の成長促進は、この菌により生産された IAA によるものであることが示唆された。

この研究で示されたエンドファイトの植物組織内における高い菌密度での生息は、それらが宿主植物と共生関係を確立する能力を有していることを示唆している。実際の農業場面における適用を考慮すると、植物にもともと生息している内生菌群集との間で生じるであろう正および負の相互作用について考慮する必要がある。

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List of publications

Major Publications

Dhungana, S.A., Adachi, F., Hayashi, S., Puri, R.R., Itoh, K., 2018. Plant growth promoting effects of Nepalese sweet potato endophytes. Horticulturae 4, 53. doi.org/10.3390/horticulturae4040053. (Chapter 2)

Dhungana, S.A., Itoh, K., 2019. Effects of co-inoculation of indole-3-acetic acid (IAA) producing and degrading bacterial endophytes on plant growth. Horticulturae 5, 17. doi.org/10.3390/horticulturae5010017. (Chapter 3)

Sub-publication

Puri, R.R., Dangi, S., Dhungana, S.A., Itoh, K., 2018. Diversity and plant growth promoting ability of culturable endophytic bacteria in Nepalese sweet potato. Advances in Microbiology 8, 734-761. doi. 10.4236/aim.2018.89049.