STUDY ON CHARACTERIZATION OF ENDOPHYTIC BACTERIAL COMMUNITIES IN SWEET POTATO (*Ipomoea batatas*) CULTIVATED IN DIFFERENT CLIMATIC CONDITIONS

（異なる気候条件下で栽培されたサツマイモ(*Ipomoea batatas*) の

内生細菌群集に関する研究）

RAMESH RAJ PURI

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THESIS BY

RAMESH RAJ PURI

(D16A2001Y)

In Partial Fulfillment of the Requirement for the Award of Degree of
DOCTOR OF PHILOSOPHY

At

THE COURSE OF BIOENVIRONMENTAL SCIENCE
THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES,
TOTTORI UNIVERSITY
2019

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. The content of my thesis is the result of work I have carried out since the commencement of my PhD studies.

Approval Sheet

This thesis enclosed herewith, **"STUDY ON CHARACTERIZATION OF ENDOPHYTIC BACTERIAL COMMUNITIES IN SWEET POTATO (*Ipomoea batatas*) CULTIVATED IN DIFFERENT CLIMATIC CONDITIONS"** prepared and submitted by RAMESH RAJ PURI in partial fulfillment of the requirement for the award of degree of Doctor of Philosophy, is hereby approved as to style and contents.

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Acknowledgement

I would like express my appreciation and sincere gratitude to my supervisor *Prof. Kazuhito Itoh*, for his guidance during my PhD research and study.

I owe a dept of gratitude to the members of my Advisory committee for their encouragements and suggestions. Furthermore, I am grateful to *Associate Prof. Kousuke Suyama* and *Assistant Prof. Shohei Hayashi* for their technical help during the study. Additionally, *Dr. Dinesh Adhikari* is highly acknowledged for his support and companionship from the beginning to the end of my PhD studies.

With Profound gratitude and deep sense of devotions I wish to thank *Prof. Makoto Ueno*, Laboratory of Plant Pathology, Faculty of Life and Environmental Science, and *Prof. Tsugiyuki Masunaga*, *Mr. Akira Fukuda* and *Ms. Adha Fatmah Siregar*, Laboratory of Soil and Ecological Engineering, Shimane University, for their technical help during the research period.

I sincerely thank to Sangharash Raj Dangi, Binita Rai, Homan Regmi and Dinesh Khadka for providing assistance during sweet potato and soil samples collection in Nepal. I would like to thank farmers for providing the samples. Special thanks are also extended to all of my laboratory friends who contributed to the success of my study.

I am grateful to the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for the financial support to my studies.

I am extremely appreciative to my wife Bishnu (Nanu) for her encouragement and unconditional support during the study. I am equally grateful to my kids Abishkar and Bela for providing me the joys and happiness. Additionally, I am thankful to my parents and family members for their moral support and inspiration during my study.

Ramesh Raj Puri
March 2019
Matsue, Shimane, Japan

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List of Abbreviations and their fullforms

Abbreviations	Fullforms
AHL	Acyl-homoserine lactones
ANOVA	Analysis of variance
ARA	Acetylene reduction assay
ARDRA	Amplified ribosomal DNA restriction analysis
BLAST	Basic local alignment search tool
C	Carbon
CMC	Carboxy methyl cellulose
CTAB	Cetyl trimethyl ammonium bromide
CWDE	Cell wall degrading enzyme
DAPG	2,4-diacetylphloroglucinol
DDBJ	DNA Data Bank of Japan
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DTR	Dye terminator removal
EPS	Exopolysaccharides
ET	Ethylene
FAO	Food and agriculture organization of the United Nations
FISH	Fluorescence *in-situ* hybridization
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
IAA	Indole 3-acetic acid
LPS	Lipopolysaccharide
MAMP	microbial-associated molecule patterns
N	Nitrogen
NGS	Next generation sequencing
NH4+	Ammonium
nifH	Nitrogen fixing gene
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
Q-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
rpm	revolutions per minute
rRNA	Ribosomal RNA
SA	Salicylic acid
T-RFLP	Terminal restriction fragment length polymorphism
VOCs	Volatile organic compounds

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Chapter 1

General introduction

1.1 What are Endophytes?

The term “endophyte” is derived from the Greek words “endon” meaning within, and “phyton” meaning plant. Bacterial endophytes were first discovered in Germany in 1903 (Tan and Zou, 2001) and, as the name indicates, Hallmann et al. (1998) suggested that the endophytes are a class of microbes that can be isolated from surface-disinfected plant tissues or extracted from within the plants and that are not observed to harm the host plants. However, due to the suspected lack of sufficient removal of surface bacteria after sterilization of plant surfaces, and the presence of noncultured species, the definition appeared to be less suitable (Garbeva et al., 2001). Additionally, Kloepper et al. (2013) reported that the group of endophytes, fluorescent Psedudomonads, turned out to be harmful to ferns, when the population reached at maximum level. This suggests that the potential plant mutualists can become deleterious for their hosts. Considering these, Hardoim et al. (2015) gave his opinion on endophytes as microbes that colonize the plant interior regardless of the outcome of the association.

Nearly 0.3 million plant species exist on the earth, and each individual plant is host to one or more endophytes (Strobel et al., 2004). Although microorganisms other than bacteria (e.g. archaea, fungi, and protists) can also act as endophytes in plants, this thesis deals exclusively with bacterial endophytes.

1.1.1 Classification of endophytic bacteria

Endophytes can be classified into three main categories, depending on their colonization habit if they require plant to live and reproduce, namely, obligate endophytes, facultative endophytes and passive endophytes (Hardoim et al., 2008). Obligate endophytes are unable to proliferate outside of plants and are likely transmitted via seed rather than originating from the rhizosphere, whereas, facultative endophytes are free living in soil and colonize the plant tissues when the conditions are suitable (Hardoim et al., 2008). Bacteria lacking the capability to colonize and infect the plants can enter into the plant endophytic niches through wounds and

cracks on the plant, which is referred as the passive mode of endophytic colonization (Liu et al., 2017).

1.2 Endophytic bacterial colonization and distribution in plants

Bacterial endophytes enter and colonize internal plant tissues, using organic plant metabolites for growth and existence, and avoiding host defense responses. The main site for endophytes entry into plants tissues is the root zone (Compant et al., 2005; Meneses et al., 2011; Meneses et al., 2017; Gaiero et al., 2013), but they can also invade aerial tissues as, stem base, leaf sheath, and leaves (Chi et al., 2005). The illustration of endophytic bacterial colonization and their distribution pattern is depicted in **Figure 1.1**.

1.2.1 Recruitment of bacterial endophytes by host plants

The term ‘rhizosphere’ describes the zone of soil in which microbial activity is influenced by plant roots (Russell, 1982), distinguishing it from the ‘bulk’ soil which is not directly affected by root derived carbon substrates (termed ‘rhizo deposition’). Root exudates including organic acids, amino acids, and proteins may be involved in acquiring bacterial endophytes from the rhizosphere (Turner et al., 2013; Pétriacq et al., 2017). Root exudates likely contain substrates that initiate early communication between host plants and bacterial endophytes, and consequently steer the colonization process. For example, evidence of the involvement of oxalate in the recruitment of the beneficial bacterial strain *Burkholderia phytofirmans* strain PsJN by host plants has been reported (Kost et al., 2013). In this study, a *Burkholderia phytofirmans* strain defective in oxalate utilization was used to inoculate lupine and maize plants that secrete moderate and low levels of oxalate, respectively. The mutant was observed in significantly less numbers in both maize and lupine plants 3 days after inoculation as compared to the wild type strain. Similarly, Balachandar et al. (2006) reported that the flavonoids and IAA in the plant medium were effective to improve *Serratia* sp. rice seedlings endophytic colonization.

Furthermore, the quorum sensing system (QS) of potential endophytes have a role in plant tissue colonization, since it regulates the expression of bacterial genes

involved in the colonization process. The bacterial quorum sensing compounds are likely involved in communication with the plant root and the subsequent colonization process. The most common QS signals found in Gram-negative bacteria are N-acyl homoserine lactones (AHLs) while in Gram-positive bacteria are peptides (Gaiero et al., 2013). The importance of these compounds in the colonization and growth promotion of plants by endophytes is supported by a recent study that a quorum sensing mutant of *Bukholderia phytofirmans* strain PsJN, which is normally a plant growth-promoting bacterium and efficient colonizer in rhizospheric and endophytic environment (Nowak et al., 1998), could no longer colonize *Arabidopsis thaliana* and did not promote its growth (Zúñiga et al., 2013).

The native soil composition and plant genotype are also considered important in the recruitment of bacterial endophytes by the host plant. A detailed study of root endophytes of *Arabidopsis* plants grown in different soils concluded that soil type likely influences the composition of the bacterial endophyte community found in the host roots. This indicates that different soil types may be inhabited by variable bacterial populations that serve as the initial inocula (Bulgarelli et al., 2012). Likewise, the sweet potato genotypes was the main factor influencing the endophytic bacterial community where, Firmicutes phyla was the most isolated with *Bacillus* strains abundantly detected in the roots of IPB-052 and IPB-149 (72% and 79%, respectively) and γ-Proteobacteria (47%)/Enteobacter (35%) being the most abundant in IPB-137 roots (Marques et al., 2015)

1.2.2 Attachment of bacterial endophytes to the host plant surface

Bacteria in the proximity of the plant roots most likely swim towards the roots, using chemotactic affinities for root exudates. This is followed by attachment to the root surface, which is likely important in getting access to potential entry sites at lateral root emergence areas or other openings caused by wounds or mechanical injuries. The exopolysaccharide (EPS) produced by endophytic bacterium *Gluconacetobacter diazotrophicus* Pal5 was responsible for rice root surface attachment and internal colonization while the EPS knockout *G. diazotrophicus* Pal5 showed no attachment and colonization in the roots. But, the mutant strain was attached on the rice root

surface and colonized internally when the EPS produced by the wild type strain was added (Meneses et al., 2017). Similarly, *Herbaspirillum seropedicae* isolated from grass roots demonstrated that the bacterial lipopolysaccharide (LPS) was necessary for attachment and subsequent endophytic colonization in maize roots as revealed by inoculating the wild type and LPS knockout strains (Balsanelli et al., 2014)

1.2.3 Biofilm formation

After the potential endophyte bacteria are attracted to the root and attached to its surface, they multiply and attain a population density that allows them to form biofilms. Biofilms are bacterial communities enclosed within an extracellular matrix of polysaccharides produced by the bacteria, which adhere to a living or an inert macrosurface. For instance, Meneses et al. (2017) demonstrated that EPS biosynthesis is required for *G. diazotrophicus* PAL5 biofilm formation and rice endophytic root colonization, since when they knocked out a gene involved in EPS biosynthesis, mutant bacteria were defective in biofilm formation, root surface attachment, and endophytic colonization.

1.2.4 Entry of Bacterial endophytes into the host plants

Two distinct mechanisms namely, active or passive, have been reported for translocation processes of endophytic bacteria inside their plant hosts allowing them to move from the rhizoplane to the cortex of the root system. Once a bacterium reaches the root cortical zone, a barrier such as the endodermis can block further colonization (Gregory, 2007). It is likely that endophytes able to pass through the endodermis can secrete CWDEs (cell wall degrading enzymes) allowing them to continue colonization inside the endorhiza (James et al., 2002; Hallmann et al., 1997). The mechanism is known as “crack entry” and allows some endophytes to passively gain entry into the interior part of plant using epidermal junctions between root hair and adjacent epidermal cells, or disrupted endodermal cell layers resulting from the emergence of developing lateral roots. This mode of entry has been suggested for different bacterial species such as *Burkholderia* (Compant et al., 2005), *Bacillus* (Ji et

al., 2014), and *Herbaspirillum* (James et al., 2002) but the mechanisms of entry is unclear.

Additionally, bacterial endophytes with potentials to produce cellulolytic enzymes, as cellulase, xylanases, pectinase and endoglucanases, facilitate bacterial entry and spread within the plant tissues (Reinhold-Hurek et al., 2006; Naveed et al., 2013). A study supported this hypothesis by observing that the frequency of entry of an endoglucanase mutant of *Azoarcus* sp. BH72 into rice roots was decreased as compared to the wild type strain and the mutant was unable to spread to the aerial plant parts (Reinhold-Hurek et al., 2006). Alternatively, some bacteria may passively enter as a portion of this endodermal cell layer is often disrupted, such as during the growth of secondary roots, situated just below the endodermis barrier (Gregory, 2007).

1.3 Distribution of endophytes within the plants

Endophyte distribution in interior of plants depends on their ability to colonize and the allocation of plant resources. Some of the approaches to enumerate and visualize colonization of bacteria in plant tissues include fluorescence *in-situ* hybridization (FISH) and using reporter gene- (e.g., *gfp* or *gus*) modified bacterial strains combined with microscopy.

Root endophytes often colonize and penetrate the epidermis at sites of lateral root emergence, below the root hair zone and in root cracks (Compant et al., 2005; Zakria et al., 2007). These colonizers are capable of establishing populations both inter- and intracellularly (Hurek et al., 1994). After initial colonization, some endophytes can move to other areas of the plant by entering the vascular tissues and spreading systemically (Terakado-Tonooka et al., 2008; Compant et al., 2005). A study on the infection, dissemination, and colonization of healthy rice plant tissues by four species of *gfp*-tagged *rhizobia* and their influence on the growth physiology of rice indicated a dynamic infection process beginning with surface colonization of the rhizoplane (especially at lateral root emergence), followed by endophytic colonization within roots, and then ascending endophytic migration into the stem base, leaf sheath, and leaves where they developed high populations (Chi et al., 2005). Similarly, Johnston-Monje and Raizada (2011) used green-fluorescent-protein (GFP) labeled

maize endophytes and inoculated into stems of maize seedlings, and revealed that the transport of the endophytes from stems moved into the roots and rhizosphere, and authors suggested that there may be a continuing movement of organisms throughout the root microbiome. Furthermore, Kandel et al. (2017) inoculated poplar endophyte tagged with *gfp* (WP5gfp) in maize and found that it colonized maize roots, leaves, and stems with high populations and resulted in increased plant growth as compared to mock-inoculated control plants.

Additionally, Elbeltagy et al. (2001) conducted a study to examine whether *Herbaspirillum* sp. strain B501 isolated from wild rice, *Oryza officinalis*, endophytically colonizes rice plants. For this purpose, the *gfp* gene encoding green fluorescent protein (GFP) was introduced into the bacteria. Observations by fluorescence stereomicroscopy showed that the GFP-tagged bacteria colonized shoots and seeds of aseptically grown seedlings of the host plant after inoculation of the seeds.

1.4 Plant growth-promoting properties of endophytic bacteria

Some of the endophytes associated with the crop plants can improve the host plant development and are referred as “Plant growth promoting bacteria (PGPB)”. Agricultural crops in the world are often deficient in macro and micronutrients and are prone to several pathogens, resulting decreased plant production. To overcome these problems and obtain crop yield increase, agricultural practices have become increasingly dependent on the use of chemical fertilizers, herbicides, insecticides, and fungicides for either supplementing soils with plant nutrients or to get rid of weeds, pathogens and insects. Therefore, Sustainability issues in agriculture are a priority for several countries in the world; in this regard, the use of microbial inoculants to the agriculture farming might contribute to ensure sustainable agriculture.

The increasingly recognized plant growth promoting traits of endophytic bacteria and their potential applications in agriculture can enhance plant growth via biofertilization, phytostimulation, biocontrol, control stress, and cold or drought tolerance (Gaiero et al., 2013; Compant et al., 2005).

1.4.1 Biofertilization

The plant growth promotion by increasing the accessibility or supply of major nutrients is termed as biofertilization (Gaiero et al., 2013). A well-studied form of biofertilization is nitrogen fixation, which is the conversion of atmospheric inert nitrogen to ammonia, the available form. Several root endophytes with N-fixing potentials are reported in non-legumes as *Herbaspirillum* sp., *Enterobacter* sp. and *Azospirillum* sp. in rice (Elbeltagy et al., 2001), *Azoarcus* sp. in Kallar grass (Hurek et al., 2002), *Klebsiells oxytoca* (Adachi et al., 2002), *Pantoea agglomerans* (Asis and Adachi, 2003) and *Bradyrhizobium* sp. (Terakado-Tonooka et al., 2013) in sweet potato.

The nitrogen-fixing bacterial endophyte *Acetobacter diazotrophicus*, isolated from the sugarcane was inoculated in the host plant under N-limiting conditions; as a result the growth of sugarcane was enhanced as compared to uninoculated plants. The transfer of fixed N from *A. diazotrophicus* to sugarcane was verified by a $^{15}N_2$ incorporation experiment and reported that the sugarcane plants inoculated with *A. diazotrophicus* incorporated $^{15}N_2$ into 0.4 and 0.2% of total N in shoots and roots, respectively, from nitrogen fixation over the 24-h period, whereas the values for uninoculated plants were ca. 0.05% (Sevilla et al., 2001).

Similarly, the nitrogen fixing endophyte *Methylobacterium* sp. isolated from *Jatropha curcas* root, increased the growth and seed yield when inoculated in the host plant (Madhaiyan et al., 2015). Authors suggested that the nitrogen fixation might be one of the contributing factors for the growth promotion in *J. curcas*.

In addition, inoculation with multiple N-fixing bacteria has higher potential than inoculation with a single bacteria (Oliveira et al., 2009). For example, sugarcane inoculation with a consortium of five N-fixing bacteria (*Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Herbaspirillum rubrisubalbicans, Azospirillum amazonense,* and *Paraburkholderia tropica*) isolated from sugarcane, showed higher stem production than mono-inoculated plants in two soils with low -medium levels of chemical fertilizer (Oliveira et al., 2009), while the study did not elucidate the mechanisms of growth promotion and authors suspected that the growth might be due to the N-fixing ability of the inoculants.

1.4.2 Phytostimulation

Phytostimulation is the direct promotion of plant growth through the production of phytohormones. There are five groups of phytohormones: cytokinins, auxins, gibberellins, abscisic acid and ethylene (Opik and Rolfe, 2005). The endophytic bacteria are known to contribute to the plants growth and development by producing plant growth hormones.

The IAA producing endophytic bacteria are reported in different crops as, *Rahnella aquatilis* (Khan and Doty, 2009), *Bacillus* sp. and *Enterobacter* sp. (Marques et al., 2015), *Achromobacter xylosoxidans* and *Bacillus cereus* (Dawwam et al., 2013) in sweet potato, *Bacillus* sp. and *Sphingopyxis* sp. in strawberry (Dias et al., 2009), *Bacillus* sp. in rice (Ji et al., 2014) and *Enterobacter* sp. in poplar trees (Taghavi and van der Lelie, 2013). Similarly, rice endophyte *Pantoea agglomerans* produced indole-3-acetic acid, abscisic acid, gibberellic acid and cytokinin in Luria-Bertani medium (Feng et al., 2006). Furthermore, Umamaheswari et al. (2013) isolated endophytes as, *Bacillus* sp, *Micrococcus* sp, *Pseudomonas* sp, *Flavobacterium* sp and *Serratia* sp. from legume crops (Redgram, Blackgram, Greengram, Chickpe and Cowpea), and reported that all the isolates possess IAA, Gibberellins and Cytokinin producing ability under culture conditions.

Inoculation with phytohormones producing endophytic bacteria provided plant growth promoting effects. For instance, endophytes *Bacillus* sp. isolated from strawberry plants with IAA production potentials promoted root development of strawberry plants (Dias et al., 2009). Similarly, the endophytic bacterium *Sphingomonas* sp. LK11 isolated from *Tephrosia apollinea* (Papilionaceae), with Gibberellins and IAA producing potentials enhanced the tomato growth (Khan et al., 2014). Furthermore, when *Rahnella aquatilis* isolated from sweet potato tuber with IAA producing ability was inoculated to poplar cuttings, the roots grew faster and the plant growth was promoted as compared to the uninoculated ones (Khan and Doty, 2009). But the IAA production and its contribution was not clarified under plant cultivation conditions.

1.4.3 Biocontrol

1.4.3.1 Antibiotics production

The antibiotics produced by endophytic bacteria exemplify a promising alternative protection to plants against phytophathogens. For instance, Jasim et al. (2016) reported that, *Bacillus* sp., an endophytic bacterium, isolated from *Capsicum annuum* was found to possess surfactin derivatives and iturin as the basis of its antipathogenic activity. Similarly, Marques et al. (2015) reported that *Bacillus* sp. isolated as endophytes from sweet potato tubers showed antimicrobial activity against a fungal pathogen, *Plenodomus destruens,* but the mechanism was not elucidated.

The endophytic strains, *Alcaligenes faecalis*, isolated from *Abelmoschus esculentus* produced hydrogen cyanide (HCN) and demonstrated antagonistic effect against *Sclerotium rolfsii* under in vitro conditions (Ray et al., 2016).

Likewise, eggplant wilt caused by *Ralstonia solanacearum* was reduced by 70% after seeds were inoculated with 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas* sp., an endophyte isolated from eggplant. (Ramesh et al., 2009).

1.4.3.2 Interruption of quorum sensing (QS) in plant pathogens

Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density. Interruption of QS by degrading autoinducers of pathogens is also among biocontrol of endophytic bacteria (Melissa B. Miller and Bonnie L. Bassler, 2001). QS bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. QS is one of the intrinsic chemical cell-to-cell signaling cascades in bacteria that regulates the physiological activities of bacteria, involving cell-to-cell communication, reproduction, biofilm formation, competence and adaptation (Melissa B. Miller and Bonnie L. Bassler, 2001). N-acylated L-homoserine lactones (AHLs) of Gram-negative bacteria and oligopeptides of Gram-positive bacteria are released as autoinducers to facilitate quorum sensing (LaSarre and Federle, 2013). These in turn coordinate responses across a population to establish crosstalk, the important being able to prevent chemical defenses (e.g., production of antibiotic compounds) of other organisms (Teplitski et al., 2011). Inhibition of quorum sensing in pathogenic bacteria, a process

known as “quorum quenching”, has a fundamental advantage over other disease-management strategies (chemical fungicides, bactericides etc.) and opens new approaches to tackle drug-resistant bacteria.

Some endophytic bacteria employ QS quenching as an antivirulence strategy to control phytopathogens. For instance, endophytes *Bacillus* sp. strain B3, *Bacillus megaterium* strain B4, *Brevibacillus borstelensis* strain B8, and *Bacillus* sp. strain B11 in *Cannabis sativa* disrupt cell-to-cell communication in the pathogenic strain *Chromobacterium violaceum* by quenching its QS signals (Kusari et al., 2014) which was elucidated by using high-performance liquid chromatography high-resolution mass spectrometry (HPLC-ESI-HRMSn) and matrix-assisted laser desorption ionization imaging high- resolution mass spectrometry (MALDI-imaging-HRMS).

1.4.3.3 Induced systemic resistance

Endophytic bacteria are also able to produce resistance-conferring volatile organic compounds (VOCs) (Chung et al., 2016). Maize plants inoculated with endophytic *Enterobacter aerogenes* that produce VOC 2,3-butanediol (2,3-BD) showed enhanced resistance against the northern corn leaf blight whose causative agent is the fungus *Setosphaeria turcica* (D’Alessandro et al., 2014).

In the next study, the endophytic bacterium *Bacillus pumilus* strain SE34, isolated from cotton plants, was inoculated in transformed pea plants which was infected with the pea root-rotting fungus *Fusarium oxysporum* f. sp. *pisi* to observe plant defense reactions at the structural level (Benhamou et al., 1996). They reported that the pathogen multiplied abundantly through much of the tissue including the vascular stele in uninoculated plants, whereas in inoculated ones, pathogen growth was restricted to the epidermis and the outer cortex, as revealed by light microscope and cytochemical observations.

1.4.4 Control stress

The production of ethylene (ET) in stressed plants may lead to decreased plant growth or even cell death when present at high concentrations (Glick, 2014). Some microbes including bacterial endophytes utilize 1-aminocyclopropane-1-carboxylate

(ACC), the immediate precursor of ET, as a carbon and nitrogen source by producing ACC deaminase. Increasing global warming, desertification, soil salinization as well as extreme weather events of drought, flood and cold may exert greater stress on plants leading to reduced crop yields (Miraglia et al., 2009).

A recent study found that endophytic bacteria namely, *Bacillus flexus, Arthrobacter soli*, *Streptomyces pactum* and *Isoptericola dokdonensis,* isolated from the *Limonium sinense* possessed efficient ACC deaminase activity and when inoculated to the host plants they were able to increase seed germination, root and shoot length, leaf area and numbers of *L. sinese* seedlings under salinity stress. In addition, the inoculants were re-isolated from the inoculated plant interior tissues (Qin et al., 2014). Similarly, *Burkholderia* sp. strain PsJN, is an effective plant growth-promoting bacterium that was isolated as a contaminant from *Glomus vesiculiferum*-infected onion roots (Nowak et al., 1998). This bacterium promoted the growth of potatoes (Frommel et al., 1991) via reduction of the level of the inhibitory hormone ethylene by producing ACC deaminase.

1.4.5 Tolerance to cold or drought Stress

The cold or drought stress tolerance provided by the inoculated endophytic bacteria provided indirect effect on plant growth promotion. Inoculation of the endophyte *Burkholderia phytofirmans* strain PsJN on *Arabidopsis* led to increase *Arabidopsis* growth and strengthened cell wall as a result plant tolerated the cold temperature conditions (Su et al., 2015).

Endophytic bacteria are also able to protect the plants from drought conditions. Using a transcriptomics approach, approach, it was found that endophytic *B. phytofirmans* strain PsJN displayed a diverse range of functionalities when inoculated on potato plants (Sheibani-Tezerji et al., 2015). Transcripts involved in transcriptional regulation, cellular homeostasis and reactive oxygen species (ROS) detoxification were upregulated in *B. phytofirmans* strain PsJN in drought stress-affected potato. This suggests that endophytes sense physiological changes in plants and adjust gene expression to adapt to the drought environment. Endophytic bacteria have therefore

the potential to be used as protective agents in agricultural systems under extreme climatic environments as they can influence plant physiological responses to stresses.

1.5 Endophytic bacterial community and influencing factors

1.5.1 Endophytic bacterial communities

Different plants are accompanied with different endophytic bacteria in terms of community and composition. Endophytic bacterial community analysis is conducted using culture-dependent and independent approaches.

1.5.1.1 Culture-dependent method

So far, most of the studies on endophytic bacterial communities have been obtained by using culture-dependent approaches. For instance, it was observed that the isolates *Enterobacter oryziphilus* and *Enterobacter oryzendophyticus* were the main bacterial inhabitants in the rice root endosphere (Hardoim et al., 2013). Similarly, Jackson et al. (2013) isolated endophytes from salad leaf belonging to Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes phyla. Likewise, the culturable endophytic bacterial community in maize was also assessed, revealing that *Achromobacter* (67.78%) genera in β-Proteobacteria class was the most dominant, followed by *Bacillus* (30.02%) in Firmicutes phyla and *Pseudomonas* (2.2%) in γ-Proteobacteria class (Pereira et al., 2011). Furthermore, 102 endophytic bacteria were isolated from banana roots representing 10 genera, (*Agrobacterium, Aneurinibacillus, Bacillus, Enterobacter, Klebsiella, Lysinibacillus, Micrococcus, Paenibacillus, Rhizobium and Sporolactobacillus*), among which the genus *Bacillus* was the most abundant (87.3% of isolates), followed by the genus *Lysinibacillus* (3.9% of the isolates) (Souza et al., 2013).

There have been a few studies on endophytic bacterial community from sweet potato crops. For example, the community was examined for samples collected in Brazil (Marques et al., 2015) and in USA (Khan and Doty, 2009), and was shown that γ-Proteobacteria was common dominating group in both studies.

1.5.1.2 Culture-independent method

Due to the unknown conditions for growth requirements of many bacteria and the presence of cells which are in a viable but noncultivable state (Tholozan et al., 1999), the portion of bacterial communities detected by culture-dependent approach is a small amount of the whole community. So, culture-independent studies have been successfully used for detailed endophytic bacterial community analysis.

Metagenomics study of endophytic bacteria in *Aloe vera* using next-generation technology revealed that the Proteobacteria, Firmicutes, Actinobacteria and Bacteriodetes were the predominant phyla. In the same study, *Klebsiella* genus detected in NGS was not isolated (Akinsanya et al., 2015), which shows that the uncultured endophytes are detected by NGS. Similarly, the maize root DNA analyzed by applying culture-independent methods revealed that the γ-Proteobacteria was dominant (79.57%) class represented by *Pseudomonas, Enterobacter, Klebsiella, Erwinia and Strenotrophomonas* genera, followed by Firmicutes (20.43%) and *Bacillus* genus. Furthermore, Ikenaga and Sakai (2014) applied LNA-PCR clamping technique and DGGE- analysis to detect bacterial community in rice roots and reported that the novel bacteria which showed low similarity with known bacteria in the data base were identified. Authors suggested that the LNA oligonucleotide-PCR clamping technique enabled the detection of bacterial genes that were hidden by low amplification due to the predominant ribosomal sequences of plant plastid and mitochondria. Therefore, the application of culture-independent approaches have also accelerated investigation of the community structures of plant associated bacteria (Ikenaga et al., 2015), and can detect unculturable bacterial colonizers of plants, as well as those bacteria that are in low abundance or grow so slowly that they are missed by traditional culture based protocols.

1.5.1.2.1 Locked nucleic acid (LNA) and its application

LNA is an artificial nucleotide analogue that contains a methylene bridge connecting the 2'-oxygen of ribose with the 4'-carbon (**Figure 1.2**) (Koshkin et al., 1998; Obika et al., 1998). This bridge locks the ribose in 3'-endo structural conformation resulting with reduced conformational flexibility (Latorra et al., 2003).

LNA oligonucleotides, which are homologous to the plant organelle SSU rRNA genes, can be annealed to the organelle genes with high temperature (70^0C) in which bacterial primers are inactive prior to the annealing step of bacterial primers (54^0C). Then, LNA oligonucleotides block the annealing of primers by designing in the positions to compete with primers in the organelle genes, and are inoperative as primers upon phosphorylation of the 3' end. Finally, this technique specifically inhibits the amplification of the plant organelle SSU rRNA genes, while allowing the amplification of bacterial genes. Subsequent next generation sequencing (NGS) generates the bacterial sequences from the plant samples.

1.5.2 Factors determining the endophytic bacterial community

Studies performed on the endosphere microbiome of different plants, have shown that host plant species (Shen and Fulthorpe, 2015), genotype (Hardoim et al., 2011; Ferreira Da Silva et al., 2014; Marques et al., 2015), growing season (Shen and Fulthorpe, 2015), cultivation conditions (Xia et al., 2015), cultivation history (Correa-Galeote et al., 2018), soil (Bala et al., 2003) and climate (Nissinen et al., 2012) influenced the endophytic community.

1.5.2.1 Host plants

It was reported that transgenic glyphosate-resistant cultivars of soybean had a higher diversity and abundance of culturable endophytic bacteria than wild-type plants (de Almeida Lopes et al., 2016). In a study, Dong et al. (2003) applied culture-independent methods and inoculated gfp tagged *Klebsiella pneumonia* Kp342, isolated from maize, into monocots (*Triticum aestivum* and *Oryza sativa*) and dicots (*Medicago sativa, Medicago truncatula, Arabidopsis thaliana*) and reported that the strain colonized 100 fold higher in monocots as compared to the dicots. Authors mentioned that, the mechanisms behind such differential influences are unknown, but differences in root architecture (larger apoplast in monocot provides larger habitat for bacterial endophytes) and nutrient availability in the apoplast (monocots secrete more sucrose into the apoplast than dicots) or the types of root exudates attracting specific endophytes may be the influencing factors.

1.5.2.2 Plant genotype

Applying culture-dependent method, Marques et al. (2015) depicted that the plant genotype affected the functional diversity of endophytic bacteria, as IAA-producing strains were predominantly detected from IPB-137 genotype as compared to IPB-149 and IPB-052 genotypes in sweet potato. Similarly, when culture-independent approach was applied, the endophytic bacterial community of the transgenic maize genotype TC1507 differed from the communities of the maize genotype MON810 (Ferreira Da Silva et al., 2014). In another study applying culture-independent method, maize genotype had a clear effect on the number and diversity of endophytic communities, the NK940 maize genotype community being more abundant and diverse than that of PAU871 (Rodríguez-Blanco et al., 2015).

1.5.2.3 Cultivation conditions

Culturable endophytic bacterial species in four vegetable crops were abundant and diverse in the organically grown plants compared to those grown using conventional practices (Xia et al., 2015). Similarly, culturable endophytic bacterial communities were more diverse in tobacco roots from organic soils compared to those grown in mineral soils (Long et al., 2010), indicating that organic management practices may increase the rhizospheric bacterial diversity, the potential endophytes. In addition, culture-independent approach revealed that the endophytic nitrogen-fixing *Azoarcus* sp. are more abundant in rice (*Oryza sativa*) and related grass species in flooded soils compared to dry soils (Engelhard et al., 2000).

1.5.2.4 Cultivation history

Correa-Galeote et al. (2018) reported that plant cultivation history could have a fundamental role responsible for selection of root endophytes from rhizospheric bacterial community as revealed by culture independent approach. They reported that the maize plant grown in a cultivated land continously for long time (5 years) demonstrated higher diversity of endophytes than the plants grown in a fallow soil.

1.5.2.5 Soil

Soil pH is a major determinant of bacterial species composition in bulk soil (Fierer and Jackson, 2006) and therefore influences the pool of potential endophytes available for plant recruitment. As revealed by culture-independent method, increased soil acidity has resulted in lower endophyte richness and diversity and a greater abundance of acid-tolerant species of rhizobia in legumes (*Calliandra calothyrsus, Leucaena leucocephala and Gliricidia sepium*) (Bala et al., 2003).

1.5.2.6 Climate

Psychrophilic bacterial endophytes were isolated abundantly in cold environments from the arcto-alpine plant species (Nissinen et al., 2012) as revealed by using culture-independent approach, and it seemed to be the selection of psychrophile already adapted in the soil. There are limited reports on the influence of soil and climatic conditions on the endophytic bacterial community.

1.6 Objectives of the study

Sweet potato (*Ipomoea batatas* L.) is a resilient, easily propagated crop which grows well in marginal lands. Reports suggest that endophytic bacteria are present in the sweet potato tubers (Marques et al., 2015; Dawwam et al., 2013, Terakado-Tonooka et al., 2013, Khan and Doty, 2009, Adachi et al., 2004, Asis and Adachi, 2003, Adachi et al., 2002). So, we choose sweet potato as an experimental plant in this study.

As mentioned earlier, the reports on endophytic bacterial community as affected by the climatic conditions is limited (Nissinen et al., 2012). Nepal is rich in biodiversity as it varies greatly in topography and climate; the elevation ranges from 68 to 8,848 masl in a just 150 to 250-km south–north transect. So, the study of endophytic bacterial community in sweet potato cultivated under different climatic conditions in Nepal would clarify the influence of the climate on the endophytic community compositions.

On the other hand, endophytic bacterial communities are reported to be influenced by several parameters, such as host plant species (Shen and Fulthorpe, 2015), genotype (Hardoim et al., 2011; Ferreira Da Silva et al., 2014; Marques et al.,

2015), growing season (Shen and Fulthorpe, 2015), cultivation conditions (Xia et al., 2015), cultivation history (Correa-Galeote et al., 2018). In addition soils (Bala et al., 2003) and climatic (Nissinen et al., 2012) conditions are also considered as important factors for determining endophytic community. For example, psychrophilic bacterial endophytes were isolated abundantly in cold environments from the arcto-alpine plant species (Nissinen et al., 2012), which seemed to be the selection of psychrophile already adapted in the soil. However, it was unclear which factor was more responsible, the soil or the climate, in determining the bacterial endophytic community. To clarify the influencing factors, the soil or the climate, we used the same soil at different locations in Japan and cultivated sweet potato, and the endophytic community compositions were compared applying culture-dependent and independent methods.

There are several studies on sweet potato bacterial endophytes and some isolates are reported to possess plant growth promoting potentials (Khan and Doty, 2009, Marques et al., 2015, Asis and Adachi, 2003, Adachi et al., 2002, Terakado-Tonooka et al., 2013). Inoculation of these plant growth promoting potential isolates may enhance the plant growth. For instance, *Rahnella aquatilis* isolated from sweet potato tuber with IAA producing ability was inoculated to poplar cuttings, the roots grew faster and the plant growth was promoted as compared to the uninoculated ones (Khan and Doty, 2009). Furthermore, synergistic effect of mixed cultures of plant growth promoting bacteria was also reported (Oliveira et al., 2002, Molina-Romero et al., 2017). So, we inoculated the mixtures of isolates, from each location of Nepalese sweet potato, on the host plant and assess their plant growth ability. The objectives of this study are as follows:

- To examine and characterize the bacterial community of sweet potato endophytes in Nepal in relation to the climatic conditions and their plant growth promoting ability, (**Chapter 2)**
- To examine the effects of the soil and the climatic conditions on the endophytic bacterial communities of sweet potato by using the same soil at different locations and applying culture-dependent **(Chapter 3)** and independent **(Chapter 4)** approaches.

Figures

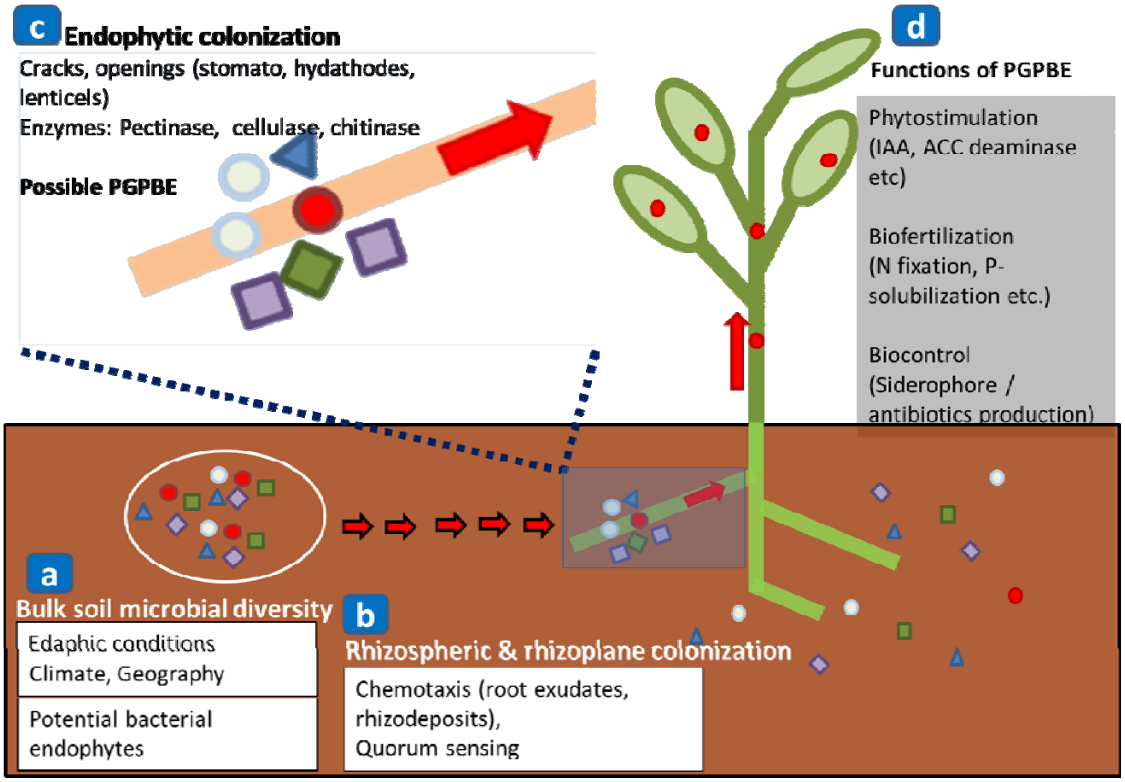


Figure 1.1 Illustration of endophytic bacterial colonization and their distribution within the plants. (a) Bulk soil microbes are diverse determined by the climate, soil and geography (Gaiero et al., 2013). (b) Bulk microbes colonize rhizospheric zone and attach root surface through chemotaxis mediated by root exudates/ rhizodeposits, quorum sensing (Wei and Zhang, 2006; Kandel et al., 2017) (c) Some of those rhizospheric bacteria enter to the root through the lateral cracks and wounds (Sprent and de Faria, 1998) or by hydrolyzing the cell walls using cellulolytic enzymes (Hallmann et al., 1997). Then the endophytes move upward to different tissues, (Chi et al., 2005) and (d) some of which are beneficial for the plant growth (Elbeltagy et al., 2001). The figure is inspired by the Vurukonda et al., 2018 and Gaiero et al., 2013.

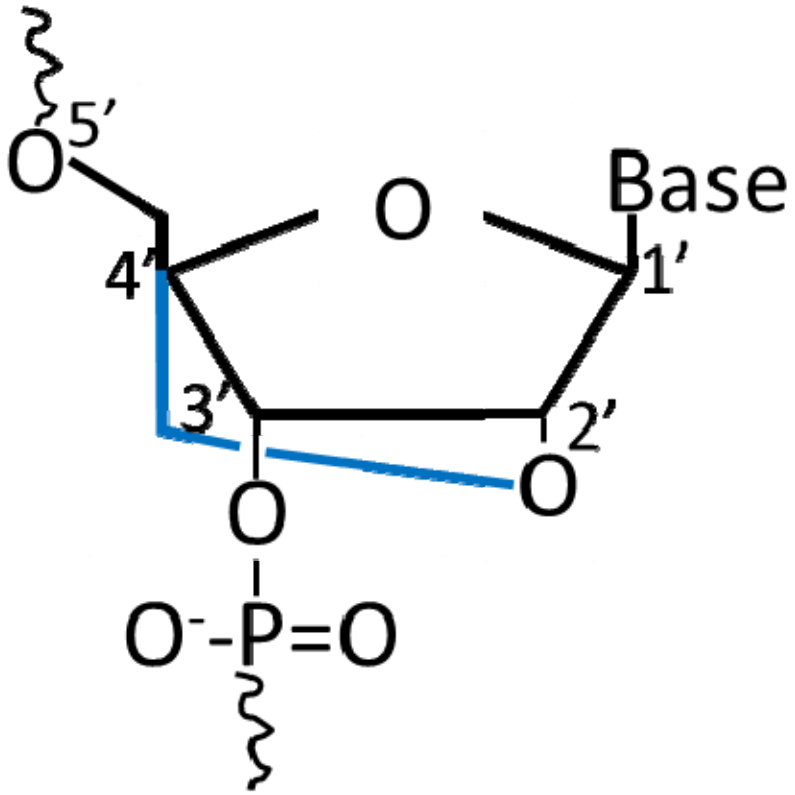


Figure 1.2 Structure of LNA

Chapter 2

Diversity and plant growth promoting ability of culturable endophytic bacteria in Nepalese sweet potato

2.1 Introduction

Sweet potato (*Ipomoea batatas* L.) is a resilient, easily propagated crop which grows well in marginal lands. The plant can be cultivated in low-fertile soils, takes up more nitrogen than other root crops (Hartemink et al., 2000; Hill et al., 1990). The capacity of sweet potato to grow well in low fertile soils might be due to the endophytic bacteria with plant growth promoting traits. Endophytic diazotrophic bacteria such as *Klebsiella*, *Pantoea* and *Gluconacetobacter* have been isolated from sweet potatoes (Adachi et al., 2002; de Araújo, 2004; Dobereiner et al., 1995). Similarly, sweet potato bacterial endophytes with auxin production, antagonistic effect, phosphate solubilization and siderophore production abilities have also been isolated (Marques et al., 2015; Khan and Doty, 2009). On the other hand, there have been a few studies on endophytic bacterial community from sweet potato crops. For instance, the community was examined for samples collected in Brazil (Marques et al., 2015) and in USA (Khan and Doty, 2009), and it was shown that γ-Proteobacteria was common dominating group in both studies.

Nepal, a small Himalayan country, lies along the southern slopes of the Himalayan Mountains between China and India. It varies greatly in topography, climate and vegetation; the elevation ranges from 68 to 8,848 masl in a just 150 to 250-km south–north transect. In Nepal, sweet potato is cultivated from terai (60-300 masl) to mid hills (300-2000 masl) and the average productivity is 5-6 tons ha^{-1} (Bhattarai, 2015), while the world productivity is 12.2 tons ha^{-1} (FAOSTAT, 2018). Till date, there is no information on Nepalese sweet potato endophytes. Adhikari et al. (2013) reported that the diverse climate and soils in Nepal was suspected to be conducive for the occurrence of diverse soybean rhizobial strains. So, we expect that diverse endophytic bacterial isolates with the potentials for plant growth promotion could be isolated from the Nepalese sweet potato.

Sustainability issues in agriculture are a priority for several countries in the world; in this regard, the use of microbial inoculants to the agriculture farming might

contribute to ensure sustainable production. In this study, we aimed to examine bacterial community of sweet potato endophytes in Nepal in relation to the environmental parameters and characterize their plant growth promoting traits. As synergistic effect of mixed cultures of plant growth promoting bacteria was reported (Oliveira et al., 2002; Molina-Romero et al., 2017), we also examined their potential by inoculating combined isolates from each location.

2.2 Materials and methods

2.2.1 Sample collection and study sites

Sweet potato tubers were collected from three months old plants during the autumn of 2015 representing 12 sweet potato growing sites in Nepal, six from subtropical and six from temperate regions. Sampling sites, climate and soil properties are presented in **Table 2.1**.

The sweet potato samples were washed with tap water, shade dried and kept at room temperature until the isolation of the endophytic bacteria. Soil samples collected from the same field during the spring of 2016 were air-dried and crushed to pass through a 2 mm sieve. The pH was measured using the glass electrode method with a soil: water ratio of 1:2.5 (McLean, 1982). Total carbon (TC) and total nitrogen (TN) were determined by the dry combustion method using an NC analyzer (MT-700, J-Science, Kyoto, Japan). Available phosphorus (P) was determined by Olsen`s bicarbonate method (Olsen, 1954).

2.2.2 Isolation and identification of endophytic bacteria

The sweet potato samples were washed again with running tap water for 10 min. Each sample was cut transversely when its diameter was more than 10 mm otherwise cut longitudinally. Then, the cut surface was stamped on the modified MR agar medium (de Araújo, 2004), and incubated for 2 days at 26^0C for further analysis. The appeared colonies were grouped based on their morphologies and the representative colonies reflecting their relative abundance were purified for further analysis as endophytes.

The partial 16S rRNA genes of the isolated endophytic bacteria were amplified using the universal primers (fD1 and rP2) to the domain bacteria (William G Weisburg et al., 1991). The PCR mixture was prepared by mixing MilliQ water, 10x reaction buffer, 10mM dNTPs, *Taq* DNA polymerase (GENETBIO Inc., Daejeon, Korea), fD1 and rP2 primers together with the template. The PCR reaction was carried out with a pre-run at 94^0C for 3 min, 30s at 94^0C, 30s at 50^0C, 1 min at 72^0C for 30 cycles and final run at 72^0C for 5 min. The PCR products were sequenced as described by (Adhikari et al., 2012). In brief, the respective PCR products were purified by using SOPE^{TM} resin (Edge Biosystems Inc. USA) and a Performa Dye Terminator Removal (DTR) Gel Filtration Cartridge (Edge Biosystems Inc. USA). Then, their nucleotide sequences were analyzed by an ABI Prism, 3100-Avant-100D2 (3130xl/Genetic Analyzer, Hitachi, Tokyo, Japan). Close relatives for each isolate was assigned using the data base (www.ddbj.nig.ac.jp) by a BLAST search (Altschul et al., 1997).

2.2.3 Distribution of endophytic bacteria in relation to environmental conditions

Correlation between bacterial class compositions and the environmental parameters (**Table 2.1**) of the sampling sites was analyzed by the principal component analysis (PCA). Bacterial class compositions as expressed by relative percentage were used for the calculation.

2.2.4 Characterization of endophytic bacteria

Sixty representative isolates of 34 genera in 6 classes were selected by their phylogenies (**Table S2.1 & Figure S2.1**) and used for the characterization of their plant growth promoting traits, antagonistic effect and endophytic traits.

For indole-3-acetic acid (IAA) production assay, the isolates were cultivated in the modified MR liquid media supplemented with 200 µg/mL of L-tryptophan at 26^0C for 3 days with shaking (150 rpm). After centrifugation at 8000 g for 15 min, the supernatant was applied for quantification of IAA according to the method described by (Gordon and Weber, 1951).

For detection of nitrogen fixing gene (*nifH*), primers PolF and PolR which were designed to match a broad range of bacterial *nifH* gene (Poly et al., 2001) were used

for PCR. The PCR components and conditions were as described in **sub section 2.2**. Nitrogenase activity of the *nifH* gene containing isolates was evaluated using the acetylene reduction assay (ARA). The isolates were cultivated in N-free modified MR media (Elbeltagy et al., 2001) for 3 days at 26^0C with shaking (150 rpm). Then, the culture was washed and suspended in sterile distilled water at OD_{660nm} 0.2. Then, 50µl of the suspension was inoculated on a slant of semi-solid (1.3%) N-free modified MR agar media in 60 mL vials in triplicate. The vials were sealed with a butyl-rubber and an aluminium stopper, and 10% of the headspace volume was replaced with pure acetylene. Vials without acetylene and without inoculants served as controls. Ethylene concentrations in the vials were measured after 3 days of incubation in dark at 28^0C, using a gas chromatograph (Shimadzu GC-14B; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and Porapak N (50/80 mesh; GL Sciences, Tokyo, Japan).

For assay of antagonistic effects, the point inoculation method (Zhao et al., 2011) was used against the fungal pathogen (*Pythium ultimum* var *ultimum* Strain OPU744). Briefly, each test strain was streaked on the PDA plate (dextrose: 1g l^{-1}, peptone: 5g l^{-1}, yeast extract: 2.5g l^{-1} and agar: 15g l^{-1}). After one day of cultivation at 28^0C, a 5 mm of fungal mycelial disk was placed at approximately 30 mm from the bacterial streaked line and continuously cultivated for 7 days. The corresponding fungal disk without endophyte strain served as control. To test antagonistic effect of endophytes against bacterial pathogens, each test isolates were streaked on a half part of PDA plates and incubated for 2 days at 28^0C. Then each bacterial pathogens (ECa: *Erwinia chrysanthemi* Strain NARCB200126, AZ9702, causing stem and root rot in sweet potato, ECb: *Erwinia chrysanthemi* Strain E7725, causing stem rot in potato and ECc: *Erwinia chrysanthemi* Strain Ech T5-2, causing root rot in Taro); was streaked approximately 5mm to the endophytes and incubated at 28^0C for 9 days. For both assays, antagonistic effects were categorized based on the distance between the test isolates and the pathogen as follows: no (0 mm), weak (1-3 mm) and strong (>3 mm) activities.

For the cellulase assay, the isolates were spotted on a carboxymethyl cellulose (CMC) agar medium (Someya, 1980). Plates were incubated at 28^0C for 6 days. The

clear zone around the point of inoculation was examined by staining remaining CMC with Congo red (Suyama, 1993). For the pectinase assay, the test strains were spotted on a nutrient agar (DIFCO laboratories, USA) medium supplemented with 0.5% pectin and incubated at 28^0C for 3 days, then remaining pectin was stained with cetyltrimethylammonium bromide (CTAB) to visualize the clear zone around the bacterial culture (Ma et al., 2011). For both assays, the activities were categorized based on the clear zones around the bacterial colony as follows: no (0 mm), weak (1-3 mm) and strong (>3 mm) activities.

2.2.5 Evaluation of plant growth promotion in sweet potato with endophytes

The endophytes within the same location were selected for the inoculation experiment based on their phylogenies (**Table S2.1**). Each strain was cultivated separately in nitrogen containing modified MR liquid medium at 26^0C with shaking for 5 days. The cells were harvested by centrifugation (10,000 rpm for 10 minutes at 15^0C), washed twice and suspended with sterile distilled water, and OD_{660} was adjusted to 0.2. Then, the cell suspensions of the same location were mixed together to make the inoculants in 10 mL final volume. The experiment was conducted using a Leonard jar (Leonard, 1943). The upper pot was filled with water-soaked sterile vermiculite and the lower pot was supplied with 150 mL of sterile 1/5N plant medium (Hoagland and Arnon, 1950). The cotton wick was set to connect the upper pot and the lower reservoir. The whole pot was autoclaved before use.

Micro-propagated sweet potato plantlets cv. *Koukei* (3-4 leaves) was used for the experiments. Two consecutive experiments were conducted in duplicate. After measuring initial fresh weight, vine and root lengths (except for root length in the first experiment due to the absence of roots), the root part was dipped into the inoculants for 3 min and transplanted to the sterile pot, and 5 mL inoculant was poured on the vermiculite around the plant. The inoculated plants were aseptically grown in a plant growth chamber (LH240S, Nippon medical and chemical instruments co., ltd, Japan) with a 14 hour photo period, 28^0C/25^0C (day/night) at 7000 lux, which was provided by white fluorescent tubes. The same plant media without nitrogen was supplied to the bottom pot, as per the requirement. For control, sterile distilled water was

inoculated. First experiment was conducted with nine inoculants and the better performing six inoculants were used in the second experiment.

The plants were harvested at 30 days after inoculation. The whole plant was carefully pulled to avoid damage and shaken to release loosely attached vermiculite, then strongly adhered vermiculite was manually removed with tweezers. After blotting excess moisture from the roots with absorbent paper, whole plant fresh weight, vine and root lengths were measured. Then, nitrogenase activity for the fresh roots was assayed by ARA using 100 mL vial. Uninoculated plant roots with/without acetylene served as controls.

2.2.6 Statistical analysis

All the statistical analyses were carried out using SPSS version 16.

2.2.7 Nucleotide sequence accession numbers

All sequences are deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC389337 to LC389579 (16S rRNA) and LC389580 to LC389582 (*nifH* gene).

2.3 Results

2.3.1 Isolation and identification of endophytic bacteria

Different morphologies were observed among the appeared bacterial colonies, ranging from one to nine morphologies in Gulmi and Rupandehi locations, respectively. Two to 43 endophytic bacterial isolates per location, making a total of 243 isolates, were isolated and examined (**Table S2.1**).

Based on partial 16S rRNA gene sequencing analysis, 243 endophytic bacterial isolates were assigned to their close relatives, belonging to 34 bacterial genera in 6 classes (**Table 2.2**). Among the classes, Bacilli represented the highest relative abundance (28%), and *Bacillus* sp. was the most dominant genus (25%), followed by γ-Proteobacteria (22%)/ *Enterobacter* sp. (5.3%), β-Proteobacteria (17%)/ *Burkholderia* sp. (8.6%), Actinobacteria (16%)/ *Microbacterium* sp. (6.8%), α-

Proteobacteria (14%)/ *Rhizobium* sp. (6.3%) and Flavobacteriia (4.4%)/ *Flavobacterium* sp. (4.4%).

2.3.2 Distribution of bacterial genera

Endophytic bacterial genera were distributed unevenly among the sweet potato samples (**Table 2.2**). Four bacterial genera commonly detected in at least five sampling sites were applied to the distribution analysis (**Table 2.3**). *Enterobacter* sp. and *Microbacterium* sp. were detected frequently in nutrient rich (copiotrophic) and poor (oligotrophic) soils, respectively. *Bacillus* sp. showed acidophilic nature while *Rhizobium* sp. and *Microbacterium* sp. were alkaliphilic. Similarly, *Enterobacter* sp. showed neutralophilic property. On the other hand, distribution of these four genera was unaffected by the temperature conditions.

PCA of the environmental parameters explained 41.3% and 40.6% of the variation in the first and second principal component factors, respectively, and showed that there are approximately two groupings, first being the high temperature and alkaline soils (Rupandehi, Banke-a and Banke-b) and second with the others **(Figure 2.1-a)**. PCA of bacterial class compositions explained 31.0% and 25.2% of the variation in the first and second principal component factors, respectively, and revealed that the endophytic bacterial composition did not group as the environmental conditions **(Figure 2.1-b)**.

2.3.3 Characterization of the endophytic bacterial isolates

Eighty three percent of the bacterial strains presented at least one of the characteristics examined. Within all the strains, 57% produced IAA, 5.0% had *nifH* gene and showed ARA activity, 37% and 2.0% possessed antagonistic effect against the bacterial and the fungal pathogens, respectively. In addition, 17% and 8.0% showed cellulase and pectinase activities, respectively **(Table 2.4)**.

Proportions of the bacterial class representing the examined traits were different (**Table 2.4**). IAA production was detected in the strains from Bacilli, Actinobacteria, α- and γ-Proteobacteria classes, and ARA activity was from β- and γ-Proteobacteria. All the classes possessed antagonistic effect against the bacterial

pathogens, while only Bacilli class showed the effect against the fungal pathogen. Almost all the bacterial classes showed cellulase activity while α-, β- and γ-Proteobacteria classes had pectinase activity.

Bacterial strains exhibiting at least one of the following plant growth promoting or endophytic traits are presented in **Table 2.5**: high IAA production (≥30 µg/mL), ARA activity, strong antagonistic and cellulase/pectinase activities. Among the bacterial strains 8 of them showed higher IAA production potential with Ban-b 4 being the highest (65 µg/mL). Strains belonging to the same genus showed varying levels of IAA producing ability. In addition, Sal 1, Sal 6 and Rol 5 had *nifH* gene and showed the ARA activity with 54.5±7.3 nmol C_2H_4/h/vial, 39.9±1.9 nmol C_2H_4/h/vial and 8.9±0.8 nmol C_2H_4/h/vial, respectively.

Likewise, 11 bacterial strains showed strong antagonistic effect against at least one pathogen tested. Among them, Chi 2 and Gul 1 possessed strong activity against the tested bacterial pathogens and the latter showed strong activity against the fungal pathogen assayed. However, these two isolates did not show IAA producing ability. On the other hand, 5 bacterial strains showed cellulase activity while Ban-b 6 showed both cellulase and pectinase activity.

2.3.4 Effect of mixture of endophytes on plant growth promotion

Fresh weight (g), vine and root lengths (cm) were considered for the assessment of plant growth promotion. Plantlets used in the experiments were non-uniform in size, and this might affect the parameters. So, times increase as compared to the control were used for the assessment of the plant growth promotion of the inoculants.

In the first experiment, the inoculated sweet potatoes showed higher values than control (**Table 2.6**). Mixture of isolates from Salyan, Palpa, Banke gained 3.18-3.51 times their initial weight whereas it was 1.6 in control. Likewise, gain in vine length ranged from 1.33-1.50 times for Kavre, Chitwan, Banke and Salyan inoculants, while it was 1.17 in control. Finally, the roots were longer in almost all inoculated plants than control.

Further, we selected six inoculants based on the first experiment and again evaluated. In the second experiment, the growth promoting effects were observed in

fresh weight and vine length, but root lengths were shorter in the inoculated plants than control (**Table 2.6**). Among all the plants assessed, only Salyan isolates inoculated plants showed ARA activity (0.09 nmol/h/g).

2.4. Discussion

In the present study, culture dependent method was used to learn more about the endophytic bacterial community in sweet potato collected from Nepal. In analyzing endophytic communities by culture dependent methods, most researchers selected dominant single colonies representing distinct morphology and ignored minor ones and therefore their diversity (Fredrickson et al., 1991; McInroy and Kloepper, 1995; Lebaron et al., 1998; Tiwari et al., 2010; Jackson et al., 2013). On the other hand, we examined all colonies in the plates and grouped based on their morphologies, and the representative colony (s) in each group was selected on the basis of their relative abundance for further analysis. Our method would be more reliable in examining the endophytic communities.

In culture dependent methods, media components are the most influential parameter. Marques et al. (2015) used three media conditions (TSA, PDA and modified RM) and isolated 93 endophytic bacteria belonging to 17 genera for three sweet potato cultivars collected in Brazil. Although the media used were different, the following common genera were mainly detected: *Bacillus* sp. and *Paenibacillus* sp. in Bacilli class, *Arthrobacter* sp. and *Microbacterium* sp. in Actinobacteria, *Sphingomonas* sp. and *Rhizobium* sp. in α-Proteobacteria and *Enterobacter* sp., *Pantoea* sp. and *Pseudomonas* sp. in γ-Proteobacteria. This result was similar to our result even though the media and cultivation locations were different. Khan and Doty (2009) isolated 11 endophytes in seven genera by MS medium from sweet potatoes collected from grocery store in USA, where *Stenotrophomonas* sp., *Pseudomonas* sp., *Enterobacter* sp. and *Xanthomonas* sp. in γ-Proteobacteria were dominated. γ-Proteobacteria have been reported as commonly dominant endophyte in plants (Hardoim et al., 2015). On the other hand, isolates from Bacilli class was not detected (Khan and Doty, 2009) demonstrating that the Bacilli might not always be dominated in sweet potato. More studies are necessary to make better conclusions for

endophytic dominancy of Bacilli in sweet potato. Hardoim et al. (2015) reported that Bacilli is not dominantly detected as endophyte in many plants, whereas, they have been dominantly detected in several crops as canola (Germida et al., 1998), banana (Souza et al., 2013), switch grass (Xia et al., 2012) and tobacco (Long et al., 2010). The determining factors of Bacilli are still unclear and needs to be explored.

Culture dependent methods have a limitation of analyzing microbial communities due to unknown conditions for growth requirements of many bacteria and presence of the viable but noncultivable state (J L Tholozan et al., 1999). As a result, the dominant bacterial endophytes could not always be isolated. For example, *Ralstonia* sp. was dominant in culture independent methods in salad crops (Jackson et al., 2013) and sweet potato (Marques et al., 2015), but it was not isolated from the samples. Likewise, *Enterobacter* sp. dominantly detected in culture independent methods in maize was not isolated (Pereira et al., 2011). In our study, we could not successfully amplify the bacterial DNA from the sweet potato DNA using LNA-PCR technique (Ikenaga and Sakai, 2014) and the possible reason is unknown. The culture dependent method has its own limitation on determining the bacterial community but it is only the option to isolate the bacteria for their functional analysis. It is important to find the suitable culture conditions for the endophytes. Modification of media components considering their natural habitat could be one of the options.

There have been relatively a few studies that have analyzed the effects of environmental variables on endophyte diversity (Santoyo et al., 2016). For example, culturable endophytic bacterial communities were more diverse in tobacco roots from organic soils compared to those grown in mineral soils (Long et al., 2010). Similarly, psychrophilic bacterial endophytes were isolated abundantly in cold environments from the arcto-alpine plant species (Nissinen et al., 2012). Likewise, culturable endophytic bacterial communities in four vegetable crops were more diverse in organic farming practices as compared to conventional ones (Xia et al., 2015).

The colonization of endophytic bacterial community could be influenced by environmental conditions through the following two processes. Firstly, environmental variables affect the plant physiology thereby influencing the root exudates which might determine the microbial communities in the rhizosphere, the potential

endophytic candidates (Germida et al., 1998; Marquez-Santacruz, 2010; Pereira et al., 2011). It was reported that environmental factors such as temperatures and photon flux density influenced root exudates of tomato and clover (Rovira, 1959). Likewise, low photon flux density increased the release of carbon in root exudates of rye grass (Hodge et al., 1997). In addition, it was also reported that oxalate in the root exudates enriched Oxalobacteraceae family in the rhizosphere of stiff brome plant (Kawasaki et al., 2016). Similarly, Haichar et al.(2008) reported that the rape plant root exudates enriched the rhizospheric zone with α-, δ-, β- and γ-roteobacteria and Actinobacteria, barrel clover with α- and γ-Proteobacteria, and maize with α-, β-, γ-Proteobacteria and Actinobacteria.

Secondly, environmental variables influence the bacterial composition in the bulk soil which ultimately determines the possible endophytic community (Hallmann et al., 1997). Although several environmental factors are responsible in determining the soil bacterial community, soil pH is one of the influencing parameters. In a diverse set of ecosystems across South and North America, soil bacterial community was strongly shaped by soil pH at the continental scale, where bacterial diversity was highest in neutral soils and lower in acidic soils (Fierer and Jackson, 2006). Similarly, the relative abundance of Actinobacteria, Bacterioidetes, Fibrobacteres and Firmicutes was higher at near-neutral pH and lower at acidic and alkaline pH (Zhang et al., 2017). In addition, temperature is one of the environmental factors determining the composition of the soil bacterial community. Studies conducted applying culture independent methods revealed that the relative abundances of Actinobacteria and Firmicutes increased at higher temperatures, while Bacteroidetes and δ-Proteobacteria showed the opposite pattern when the soils collected from an alpine meadow were incubated at different temperatures (Wu et al., 2015). Similarly, Lin et al. reported that the relative abundance of soil Acidobacteria decreased with increasing temperature while γ-Proteobacteria increased (Lin et al., 2017). Thus, environmental conditions influenced on the endophytic bacterial community by changing the profile of plant exudates resulting in selection of distinct rhizobacterial community and by influencing the soil microbial community; the main sources for endophytic community.

There are reports that the plant determines the endophytic bacterial communities and soil factors played a minor role (Germida et al., 1998). Other researchers, however, have reported that soil type (Gaiero et al., 2013; Prischl et al., 2012) and environmental factors (Gaiero et al., 2013) determine the endophytic communities. Our results indicate that endophytic diversity is independent on soil and environmental factors. Hence, it was suggested that the plant and other unknown factors would be responsible in determining the endophytic bacterial community.

Plant growth promoting and endophytic characteristics of the selected bacterial isolates were analyzed in this study using *in vitro* tests. IAA is the main phytohormone in plants, regulating many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light (Gordon and Weber, 1951). In our study, 57% of endophytic bacteria isolated from sweet potato synthesized IAA from tryptophan, and the ability was distributed to Bacilli, Actinobacteria, α- and γ-Proteobacteria classes. Similarly, IAA producing endophytes in the same classes have been reported in sweet potato (Marques et al., 2015; Khan and Doty, 2009) and the other crops as rice (Ji et al., 2014), ginseng (Vendan et al., 2010), semi-aquatic grass (Jha and Kumar, 2007) and poplar trees (Taghavi and van der Lelie, 2013). The IAA producing endophytes can be used as plant growth promoting agent, but the ability should be confirmed in *in situ* conditions.

Among the tested endophytes, only three strains (5%) showed the N_2-fixing potential. Similarly, detection of *nifH* in endophytic isolates was negative in sweet potato (Marques et al., 2015) or not often in rice (Ji et al., 2014) and ginseng (Vendan et al., 2010), representing 2% and 4% of the total isolates, respectively. As their presence might play a role in the growth of sweet potato plants, it is thus, necessary to determine how much nitrogen they fix in the host plant.

Endophytic bacteria from all the classes demonstrated the antagonistic effect against the bacterial pathogens, while antagonism against the fungal pathogen was observed only for Bacilli class represented by *Bacillus* sp. Gul 1. In congruent to our findings, Marques et al. (2015) reported that *Bacillus* sp. isolated as endophytes from sweet potato tubers showed antimicrobial activity against a fungal pathogen, *Plenodomus destruens*. Because it was reported that some of the *Bacillus* strains

produce antimicrobial compounds as iturins that affect fungal signaling pathways (Han et al., 2015) and surfactin, an antifungal lipopeptide (Tendulkar et al., 2007), *Bacillus* sp. Gul 1 might produce antifungal compounds.

Besides all these plant growth promoting properties, endophytes need to colonize inside the host plants. Except for already established seed endophytes (Truyens et al., 2015), common points of entry are through stomata (Roos and Hattingh, 1983), primary and lateral root cracks and tissues wounds created as a result of plant growth (Sprent and De Faria, 1989). Besides these pathways, presence of the hydrolytic enzymes in sweet potato endophytes suggests enzyme based penetration of these endophytes to the plant.

The inoculation of crop plants with beneficial microbes is a practice used in agriculture and provides advantages to crops by enhancing plant growth and triggering protection to diseases (Ji et al., 2014). It was also reported that the inoculation with multiple beneficial bacteria have higher potential than inoculation with a single bacterial inoculant (Molina-Romero et al., 2017; Oliveira et al., 2009). Our study also showed the positive effects when the isolates were applied as a mixture from each location. Mixture of inoculants might interact synergistically to provide nutrients, remove inhibitory products and stimulate one another.

Plant growth promotions by the IAA producing endophytes have been reported in sweet potato (Khan and Doty, 2009) and other crops as tomato (Khan et al., 2014) and strawberry (Dias et al., 2009). In our study, all inoculants with plant growth promoting activity included the IAA producing endophytic bacteria suggesting that IAA produced influenced the growth. In addition, sweet potato inoculated with Salyan mixture including *nifH* gene containing *Klebsiella* sp. Sal 1 and *Herbaspirillum* sp. Sal 6 showed ARA activity suggesting that N-fixation would be one of the reasons for the higher fresh weight of the inoculated plants.

In this study, it was difficult to prepare the test plants with similar initial size, and the bigger the initial size produced the bigger plant. Therefore, times increase of fresh weight, vine length and root length were used to compare the plant growth promoting effect. Although, the times increase were not constant in the repeated

experiments, we could observe the positive effect of the inoculants. This suggests the endophytic community possess potential for plant growth promotion.

2.5 Conclusion

Further studies will be necessary to determine the responsible endophytes and their mechanisms of the plant growth promotion, but community might be important and necessary for the ability. Besides this, scope still exists to unravel the endophytic community structure by culture independent method and to cultivate the uncultured endophytes by modifying the culture conditions. Although the *in vitro* assays used may not reproduce exactly the conditions of natural environment, they can provide rapid screening of the potential strains, which can save time and costs, and further screening for the candidates *in situ* is necessary.

Tables

Table 2.1 Climate, land and soil properties of the sweet potato sampling sites in Nepal

Climate	Location (District)	Temperature[a] Max	Min	Latitude	Longitude	Annual Rainfall (mm)[a]	Altitude (masl)	Soil type[b]	Soil pH	Soil P[c]	Soil C[c]	Soil N[c]
Temperate	Rolpa	32	3.4	28.30^{0}N	82.63^{0}E	1261	1200	Inceptisols	7.3	331	16	1.5
(Cooler	Salyan	31	6.5	28.42^{0}N	82.00^{0}E	987	1300	Entisols	6.2	4.8	11	0.8
highland)	Gulmi	28	4.3	28.02^{0}N	83.24^{0}E	1860	1500	Entisols	6.7	12	7.0	0.7
	Palpa	33	6.1	27.89^{0}N	83.50^{0}E	1564	1219	Entisols	6.4	37	20	1.7
	Kavre-a	28	2.8	27.62^{0}N	85.58^{0}E	1190	1408	Entisols	5.8	111	8.0	0.6
	Kavre-b	28	2.8	27.61^{0}N	85.59^{0}E	1190	1116	Entisols	5.5	87	7.0	0.6
Subtropical	Banke-a	39	8.0	28.02^{0}N	81.76^{0}E	1230	181	Alfisols	8.0	87	11	1.0
(warmer foot-	Banke-b	39	8.0	28.11^{0}N	81.59^{0}E	1230	179	Alfisols	8.4	6.2	8.0	0.7
hills & plains)	Rupandehi	38	7.8	27.58^{0}N	83.31^{0}E	1572	107	Alfisols	8.3	11	4.0	0.3
	Chitwan	36	6.2	27.65^{0}N	84.39^{0}E	1960	228	Alfisols	6.6	137	13	1.0
	Sunsari-a	34	8.0	26.71^{0}N	87.25^{0}E	1816	107	Alfisols	6.7	379	20	1.7
	Sunsari-b	34	8.0	26.70^{0}N	87.28^{0}E	1816	108	Alfisols	5.9	20	16	1.3

[a] 5 years average of maximum, minimum annual temperature and annual rainfall (www.dhm.gov.np)

[b]Based on USDA classification (Soil survey staff, 1999).

[c]Soil P in mg kg^{-1}, and Soil C and Soil N in g kg^{-1}

Table 2.2 Relative abundance of bacterial endophytes of sweet potato in Nepal

Sampling sites	Rolpa	Salyan	Gulmi	Palpa	Kavre-a	Kavre-b	Banke-a	Banke-b	Rupandehi	Chitwan	Sunsari-a	Sunsari-b	Total
No. of colonies	301	440	20	70	54	165	320	327	384	150	172	87	2490
No. of morphologies	7	5	1	3	2	5	7	7	9	4	3	2	55
No. of isolates	30	43	2	6	5	17	31	32	37	14	18	8	243
Class/genera													Average (%)
Flavobacteriia	23	25					4.0						4.4
Flavobacterium sp.	23	25					4.0						4.4
Bacilli	16	3.0	100	32	22	51	3.0		8.0	48	44	12	28
Bacillus sp.		3.0	100	43	22	37			3.0	48	44	12	25
Staphylococcus sp.	16					13	3.0						2.7
Exiguobacterium sp.									3.0				0.3
Paenibacillus sp.									3.0				0.3
Actinobacteria	37	5.0					61	13	67				16
Microbacterium sp.	7.0	5.0					40	3.0	27				6.8
Curtobacterium sp.	23						11	10	12				4.6
Cellulomonas sp.							3.0						2.0
Arthrobacter sp.							6.8		10				1.0
Glutamicibacter sp.									13				1.0
Pseudarthrobacter sp.									5.0				0.4
Streptomyces sp.	4.0												0.3
Brachybacterium sp.	3.0												0.2
α-Proteobacteria		11		16	37	13	24	44	6.0	7.0	6.0		14
Rhizobium sp.		9.0		16		13	6.0	19	6.0		6.0		6.3
Agrobacterium sp.		2.0			37		6.0	13					4.8
Sphingobium sp.								13					1.0
Sphingomonas sp.							12						1.0
Neorhizobium sp.										7.0			0.6
β-Proteobacteria	7.0	7.0			22	32		10	13	22		88	17
Burkholderia sp.										15		88	8.6
Achromobacter sp.						19			13				3.0
Herbaspirillum sp.		7.0			22	7.0							3.0
Xenophilus sp.								10					0.8
Massilia sp.	7.0												0.6
Paraburkholderia sp.										7.0			0.6
Caballeronia sp.						6.0							0.5
γ-Proteobacteria	17	48		51	19	4.2	6.0	33	6.0	23	50		22
Enterobacter sp.	7.0	2.0		33			3.0			7.0	11		5.3
Pseudomonas sp.					19						39		4.8
Stenotrophomonas sp.	3.0	28						12		9.0			4.3
*Luteibacte*r sp.				19		1.0							1.7
Pantoea sp.						3.0	3.0		6.0	7.0			1.6
Klebsiella sp.		18											1.5
Xanthomonas sp.								15					1.3
Pseudoxanthomonas sp.								6.0					0.5
Yokenella sp.	6.0												0.5

Table 2.3 Detection frequency (%) of the bacterial genera in different environmental conditions[a].

Genera	Phosphorus		Carbon		Nitrogen		pH			Temperature	
	Low	High	Low	High	Low	High	Acidic	Neutral	Alkaline	Low	High
Bacillus sp.	83	67	71	80	83	67	100	83	33	83	67
Rhizobium sp.	67	50	71	40	67	50	33	50	100	50	67
Microbacterium sp.	50	33	57	20	50	33	0	33	100	33	50
Enterobacter sp.	33	67	29	80	17	83	0	83	33	50	50

[a]: Low P (4.8-37 mg kg^{-1} soil) and high P (87-379 mg kg^{-1} soil), low C (4-11 g kg^{-1} soil) and high C (13-20 g kg^{-1} soil), low N (0.3-0.8 g kg^{-1} soil) and high N (1.0-1.7 g kg^{-1} soil), acidic (pH 5.5-5.9), neutral (pH 6.2-7.3) and alkaline (pH 8.0-8.4), high temperature (34-39^{0}C) and low temperature (28-32^{0}C)

Table 2.4 Bacterial class and their proportions having the plant growth promoting traits, antagonistic effect and endophytic traits.

Class	Number of genera	Number of strains	Plant growth promoting traits		Antagonistic effect				Endophytic traits	
			IAA[a]	ARA activity[b]	ECa[c]	ECb[d]	ECc[e]	Fungal[f]	Cellulase	Pectinase
Flavobacteriia	1	1	0	0	100	100	100	0	0	0
Bacilli	4	7	43	0	29	43	28	14	14	0
Actinobacteria	8	20	63	0	21	16	16	0	16	0
α-Proteobacteria	5	9	78	0	22	33	22	0	11	11
β-Proteobacteria	7	8	0	13	25	25	25	0	13	13
γ-Proteobacteria	9	15	75	13	19	25	6	0	25	19
Total	34	60	57	5	27	58	25	2	17	8

[a] Indole-3-acetic acid (μg mL^{-1})

[b] Acetylene reduction activity (nmol/h/vial)

[c] *Erwinia chrysanthemi* NARCB200126, AZ9702

[d] *Erwinia chrysanthemi* E7725

[e] *Erwinia chrysanthemi* T5-2

[f] *Pythium ultimum* var *ultimum* OPU744

Table 2.5 Plant growth promoting traits, antagonistic effect and endophytic traits of bacterial endophytes of sweet potato in Nepal.

Class	Strains	Most similar 16S rRNA gene sequence	Plant growth promoting traits		Antagonistic effect[c]				Endophytic traits[c]	
			IAA / OD[a]	ARA activity[b]	ECa[d]	ECb[e]	ECc[f]	Fungal[g]	Cellulase	Pectinase
Bacilli	Gul 1	*Bacillus* sp.	0	-	++	++	++	++	++	-
	Sun-a 3	*Bacillus* sp.	34	-	-	-	-	-	-	-
Actinobacteria	Rol 1	*Curtobacterium* sp.	0	-	-	-	-	-	++	-
	Sal 8	*Microbacterium* sp.	2	-	+	+	++	-	-	-
	Ban-a 5	*Arthrobacter* sp.	35	-	-	-	-	-	-	-
	Rup 2	*Microbacterium* sp.	11	-	++	+	++	-	++	-
	Rup 6	*Microbacterium* sp.	17	-	++	+	-	-	-	-
α-Proteobacteria	Sal 7	*Agrobacterium* sp.	30	-	+	+	-	-	-	-
	Ban-a 3	*Agrobacterium* sp.	26	-	-	++	-	-	-	-
	Ban-a 4	*Rhizobium* sp.	28	-	-	++	++	-	-	-
	Ban-a 9	*Sphingomonas* sp.	8	-	-	+	+	-	++	-
	Ban b 4	*Sphingobium* sp.	65	-	++	+	+	-	-	-
β-Proteobacteria	Sal 6	*Herbaspirillum* sp.	0	9	-	+	-	-	-	-
	Chi 2	*Burkholderia* sp.	0	-	++	++	++	-	-	-
	Sun-b 1	*Burkholderia* sp.	0	-	++	+	+	-	+	-
γ-Proteobacteria	Rol 5	*Yokenella* sp.	36	40	-	-	-	-	-	-
	Sal 1	*Klebsiella* sp.	48	55	-	+	-	-	+	-
	Sal 3	*Enterobacter* sp.	61	-	-	+	-	-	-	+
	Kav-b 3	*Luteibacter* sp.	15	-	++	-	-	-	-	-
	Ban-a 7	*Pantoea* sp.	29	-	++	++	-	-	+	-
	Ban-b 6	*Pseudoxanthomonas* sp.	13	-	-	+	-	-	++	++
	Chi 1	*Pantoea* sp.	36	-	-	+	-	-	++	-

[a] Indole-3-acetic acid (μg mL^{-1}) optical $density^{-1}$, [b] Acetylene reduction activity (nmol/h/vial), [c] -, + and ++ denote no, weak and strong activities, respectively. , [d] *Erwinia chrysanthemi* NARCB200126, AZ9702, [e] *Erwinia chrysanthemi* E7725, [f] *Erwinia chrysanthemi* Ech T5-2, and [g]*Pythium ultimum* var *ultimum* OPU744

Table 2.6 Times increase in growth parameters of sweet potato plants (n=2)

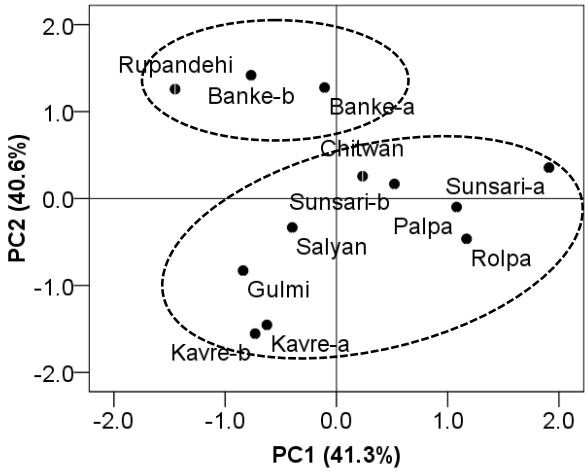
Inoculants	First Experiment			Second experiment		
	Fresh weight	Vine length	Root length[b]	Fresh weight	Vine length	Root length
Rolpa	2.63	1.17	59	-	-	-
Salyan	3.51	1.33	73	5.70	1.53	7.2
Gulmi	2.31	1.28	68	-	-	-
Palpa	3.33	1.26	76	7.56	1.45	18.7
Kavre	2.59	1.50	66	10.20	2.33	22.4
Banke	3.18	1.33	50	9.45	1.50	34.2
Rupandehi	2.25	1.21	75	5.86	2.14	21.0
Chitwan[a]	2.99	1.35	74	4.98	1.00	25.0
Sunsari[a]	2.80	1.16	70	-	-	-
Control	1.60	1.17	47	4.68	1.24	38.0

[a] Data from one replication is considered for Sunsari and Chitwan in first and second experiment respectively, as one replication plant was dead

[b] Final root length (cm).

Figures

(a)



(b)

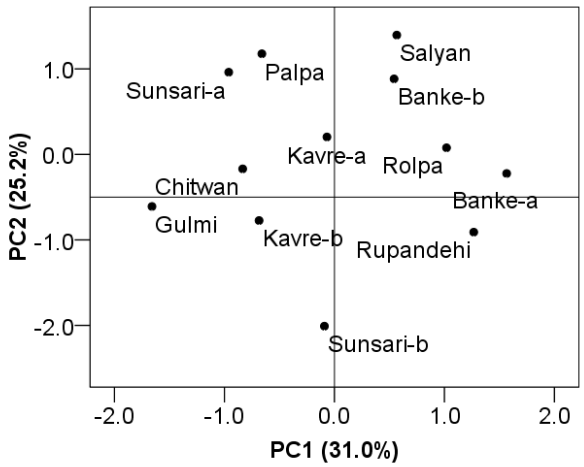


Figure 2.1 2-D plot of principal component analysis of sampling locations based on (a) environmental parameters and (b) bacterial class composition in each location.

Supplementary Tables

Table S2.1 List of endophytic bacterial strains isolated from Nepalese sweet potato tubers.

Locations	Strains	Close relatives based on 16S	Class	a	b
Rolpa	Rol 1	*Curtobacterium luteum*	Actinobacteria	×	×
	Rol 2	*Stenotrophomonas rhizophila*	γ-Proteobacteria	×	×
	Rol 3	*Brachybacterium rhamnosum*	Actinobacteria	×	×
	Rol 4	*Staphylococcus sciuri*	Bacilli	×	×
	Rol 5	*Yokenella regensburgei*	γ-Proteobacteria	×	×
	Rol 6	*Streptomyces viridochromogene*	Actinobacteria	×	×
	Rol 7	*Massilia haematophila*	β-Proteobacteria	×	×
	Rol 8	*Enterobacter cloacae*	γ-Proteobacteria	×	
	Rol 9	*Enterobacter asburiae*	γ-Proteobacteria		
	Rol 10	*Curtobacterium luteum*	Actinobacteria		
	Rol 11	*Curtobacterium luteum*	Actinobacteria		
	Rol 12	*Curtobacterium luteum*	Actinobacteria		
	Rol 13	*Flavobacterium johnsoniae*	Flavobacteriia		
	Rol 14	*Yokenella regensburgei*	γ-Proteobacteria		
	Rol 15	*Microbacterium paraoxydans*	Actinobacteria	×	
	Rol 16	*Massilia haematophila*	β-Proteobacteria		
	Rol 17	*Flavobacterium johnsoniae*	Flavobacteriia		
	Rol 18	*Flavobacterium johnsoniae*	Flavobacteriia		
	Rol 19	*Curtobacterium luteum*	Actinobacteria		
	Rol 20	*Curtobacterium luteum*	Actinobacteria		
	Rol 21	*Curtobacterium luteum*	Actinobacteria		
	Rol 22	*Microbacterium paraoxydans*	Actinobacteria		
	Rol 23	*Staphylococcus xylosus*	Bacilli		
	Rol 24	*Staphylococcus saprophyticus*	Bacilli		
	Rol 25	*Staphylococcus saprophyticus*	Bacilli		
	Rol 26	*Staphylococcus saprophyticus*	Bacilli		
	Rol 27	*Flavobacterium johnsoniae*	Flavobacteriia		
	Rol 28	*Flavobacterium johnsoniae*	Flavobacteriia	×	
	Rol 29	*Flavobacterium johnsoniae*	Flavobacteriia		
	Rol 30	*Flavobacterium johnsoniae*	Flavobacteriia		
Salyan	Sal 1	*Klebsiella variicola*	γ-Proteobacteria	×	×
	Sal 2	*Flavobacterium johnsoniae*	Flavobacteriia	×	×
	Sal 3	*Enterobacter asburiae*	γ-Proteobacteria	×	×
	Sal 4	*Rhizobium pusense*	Aproteobacteria	×	×
	Sal 5	*Stenotrophomonas rhizophila*	γ-Proteobacteria	×	×
	Sal 6	*Herbaspirillum huttiense*	β-Proteobacteria	×	×
	Sal 7	*Agrobacterium larrymoorei*	α-Proteobacteria	×	×
	Sal 8	*Microbacterium testaceum*	Actinobacteria	×	×
	Sal 9	*Stenotrophomonas rhizophila*	γ-Proteobacteria		
	Sal 10	*Flavobacterium johnsoniae*	Flavobacteriia		
	Sal 11	*Klebsiella variicola*	γ-Proteobacteria		
	Sal 12	*Rhizobium cellulosilyticum*	α-Proteobacteria		
	Sal 13	*Bacillus megaterium*	Bacilli	×	
	Sal 14	*Flavobacterium anhuiense*	Flavobacteriia		
	Sal 15	*Stenotrophomonas rhizophila*	γ-Proteobacteria		
	Sal 16	*Klebsiella variicola*	γ-Proteobacteria		
	Sal 17	*Stenotrophomonas maltophilia*	γ-Proteobacteria		
	Sal 18	*Stenotrophomonas maltophilia*	γ-Proteobacteria		
	Sal 19	*Stenotrophomonas maltophilia*	γ-Proteobacteria		
	Sal 20	*Flavobacterium johnsoniae*	Flavobacteriia		

	Sal 21	*Stenotrophomonas rhizophila*	γ-Proteobacteria		
	Sal 22	*Stenotrophomonas rhizophila*	γ-Proteobacteria		
	Sal 23	*Flavobacterium johnsoniae*	Flavobacteriia		
	Sal 24	*Klebsiella variicola*	γ-Proteobacteria		
	Sal 25	*Microbacterium testaceum*	Actinobacteria		
	Sal 26	*Rhizobium pusense*	Aproteobacteria		
	Sal 27	*Herbaspirillum huttiense*	β-Proteobacteria		
	Sal 28	*Stenotrophomonas rhizophila*	γ-Proteobacteria		
	Sal 29	*Flavobacterium johnsoniae*	Flavobacteriia		
	Sal 30	*Klebsiella pneumoniae*	γ-Proteobacteria		
	Sal 31	*Klebsiella pneumoniae*	γ-Proteobacteria		
	Sal 32	*Klebsiella variicola*	γ-Proteobacteria		
	Sal 33	*Flavobacterium johnsoniae*	Flavobacteriia		
	Sal 34	*Flavobacterium anhuiense*	Flavobacteriia		
	Sal 35	*Stenotrophomonas rhizophila*	γ-Proteobacteria		
	Sal 36	*Herbaspirillum huttiense*	β-Proteobacteria		
	Sal 37	*Klebsiella pneumoniae*	γ-Proteobacteria		
	Sal 38	*Rhizobium pusense*	α-Proteobacteria		
	Sal 39	*Stenotrophomonas maltophilia*	γ-Proteobacteria		
	Sal 40	*Stenotrophomonas rhizophila*	γ-Proteobacteria		
	Sal 41	*Flavobacterium johnsoniae*	Flavobacteriia		
	Sal 42	*Flavobacterium johnsoniae*	Flavobacteriia		
	Sal 43	*Flavobacterium johnsoniae*	Flavobacteriia		
Gulmi	Gul 1	*Bacillus pumilus*	Bacilli	×	×
	Gul 2	*Bacillus safensis*	Bacilli		
Palpa	Pal 1	*Rhizobium cellulosilyticum*	α-Proteobacteria	×	
	Pal 2	*Enterobacter cloacae*	γ-Proteobacteria		
	Pal 3	*Luteibacter yeojuensis*	γ-Proteobacteria	×	
	Pal 4	*Enterobacter cloacae*	γ-Proteobacteria	×	
	Pal 5	*Bacillus wiedmannii*	Bacilli	×	
	Pal 6	*Bacillus thuringiensis*	Bacilli		
Kavre-a	Kav-a 1	*Herbaspirillum seropedicae*	β-Proteobacteria	×	×
	Kav-a 2	*Pseudomonas oryzihabitans*	γ-Proteobacteria	×	×
	Kav-a 3	*Agrobacterium tumefaciens*	α-Proteobacteria	×	
	Kav-a 4	*Agrobacterium fabrum*	α-Proteobacteria		
	Kav-a 5	*Bacillus wiedmannii*	Bacilli	×	
Kavre-b	Kav-b 1	*Caballeronia temeraria*	β-Proteobacteria	×	×
	Kav-b 2	*Staphylococcus succinus*	Bacilli	×	×
	Kav-b 3	*Luteibacter yeojuensis*	γ-Proteobacteria	×	×
	Kav-b 4	*Rhizobium miluonense*	α-Proteobacteria	×	×
	Kav-b 5	*Bacillus cereus*	Bacilli	×	×
	Kav-b 6	*Caballeronia temeraria*	β-Proteobacteria		
	Kav-b 7	*Herbaspirillum huttiense*	β-Proteobacteria	×	
	Kav-b 8	*Staphylococcus saprophyticus*	Bacilli		
	Kav-b 9	*Rhizobium pusense*	α-Proteobacteria		
	Kav-b 10	*Achromobacter xylosoxidans*	β-Proteobacteria	×	
	Kav-b 11	*Pantoea stewartii*	γ-Proteobacteria	×	
	Kav-b 12	*Achromobacter xylosoxidans*	β-Proteobacteria		
	Kav-b 13	*Bacillus cereus*	Bacilli		
	Kav-b 14	*Bacillus cereus*	Bacilli		
	Kav-b 15	*Bacillus cereus*	Bacilli		
	Kav-b 16	*Bacillus wiedmannii*	Bacilli		
	Kav-b 17	*Bacillus cereus*	Bacilli		
Banke-a	Ban-a 1	*Microbacterium radiodurans*	Actinobacteria	×	×
	Ban-a 2	*Curtobacterium citreum*	Actinobacteria	×	×
	Ban-a 3	*Agrobacterium larrymoorei*	α-Proteobacteria	×	×
	Ban-a 4	*Rhizobium cellulosilyticum*	α-Proteobacteria	×	×

	Ban-a 5	*Arthrobacter pokkalii*	Actinobacteria	×	×
	Ban-a 6	*Cellulomonas hominis*	Actinobacteria	×	×
	Ban-a 7	*Pantoea stewartii*	γ-Proteobacteria	×	×
	Ban-a 8	*Microbacterium radiodurans*	Actinobacteria	×	×
	Ban-a 9	*Sphingomonas yantingensis*	α-Proteobacteria	×	×
	Ban-a 10	*Enterobacter cloacae*	γ-Proteobacteria	×	
	Ban-a 11	*Curtobacterium citreum*	Actinobacteria		
	Ban-a 12	*Curtobacterium citreum*	Actinobacteria		
	Ban-a 13	*Staphylococcus gallinarum*	Bacilli	×	
	Ban-a 14	*Sphingomonas koreensis*	α-Proteobacteria		
	Ban-a 15	*Flavobacterium johnsoniae*	Flavobacteriia	×	
	Ban-a 16	*Microbacterium radiodurans*	Actinobacteria		
	Ban-a 17	*Microbacterium radiodurans*	Actinobacteria		
	Ban-a 18	*Microbacterium oleivorans*	Actinobacteria		
	Ban-a 19	*Microbacterium oleivorans*	Actinobacteria		
	Ban-a 20	*Rhizobium cellulosilyticum*	α-Proteobacteria		
	Ban-a 21	*Arthrobacter enclensis*	Actinobacteria		
	Ban-a 22	*Microbacterium radiodurans*	Actinobacteria		
	Ban-a 23	*Microbacterium radiodurans*	Actinobacteria		
	Ban-a 24	*Microbacterium radiodurans*	Actinobacteria		
	Ban-a 25	*Sphingomonas yantingensis*	α-Proteobacteria		
	Ban-a 26	*Agrobacterium larrymoorei*	α-Proteobacteria		
	Ban-a 27	*Sphingomonas yantingensis*	α-Proteobacteria		
	Ban-a 28	*Microbacterium paraoxydans*	Actinobacteria		
	Ban-a 29	*Microbacterium oleivorans*	Actinobacteria		
	Ban-a 30	*Microbacterium oxydans*	Actinobacteria		
	Ban-a 31	*Microbacterium oleivorans*	Actinobacteria		
Banke-b	Ban-b 1	*Xenophilus aerolatus*	β-Proteobacteria	×	×
	Ban-b 2	*Stenotrophomonas maltophilia*	γ-Proteobacteria	×	×
	Ban-b 3	*Xanthomonas campestris*	γ-Proteobacteria	×	×
	Ban-b 4	*Sphingobium yanoikuyae*	α-Proteobacteria	×	×
	Ban-b 5	*Microbacterium lemovicicum*	Actinobacteria	×	×
	Ban-b 6	*Pseudoxanthomonas spadix*	γ-Proteobacteria	×	×
	Ban-b 7	*Xanthomonas translucens*	γ-Proteobacteria	×	×
	Ban-b 8	*Rhizobium pusense*	α-Proteobacteria	×	
	Ban-b 9	*Curtobacterium luteum*	Actinobacteria	×	
	Ban-b 10	*Stenotrophomonas maltophilia*	γ-Proteobacteria		
	Ban-b 11	*Rhizobium giardinii*	α-Proteobacteria		
	Ban-b 12	*Rhizobium pusense*	α-Proteobacteria		
	Ban-b 13	*Xanthomonas campestris*	γ-Proteobacteria		
	Ban-b 14	*Agrobacterium fabrum*	α-Proteobacteria		
	Ban-b 15	*Agrobacterium larrymoorei*	α-Proteobacteria		
	Ban-b 16	*Stenotrophomonas panacihumi*	γ-Proteobacteria		
	Ban-b 17	*Sphingobium yanoikuyae*	α-Proteobacteria		
	Ban-b 18	*Curtobacterium citreum strain*	Actinobacteria		
	Ban-b 19	*Rhizobium cellulosilyticum*	α-Proteobacteria		
	Ban-b 20	*Xenophilus aerolatus*	β-Proteobacteria		
	Ban-b 21	*Sphingobium yanoikuyae*	α-Proteobacteria		
	Ban-b 22	*Sphingobium yanoikuyae*	α-Proteobacteria		
	Ban-b 23	*Curtobacterium luteum*	Actinobacteria		
	Ban-b 24	*Agrobacterium tumefaciens*	α-Proteobacteria		
	Ban-b 25	*Rhizobium pusense*	α-Proteobacteria		
	Ban-b 26	*Stenotrophomonas maltophilia*	γ-Proteobacteria		
	Ban-b 27	*Pseudoxanthomonas spadix*	γ-Proteobacteria	×	
	Ban-b 28	*Xanthomonas translucens*	γ-Proteobacteria		
	Ban-b 29	*Agrobacterium larrymoorei*	α-Proteobacteria	×	
	Ban-b 30	*Rhizobium pusense*	α-Proteobacteria		

	Ban-b 31	*Xanthomonas translucens*	γ-Proteobacteria		
	Ban-b 32	*Xenophilus aerolatus*	β-Proteobacteria		
Rupandehi	Rup 1	*Microbacterium binotii*	Actinobacteria	×	×
	Rup 2	*Microbacterium arborescens*	Actinobacteria	×	×
	Rup 3	*Microbacterium hydrothermale*	Actinobacteria	×	×
	Rup 4	*Achromobacter xylosoxidans*	β-Proteobacteria	×	×
	Rup 5	*Curtobacterium citreum*	Actinobacteria	×	×
	Rup 6	*Microbacterium oleivorans*	Actinobacteria	×	×
	Rup 7	*Glutamicibacter nicotianae*	Actinobacteria	×	×
	Rup 8	*Microbacterium phyllosphaerae*	Actinobacteria	×	×
	Rup 9	*Rhizobium vallis*	α-Proteobacteria	×	×
	Rup 10	*Paenibacillus taichungensis*	Bacilli	×	×
	Rup 11	*Microbacterium paraoxydans*	Actinobacteria	×	×
	Rup 12	*Exiguobacterium indicum*	Bacilli	×	×
	Rup 13	*Glutamicibacter nicotianae*	Actinobacteria	×	×
	Rup 14	*Curtobacterium luteum*	Actinobacteria		
	Rup 15	*Glutamicibacter nicotianae*	Actinobacteria		
	Rup 16	*Curtobacterium citreum*	Actinobacteria		
	Rup 17	*Pseudarthrobacter niigatensis*	Actinobacteria		
	Rup 18	*Rhizobium pusense*	α-Proteobacteria		
	Rup 19	*Achromobacter xylosoxidans*	β-Proteobacteria		
	Rup 20	*Pseudarthrobacter niigatensis*	Actinobacteria	×	
	Rup 21	*Achromobacter xylosoxidans*	β-Proteobacteria		
	Rup 22	*Bacillus aryabhattai*	Bacilli	×	
	Rup 23	*Pantoea dispersa*	γ-Proteobacteria	×	
	Rup 24	*Pantoea dispersa*	γ-Proteobacteria		
	Rup 25	*Glutamicibacter nicotianae*	Actinobacteria		
	Rup 26	*Glutamicibacter nicotianae*	Actinobacteria		
	Rup 27	*Curtobacterium luteum*	Actinobacteria		
	Rup 28	*Microbacterium paraoxydans*	Actinobacteria		
	Rup 29	*Microbacterium hydrocarbonoxy*	Actinobacteria		
	Rup 30	*Microbacterium hydrocarbonoxy*	Actinobacteria		
	Rup 31	*Arthrobacter nicotianae*	Actinobacteria		
	Rup 32	*Arthrobacter nicotianae*	Actinobacteria		
	Rup 33	*Arthrobacter nicotianae*	Actinobacteria		
	Rup 34	*Microbacterium hydrocarbonoxy*	Actinobacteria		
	Rup 35	*Achromobacter xylosoxidans*	β-Proteobacteria		
	Rup 36	*Achromobacter xylosoxidans*	β-Proteobacteria		
	Rup 37	*Achromobacter xylosoxidans*	β-Proteobacteria		
Chitwan	Chi 1	*Pantoea dispersa*	γ-Proteobacteria	×	×
	Chi 2	*Paraburkholderia caribensis*	β-Proteobacteria	×	×
	Chi 3	*Neorhizobium alkalisoli*	α-Proteobacteria	×	×
	Chi 4	*Bacillus megaterium*	Bacilli	×	
	Chi 5	*Bacillus aryabhattai*	Bacilli		
	Chi 6	*Stenotrophomonas maltophilia*	γ-Proteobacteria	×	
	Chi 7	*Enterobacter cloacae*	γ-Proteobacteria	×	
	Chi 8	*Burkholderia vietnamiensis*	β-Proteobacteria		
	Chi 9	*Bacillus safensis*	Bacilli		
	Chi 10	*Burkholderia caribensis*	β-Proteobacteria		
	Chi 11	*Bacillus pumilus*	Bacilli		
	Chi 12	*Bacillus safensis*	Bacilli		
	Chi 13	*Bacillus safensis*	Bacilli		
	Chi 14	*Bacillus safensis*	Bacilli		
Sunsari-a	Sun-a 1	*Pseudomonas nitroreducens*	γ-Proteobacteria	×	×
	Sun-a 2	*Pseudomonas nitroreducens*	γ-Proteobacteria	×	×
	Sun-a 3	*Bacillus megaterium*	Bacilli	×	×
	Sun-a 4	*Pseudomonas nitroreducens*	γ-Proteobacteria	×	×

	Sun-a 5	*Pseudomonas nitroreducens*	γ-Proteobacteria		
	Sun-a 6	*Enterobacter asburiae*	γ-Proteobacteria	×	
	Sun-a 7	*Pseudomonas aeruginosa*	γ-Proteobacteria		
	Sun-a 8	*Enterobacter cloacae*	γ-Proteobacteria		
	Sun-a 9	*Bacillus safensis*	Bacilli		
	Sun-a 10	*Rhizobium etli*	α-Proteobacteria	×	
	Sun-a 11	*Bacillus megaterium*	Bacilli		
	Sun-a 12	*Pseudomonas nitritireducens*	γ-Proteobacteria		
	Sun-a 13	*Pseudomonas nitroreducens*	γ-Proteobacteria		
	Sun-a 14	*Bacillus megaterium*	Bacilli		
	Sun-a 15	*Bacillus megaterium*	Bacilli		
	Sun-a 16	*Bacillus megaterium*	Bacilli		
	Sun-a 17	*Bacillus megaterium*	Bacilli		
	Sun-a 18	*Bacillus megaterium*	Bacilli		
Sunsari-b	Sun-b 1	*Burkholderia cenocepacia*	β-Proteobacteria	×	×
	Sun-b 2	*Burkholderia cepacia*	β-Proteobacteria		
	Sun-b 3	*Burkholderia ambifaria*	β-Proteobacteria		
	Sun-b 4	*Burkholderia cenocepacia*	β-Proteobacteria		
	Sun-b 5	*Burkholderia cenocepacia*	β-Proteobacteria		
	Sun-b 6	*Bacillus safensis*	Bacilli		
	Sun-b 7	*Burkholderia cepacia*	β-Proteobacteria		
	Sun-b 8	*Burkholderia territorii*	β-Proteobacteria		

a: Strains used for the inoculation experiment,

b: Strains selected for characterizing their plant growth promoting and endophytic traits.

Supplementary Figures

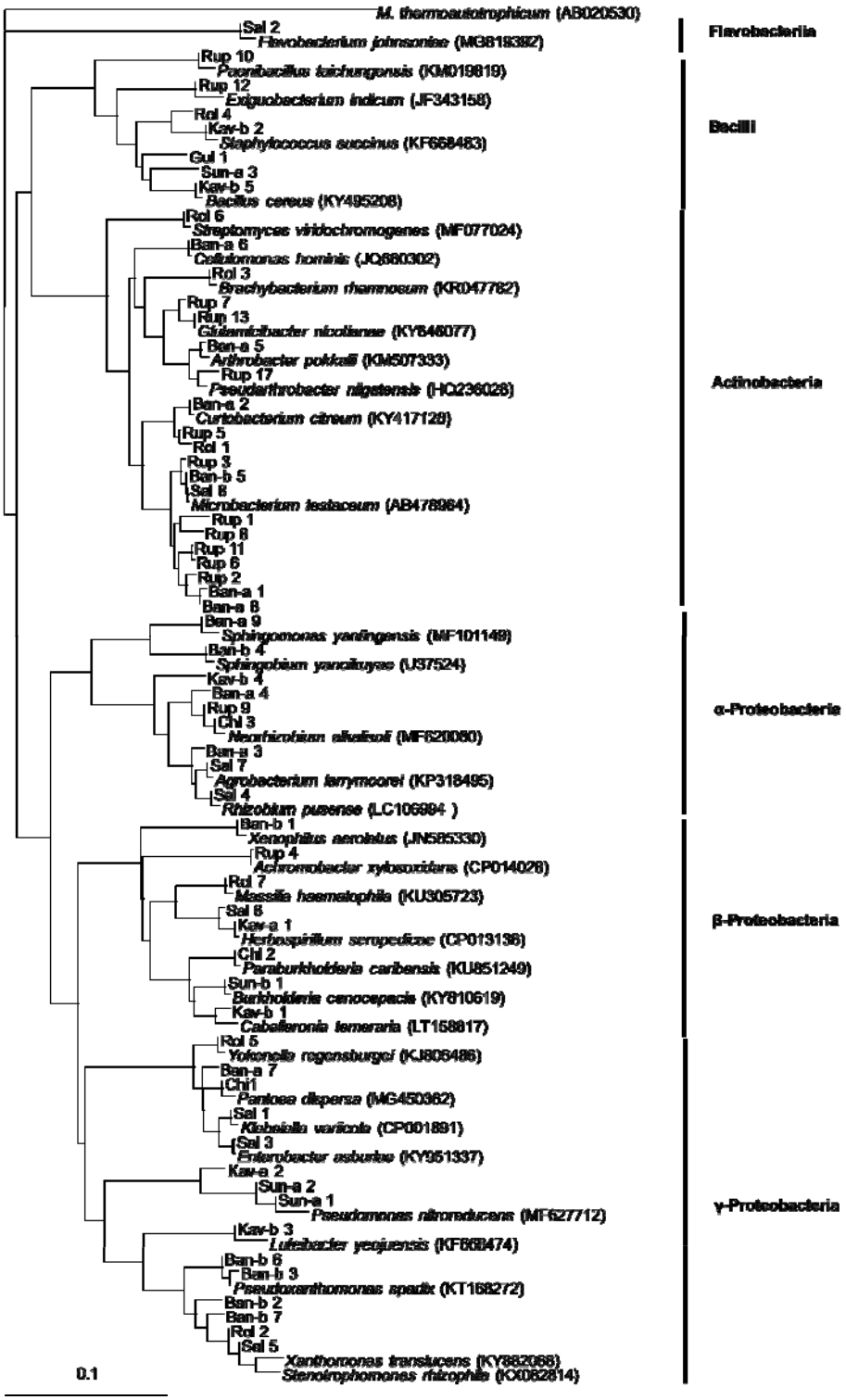


Figure S2.1 Phylogenetic relationship of 60 selected endophytic bacterial strains from Nepalese sweet potato based on partial 16S rRNA gene sequences.The sequence of *Methanobacterium thermoautotrophicum* (AB020530) served as an outgroup. Strain names are listed in Table S1. Strain names followed by accession numbers represent the sequences from database. The scale bar indicates the number of substitutions per site.

Chapter 3

Culture-Dependent Analysis of Endophytic Bacterial Community of Sweet potato (*Ipomoea batatas*) in Different Soils and Climates

3.1 Introduction

Endophytic bacteria are a class of microbes that resides within the interior tissues of plants without harming the host plants, and they have been isolated from a broad range of plants (Lodewyckx et al., 2002). Many endophytic bacteria have been reported to possess plant growth abilities, anti-plant pathogenic and phytoremediation abilities (Puri et al., 2018b; Feng et al., 2006; Ryan et al., 2008; Kandel et al., 2017). Therefore, understanding the effects of environmental conditions on the endophytic community is important to utilize their functions for developing sustainable systems of crop production.

Previous studies have analyzed endophytic bacterial community in sweet potato by the culture dependent method and revealed that the plant was colonized by diazotrophic *Pantoea agglomerans* and nondiazotrophic *Enterobacter asburiae* (Asis and Adachi, 2003). Similarly, Khan and Doty (2009) isolated 11 endophytes belonging to *Enterobacter, Rahnella, Rhodanobacter, Pseudomonas, Stenotrophomonas, Xanthomonas* and *Phyllobacterium* from sweet potatoes collected from grocery store. Likewise, (Puri et al., 2018b) isolated 243 endophytic bacteria belonging to 34 genera in six classes from 12 locations of Nepal.

Endophytic communities are reported to be influenced by several parameters, such as plant genotype (Marques et al., 2015), growth stage, physiological status and tissue of plant (Yang et al., 2017), as well as agricultural practices (Xia et al., 2015). In addition, climatic conditions are also considered as important factors for determining endophytic community. For example, psychrophilic bacterial endophytes were isolated abundantly in cold environments from the arcto-alpine plant species (Nissinen et al., 2012), which seemed to be the selection of psychrophile already adapted in the soil. In a previous study, we examine the diversity of sweet potato endophytes isolated in 12 locations of Nepal, and revealed that the endophytic communities were not related to the climatic conditions. However, it was unclear which factor was more responsible, the soil or the location, in determining the

bacterial endophytic community. In this study, we aimed to examine the effects of soil and climatic conditions on the endophytic bacterial communities of sweet potato by using the same soil at different locations and applying culture dependent approach.

3.2 Materials and Methods

3.2.1 Sweet potato cultivation

The experiment was conducted in Fukagawa (Fuk) in Hokkaido prefecture, Matsue (Mat) in Shimane prefecture and Miyazaki (Miy) in Miyazaki prefecture in Japan. Soils of Fukagawa, Matsue and Miyazaki were exchanged and used for cultivation of sweet potato. Briefly, the soils from 3 above mentioned locations were collected in sterile plastic bags and transported to the other locations, and the soils were immediately used for the experiment in the respective sites. The pots were placed in the open field, and placed on a wooden palette or a plastic sheet. Each one sweet potato cv. Beni Azuma slips, received from same nursery farm, were planted in a plastic pot (25 cm in diameter and 25 cm high) containing each soil sample, fertilized with chemical fertilizer Silicamap 555 (Central Kasei Co. Ube, Japan) containing N : P_2O_5 : K_2O=5:15:15 % at 6.6 g/ pot, and cultivated in triplicate, from June to September in 2017. After harvesting, the tubers were used for the isolation of endophytic bacteria. The precise location, climatic parameters and soil nutrients of the cultivation sites are presented in **Table 3.1**.

3.2.2 Culturable endophytic bacterial community

One tuber from each cultivation conditions, making a total of 9, was considered for culture dependent analysis. The tubers were washed in a running tap water for 10 min and then rinsed with sterilized distilled water. Then, cork-borer was perpendicularly inserted into the six different parts across the longitudinal axis of the tuber, each ca. 0.5 g making a total of ca. 3 g tuber samples. The samples were then placed in a sterilized mortar and macerated with 6 mL sterilized distilled water under aseptic conditions. Further, serial 10-fold dilutions were prepared up to 10^{-7}, and each 0.1 mL aliquot was taken and spread on modified MR media (Elbeltagy et al., 2001) supplemented with 0.1 g NH_4NO_3/L and incubated at 26^0C. Efficiency of the washing

was confirmed by stamping the surface of the washed tubers on the agar media, and a few culturable bacteria (colony forming unit) were expected on the surface of the macerated samples, which was considered to be negligible as the dilutions 10^{-4} to 10^{-6} were used for the endophytic bacterial community analysis (data not shown).

To isolate fast and slow growing bacteria, colonies were selected at two and ten days of cultivation, respectively. From both the groups, appeared colonies were pooled based on their morphologies and one representative colony of each morphology was purified for identification by analyzing the partial 16S rRNA gene sequences using universal primers fD1 and rP2 (W G Weisburg et al., 1991). Then a phylogenetic tree of bacterial genera was constructed using Clustal W (Thompson et al., 1994). Endophytic bacterial community was analyzed based on phylum/class and genus levels.

3.2.3 Nucleotide sequence accession numbers

The sequence data generated in this study were deposited in the DDBJ Nucleotide Submission System under the accession numbers LC430019 to LC430094.

3.2.4 Statistical Analysis

Tukey's test after one-way analysis of variance (ANOVA) was used to test the effect of the locations and the soils on the endophytic bacterial populations and compositions. ANOVA was performed by MINITAB (version 14.0).

3.3 Results

3.3.1 Isolation and identification of endophytic bacteria

Fast and slow growing endophytic bacterial isolates were detected from 9 sweet potato samples cultivated in different locations and soils **(Table S3.1)**. For fast growers, 3-9 morphologies in 27-80 colonies per plate, while 1-4 morphologies in 1-9 colonies per plate appeared in slow growers. Due to the smaller numbers of slow growing colonies in a plate, populations were calculated only for the fast growers. The bacterial populations were different among the locations regardless of the soils as the highest at Fukagawa location at 1.1-2.0 × 10^6, then Miyazaki at 8.1-18 × 10^4, and

Matsue location possessed the lowest at 1.7-2.4 × 10^4 CFU/g fresh weight (fw) **(Figure 3.1)**. The populations of Fukagawa location was significantly higher than Matsue and Miyazaki locations (P=0.001), and Matsue and Miyazaki locations were also significantly different (P=0.017) but not among the soils.

Based on the partial 16S rRNA gene sequence, 47 endophytes belonged to four bacterial phyla representing 25 genera. The endophytic compositions clearly showed that the phyla and genera differed among samples and shifted by changing the cultivating locations **(Table 3.2 & Figure 3.2)**.

Proteobacteria was the most dominant in 8 samples in Fukagawa (92-100%), Matsue (63-94%) and Miyazaki (56-63%) locations. For the Miyazaki location and Miyazaki soil sample, it was 10%. Compositions of Proteobacteria were dominated by only 1 or 2 classes in each sample. In Fukagawa location, γ-Proteobacteria (Fuk-Fuk, Fuk-Mat) or γ- and β-Proteobacteria (Fuk-Miy) dominated. In Matsue and Miyazaki locations, β-Proteobacteria (Mat-Fuk, Mat-Mat, Miy- Fuk) or α- and β-Proteobacteria (Mat-Miy, Miy-Mat) dominated. In the Miy-Miy sample, Actinobacteria dominated (88%) under the lower composition of Proteobacteria and this phylum was detected as second highest component in the other samples of Miyazaki location (23-26%) and 2 samples of Matsue location (11-23%). Phylum Firmicutes was detected in 7 samples as a minor component (2-18%). Bacteroidetes was detected only from Mat-Fuk sample, representing 14%.

The relative abundance of γ-Proteobacteria in Fukagawa location was significantly higher than those in Matsue and Miyazaki locations (*P*=0.003). The relative abundance of β-Proteobacteria and Actinobacteria were relatively higher in Matsue and Miyazaki locations, respectively, but the differences were not significant. The difference was also not significant among the soils.

3.3.2 Shift in composition of endophytic bacterial phyla

The endophytic bacterial compositions showed dominancy of specific bacterial phyla at the original sites but changed when the soils were used in the different locations (Figure 3.2).

For Fukagawa soil, the endophytic bacterial populations was dominated by γ-Proteobacteria (96%) in original Fukagawa location, but it reduced when used in

Matsue (13%) and Miyazaki (12%) locations, while β-Proteobacteria (50% and 44%) and Actinobacteria (23% and 26%) increased in Matsue and Miyazaki locations, respectively.

Similarly, Matsue soil was dominated by β-Proteobacteria (87%) in Matsue location, but it reduced when the soil was used in Fukagawa (8%) and Miyazaki (42%) locations, while γ-Proteobacteria dominated in Fukagawa location (92%), and Actinobacteria (23%) and α-Proteobacteria (21%) increased in Miyazaki location.

Finally, Miyazaki soil was dominated by Actinobacteria (88%) in Miyazaki location, while it was absent in Fukagawa and minor in Matsue (11%) locations, whereas β-Proteobacteria increased when Miyazaki soil was used in Fukagawa (46%) and Matsue (38%) locations. In addition γ- (46%) and α- (49%) Proteobacteria were dominant in Fukagawa and Matsue locations, respectively.

In summary, when the soil samples were used in different locations, γ-, β-Proteobacteria and Actinobacteria showed tendency to dominate in Fukagawa, Matsue and Miyazaki locations, respectively.

3.3.3 Shift in composition of endophytic bacterial genera

While the same phyla and class increased by changing the cultivating locations, the genera appeared were not the same among the samples **(Figure 3.3)**.

In γ-Proteobacteria, *Stenotrophomonas* (69%) and *Pseudomonas* (27%) were detected as major genera in tubers cultivated in Fuk-Fuk. When Miyazaki soil was used in Fukagawa location γ-Proteobacteria increased, and the main component was *Pseudomonas* (44%). On the other hand, in the case of Matsue soil, *Dyella* (58%) and *Pantoea* (34%) dominated **(Figure 3.3a)**.

β-Proteobacteria was dominant as *Variovorax* (31%), *Roseateles* (29%) and *Paraburkholderia* (25%) in Mat-Mat. When Matsue soil was used in Miyazaki location *Roseateles* (21%) was re-isolated but the other genera disappeared, and *Ralstonia* (21%) was newly detected. In Fukagawa and Miyazaki soils endophytic β-Proteobacteria were not detected and minor (10%) in each site, respectively. But, when Fukagawa soil was used in Matsue and Miyazaki locations, *Variovorax* (39%) and *Pelomonas* (11%), and *Acidovorax* (37%) appeared, respectively. Appearance of

different genus in different locations was also observed as *Mitsuaria* (38%) in Matsue and *Janthinobacterium* (46%) in Fukagawa when Miyazaki soil was used in each location **(Figure 3.3b)**.

Actinobacteria was dominant in tubers cultivated in Miy-Miy location, representing *Streptomyces* (38%) and *Microbacterium* (50%). When Fukagawa soil was used in Matsue and Miyazaki locations, *Curtobacterium* (23%) and *Microbacterium* (26%) were detected, respectively. *Paenarthrobacter* (23%) was detected when Matsue soil was used in Miyazaki location **(Figure 3.3c)**.

3.3.4 Phylogenetic relationships of endophytic bacterial genera

Although the quantitative information on the slow growing endophytes was less due to the small number of colonies on the plate and lower populations than the fast growers, whole community of the isolates was expressed in phylogenetic tree **(Figure 3.4)**.

β-, γ-Proteobacteria, Bacilli and Flavobacteriia consisted mainly of the fast growers while α-Proteobacteria of the slow growers, and the fast and slow growers were phylogenetically separated in Actinobacteria.

3.4 Discussion

To our knowledge, this is the first implementation of culture dependent approach to investigate the effects of environmental conditions on the endophytic bacterial community in sweet potato tubers cultivated in the different combinations of soil and location.

The sweet potato endophytic population was affected by the cultivating location rather than the soil, ranging from around 10^6 CFU/g fw to 10^5 and 10^4 CFU/g fw in Fukagawa, Miyazaki and Matsue locations, respectively **(Figure 3.1)**. Information on endophytic population and affecting factors is limited. Nissinen *et al.* (Nissinen et al., 2012) reported that the endophytic populations were 10^4-10^6 CFU/g fw in arcto-alpine plants depending on their species, and suggested that the plant type affected on the population. In our study, we expected that the unknown location-specific factors affected on the plant physiology, which could determine the

endophytic and/or rhizospheric population, a major source from which endophytic bacterial populations originate.

In the present study, some specific phyla, γ- and β-Proteobacteria, and Actinobacteria were dominantly isolated in sweet potato tubers collected from Fukagawa, Matsue and Miyazaki locations, respectively **(Figure 3.2)**. It was reported that endophytic bacteria generally belonged to Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes, among which γ-Proteobacteria was reported as the most dominant endophytes (Hardoim et al., 2015). This has also been the case for the endophytes of sweet potato, as reported by Khan and Doty (2009), Marques et al. (2015) and Puri et al. (2018b), and also in this study.

When the soil samples were used in the different locations, the above mentioned location-specific phyla increased in the new sites **(Figure 3.2)**. From these results, we assumed that the climatic conditions as, temperature and rainfall **(Table 1)** of the specific locations might influence the physiology of sweet potato plant. Then the physiological changes might effect on the internal plant environment and/or root exudates profile. The former would directly impact on the endophytic community and the later on the rhizospheric conditions, which influence on the rhizospheric community, the potential endophytes. It was reported that temperature influenced root exudates profile of tomato and clover (Rovira, 1959). In a previous study, it was unclear to specify which factor, the soil or the climate, was more important in determining the endophytic community (Puri et al., 2018b). In this study, by exchanging the soil samples among the different locations, it was suggested that the climatic conditions would determine the endophytic community. The mechanisms and the determining factors of the specific domination have been unclear and need to be investigated.

While the endophytic community is characterized by the location-specific phyla, dissimilar genera generally appeared among the samples **(Figure 3.3)**. It was reported that some microbial characteristics were phyla basis. For example, the soil Acidobacteria had a negative relationship with carbon concentration and were classified as oligotrophs, while β-Proteobacteria and Bacteroidetes had an opposite relationship and classified as copiotrophs (Fierer et al., 2007). In another example,

Kurm et al. (2017) reported that γ-Proteobacteria grew faster and used more substrates in high nutrient tryptone soy broth media, whereas α-Proteobacteria grew slowly and used fewer substrates among the soil bacterial isolates. In relation to the cultivation conditions, the relative abundances of Acidobacteria, Verrucomicrobia and Gemmatimonadetes decreased in the soil with N-fertilization (Cederlund et al., 2014). Thus, it was expected that these characteristics might be responsible for the phyla-specific endophytic community.

Culture-dependent methods have been used to characterize the endophytic bacterial community. However, the community is influenced mainly by the media conditions (Marques et al., 2015), and a limited number of populations are culturable (Amann et al., 1995; Torsvik et al., 1990; Oliver, 2010). Therefore, the use of culture-independent methods, such as next generation sequencing technologies using DNA extracted from the plant sample, are the possible options to provide additional information on the endophytic bacterial communities.

3.5 Conclusion

The bacterial phyla, γ-Proteobacteria, β-Proteobacteria and Actinobacteria, dominated in the sweet potato tubers cultivated in Fukagawa, Matsue and Miyazaki soils at the corresponding locations, respectively. When effects of the location-soil combinations were examined, the above mentioned location-specific phyla increased at respective sites regardless of the soils used, and the endophytic bacterial population was also affected by the locations. The results suggested that the cultivating locations were more important factor than the soils to determine the sweet potato endophytic bacterial community and population.

Tables

Table 3.1 Climate and soil nutrients of the sweet potato cultivation sites.

Location	Latitude (^{0}N)	Longitude (^{0}E)	Temperature (^{0}C)[a]		Rainfall (mm)[a]	Soil type[b]	Soil nutrients						
			Max	Min			NH_4-N (mg kg^{-1})	P_2O_5 (mg kg^{-1})	K_2O (mg kg^{-1})	Available P (mg kg^{-1})	Total C (g kg^{-1})	Total N (g kg^{-1})	pH (H_2O)
Fukagawa	43.71	142.01	23	13	501	Andisol	16	472	369	3.3	5.2	0.4	6.0
Matsue	35.48	133.06	29	21	611	Inceptisol	12	288	86	2.5	1.2	0.1	6.2
Miyazaki	31.82	131.41	30	23	1252	Andisol	22	160	220	2.2	4.4	0.3	6.4

[a] Average maximum and minimum monthly temperatures and total rainfall during the cultivation period (https://www.jma.go.jp),

[b] Based on USDA classification (Staff, 1999).

Table 3.2 Relative abundance of culturable sweet potato endophytic bacteria isolated from different location and soil conditions

Phyla/Genus	Location-Soil								
	Fuk-Fuk	Fuk-Mat	Fuk-Miy	Mat-Fuk	Mat-Mat	Mat-Miy	Miy-Fuk	Miy-Mat	Miy-Miy
Firmicutes	4		8		6	2	18	13	2
Bacillus sp.	4		8		6	2	11	13	2
Exiguobacterium sp.							7		
Actinobacteria				23		11	26	23	88
Streptomyces sp.						11			38
Microbacterium sp.							26		50
Curtobacterium sp.				23					
Paenarthrobacter sp.								23	
Bacteroidetes				14					
Chryseobacterium sp.				14					
Proteobacteria	96	100	92	63	94	87	56	63	10
α-Proteobacteria					4	49		21	
Sphingobium sp.					4	49			
Caulobacter sp.								21	
β-Proteobacteria		8	46	50	87	38	44	42	10
Variovorax sp.				39	31				
Roseateles sp.					29			21	4
Janthinobacterium sp.			46						
Mitsuaria sp.						38			
Acidovorax sp.							37		
Paraburkholderia sp.					25				
Pelomonas sp.				11			7		4
Ralstonia sp.								21	
Burkholderia sp.		8							
Herbaspirillum sp.					2				
Chitinimonas sp.									2
γ-Proteobacteria	96	92	46	13	3		12		
Stenotrophomonas sp.	69		2						
Pseudomonas sp.	27		44						
Dyella sp.		58							
Pantoea sp.		34			3		12		
Kosakonia sp.				13					

Figures

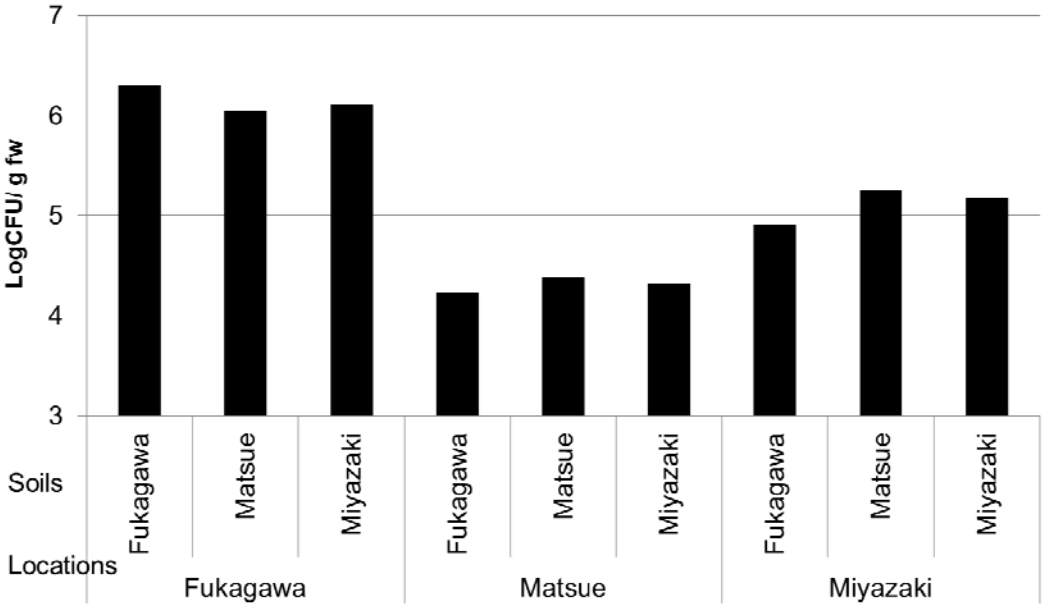


Figure 3.1 Endophytic bacterial populations in different locations and soils conditions.

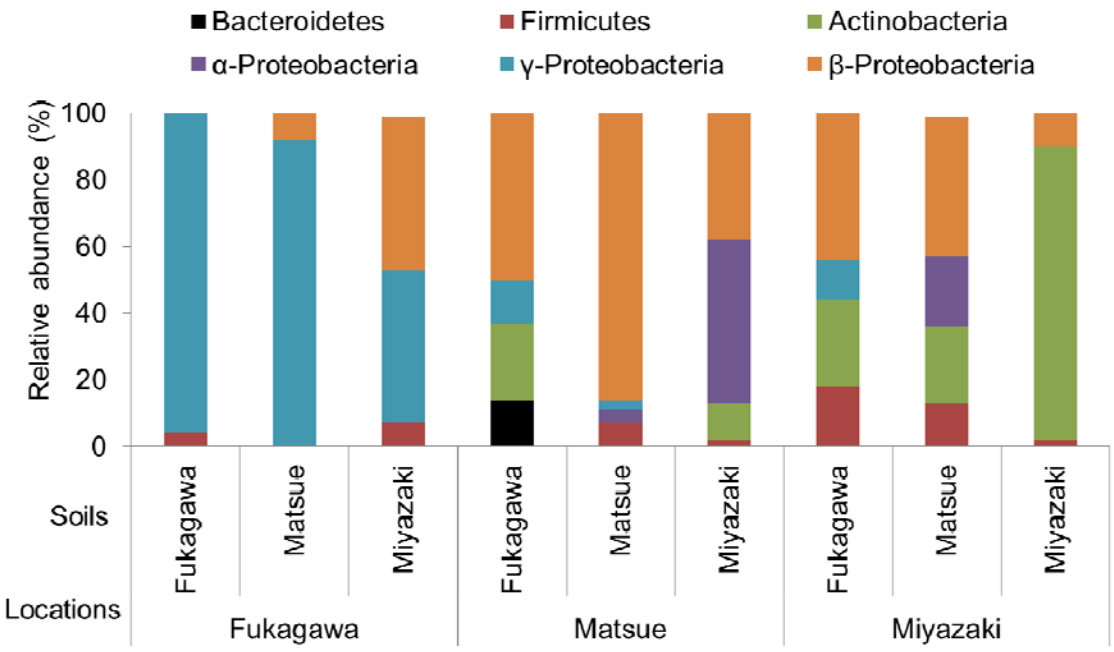


Figure 3.2 Sweet potato endophytic bacterial composition cultivated in different location and soil combinations.

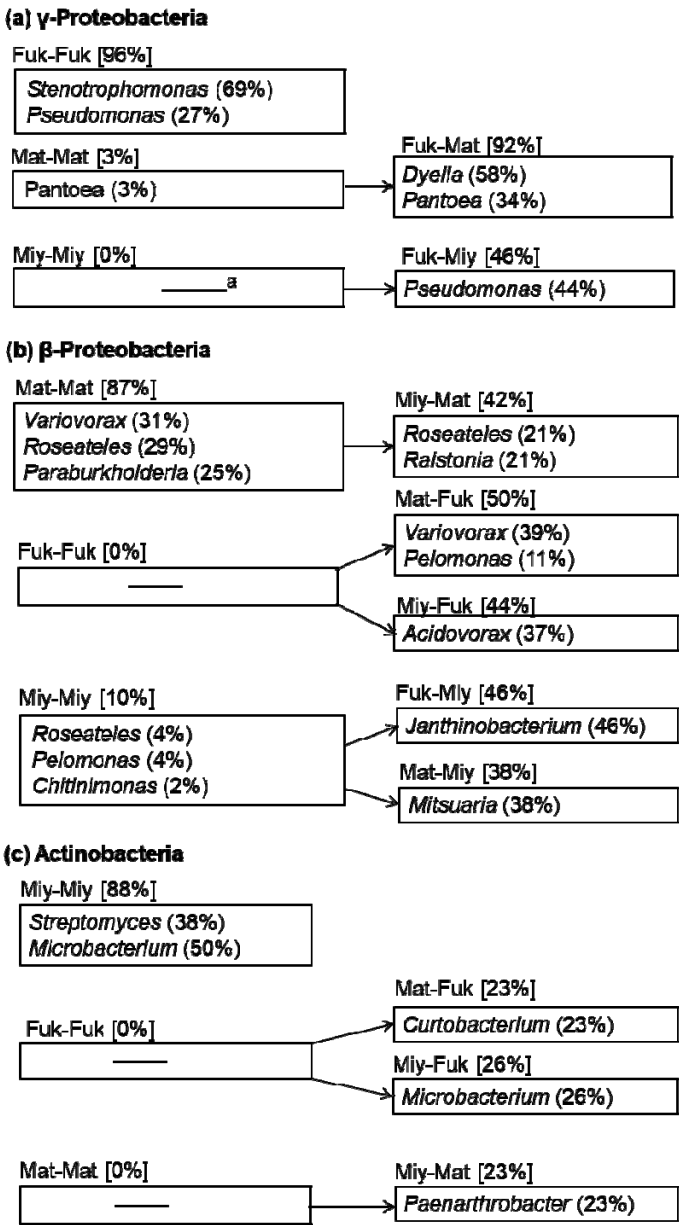


Figure 3.3 Shift in endophytic genera composition under different location-soil conditions. [ ] and ( ) indicate relative percentages of class and genera, respectively. [a] the bar indicates absence of corresponding isolates.

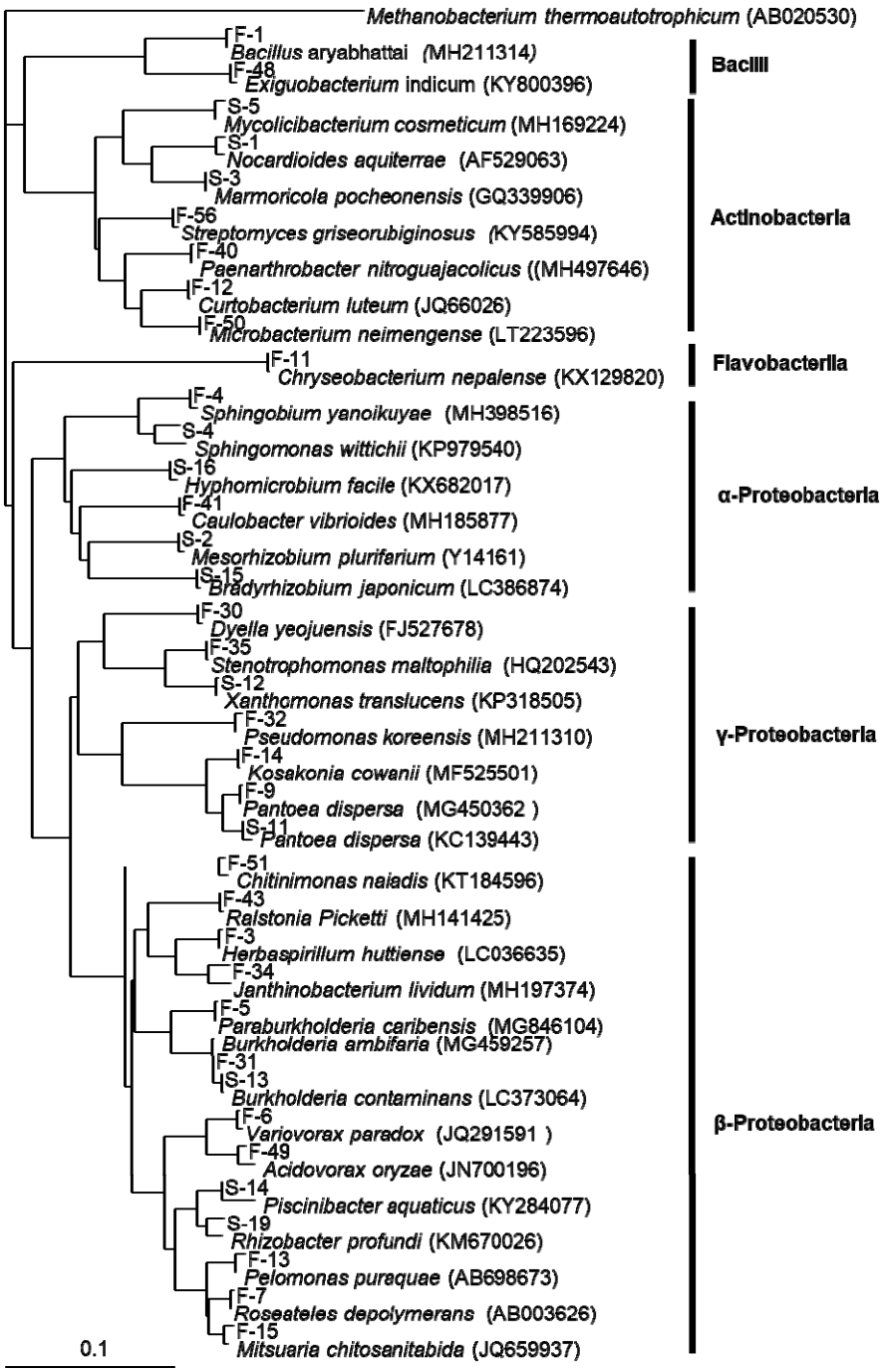


Figure 3.4 Phylogenetic relationship of the fast and slow growing sweet potato endophytic bacteria. The sequence of *Methanobacterium thermoautotrophicum* (AB020530) served as an outgroup. Strain names are listed in Table S3.1 and the name of the strains designated as F and S for the fast and slow growers, respectively. The scale bar indicates the number of substitutions per site.

Supplementary Tables

Table S3.1 Endophytic bacterial isolates identified from sweet potato cv. Beni Azuma tubers when soil samples were used in different locations in Japan.

Location	Soil	Strain	Growth	Closest candidate based on 16S rRNA gene sequencing	Accession	Identity (%)	Class
Fukagawa	Fukagawa	F-36	Fast	*Pseudomonas frederiksbergensis*	MG571721	100	γ-Proteobacteria
Fukagawa	Fukagawa	F-37	Fast	*Stenotrophomonas maltophilia*	X95924	100	γ-Proteobacteria
Fukagawa	Fukagawa	F-38	Fast	*Bacillus aryabhattai*	MH211282	100	Bacilli
Fukagawa	Fukagawa	F-39	Fast	*Bacillus mycoides*	MH169305	99	Bacilli
Fukagawa	Fukagawa	S-13	Slow	*Burkholderia contaminans*	LC373064	100	β-Proteobacteria
Fukagawa	Matsue	F-29	Fast	*Pantoea rodasii*	MG571723	99	γ-Proteobacteria
Fukagawa	Matsue	F-30	Fast	*Dyella yeojuensis*	FJ527678	99	γ-Proteobacteria
Fukagawa	Matsue	F-31	Fast	*Burkholderia ambifaria*	MG459257	99	β-Proteobacteria
Fukagawa	Matsue	S-11	Slow	*Pantoea dispersa*	KC139443	100	γ-Proteobacteria
Fukagawa	Miyazaki	F-32	Fast	*Pseudomonas koreensis*	MH211310	100	γ-Proteobacteria
Fukagawa	Miyazaki	F-33	Fast	*Bacillus megaterium*	FJ613537	99	Bacilli
Fukagawa	Miyazaki	F-34	Fast	*Janthinobacterium lividum*	MH197374	97	β-Proteobacteria
Fukagawa	Miyazaki	F-35	Fast	*Stenotrophomonas maltophilia*	HQ202543	100	γ-Proteobacteria
Fukagawa	Miyazaki	S-12	Slow	*Xanthomonas melonis*	KP318505	100	γ-Proteobacteria
Matsue	Fukagawa	F-10	Fast	*Variovorax paradoxus*	JQ291591	99	β-Proteobacteria
Matsue	Fukagawa	F-11	Fast	*Chryseobacterium nepalense*	KX129820	100	Flavobacteriia
Matsue	Fukagawa	F-12	Fast	*Curtobacterium luteum*	JQ660269	100	Actinobacteria
Matsue	Fukagawa	F-13	Fast	*Pelomonas puraquae*	AB698673	99	β-Proteobacteria
Matsue	Fukagawa	F-14	Fast	*Kosakonia cowanii*	MF525501	100	γ-Proteobacteria
Matsue	Fukagawa	S-2	Slow	*Mesorhizobium plurifarium*	Y14161	100	α-Proteobacteria
Matsue	Fukagawa	S-3	Slow	*Marmoricola pocheonensis*	GQ339906	100	Actinobacteria

Matsue	Matsue	F-1	Fast	*Bacillus aryabhattai*	MH211314	100	Bacilli
Matsue	Matsue	F-2	Fast	*Bacillus aryabhattai*	MH211314	100	Bacilli
Matsue	Matsue	F-3	Fast	*Herbaspirillum huttiense*	LC036635	100	β-Proteobacteria
Matsue	Matsue	F-4	Fast	*Sphingobium yanoikuyae*	MH398516	100	α-Proteobacteria
Matsue	Matsue	F-5	Fast	*Paraburkholderia caribensis*	MG846104	100	β-Proteobacteria
Matsue	Matsue	F-6	Fast	*Variovorax paradoxus*	JQ291591	99	β-Proteobacteria
Matsue	Matsue	F-7	Fast	*Roseateles depolymerans*	AB003626	100	β-Proteobacteria
Matsue	Matsue	F-8	Fast	*Roseateles depolymerans*	AB003626	100	β-Proteobacteria
Matsue	Matsue	F-9	Fast	*Pantoea dispersa*	MG450362	100	γ-Proteobacteria
Matsue	Matsue	S-1	Slow	*Nocardioides aquiterrae*	AF529063	99	Actinobacteria
Matsue	Miyazaki	F-15	Fast	*Mitsuaria chitosanitabida*	JQ659937	99	β-Proteobacteria
Matsue	Miyazaki	F-16	Fast	*Streptomyces turgidiscabies*	KT363057	100	Actinobacteria
Matsue	Miyazaki	F-17	Fast	*Bacillus aryabhattai*	MH538124	100	Bacilli
Matsue	Miyazaki	F-18	Fast	*Mitsuaria chitosanitabida*	JQ659937	99	β-Proteobacteria
Matsue	Miyazaki	F-19	Fast	*Sphingobium yanoikuyae*	MH211258	100	α-Proteobacteria
Matsue	Miyazaki	S-4	Slow	*Sphingomonas wittichii*	KP979540	96	α-Proteobacteria
Matsue	Miyazaki	S-5	Slow	*Mycobacterium cosmeticum*	MH169224	99	Actinobacteria
Miyazaki	Fukagawa	F-45	Fast	*Bacillus aryabhattai*	MH211314	100	Bacilli
Miyazaki	Fukagawa	F-46	Fast	*Pantoea agglomerans*	EU360112	99	γ-Proteobacteria
Miyazaki	Fukagawa	F-47	Fast	*Pelomonas puraquae*	AB698673	99	β-Proteobacteria
Miyazaki	Fukagawa	F-48	Fast	*Exiguobacterium indicum*	KY800396	99	Bacilli
Miyazaki	Fukagawa	F-49	Fast	*Acidovorax oryzae*	JN700196	98	β-Proteobacteria
Miyazaki	Fukagawa	F-50	Fast	*Microbacterium neimengense*	LT223596	99	Actinobacteria
Miyazaki	Fukagawa	S-18	Slow	*Piscinibacter aquaticus*	KY284087	98	β-Proteobacteria
Miyazaki	Matsue	F-40	Fast	*Paenarthrobacter nitroguajacolicus*	MH497646	100	Actinobacteria
Miyazaki	Matsue	F-41	Fast	*Caulobacter vibrioides*	MH185877	100	α-Proteobacteria
Miyazaki	Matsue	F-42	Fast	*Roseateles depolymeran*	CP013729	99	β-Proteobacteria

Miyazaki	Matsue	F-43	Fast	*Ralstonia pickettii*	MH141425	100	β-Proteobacteria
Miyazaki	Matsue	F-44	Fast	*Bacillus aryabhattai*	MH538124	99	Bacilli
Miyazaki	Matsue	S-14	Slow	*Piscinibacter aquaticus*	KY284077	98	β-Proteobacteria
Miyazaki	Matsue	S-15	Slow	*Bradyrhizobium japonicum*	LC386874	100	α-Proteobacteria
Miyazaki	Matsue	S-16	Slow	*Hyphomicrobium facile*	KX682017	100	α-Proteobacteria
Miyazaki	Matsue	S-17	Slow	*Rhizobium alamii*	KU305699	100	α-Proteobacteria
Miyazaki	Miyazaki	F-51	Fast	*Chitinimonas naiadis*	KT184596	98	β-Proteobacteria
Miyazaki	Miyazaki	F-52	Fast	*Pelomonas puraquae*	AB698673	99	β-Proteobacteria
Miyazaki	Miyazaki	F-53	Fast	*Roseateles depolymerans*	CP013729	100	β-Proteobacteria
Miyazaki	Miyazaki	F-54	Fast	*Bacillus drentensis*	KT719469	100	Bacilli
Miyazaki	Miyazaki	F-55	Fast	*Microbacterium binotii*	JQ659823	100	Actinobacteria
Miyazaki	Miyazaki	F-56	Fast	*Streptomyces phaeopurpureus*	KY585994	100	Actinobacteria
Miyazaki	Miyazaki	S-19	Slow	*Rhizobacter profundi*	KM670026	97	β-Proteobacteria
Miyazaki	Miyazaki	S-20	Slow	*Piscinibacter aquaticus*	KY284087	98	β-Proteobacteria

Chapter 4

Metagenomic study of endophytic bacterial community of sweet potato (*Ipomoea batatas*) cultivated in different soil and climatic conditions

4.1 Introduction

Endophytic bacteria inhabit most plant species and have been reported to possess plant growth promoting abilities (Ryan et al., 2008). Culture-dependent methods have been used to isolate endophytic bacteria however the isolation processes are influenced by cultivation media conditions (Marques et al., 2015), and a limited number of populations are culturable (Amann et al., 1995). Additionally, some of the naturally occurring bacteria remains in a nonculturable state (Torsvik et al., 1990; Oliver, 2010). Therefore, the use of culture-independent methods is an option to provide additional information on the diversity of bacterial communities (Yang et al., 2017; Pei et al., 2017; Shi et al., 2014; Jackson et al., 2013).

Despite the advantages of culture-independent molecular techniques, there is a major limitation when investigating the community structures of plant-associated bacteria because of the presence of plant organelle (mitochondria and plastid) (Garbeva et al., 2001). In PCR, primer sets for bacterial rRNA gene also amplify the organelle rRNA genes, resulting in abundant PCR products derived from the plant organelle. To inhibit the amplification of organelle genes, the locked nucleic acid (LNA) oligonucleotide-PCR clamping technique has been developed as an effective approach for the selective amplification of endophytic bacterial genes from plant samples (Ikenaga and Sakai, 2014).

The endophytic bacterial communities are influenced by several factors, such as plant genotype (Marques et al., 2015), growing season (Shen and Fulthorpe, 2015), physiological status and tissue of plant (Yang et al., 2017) as well as agricultural practices (Xia et al., 2015). In our previous study, the diversity of sweet potato endophytes isolated in 12 locations of Nepal revealed that the endophytic communities were not related to the climatic conditions (Puri et al., 2018b). However, it was unclear which factor was more responsible, the soil or the location, in

determining the bacterial endophytic community. To address this issue, we examined the effects of soil and climatic conditions on the culturable endophytic bacterial communities of sweet potato by exchanging the soils among the locations and revealed that the location rather than the soil influenced on the endophytic community and population (Puri et al., 2018a). However, the effects of the soils and the locations on the entire endophytic community have not been clearly understood. In this study, we aimed to clarify the influencing factor, the soil or the location, on the endophytic community by analyzing the DNA extracted from sweet potato tubers, including the tubers used in the culture-dependent analysis, using LNA-PCR clamping technique and metagenomic sequencing.

4.2 Materials and Methods

4.2.1 Sweet potato cultivation

The experimental procedures were described in our previous study (Puri et al., 2018a). Briefly, Sweet potatoes were cultivated in triplicate, from June to September, 2017 in Fukagawa (Fuk) in Hokkaido prefecture, Matsue (Mat)/Shimane prefecture and Miyazaki (Miy)/Miyazaki prefecture in Japan. Soils of Fukagawa, Matsue and Miyazaki were exchanged and used for cultivation of sweet potato. Each one sweet potato cv. Beni Azuma slips, received from same nursery farm, were planted in a plastic pot (25 cm in diameter and 25 cm high) containing each soil sample and fertilized as reported. The tubers from all the pots were harvested and stored at -20^{0}C until DNA extraction. The precise location, climatic parameters and soil nutrients of the cultivation sites were also presented previously.

4.2.2 DNA extraction from sweet potato tubers

Each ca. 5 g of frozen sweet potato tuber was taken from each pot, and macerated to a fine powder with a sterile mortar and pestle in liquid nitrogen, and then total DNA was extracted using ISOPLANT II plant DNA extraction kit (Nippon Gene Co., Ltd., Japan) according to the manufacturer's instruction. The extracted

DNA was applied to selective amplification of bacterial DNA using LNA-PCR clamping technique (Ikenaga and Sakai, 2014).

4.2.3 LNA-PCR for endophytic bacteria

As any mitochondrial 16S rRNA gene sequence for sweet potato was not available in the data base, the genes were amplified using fD1 and modified 1525r primers for the sweet potato tubers (Japan cv. Koukei and Nepal cv. C6). In addition, plastid DNA of the sweet potato tubers was also amplified using fD1 and rP2 primers. Then DNA sequences of the fragments were determined using internal primers: 338f, 926f, 518r, r1L, 907r and r3L. Details of the primers are presented in **Table S4.1**.

The sweet potato mitochondria and plastid DNA sequences examined were identical to those of *Poaceae* plants mitochondria (Ikenaga and Sakai, 2014) and group 63b/1492b plastid sequences (Ikenaga et al., 2015), respectively. Therefore, the LNA oligonucleotides were designed according to Ikenaga et al. (2015) and Ikenaga and Sakai (2014). The 3'-end of the LNA oligonucleotides were phosphorylated to avoid extension during PCR. The conditions were 94^0C for 3 min (initial denaturation), followed by 30 cycles at 94^0C for 1 min, 70^0C for 1 min (annealing for LNA), 54^0C for 1 min (annealing for primers) and 72^0C for 2 min (extension) with a final extension step at 72^0C for 10 min. DNA extracted from sweet potato root was used as template. The PCR mixture contained 0.25 µL of 10mM dNTPs, 1 µL of 10x Reaction buffer, 0.5 µL of *Taq* DNA polymerase (GENETBIO Inc., Daejeon, South Korea) and 0.4 µL of bacterial primers modified 63f and 1492r (12.5 pmol μL^{-1}), and LNA primers (12.5 pmol μL^{-1}). Milli Q water was added to a total volume of 10 µL. The PCR mixtures were prepared to contain serial concentrations of LNA oligonucleotides at 0, 0.5, 1, 2, 3 and 4 µM. The amplified PCR products were sequenced to confirm the amplification of bacterial DNA.

To detect the possible interference of LNA oligonucleotides against the amplification of bacterial DNA, the endophytic bacterial 16S rRNA gene was amplified with and without LNA at 0.5 µL, based on the above mentioned experiment.

4.2.4 16S rRNA amplification and MiSeq sequencing

The LNA PCR products were applied for metagenomic analysis and the 16S rRNA gene amplicon libraries were prepared following the “16S Metagenomic Sequencing Library Preparation Protocol” (Illumina) using the Nextera XT index kit (Amplicon et al., 2013).

The samples were prepared by amplifying V3 and V4 regions of the 16S rRNA gene using the locus specific primers 341f and 785r with the Illumina overhang adapter attached at 3’-end. Each PCR reaction contained DNA template (~10–12 ng), 2.5 µL primers (1 µM), 6.25 µl 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, cat no. KK2601) and Milli Q water to a final volume of 25 µL. PCR amplification was carried out as follows: 95°C 3 min, 25 cycles of 95°C 30 s, 55°C 30 s and 72°C 30 s, then 72°C 5 min. The PCR products were visualised using gel electrophoresis followed by purification using Agencourt Ampure XP magnetic beads (Beckman Coulter, USA).

The second PCR was performed using the purified PCR products as template and index primers with unique barcode sequences for Illumina MiSeq sequencing. Each PCR reaction contained template DNA (~1-2 ng), 5 µL of index primers, 25 µL 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, cat no. KK2601) and Milli Q water to 50 µl volume. The PCR protocol was as described above, except that the number of cycles was eight. PCR products were visualised using gel electrophoresis and subsequently purified as the first PCR. Concentrations of the purified samples were adjusted and sequenced on the MiSeq sequencing platform in the Hokkaido System Science Co., Ltd. using MiSeq Reagent Kit v3 with 2 x 301 cycles.

In bioinformatic analysis of the sequences, the adapter sequences and low quality regions were trimmed by Cutadapt-1.1 and Trimmomatic-0.32, respectively. Paired-end sequences reads were assembled using the script fastq-join-1.1.2-537. Further data processing were performed using the open-source software pipeline “Quantitative Insights into Microbial Ecology” (QIIME) version 1.8.0. Reads were trimmed of primers and sequence quality control was performed using QIIME’s script (sequences length 200-1000 nucleotides; minimum average quality score 25; maximum length of homopolymer runs 8). Operational taxonomic units (OTUs) were

performed at 97% similarity using UCLUST (*denovo* OTU picking). The phylogenetic assignment of representative sequences from each OTU was carried out with a 16S reference data set obtained from Greengenes version 13_8 and chimeric sequences were removed using ChimerSlayer.

4.2.5 Nucleotide sequence accession numbers

The sweet potato plastids and mitochondrial sequences generated in this study were deposited in the DDBJ Nucleotide Submission System under the accession numbers LC431503 to LC431505 and LC431504 to LC431506, respectively, and the metagenomic data in the DDBJ Sequence Read Archive under the accession numbers DRA007544-007546 and DRA007549-007554.

4.2.6 Statistical analysis

Tukey's test after one-way analysis of variance (ANOVA) and PCA were used to test the effect of the locations and the soils on the endophytic bacterial diversity and compositions, respectively. Shannon diversity index were calculated by QIIME.

4.3 Results

4.3.1 Design of LNA oligonucleotides and their effective concentrations

As the organelle rRNA gene sequences of sweet potato were identical to those of plants mitochondria (Ikenaga and Sakai, 2014) and plastid (Ikenaga et al., 2015) sequences, the LNA oligonucleotides were designed according to (Ikenaga et al., 2015) (Ikenaga and Sakai, 2014) **(Table S4.2).**

The length of mitochondria and plastid PCR products of sweet potato were 1823 bp and 1375 bp based on our study, respectively, while bacterial genes generate different sizes ca. 1470 bp as amplified by the modified 63f and 1492 primer set. Then, the PCR products from bacterial and plant rRNA genes could be visually distinguished by agarose gel electrophoresis. Mitochondria and plastid DNA bands were dominantly observed in the products amplified without LNA oligonucelotides. In contrast, these bands were not observed at 0.5 to 4 μM while bacterial products were

observed **(Figure 4.1)**. When the PCR products were sequenced using modified 63f primer, the products were a mixture of bacterial DNA based on BLAST search (data not shown). **Figure 4.2** shows that the bacterial PCR products were amplified with and without LNA oligonucleotides with the same band intensities, suggesting no interference for the bacterial DNA amplification.

4.3.2 Analysis of metagenomic data

After the removal of plastid, mitochondria and undefined sequences, the averaged numbers of retained sequences, mean sequence length and OTUs were 20891, 458 and 846, respectively, across all 26 samples **(Table S4.3)**. The plant of Mat-Fuk (3) was dead during the cultivation and due to the lower numbers of sequences of Fuk-Miy (3) and Miy-Miy (1), they were not considered in further analysis. The rarefaction curves tended to approach the saturation plateau and indicated that the libraries could reflect the main bacterial compositions in the samples **(Figure S4.1)**. The diversity was significantly lower in Fukagawa location as compared to Matsue (P=0.044) and Miyazaki (P=0.009) **(Figure 4.3)**. On the other hand, the differences were not significant among the soils.

4.3.3 Unculturable endophytic bacterial composition

The sequences were classified into 19 different phyla, 51 classes, 77 orders, 118 families, and 302 genera. The overall endophytic bacterial compositions of the different samples were similar and there was no distinct difference among the soils and locations. The shift of phyla was not observed at different locations **(Figure 4.4; Figure S4.2)**.

Proteobacteria (85.0%), Bacteroidetes (6.6%) and Actinobacteria (6.3%) were the three most dominant phyla, accounting for 97.9% of the total reads. Among them γ-Proteobacteria (66.3%) was the most abundant, followed by β-Proteobacteria (10.7%) and α-Proteobacteria (8.0%) **(Table S4.4)**.

The overall distribution of endophytic genera was also similar among the sweet potato tuber samples, and top 10 genera represented 81.2% of the overall reads in

which *Pseudomonas* (36.7%)*, Enterobacteriaceae; g_* (11.9%)*, Erwinia* (7.9%) and *Burkholderia* (6.9%) contributed 63.3 % **(Table 4.1)**.

To further elucidate the influence of the soils and the locations on the composition of genera, PCA was conducted considering the relative abundance of the endophytic genera in **Table 4.1**. The first and second component factors explained 35.0% and 24.6% of the variation, respectively **(Figure 5)**, and the samples of Matsue and Miyazaki locations positioned closely except Miy-Mat which positioned close to the Matsue cluster, while the samples of Fukagawa location were distantly positioned.

4.4 Discussion

To our knowledge, this is the first implementation of LNA-PCR based metagenomic approaches to investigate the effects of environmental conditions on the endophytic bacterial community in sweet potato tubers cultivated in the different combinations of soil and location.

The overall endophytic bacterial communities were similar among the samples which indicated that the soil and the location conditions did not considerably affect the entire endophytic community. As sweet potato cv. Beni Azuma slips from the same nursery were used, we suspected that the original endophytic bacterial community might be kept during the cultivation period. On the other hand, the culture-dependent endophytic bacterial communities were visibly affected by the locations (Puri et al., 2018a). These results suggested that there might be two distinct culture-dependent and independent endophytic communities, where the former group was affected by the cultivation conditions and the latter group was maintained throughout the cultivation period. As the change in the culture-dependent community did not effect on the whole community composition, it was suggested that the culturable population might be small within the whole community. Although the overall culture-independent communities were similar, there was a tendency that the Shannon diversity and the endophytic compositions were also affected by the location rather than the soil as in the culturable community.

In addition, the previous culture-independent studies showed that endophytes were dominated by only a few groups, as Enterobacteriales (53.5%) in sugarbeet (Shi et al., 2014), *Pseudomonas* (34%) in *Populus deltoids* (Gottel et al., 2011), and *Pseudomonas* (52%) and *Enterobacter* (36%) in sugarcane stems (Magnani et al., 2013), which is congruent to our study.

In this study, *Pseudomonas* was the most prevalent genera in all the samples (31.9-45.0%), however it was isolated only from two samples Fuk-Fuk and Fuk-Miy with relative abundancy of 27% and 44%, respectively (Puri et al., 2018a). Failure to isolate *Pseudomonas* from rest of the samples might be due to, in some parts, the presence of antibiotics-producing *Bacillus* in the culture plates (Pereira et al., 2011) that might possibly inhibit the growth of other bacteria in the plates. In addition, we suspected that the only culturable *Pseudomonas* strains detected while other strains of the same genus were unculturable.

Pseudomonas strains were found as endophytes in several plants as sweet potato (Khan and Doty, 2009; Marques et al., 2015; Puri et al., 2018b), maize (Pereira et al., 2011), poplar (Taghavi et al., 2009), tomato (Shi et al., 2014) (Tian et al., 2017) and potato (Andreote et al., 2009). They are known as plant growth-promoting bacteria (PGPB), for example, by producing indole-3-acetic acid (IAA) (Moronta-Barrios et al., 2018; Taghavi et al., 2009), releasing 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Gaiero et al., 2013), providing cold tolerance to plants (Subramanian et al., 2015), solubilizing inorganic phosphate (Oteino et al., 2015), fixing nitrogen (Rediers et al., 2003) and producing antipathogenic compounds (Duffy et al., 1999; Miller et al., 1998) and so on. If the predominant *Pseudomonas* strains detected in our study have above-mentioned plant growth-promoting properties, their applications would be an advantage for the sustainable and ecofriendly agricultural production systems. Development and modification of the culture conditions is important to isolate the uncultured ones.

Further study on why some specific group of endophytes dominate in sweet potato, and why the overall endophytic bacterial compositions were similar in this study as compared to culture-dependent study under different soil and climate conditions need to be explored.

4.5 Conclusion

Proteobacteria, Bacteroidetes and Actinobacteria were the three most dominant phyla, and γ-Proteobacteria being the most abundant. The overall endophytic communities were similar among the samples and top 10 genera represented 81.2% of the overall reads in which *Pseudomonas* being the most predominant. Principal component analysis and Shannon diversity indices showed a tendency that the location was more important than the soil to determine the sweet potato endophytic bacterial community.

Tables

Table 4.1 Average relative abundance of top 20 genera in the endophytic community.

Phylum/class	Genus	% reads	Samples[a]								
			Fuk-Fuk	Fuk-Mat	Fuk-Miy	Mat-Fuk	Mat-Mat	Mat-Miy	Miy-Fuk	Miy-Mat	Miy-Miy
γ-Proteobacteria	*Pseudomonas*	36.7	40.0	37.2	45.0	31.9	34.7	33.1	38.1	35.1	37.1
	*Enterobacteriaceae;*g_	11.9	8.0	10.6	10.9	14.4	16.8	14.0	8.8	13.1	8.1
	Erwinia	7.9	8.2	12.9	7.4	8.8	6.3	6.9	7.0	6.3	6.8
	Stenotrophomonas	2.6	4.4	1.7	1.9	2.2	2.2	2.5	3.0	2.3	3.0
	Xanthomonas	2.0	3.3	1.5	1.4	1.8	1.7	1.8	2.1	1.6	2.3
	Enterobacter	1.9	1.1	1.3	1.4	4.5	2.1	3.4	1.2	1.4	1.1
	Klebsiella	1.5	0.2	0.5	1.0	1.0	5.1	2.1	0.4	1.7	0.3
	Others	1.8	1.0	2.4	1.2	2.1	2.5	2.5	1.2	1.6	1.2
β-Proteobacteria	*Burkholderia*	6.9	6.1	11.1	6.7	5.8	6.4	6.2	7.6	5.8	7.4
	Janthinobacterium	1.5	3.2	1.2	0.9	1.1	1.2	1.2	1.6	1.3	1.5
	*Oxalobacteraceae;*g_	1.0	1.0	1.6	1.0	1.2	0.9	0.8	0.9	0.8	1.0
	Others	1.3	1.4	1.2	1.0	1.4	1.2	1.1	1.3	1.5	1.4
α-Proteobacteria	*Rhizobium*	3.9	4.1	3.5	4.3	3.7	3.5	3.7	4.6	3.5	4.9
	Agrobacterium	1.6	1.4	1.6	1.5	2.3	1.4	1.7	1.6	1.5	1.7
	Novosphingobium	1.2	2.5	1.1	0.6	0.9	1.1	0.8	1.2	0.9	1.2
	Others	1.4	1.0	1.2	1.1	2.7	1.0	1.4	1.2	1.6	1.3
Bacteroidetes	*Sphingobacteriaceae;*g_	3.3	3.3	2.2	2.7	3.1	2.9	3.2	4.5	3.3	5.3
	Chryseobacterium	2.4	2.1	1.8	2.0	2.6	2.3	2.8	3.1	2.4	3.3
	Pedobacter	0.7	0.8	0.5	0.5	0.6	0.6	0.6	1.0	0.7	1.0
	Others	0.2	0.0	0.1	0.0	0.3	0.2	0.4	0.1	0.1	0.1
Actinobacteria	*Streptomyces*	3.6	3.4	2.3	3.7	3.5	3.0	3.5	4.9	4.0	5.0
	*Micrococcaceae;*g_	1.4	1.2	1.0	1.4	1.1	1.1	1.3	1.6	2.0	1.8
	Other;g_	0.9	0.8	0.6	0.8	0.7	0.7	0.9	1.2	0.9	1.2
	Others	0.5	0.4	0.3	0.4	0.7	0.4	0.4	0.5	0.6	0.6
Firmicutes	*Bacillus*	1.8	0.7	0.5	0.8	1.0	0.8	3.5	1.1	5.4	1.1
	Others	0.1	0.1	0.1	0.1	0.3	0.1	0.1	0.1	0.2	0.1

[a] Samples are denoted as location-soil.

‘g_’ represents genus from respective families which are not similar to the sequences in database.

‘other:g_’ represents families and genera in Actinobacteria phyla, that were not similar to database sequence.

Figures

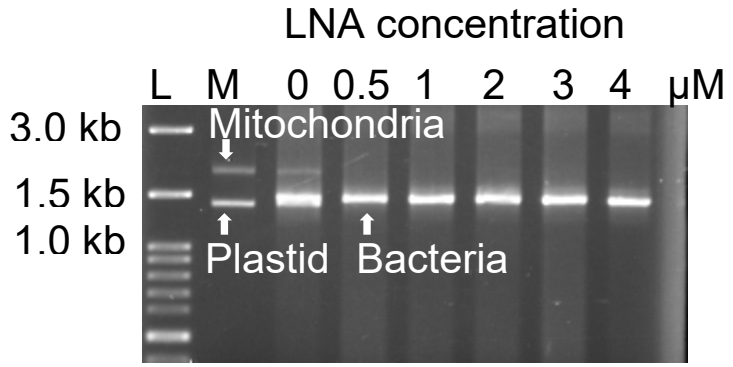


Figure 4.1 Agarose gel electrophoresis of LNA-PCR products derived from the sweet potato root DNA at different concentrations of LNA oligonucleotides. L; the marker of 100 bp ladders, M; mitochondria and plastid PCR products prepared from sweet potato root DNA.

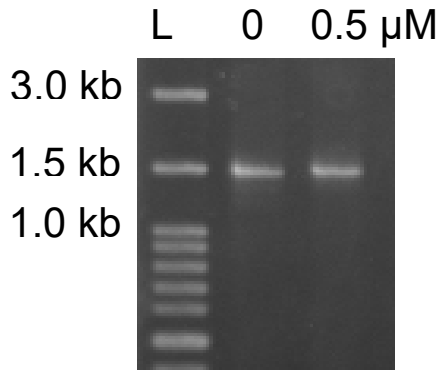


Figure 4.2 Agarose gel electrophoresis of PCR products of bacterial 16S rRNA genes fragments with and without LNA oligonucleotides. L; the marker of 100 bp ladders.

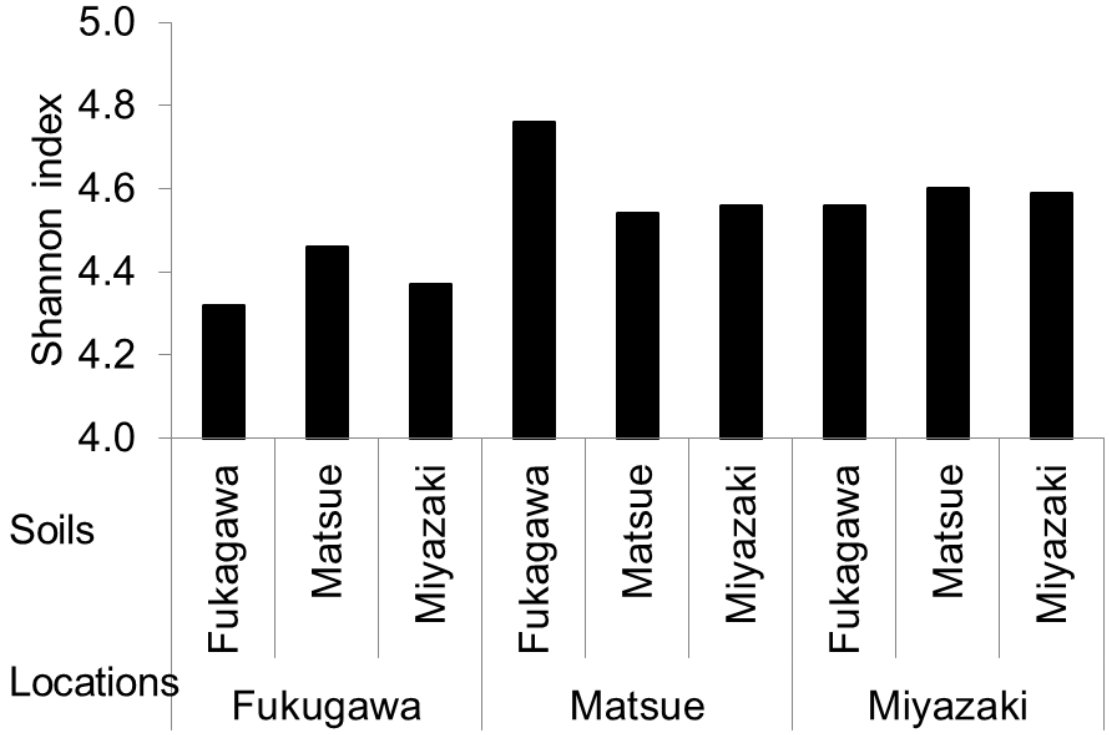


Figure 4.3 Shannon diversity indices of endophytic bacterial community when soils were used in different locations.

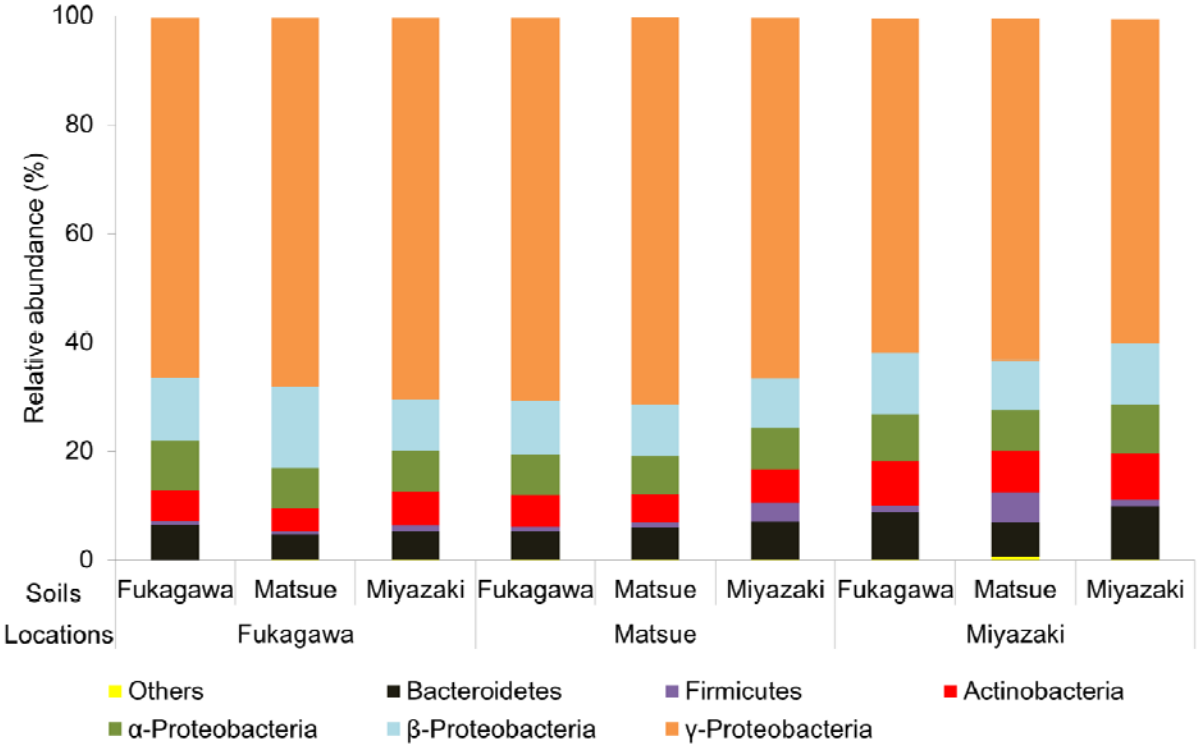


Figure 4.4 Relative abundances of unculturable endophytic bacterial phyla in sweet potatoes cultivated in different location and soil combinations.

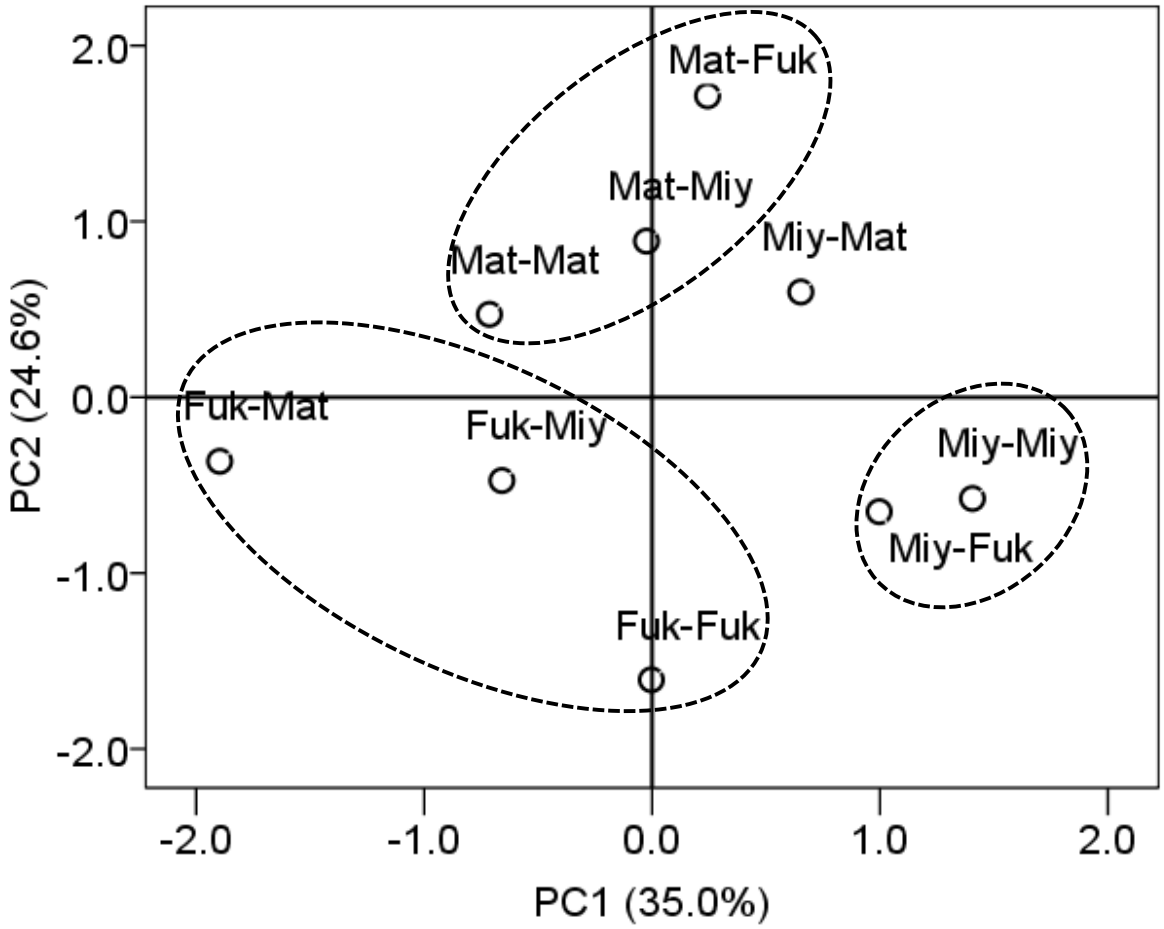


Figure 4.5 Principal component analysis of unculturable endophytic bacterial communities in sweet potatoes cultivated at different locations and soils. PCA was analyzed based on the bacterial genera in Table 3. The sample names represent location-soil.

Supplementary Tables

Table S4.1 Primers used in the experiment

Direction	Primers	Sequence (5'-3')[a]	References
Forward	fD1	AGAGTTTGATCCTGGCTCAG	(William G Weisburg et al., 1991)
	modified 63f	YRKGCYTWAYACATGCAAGTC	(Ikenaga et al., 2015)
	adapter-341f	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG** -CCTACGGGNGGCWGCAG	(Klindworth et al., 2013)
	338f	ACTCCTACGGGAGGCAGCAG	(Huong et al., 2007)
	926f	AAACTCAAAGGAATTGACGG	(Sato et al., 2008)
Reverse	518r	ATTACCGCGGCTGCTGG	(Huong et al., 2007)
	r1L	GTATTACCGCGGCTGCTGG	(Jaric et al., 2013)
	adapter-785r	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG** -GACTACHVGGGTATTAATCC	(Klindworth et al., 2013)
	907r	CCGTCAATTCCTTTGAGTTT	(Huong et al., 2007)
	r3L	TTGCGCTCGTTGCGGGACT	(Sato et al., 2008)
	rP2	ACGGCTACCTTGTTACGACTT	(William G Weisburg et al., 1991)
	1492r	GGYTACCTTGTTACGACTT	(Ikenaga et al., 2015)
	modified 1525r	AARGATTCMATCCAGCC	Ikenaga et al., Personal information

[a] bold letters are overhang adapter sequences

Table S4.2 Sequences of the designed LNA oligonucleotides specific for the 16S rRNA genes of sweet potato plastid and mitochondria.

LNA oligonucleotide	Sequence[a]	Melting temperature (^{0}C)	Reference
LNA-Mit63	5`-GTCGA**A**CG**T**TGT**TT**T**C**G**G**p-3'	70	(Ikenaga and Sakai, 2014)
LNA-Mit1492	5`-CTTC**A**CCCCA**G**TCG**AA**G**A**p-3'	70	(Ikenaga and Sakai, 2014)
LNA-Pla63b	5`-TCG**G**ACGGGAA**GT**GG**T**p-3'	70	(Ikenaga et al., 2015)
LNA-Pla1492b	5`-CTTCAC**T**CCA**G**TCA**CT**AG**C**p-3'	71	(Ikenaga et al., 2015)

[a]LNA bases were indicated with bold letters. Underlined DNA bases indicated the overlapped sequences with the bacterial primer.

Table S4.3 Number of OTUs in metagenomic analysis of endophytic bacteria in sweet potato tubers

Sample ID	Samples[a]	Number of sequences	Mean sequence length	Observed OTUs
L14198	Fuk-Fuk (1)	25,728	460	623
L14199	Fuk-Fuk (2)	20,861	458	801
L14200	Fuk-Fuk (3)[b]	19,974	457	858
L14201	Fuk-Mat (1)	19,773	457	850
L14202	Fuk-Mat (2)	22,347	459	907
L14203	Fuk-Mat (3)[b]	23,015	459	817
L14204	Fuk-Miy (1)	22,675	459	904
L14205	Fuk-Miy (2)	21,480	458	768
L14206	Fuk-Miy (3)[b,c]	12,592	459	518
L14187	Mat-Fuk (1)	21,605	458	908
L14188	Mat-Fuk (2)[b]	22,862	458	977
L14184	Mat-Mat (1)	20,972	458	766
L14185	Mat-Mat (2)	22,487	460	902
L14186	Mat-Mat (3)[b]	20,950	458	758
L14189	Mat-Miy (1)	21,185	458	805
L14190	Mat-Miy (2)	20,867	457	876
L14191	Mat-Miy (3)[b]	24,214	460	859
L14213	Miy-Fuk (1)	20,575	457	867
L14214	Miy-Fuk (2)	20,790	457	874
L14215	Miy-Fuk (3)[b]	20,104	457	925
L14210	Miy-Mat (1)	21,200	457	931
L14211	Miy-Mat (2)	20,697	457	963
L14212	Miy-Mat (3)[b]	22,354	460	1,078
L14207	Miy-Miy (1)[c]	15,245	458	622
L14208	Miy-Miy (2)	19,307	457	911
L14209	Miy-Miy (3)[b]	19,295	457	920
Average		20,891	458	846

[a] Samples are denoted as location-soil.

[b] Samples used in the culture dependent analysis

[c] Samples were not considered in the endophytic bacterial community analysis due to less number of sequences.

Table S4.4 Number of reads of bacterial sequences in the sweet potato tubers on phyla/class basis.

Sample ID	Samples[a]	Phylum/ class						
		α-Proteobaceria	*β-Proteobacteria*	*γ-Proteobacteria*	Bacteroidetes	Actinobacteria	Firmicutes	Others
L14198	Fuk-Fuk (1)	2663	3082	18008	1013	893	59	10
L14199	Fuk-Fuk (2)	1651	2371	13755	1439	1431	195	19
L14200	Fuk-Fuk (3)[b]	1785	2375	12541	1620	1409	213	31
L14201	Fuk-Mat (1)	1886	5445	10755	878	658	81	70
L14202	Fuk-Mat (2)	1405	2029	16618	965	1134	136	60
L14203	Fuk-Mat (3)[b]	1439	1986	17325	1055	1018	161	31
L14204	Fuk-Miy (1)	1632	1763	16660	1043	1356	190	31
L14205	Fuk-Miy (2)	1703	2422	14412	1267	1430	218	28
L14187	Mat-Fuk (1)	1784	2412	14185	1522	1473	196	33
L14188	Mat-Fuk (2)[b]	2509	1754	15537	1409	1173	412	68
L14184	Mat-Mat (1)	1530	2278	14165	1509	1292	168	30
L14185	Mat-Mat (2)	1466	1716	17355	1030	701	203	16
L14186	Mat-Mat (3)[b]	1506	2160	14465	1288	1325	183	23
L14189	Mat-Miy (1)	1660	2369	13954	1456	1560	165	21
L14190	Mat-Miy (2)	1797	2332	13141	1711	1618	240	28
L14191	Mat-Miy (3)[b]	1544	1267	17105	1419	720	2119	40
L14213	Miy-Fuk (1)	1733	2354	12886	1702	1629	243	28
L14214	Miy-Fuk (2)	1773	2405	12811	1822	1715	227	37
L14215	Miy-Fuk (3)[b]	1757	2252	12278	1803	1716	277	21
L14210	Miy-Mat (1)	1618	2122	14079	1567	1532	250	32
L14211	Miy-Mat (2)	1771	2272	12782	1820	1713	301	38
L14212	Miy-Mat (3)[b]	1414	1582	13626	730	1601	3140	261
L14208	Miy-Miy (2)	1731	2133	11338	2057	1776	232	40
L14209	Miy-Miy (3)[b]	1784	2172	11839	1685	1544	229	42
Average % reads		8.0	10.7	66.3	6.6	6.3	1.9	0.2

[a] Samples are denoted as location-soil.

[b] Samples used in the culture dependent analysis

Supplementary Figures

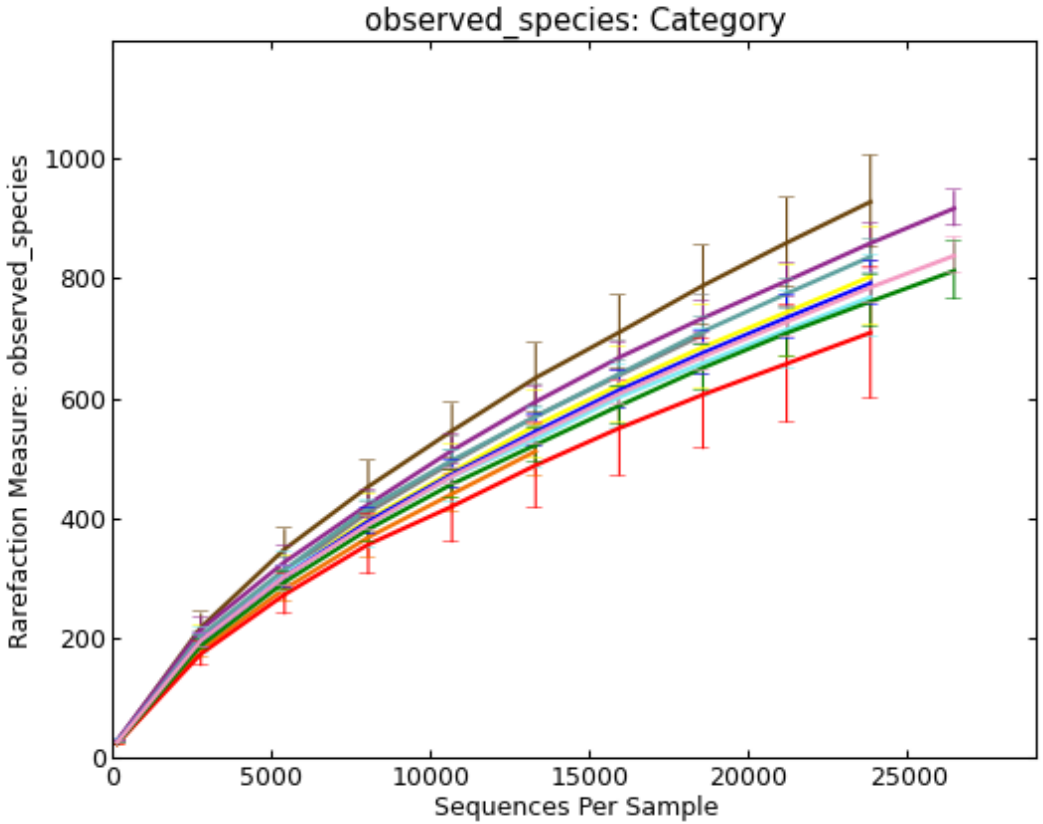


Figure S4.1 Rarefaction curves of sequences per sample and observed species.

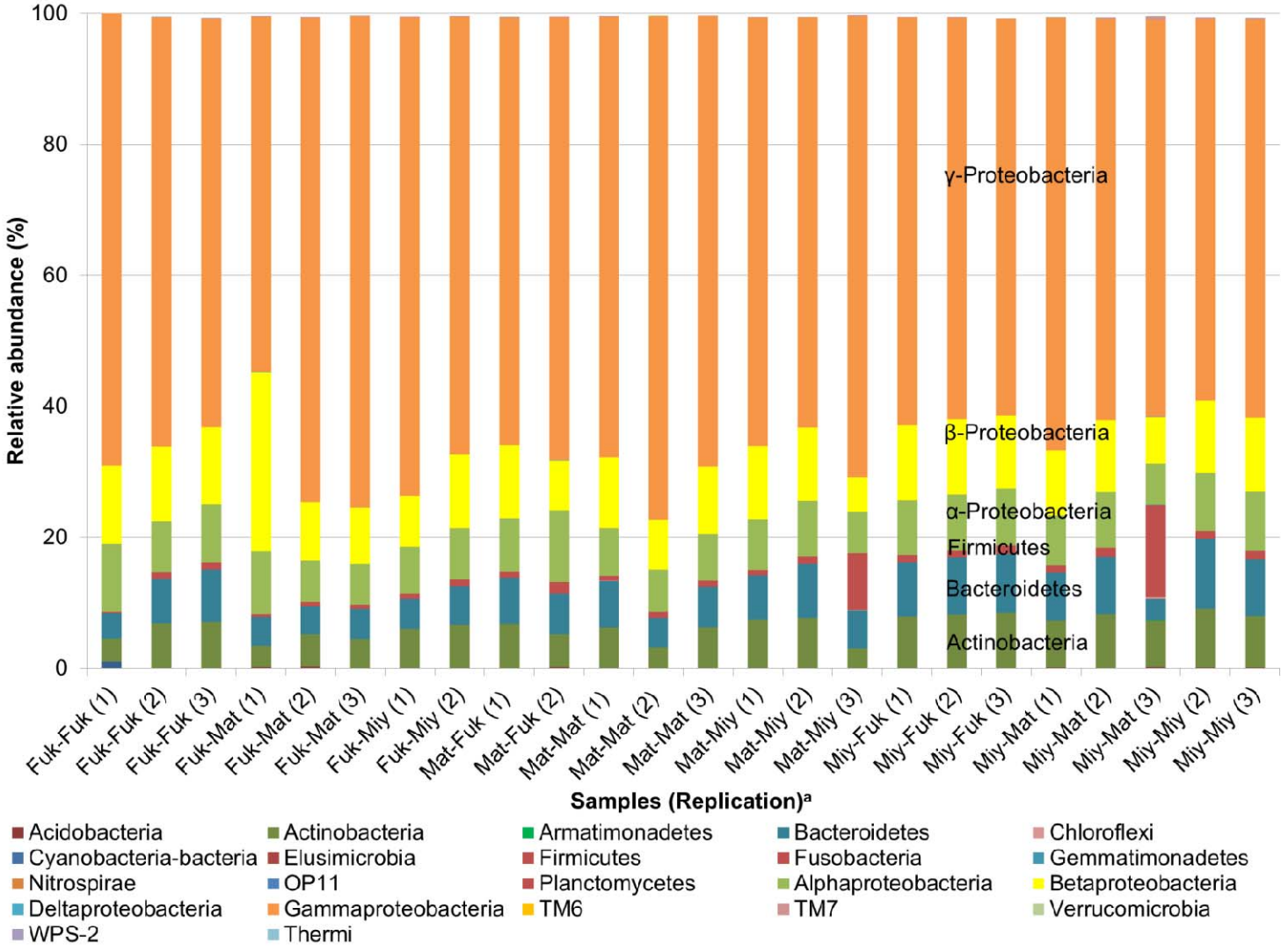


Figure S4.2 Relative abundances of unculturable endophytic bacterial phyla in sweet potatoes cultivated in different location and soil combinations. [a]: Samples represent location-soil and their respective replication.

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Summary (English)

Sweet potato (*Ipomoea batatas* L.) is a resilient, easily propagated crop which grows well in marginal lands and can be cultivated in low-fertile soils, and takes up more nitrogen than other root crops. There are reports that sweet potato harbors different types of endophytic bacteria and the effects of climatic conditions on the endophytic bacterial community compositions are limited. In the context of Nepal, there is no information on the sweet potato endophytes till date. On the other hand, Nepal possess diverse climatic conditions, the elevation ranges from 68 to 8,848 masl in a just 150 to 250-km south–north transect.

Therefore, we aimed to examine bacterial community of sweet potato endophytes in Nepal in relation to the environmental parameters and characterize their plant growth promoting traits. As synergistic effect of mixed cultures of plant growth promoting bacteria was reported, we also examined their potential by inoculating combined isolates from each location. From this study, we isolated 243 endophytic bacteria belonging to 34 genera in six classes from 12 locations of Nepal. Among them, the predominant classes were Bacilli and γ-Proteobacteria. The principal component analysis revealed that the composition of bacterial classes was unrelated to the environmental parameters of the sampling sites. Regarding their plant growth promoting potentials, 57% of the strains demonstrated indole-3-acetic acid (IAA) producing ability while 5% strains had nitrogen fixing gene (*nifH*) and acetylene reduction assay (ARA) activity. The representative strains in all six classes showed antagonistic effect against bacterial pathogens while only Bacillus strain showed the effect against fungal pathogen. For endophytic traits, cellulase activity was observed in 5 classes, while pectinase activity was only in Proteobacteria. Fresh

weight and vine length of sweet potato increased by inoculating mixed cultures of the isolates from each location. In this study, we observed that the cultivating locations did not effect on the sweet potato endophytic community and distinction of the effects between the climate and the soil was not clear.

So we designed next experiment with an aim to examine the effects of soil and climatic conditions on the endophytic bacterial communities of sweet potato by using the same soil at different locations and applying culture-dependent and independent approaches. Using culture-dependent approach, sixty two colonies were isolated and identified. γ-Proteobacteria (96%), β-Proteobacteria (87%) and Actinobacteria (88%) dominated in the samples cultivated in Fukagawa, Matsue and Miyazaki soils at the corresponding locations, respectively. When the soil samples were used in the different locations, the above mentioned location-specific phyla increased at the new sites. The endophytic bacterial population was also affected by the cultivating locations. It was suggested that the location rather than the soil influenced on the endophytic community and population.

In culture-independent approach, locked-nucleic acid-PCR clamping technique and next-generation technology were used to examine the effect of the soils and the locations on the whole community. The study revealed that Proteobacteria (85.0%), Bacteroidetes (6.6%) and Actinobacteria (6.3%) were the three most dominant phyla, accounting for 97.9 % of the reads, and γ-Proteobacteria (66.3%) being the most abundant. The overall endophytic communities were similar among the samples and top 10 genera represented 81.2% of the overall reads in which *Pseudomonas* (31.9-45.0%) being the most predominant operational taxonomic units. Principal component analysis and Shannon diversity indices showed a tendency that the location was

more important than the soil to determine the sweet potato endophytic bacterial community.

In conclusion, sweet potato tubers were dominated by specific but different endophytic bacteria with plant growth promoting potentials, and the endophytic bacterial compositions were unrelated with the cultivating locations. Effect of climate and soil on endophytic bacterial compositions was analyzed by culture-dependent and independent methods showed different results. The former method showed that the climate of the specific locations influenced on the endophytic community compositions, while the later indicated that the endophytic community compositions were similar among the samples cultivated in different climate and soil conditions.

Further study on why the specific groups of endophytes dominate the sweet potato tubers and how the climate and the soil effect on the endophytic community compositions and population is to be explored.

Summary (Japanese)

サツマイモは他の作物よりも多くの窒素を吸収することでやせた土地でもよく育つ。このようなサツマイモの能力は植物内生菌の働きによるものである。農業における持続可能性は重要であり、この点において有用微生物は安定的な生産に貢献すると考えられる。現在までにネパールのサツマイモの内生菌に関する情報はないことから、本研究では、ネパールの異なる気候条件下で栽培したサツマイモの内生菌の微生物群集を調べ、さらに、分離した内生菌を栽培地点ごとに混合接種し、それらの植物への成長促進効果を明らかにすることを目的とした。ネパールの気候条件の異なる 12 ヵ所で栽培したサツマイモをサンプルとして集め、6 綱 34 属の 243 種の内生細菌を単離した。それらの中で優占種は Bacilli と γ-Proteobacteria であったが、それらの綱レベルでの群集構造は気候条件と無関係であった。分離した内生菌の 57%が IAA 生産能を持っていたのに対し、nitrogen fixing gene（*nifH*）とアセチレン還元活性（ARA）を持つ菌株は 5%であった。植物病原細菌に対する生育抑制効果は 6 綱全ての菌株で観察されたが、真菌の病原菌に対しては *Bacillus* 属の菌株のみで認められた。また、ペクチナーゼ活性は Proteobacteria のみで観察されたが、セルラーゼ活性は 5 鋼の細菌で観察された。分離した内生菌を栽培地点ごとに混合接種した結果、サツマイモの生重と茎長を増加させる内生菌群集が認められた。

上記の結果から、気候がサツマイモの内生菌群集構成に影響を及ぼさないことが認められたが、土壌と気候条件の影響が明確に区別できないことから、深川、松江、宮崎の各地点で、それぞれの土壌を用いてサツマイモを栽培することにより、気候および土壌がサツマイモの内生細菌の群集構造に与える影響を調べることを目的として

実験を行った。なお、微生物群集の解析は、培養法と非培養法を用いて行い、それらの違いについても検討した。培養法では、62 のコロニーから内生細菌が単離・同定された。γ-Proteobacteria（96%）、β-Proteobacteria（87%）、Actinobacteria（88%）がそれぞれ深川、松江、宮崎で栽培されたサツマイモ塊根において優占していた。各土壌を異なる場所で使用してサツマイモを栽培した時、上記のそれぞれの場所に特徴的な細菌群が新しい場所において増加した。また、内生細菌のサツマイモ内での菌密度も栽培された場所の影響を受ける傾向にあった。以上の結果から、培養法で観察されたサツマイモ内生菌の群集構造は、土壌よりもむしろ気候の影響を受けることが示唆された。

非培養法では、サツマイモ内の全細菌を解析するため、locked-nucleic acid PCR clamping technique と次世代シークエンス技術が用いられた。培養法で用いたサツマイモ試料と同じものを用いて分析した結果、Proteobacteria（85％）、Bacteroidetes（6.6%）、Actinobacteria（6.3%）が優占しており、その中でも、γ-Proteobacteria（66.3%）が最も優占していた。全てのサツマイモサンプルで内生菌の群集構造は類似しており、上位 10 属の細菌が全体の 81.2%を占め、*Pseudomonas* 属が最大（31.9%～45.0%）の優占種であった。一方、属レベルの群集構造は、主成分分析と Shannon 多様性指数において、サツマイモの栽培場所に影響を受けることが示唆された。培養法と非培養法の結果は大きく異なり、一部の内生菌が気候条件の影響をより大きく受けることが明らかとなったが、内生菌群集全体でも、気候が土壌よりも影響を与えることが示唆された。

Publications included in this thesis

Publication citation – incorporated as Chapter 2.

Puri, R.R., Dangi, S.R., Dhungana, S.A. and 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

Publication citation – incorporated as Chapter 3.

Puri, R.R., Adachi, F., Omichi, M., Saeki, Y., Yamamoto, A., Hayashi, S. and Itoh, K. 2018. Culture-Dependent Analysis of Endophytic Bacterial Community of Sweet Potato (*Ipomoea batatas*) in Different Soils and Climates. Journal of Advances in Microbiology. 13(2), 1–12. DOI: 10.9734/JAMB/2018/45442