Effects of Inoculation of Plant Growth-Promoting Bacteria on Plant Growth, Bacterial Endophytic Community Structure and Colonization

(植物成長促進細菌の接種が植物成長、内生細菌の群集構造および植物内定着に及ぼす影響)

AHSANUL SALEHIN

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THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES,

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Author Declaration

I, the undersigned **AHSANUL SALEHIN** hereby declare that I am the sole author of this thesis. To the best of my knowledge, this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted as part of the requirements of any other academic degree or non-degree program, in English or any other language.

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The thesis enclosed herewith, **'Effects of Inoculation of Plant Growth-Promoting Bacteria on Plant Growth, Bacterial Endophytic Community Structure and Colonization'** prepared and submitted by **AHSANUL SALEHIN** in partial fulfillment of the requirement for the award of Doctor of Philosophy, is hereby approved as to style and contents.

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Dedicated to my beloved son ***FAYAAZ SALEHIN***

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*First and foremost, I proclaim my submission to Almighty **ALLAH**, the ever-living of everything that exists. Only He is the knower of revealed and concealed, the approver of all knowledge divulged.*

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CHAPTER ONE

General Introduction

The modern agriculture system is being intensified by using various technologies to achieve maximum efficiency and high qualify products to meet the growing need of the global demand for food supply (Tilman et al., 2011). Fertilization is one of the most important ways to increase efficiency and obtains a better quality of products in agriculture (Lin et al., 2019). However, in recent years, the use of chemical fertilizer has increased exponentially throughout the world, which causes serious environmental problems (Silva et al., 2010; Atilgan et al., 2007; Gross et al., 1998). Therefore, it is a great challenge in agriculture to find out sustainable strategies to ensure high yields, provide environmental safety, and protect the ecological balance in agro-ecosystem in relieving the detrimental effects of intensive farming practices.

1.1 Plant Growth-Promoting Bacteria

Plant growth-promoting bacteria (PGPB) are soil-borne free living bacteria that aggressively colonize the rhizosphere and/or plant roots, and enhance the growth and yield of plants during cultivation (Ashrafuzzaman et al., 2009). It is supposed that using PGPB is becoming a more widely accepted practice in intensive agriculture to enhance sustainable agricultural production in the world (Kaymak, 2010). Plant growth may be enhanced under different biotic and abiotic stresses through the microbial application, and these microbes may help in promoting plant growth in different ways such as regulating nutritional and hormonal balance, producing plant growth regulators, solubilizing nutrients, and inducing resistance against plant pathogens (Nadeem et al., 2014; Glick, 1995; Kumar et al., 2014). The mechanisms of PGPB in plant growth have not been fully understood, but it is assumed that PGPB enhance growth and yield either by direct (such as nitrogen fixation, phosphate solubilization, sequestering iron, cytokinins and gibberellins, IAA, ethylene) or indirect mechanisms (such as antibiotics and enzymes, siderophores, competition between pathogens and nonpathogens, induced systemic resistance) (Glick, 1995).

Microorganisms are important microbial resources and have gradually become a multidisciplinary research hotspot in different fields of research. Studies found that the addition of PGPB has plant growth promotion in different ways include an increase in seed germination rate, root and shoot growth, increase in yield, leaf size & chlorophyll content, nitrogen-protein content, drought tolerance, delayed leaf senescence (Cakmakci et al., 2006; Babalola et al., 2006; Yadegari et al., 2008). Cakmakci et al. (2007) found that microorganisms have great potential to reduce the need for chemical fertilizers by promoting and enhancing the availability of plant nutrients. In sustainable agriculture practices, it is assumed that inoculation is one of the most recognized techniques to confirm the effect of plant growth promotion (Zakria et al., 2008). Inoculation with PGPB has already been studied in confirming its significant role to increase the growth and yield of agronomically important crops such as *Triticum aestivum* (wheat), *Oryza sativa* L. (rice), *Brassica juncea* L. (canola) (Amara and Dahdoh, 1997; Biswas et al., 2000; Hilali et al., 2001; Asghar et al., 2002).

It is hypothesized that the inoculation of PGPB might have some synergic effects on plant growth promotion, and the inoculation with beneficial microbes may become a practice used in agriculture that may provide advantages in crops by enhancing growth and protection against diseases (Ji et al., 2014). The use of a single microbial species or strains as inoculant might not likely be active in all soil environments to improve plant growth while the studies found that inoculation with multiple beneficial bacteria may have higher potential than inoculation with a single bacterial inoculant (Molina-Romero et al., 2017). Application of binary or multiple mixtures would mimic the natural condition more diligently and might broaden the spectrum of biocontrol activity (Raupach and Kloepper, 1998). Moreover, they would enhance the efficiency and consistency of healthy effects on crops (Marimuthu et al., 2002) by allowing the combination of various mechanisms without the need for genetic engineering. The implementation of these strategies has been noticed previously, and now a growing interest has also been focused on the research where new microbial combinations might have the ability to enhance performances on plant health,

concerning the applications of single strains/species (Dandurand and Knudsen, 1993; Roberts et al., 2005; Vestberg et al., 2004; Marimuthu et al., 2002).

1.2 Endophytes

The term "endophyte" is derived from the Greek words "endon" meaning within, and "phyton" meaning plant. Thus, an endophyte is an organism that lives inside a plant. Previously, endophytes were defined as microorganisms such as bacteria and fungi that inhabit the plant endosphere during all or at least a part of their life cycle, and cause no apparent harm to the host plant (Wilson, 1995; Dobereiner, 1992). Additionally, Hallmann et al. (1997) suggested that the endophytes are a class of microbes and can be isolated from surface-disinfected plant tissues or extracted from within the plants and could establish a mutualistic association without any harm to the host plants. Due to the suspected lack of sufficient sterilization of plant surfaces for removing the surface bacteria, and the presence of non-cultured species, the definition appeared to be less appropriate (Garbeva et al., 2001). However, the definition of endophytes has been revised multiple times by different authors while more recently, Hardoim et al. (2015), defined endophytes as microbes including bacteria, archaea, fungi, and protists that colonize the plant interior regardless of the outcome of the association. Conventionally, endophytes were isolated from surface-sterilized plant tissue and cultivated in a nutrient-rich medium. Although endophytic microorganisms include archaea, fungi, and protists also act as endophytes in plants other than bacteria, our study deals with bacterial endophytes.

1.3 Enrollment of Bacterial Endophytes by Host Plants

The rhizosphere is well-defined as the soil-root border, where multifaceted interactions take place between the plant and its surrounding soil microorganisms (Bulgarelli et al., 2012; Senga et al., 2017). It has been reported that plants can release significant amounts of photosynthates or exudates from its roots, which influence microbial communities in the rhizosphere. Root exudates including organic acids, amino acids, and proteins may be involved in recruiting bacterial endophytes from the rhizosphere (Bulgarelli et al., 2012; Kawasaki et al., 2016; Pétriacq et al., 2017) and likely contain substrates that initiate early

communication between host plants and bacterial endophytesto navigate the colonization process. For example, oxalate involvement in the enrollment of bacterial strain *Burkholderia phytofirmans* PsJN by host plants has been reported where a *B. phytofirmans* strain defective in oxalate utilization was inoculated lupine and maize plants that secrete moderate and low levels of oxalate, respectively. At 3 days after inoculation, the mutant was observed in significantly fewer numbers in both maize and lupine plants as compared to the wild type strain. Interestingly, inoculation with both wild type and mutant strains resulted in significant differences in colonization by the two strains in lupine but not in maize. Oxalate was also observed in *Brachypodium* root exudates, and high numbers of Proteobacteria were detected in the *Brachypodium* rhizosphere (Kawasaki et al., 2016).

Moreover, bacterial quorum sensing compounds are likely involved in communication with the plant root and the subsequent colonization process. The importance of these compounds in the colonization and growth promotion of plants by endophytes is supported by a recent study. It was reported that a quorum-sensing mutant of *B. phytofirmans* PsJN neither efficiently colonizes *Arabidopsis thaliana* nor promotes its growth (Zúñiga et al., 2013). Plants are likely directly involved in quorum sensing as well, given that some plant extracts have been shown to have quorum quenching capabilities that could protect them against pathogens and some quorum sensing molecules have been shown to have direct plant growth-promoting effects (Schikora et al., 2016). Additionally, several endophytes of *Populus deltoides* were found to have LuxR homologs hypothesized to be involved in responding to plant derived compounds (Schaefer et al., 2013). The study was also found that many of the surveyed endophyte genomes contained LuxR-LuxI type quorum sensing gene pairs pointing to their importance in the endophytic lifestyle.

Bacterial structures such as flagella, fimbriae, or cell surface polysaccharides are also likely involved in the attachment of bacteria to the plant surface. Balsanelli et al. (2010) reported the importance of bacterial lipopolysaccharide (LPS) for attachment and subsequent endophytic colonization of plant roots by studying the colonization ability of endophyte *Herbaspirillum seropedicae* to maize plants. However, the mechanisms by which bacterial endophytes attach to plant surfaces remain relatively unexplored (Pankievicz et al., 2016).

1.4 Attachment of Bacterial Endophytes to the Host Plant Surface

The first step of the colonization process is considered the attachment or adhesion of bacterial cells to the plant surface. Bacteria in the vicinity 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 possible entry sites at lateral root emergence areas or other openings caused by wounds or mechanical injuries. The attachment of bacterial cells onto the root surface might be facilitated by the exopolysaccharides (EPS) synthesis of a bacterial cell and may be important in the early stages of endophytic colonization. The production of EPS by endophytic bacterium *Gluconacetobacter diazotrophicus* Pal5 was reported as an essential factor for rice root surface attachment and colonization (Meneses et al., 2011). Bacterial cells were shielded from oxidative damage by exopolysaccharides, by reducing free radical concentrations in planta. Colonization was reduced in an EPS knockout strain of *G. diazotrophicus* and interestingly, the addition of EPS produced by the wild-type strain rescued this reduction in colonization (Meneses et al., 2017).

Bacterial endophytes primary attachment to the root surface called rhizoplane, and explore the possible entry sites to access the internal plant tissues. Hardoim et al. (2015) reported while entering into the host plant endophytes used to consider the main entry points such as root hairs or lateral roots emerge, as well as stomata, wounds, and hydathodes in the shoots. Endophytic bacteria likely utilize these natural discontinuities in the plant body to access the internal plant tissues. Moreover, it is supposed, plant cell wall might be modified by some bacterial endophytes secreting cell wall cellulolytic enzymes such as cellulases, xylanases, pectinases, and endoglucanases, which facilitate bacterial entry and spread within the plant tissues (Compant et al., 2005; Reinhold-Hurek et al., 2006; Naveed et al., 2014). Reinhold-Hurek et al. (2006) 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. Many colonization studies suggested that natural cracks at the lateral root emergence site are the most common entry sites for endophytic bacteria (Hardoim et al.,

2015; Iniguez et al., 2004; Compant et al., 2005). Furthermore, some bacteria use root apex and root hairs as entry points followed by endophytic colonization in root cortex and vascular tissues (Prieto et al., 2011; Rangel de Souza et al., 2016).

1.5 Bacterial Niches Inside the Host Plant and Colonization

Intercellular spaces in the plant have an abundance of carbohydrates, amino acids, and inorganic nutrients, and bacterial endophytes most often inhabit these areas (Hardoim et al., 2015; Elbeltagy et al., 2001; Dong et al., 1994). They likely exclusively colonize the intercellular spaces of various plant parts including roots, leaves, stems, flowers, and seeds (Iniguez et al., 2004; Kandel et al., 2015; Compant et al., 2005; Germaine et al., 2004; Mitter et al., 2017; Glassner et al., 2017). Colonization can be localized at the tissue level or systemically throughout the plant body. Endophytes are observed first in plant root hairs, and subsequently in the root cortex, in his early stage of colonization (Rangjaroen et al., 2017; Prieto et al., 2011; Castanheira et al., 2017). Compant et al. (2005) reported, inoculated *Burkholderia* sp. strain PsJN was detected in cortical cells, endodermis, and xylem vessels, and colonization was especially high at primary and secondary roots and at the base of lateral roots and root tips where both intracellular and intercellular colonization was observed. Similarly, Fisher et al. (1992) showed bacterial endophytic colonization was stronger in the lower stem compared to the stem closer to the shoot apex in maize plants. The mobility of bacterial cells accompanied by the synthesis of the cellulolytic enzyme might help endophytes to spread to aerial plant parts including leaves and stems (Elbeltagy et al., 2001; Santi et al., 2013; Compant et al., 2005).

In the rhizosphere, there is a selection of microorganisms that can survive in the root exudates and compete with others. Studies found that *Rhizobium etli* strains in maize plants were equally competitive for colonizing the rhizosphere and inside tissues of the root (Rosenblueth and Martínez-Romero, 2004). For plant colonization, some bacteria must find their way through cracks formed at the emergence of lateral roots or at the zone of elongation and differentiation of the root. Dong et al. (2003) showed that cells of *Klebsiella* sp. strain Kp342 aggregate at lateral-root junctions of wheat and alfalfa. Similarly,

Gluconacetobacter diazotrophicus and *Herbaspirillum seropedicae* also colonize lateral-root junctions in high numbers (James and Olivares, 1997). It has been proposed that cellulolytic and pectinolytic enzymes produced by endophytes are involved in the infection process (Hallmann et al., 1997), as in *Klebsiella* strains, pectatelyase has been implicated to participate during plant colonization (Kovtunovych et al., 1999). In some assays, early endophytic colonization differed from one cultivar to another, but later endophytes were recovered in approximately similar numbers from the different cultivars (Pillay and Nowak, 1997). In general, endophytic isolates were accomplished by colonizing or recolonizing the inside plant tissues in higher numbers than isolates from the root surface (van Peer et al., 1990; Rosenblueth and Martínez-Romero, 2004). Bacterial endophytes may move all the plant parts within a short period. For example, *Salmonellae* have inoculated the roots of hydroponically grown tomato plants at around 4.55 log CFU ml^{-1} and, the next day found that hypocotyls, cotyledons, and stems had around 3 log CFU g^{-1} (Guo et al., 2002). Similarly, the systematic spread of an endophytic *Burkholderia* strain to aerial parts of *Vitis vinifera* seems to be through the transpiration stream (Compant et al., 2005). Endophytes can also play an active role in colonization. *Azoarcus* sp. type IV pili are involved in the adherence to plant surfaces, an essential step towards endophytic colonization (Dörr et al., 1998). Two *Klebsiella* strains differ significantly in their invasion capacity in different plant hosts (*Medicago sativa*, *Medicago truncatula*, *Arabidopsis thaliana*, *Triticum aestivum*, and *Oryza sativa*). One of them (Kp342) was a better colonizer in all hosts and only needed a single cell to colonize the plants substantially a few days after inoculation (Dong et al., 2003). The plant hosts also differed in their ability to be colonized endophytically by the same bacterium, further suggested an active host role in the colonization process. Some rhizospheric bacteria can colonize the internal roots and stems, showing that these bacteria are a source for endophytes (Germaine et al., 2004), but also phyllosphere bacteria might be a source of endophytes (Hallmann et al., 1997). The colonizing capacity may be overestimated in vitro, as there is no competition with indigenous soil bacteria (Cooley et al., 2003).

1.6 Endophytic Bacterial Communities

Different endophytic bacteria were accompanied by different host plants in terms of community and composition. Endophytic bacterial community analysis is conducted using culture-dependent approaches. Different studies on endophytic bacterial communities have been conducted using the same approaches. Pereira et al. (2011) reported a culturable endophytic bacterial community in maize plant where *Achromobacter* (67.78%) genera in the β-Proteobacteria class identified the most dominant followed by *Bacillus* (30.02%) and *Pseudomonas* (2.2%). Souza et al. (2013) identified 102 endophytic bacteria from banana roots representing 10 genera, among which the genus *Bacillus* was the most abundant (87.3% of isolates), followed by the genus *Lysinibacillus* (3.9% of the isolates).

There have been a few studies on the endophytic bacterial community from sweet potato crops. In most recent, Puri et al. (2018a) isolated 243 endophytic bacteria belonging to 34 genera in six classes from 12 locations in Nepal. Among the classes, Bacilli represented the highest relative abundance (28%), and *Bacillus* sp. was the most dominant genus (25%), followed by *Enterobacter* sp. (5.3%), *Burkholderia* sp. (8.6%), *Microbacterium* sp. (6.8%), *Rhizobium* sp. (6.3%) and *Flavobacterium* sp. (4.4%). Moreover, the community was examined for samples collected in Brazil (Marques et al., 2015) and the USA (Khan and Doty, 2009), and was shown that γ-Proteobacteria was the common dominating group in both studies.

1.7 Plant Growth-Promoting Properties

Bacterial endophytes that are beneficial to plant growth and development may found across many phyla, including the Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes (Hardoim et al., 2015; Bulgarelli et al., 2012; Doty et al., 2009; Wemheuer et al., 2017). Increased biomass and height in inoculated plants have been reported as a result of colonization by many endophytic genera such as *Azoarcus*, *Burkholderia*, *Gluconobacter*, *Klebsiella*, *Pantoea*, *Herbaspirillum*, *Rahnella*, *Pseudomonas*, and *Bacillus* (Elbeltagy et al., 2001; Hurek et al., 2002; Iniguez et al., 2004; Feng et al., 2006; Momose et al., 2009; Botta et al., 2013; Kandel et al., 2015; Knoth et al., 2012; Li et al., 2007). There are some

common characteristics of endophytes include the ability to synthesize plant hormones such as indole-3-acetic acid (IAA), solubilize phosphate, secrete siderophores, and confer plant tolerance to biotic and abiotic stresses (Gaiero et al., 2013; Lebeis et al., 2014; Rosenblueth et al., 2006; Bastian et al., 1998). Additionally, some bacterial endophytes carry genes necessary for biological nitrogen fixation (BNF), potentially enabling them to convert di-nitrogen gas (N_2) into usable forms of nitrogen such as ammonium within the host plant (Bhattacharjee et al., 2008; Santi et al., 2013).

Studies reported that bacterial endophyte strains promote plant growth by synthesizing phytohormones including IAA, cytokinins, and gibberellins or through regulating internal hormone levels in the plant body (Hardoim et al., 2015; Santoyo et al., 2016; Spaepen and Vanderleyden, 2011). IAA produced by endophytes within plants increases the number of lateral and adventitious roots, facilitating access to nutrients, and improving root exudation, offering more resources for soil microbes to interact with roots (Spaepen and Vanderleyden, 2011; Gamalero et al., 2011). Growth enhancement by increasing plant height and/or biomass has been reported in many studies when plants were inoculated with bacterial endophytes capable of producing IAA (Santoyo et al., 2016; Shi et al., 2009; Xin et al., 2009; Barra et al., 2016; Khan et al., 2016).

Furthermore, bacterial endophytes secrete siderophores and solubilize phosphorus while initiating the symbiotic interactions with host plants (Hardoim et al., 2015; Gamalero et al., 2011). Siderophores are organic compounds secreted by microorganisms and plants in iron-limited conditions enabling them to chelate iron from the environment for microbial and plant cells to uptake (Hardoim et al., 2015; Ahmed et al., 2014). Bacterial endophytes can confer resistance or tolerance to the host plant from biotic and abiotic stresses by releasing antimicrobial compounds, producing siderophores, competing for space and nutrients, and modulating the plant resistance response (Santoyo et al., 2016; Friesen et al., 2011; Mercado-Blanco et al., 2014). Similarly, phosphate-solubilizing bacteria can solubilize immobile phosphorus in soil, which is potentially available for plants to absorb, an important trait for plant growth promotion (Dias et al., 2009; Oteino et al., 2015; Passari et al., 2015; Joe et al., 2016).

Some bacterial strains can relieve plant stress by blocking the pathway of ethylene synthesis in plants. These bacteria utilize 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which helps to reduce ethylene concentrations accumulated in response to different stresses in plants, otherwise lethal to plant health (Glick, 2014). Endophytic strains of *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, and *Serratia* were found to be effective in suppressing the growth of pathogenic microorganisms in in-vivo and in-vitro conditions (Mercado-Blanco et al., 2014; Esmaeel et al., 2016; Larran et al., 2016; Kandel et al., 2017b).

Moreover, endophyte strains in the genera *Bacillus*, *Enterobacter*, *Pseudomonas*, *Azotobacter*, *Arthrobacter*, *Streptomyces*, and *Isoptericola* were successful in alleviating drought, heat, and salt stress in different crop plants (Kandel et al., 2017a). More importantly, symbiotic plants with these endophytes were not only capable of relieving the stress but also significantly increased plant biomass and height (Rojas-Tapias et al., 2012; Ali et al., 2014; Naveed et al., 2014; Qin et al., 2014; Yaish et al., 2015). However, the mechanisms used by bacterial endophytes to mitigate abiotic stress remain unclear.

1.8 Objectives of Our Study

Sweet potato (*Ipomoea batatas* L.) is a resilient, easily propagated crop, and its roots are largely used for food consumption. More than 95% of the global sweet potato crop is produced in developing countries having a vast economic and social importance (Reiter et al., 2003; Souza and Lorenzi, 2008). It is also well-known for its ability to grow well even in infertile and nitrogen-limited fields (Hartemink et al., 2000; Hill et al., 1990), and the contribution of nitrogen fixation by endophytic bacteria is proposed (Yonebayashi et al., 2014).

Diverse endophytic bacteria have been isolated from sweet potato as *Gluconacetobacter*, *Klebsiella*, and *Pantoea* (Adachi et al., 2002; Asis and Adachi, 2004), *Enterobacter*, *Rahnella*, *Rhodanobacter*, *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, and *Phyllobacterium* (Khan et al., 2009). Marques et al., (2015) and Puri et al., (2018a) reported 93 and 243 endophytic bacterial strains belonging to 17 and 34 genera in Brazilian and

Nepalese sweet potatoes, respectively. Among these isolates of sweet potato bacterial endophytes, many strains showed beneficial properties as nitrogen fixation, auxin production, antagonistic effect, phosphate solubilization, and siderophore production.

It is supposed that the beneficial functions of endophytes are available when a suitable endophytic community is established, and it is expected that the inoculation of PGPB might have some synergic or competitive effects on the composition and function of the endophytic community (Trabelsi and Mhamdi, 2013). However, to the best of our knowledge, only a few studies are available on this subject. Conn and Franco (2004) showed that the inoculation of a non-adapted microbial inoculum to the soil disrupted the natural actinobacterial endophyte population of the wheat plant and reduced their diversities and colonization levels, whereas the inoculation of a single actinobacterial endophyte did not affect the indigenous endophyte population. Gadhave et al. (2018) reported that seed and soil inoculations with *Bacillus* spp. changed the composition of the endophytic bacterial community of sprouting broccoli and increased their diversities as examined by the metagenomic approach.

In the application of PGPR, successful colonization of PGPR inoculant is important after inoculation, however, because many biological and environmental factors are involved in the colonization, it is a challenging subject. In addition to the individual colonization of PGPR, co-existing with other bacteria would be important to determine the colonization and plant growth-promoting potentials. Synergetic effects of the inoculation with the other PGPR have been reported in maize (Molina-Romero et al., 2017), cotton (Marimuthu et al., 2002), ryegrass (Castanheira et al., 2017), strawberry (Vestberg et al., 2004), and cucumber (Raupach and Kloepper, 1998). On the other hand, negative interaction with co-existing bacteria should also be considered. They inhibited the colonization of inoculants in sugarcane (Oliveira et al., 2008), reduced the plant growth-promoting effects in tomato (Felci et al., 2008; Dhungana et al., 2019). Though several studies have been reported on the effects of co-inoculation with multiple bacteria on plant growth, but to the best of our knowledge, their effects on colonization have not been extensively studied yet.

In the present study, we used a biofertilizer OYK (*Bacillus* sp.) (Ono et al., 2002) as plant growth-promoting bacteria (PGPB), and treated sweet potato to examine the effects of OYK inoculation on indigenous culturable endophytic bacterial communities in field conditions (**Chapter Two**). Then, we compared the colonization properties of OYK (*Bacillus* sp.) with the other *Bacillus* sp. strains, which were isolated from rhizosphere and tubers of sweet potatoes, to evaluate the colonization potential of the *Bacillus* sp. strains with different origin. In addition, the effects of co-inoculation of the endophytic *Bacillus* sp. strain with the other bacterial endophytes on their colonization and plant growth-promoting activities were examined (**Chapter Three**).

CHAPTER TWO

Effects of the biofertilizer OYK (*Bacillus* sp.) inoculation on endophytic microbial community in sweet potato

2.1 Introduction

Modern agriculture systems are being intensified through the use of various technologies to achieve maximum efficiency and high qualify products to meet the growing global demand for food supply (Tilman et al., 2011). At present, as a part of agricultural intensification, crop production depends on the large-scale use of chemical fertilizers (Adesemoye et al., 2009). However, the intensive use of chemical fertilizers can result in considerable negative environmental impacts and pollutions (Silva et al., 2010). Therefore, an alternative strategy is urgently needed to establish sustainable agriculture and ecological balance in agro-ecosystems.

Plant growth-promoting rhizobacteria (PGPR) are free-living soil bacteria that enhance plant growth by colonizing the rhizosphere (Ashrafuzzaman et al., 2009). PGPR regulate nutritional and hormonal balance, produce phytohormones, solubilize nutrients, and induce resistance to plant pathogens (Nadeem et al., 2014). Therefore, PGPR has been used as biofertilizers and/or bioenhancers as an alternative source of chemical fertilizer to improve soil quality and sustainability and to increase crop production (Li et al., 2007; Nosratabad et al., 2017; Dawwam et al., 2013). The application of PGPR has becomes a more broadly recognized practice for the enrichment of sustainable agricultural production in several parts of the world.

Sweet potato (*Ipomoea batatas* L.) is a resilient, easily propagated crop, and its roots are largely used for food consumption. More than 95% of the global sweet potato crop is produced in developing countries, and it has vast economic and social importance (Reiter et al., 2003; Souza et al., 2008). It is also well-known for its ability to grow well even in infertile and nitrogen-limited fields (Hartemink et al., 2000; Hill et al., 1990), and nitrogen

fixation by endophytic bacteria has been proposed to contribute to this attribute (Yonebayashi et al., 2014).

Endophytes are known to promote plant growth by producing phytohormones (Jacobson et al., 1994; Dhungana et al., 2018; Gamalero et al., 2015), and siderophores (O'Sullivan et al., 1992; Khan et al., 2020), and through nitrogen fixation (Terakado-Tonooka et al., 2013). It has also been reported that some endophytes can protect plants by producing antipathogenic substances (Bangera et al., 1996), ameliorating disease development (Benhamou et al., 1996), and inducing stress tolerance (Hallmann et al., 1997). Therefore, an understanding of the endophyte-plant interaction is essential for developing sustainable systems of crop production (Sturz et al., 2000).

Diverse endophytic bacteria have been isolated from sweet potato; such bacteria include *Gluconacetobacter*, *Klebsiella*, and *Pantoea* (Adachi et al., 2002; Asis et al., 2004), as well as *Enterobacter*, *Rahnella*, *Rhodanobacter*, *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, and *Phyllobacterium* (Khan et al., 2009). Marques et al. (2015) and Puri et al. (2018a) reported 93 and 243 endophytic bacterial strains belonging to 17 and 34 genera in Brazilian and Nepalese sweet potatoes, respectively. Among these isolates of sweet potato bacterial endophytes, many strains had beneficial properties, such as nitrogen fixation, auxin production, antagonistic effects, phosphate solubilization, and siderophore production.

It is speculated that the beneficial functions of endophytes are realized when a suitable endophytic community is established, and it is expected that the inoculation of PGPR has synergic or competitive effects on the composition and function of the endophytic community (Trabelsi et al., 2013). However, to the best of our knowledge, only a few studies are available on this subject. Conn and Franco (Conn et al., 2004) showed that the inoculation of a non-adapted microbial inoculum into the soil disrupted the natural actinobacterial endophyte population of wheat plants and reduced their diversities and colonization levels, whereas the inoculation of a single actinobacterial endophyte did not affect the indigenous endophyte population. Gadhave et al. (2018) reported that seed and soil inoculations of *Bacillus* spp. changed the composition of the endophytic bacterial

community of sprouting broccoli and increased its diversity, as established through the metagenomic approach.

In the present study, we treated sweet potato with a commercial biofertilizer, OYK consisting of a *Bacillus* strain, which was reported to induce plant tolerance to abiotic and/or biotic stresses, and to have antimicrobial activities against pathogens (Ono et al., 2002). We then examined culturable endophytic communities at harvest in order to obtain further information on the effects of PGPR inoculation on indigenous endophytic bacterial communities in field conditions.

2.2 Materials and Methods

2.2.1 *Growth Condition, Inoculation and Cultivation of Sweet Potato*

Two cultivars of sweet potato, Beniazuma (A) and Beniharuka (H), were used in this study. OYK Farming Ace (Hamaguchi Institute of Microbiology Inc., Kyoto, Japan, http://www.oyk.jp/) consisting of about 8E+9 CFU/ml endospores of one *Bacillus* sp. strain, was used as PGPR according to the manufacturer's instruction. One milliliter of OYK solution was diluted to 4L with sterilized distilled water, and twelve seedlings of each cultivar were dipped in the solution for 60 hours (O). The same numbers of the seedlings were soaked in distilled water as a control (C). These seedlings were transplanted at random at 20 cm intervals on ridges with 1 m spacing in a roof top experimental field (Aizaki et al., 2005) at Shimane University in Shimane, Japan. The field was filled with artificial soil (Viva soil; Toho Leo Co., Osaka, Japan) that had high porosity (45%) and contained very little nutrition, and a chemical fertilizer ($N:P_2O_5:K_2O$ = 4:8:15 g/m^2) was applied before planting. The plants were cultivated from June to November in 2015 with drip irrigation (Super Typhoon NETAFIM Co., Tel Aviv, Israel).

2.2.2 *Sample Collection and Isolation of Endophytic Bacteria*

At harvest, the fresh weights of the shoots and tubers of each sweet potato plant were measured. Culturable endophytes of sweet potato tubers were examined; among plant parts, the highest population was observed in tubers in our previous study (Itoh et al., 2019). The surface of each tuber samples was washed with running tap water for 10 minutes, and cut longitudinally with a sterilized knife at its middle part after wiping off the water with a paper towel; then, the cut surface was stamped on modified MR agar medium with and without the supplementation of ammonium nitrate as a nitrogen source (Elbeltagy et al., 2001) in a petri dish. The ingredients of the media are listed in Table S2.1. The efficiency of the washing procedure was evaluated by stamping the surface of the washed tubers on agar media. After incubation for 2 days at ca. 26 °C, all the bacterial colonies were transferred to either N-supplemented or N-free MR media for purification and then grouped based on their morphologies on the two media. Based on their relative abundance, 1-3

representative isolates from each group, comprising 30-81% of total isolates, were selected for further analysis (Table 2.1).

2.2.3 *Genetic Analysis of Endophytes*

Genomic DNA was extracted from each isolate, as described by Saeki et al. (Saeki et al., 2005) with slight modifications, and used as a template for PCR for the amplification of the partial 16S rRNA gene sequence. As an indication, of the dinitrogen-fixing potential of the isolates, *nifH* genes, which encode nitrogenase reductase, were PCR amplified, for which a small amount of culture was directly used as a template. The primers used were fD1 and rP2 (Weisburg et al., 1991) and PolF and PolR (Poly et al., 2001) for the 16S rRNA and *nifH* genes, respectively. The components of the PCR master mixtures and the PCR running conditions are summarized in Table S2.2. PCR products were purified and subjected to PCR cycle sequencing according to the procedures described previously (Adhikari et al., 2012).

The closest sequence in the database (https://www.ddbj.nig.ac.jp/) was determined by a BLAST (Altschul et al., 1997) search, and multiple sequence alignments were constructed using ClustalW 2.1 (Larkin et al., 2007). Alignments were manually edited and phylogenetic trees with the related reference genes were constructed using ClustalW 2.1 with the neighbor-joining method.

2.2.4 *Analysis of the Community Structure of Endophytes*

Based on the results of the BLAST search and phylogenetic analysis, relative abundance (%) was calculated according to the class and genus of the identified bacteria for each sample, reflecting the relative abundance on the plate (Table 2.1). These results were used to analyze the effects of OYK inoculation, the difference between the presence and absence of a nitrogen source in the medium, and the two sweet potato cultivars on the community structure of the endophytes. Principal component analysis (PCA) was applied on a genera basis using IMB SPSS Statistics ver. 25 (IBM Co., Armonk, NY, USA).

2.2.5 *Nucleotide Sequence Accession Numbers*

The sequence data generated in this study were deposited in the DDBJ Nucleotide Submission System under the accession numbers LC583148 to LC583248.

2.2.6 *Statistical Analysis*

Statistical analysis of the sweet potato cultivation data was performed using Student's *t*-test. The Shannon diversity index (*H'*) was calculated based on the identified genus to characterize diversities in the endophytic bacterial communities.

2.3 Results

2.3.1 *Effects of OYK Inoculation*

In terms of the dry weights of shoots and tubers, the growth of the sweet potato cultivar Beniharuka was better than that of Beniazuma, and there was no significant difference between samples with and without OYK inoculation in either cultivar (Figure 2.1).

2.3.2 *Isolation of Endophytic Bacterial Strains*

Originally 269 bacterial colonies appeared on the agar plates in total, of which 232 strains were successfully isolated. On the basis of their observed morphologies on the modified MR agar medium with and without nitrogen supplementation, the isolates were grouped into 6-17 groups in each sample. Based on their relative abundance, 1-3 representative isolates were selected from each group, comprising 30-81% of the original isolates; as a results, 109 isolates were selected in total for further analysis (Table 2.1).

2.3.3 *Genetic Analysis of Endophytes*

Among 109 selected endophytic bacterial isolates, 101 strains were successfully sequenced for the partial 16S rRNA gene. The results of the closest relatives in the DDBJ database are presented in Table S2.3 and Figure S2.1, and summarized in Table 2.2 and Figure 2.2. The isolates belonged to 25 bacterial genera in 9 classes, which showed 97-100% homology. Among the 101 identified bacterial strains, 55 representative strains from each genus in each sample were subjected to PCR for the *nifH* gene; however, none of the strains produced positive amplification, with *Bradyrhizobium elkanii* USDA 94 used as a positive control.

2.3.4 *Community Structure of Endophytes*

In control samples, α-Proteobacteria predominated (36-69%) in both cultivars, in which *Novosphingobium* sp. was dominant (36-54%). After the inoculation of OYK, the fate of *Novosphingobium* sp. was different between the cultivars. In Beniazuma, *Novosphingobium* sp. disappeared, while it remained (25-38%) in Beniharuka. *Rhizobium* sp. in N(+) disappeared in both cultivars after inoculation. With the disappearance of or decrease in

Novosphingobium sp. and *Rhizobium* sp., two other genera in α-Proteobacteria, *Sphingomonas* sp. (6-21%) and *Sphingobium* sp. (8-15%), newly appeared, and *Chryseobacterium* sp. (21-24%) and *Acinetobacter* sp. (21%) in Flavobacteriia also appeared in Beniazuma. Bacilli (8-10%) disappeared only in Beniazuma after inoculation, while it persisted in Beniharuka. While Sphingobacteriia tended to be detected in Beniazuma (9-21%), Actinobacteria was detected in Beniharuka (8-27%), and β-Proteobacteria was similarly detected in both cultivars (7-17%).

To further elucidate the influence of the OYK inoculation, PCA was conducted to evaluate the relative abundance of the endophytic genera in Table 2.2. The first and second component factors explained 61.1% and 13.8% of the variation, respectively (Figure 2.3). All control samples, including both cultivars and both media conditions, were positioned close to each other, while the OYK-inoculated samples were positioned farther apart for each cultivar, especially in Beniazuma. The effects of the presence or absence of nitrogen in the media were not apparent.

2.3.5 *Diversity of Endophytes*

Shannon diversity indices (*H*) calculated on the genus level, were increased with the inoculation of OYK in all conditions (Figure 2.4 and Figure S2.2). The increase appeared to be larger in endophytic communities that were isolated using nitrogen-free media, although the indices were similar among the control samples. No difference between the cultivars was apparent.

2.4. Discussion

Bacillus strains have been well recognized as PGPR for their plant growth-promoting performance in sweet potato (Dawwam et al., 2013), tomato (Felici et al., 2008; Valenzuela-Soto et al., 2010; Qiao et al., 2017; Nascimento et al., 2019), mulberry (Weifang et al., 2019), lettuce (Chowdhury et al., 2013), wheat (Zhao et al., 2015), pepper (Yu et al., 2011), potato (Khedher et al., 2015), tobacco (Dutta et al., 2013; Li et al., 2007) and saffron (Sharaf-Eldin et al., 2008), as well as their antimicrobial activities against pathogens (Marques et al., 2015; Puri et al., 2018a), and they are commercially available for their potential use in agriculture (Paulitz et al., 2001; Lacey et al., 2001). However, in our study, the PGPR properties of OYK were not observed (Figure 2.1). One possible reason might be that the inoculated OYK disappeared during the cultivation due to environmental factors and competition with indigenous rhizobacteria, as discussed below.

The endophytic community structure has been reported to be determined by several factors, such as plant genotype, soil type (Singh et al., 2009), and environmental conditions, as well as stochastic sampling factors (Hardoim et al., 2008). In the present study, analysis of the bacterial endophytes of sweet potato revealed that Proteobacteria was the dominant phylum in the communities, followed by Flavobacteria, Sphingobacteria, Actinobacteria, and Bacilli. α-Proteobacteria was the dominant class in Proteobacteria, followed by β- and γ-Proteobacteria (Table 2.2). In previous studies of sweet potato endophytes, Proteobacteria, including α-, β-, and γ-Proteobacteria, Flavobacteria, Actinobacteria, and Bacilli were also predominant among isolates (Marques et al., 2015; Puri et al., 2018a; Puri et al., 2018b). These results suggest that the endophytic community of sweet potato consists of bacteria belonging to common phyla.

Almost all of the detected genera in Proteobacteria, Actinobacteria, and Bacilli have been reported as endophytes in sweet potato (Marques et al., 2015; Puri et al., 2018a; Puri et al., 2018b) except for *Novosphingobium* sp., which was the dominant genus in most samples. The other dominant genera in our study, *Chryseobacterium* sp., *Acinetobacter* sp., *Mucilaginibacter* sp., and *Sphingobacterium* sp., have not been reported as endophytes. The

genera in *Flavobacteria* and *Sphingobacteria* were isolated from the cultivar Beniazuma, suggesting that these isolates were sweet potato cultivar-dependent. Differences in endophytic and rhizosphere bacterial communities among sweet potato cultivars have also been demonstrated (Marques et al., 2104; Marques et al., 2015). On the other hand, the common dominant genera in the other studies, *Enterobacter* sp., *Pantoea* sp., *Luteibacter* sp., *Herbaspirillum* sp., and *Curtobacterium* sp., were not isolated in our study, suggesting the presence of diverse bacterial endophytes of sweet potato, with some common genera.

The inoculation of OYK changed the composition of the indigenous bacterial endophytic communities on both the phylum and genus levels, though OYK itself failed to maintain a population as an endophyte. The effects were similar between N(+) and N(−) media, while they were different between the Beniazuma (A) and Beniharuka (H) cultivars, especially for *Novosphingobium* sp., which was dominant in all control samples and disappeared in Beniazuma (A) while remaining predominant in Beniharuka (H). *Flavobacteria* and *Sphingobavteria* in Beniazuma (A) only appeared after the inoculation of OYK, which could have caused the change in the community structures found in PCA (Figure 2.3). Although only one sample of the sweet tuber was used for each cultivar and media condition, the closer positions of the control samples indicate that variability in the community structures of the control samples was within a certain range and that the different positions in PCA were caused by the inoculation of OYK. These results suggest that interactive endophytic bacterial behavior might be influenced by the cultivar of sweet potato. It has been reported that the plant cultivar and genotype affect communities of rhizobacteria, presumably as a result of competition for different root exudates (Dalmastri et al., 1999; Miller et al., 1989; Fromin et al., 2001). Differences in a rhizobacterial community might affect the corresponding endophytic community as a result. Germida et al. (1998) compared rhizoplane and endophytic bacteria strains that were isolated from canola plants and suggested that endophytes are a subset of the rhizoplane community. Additionally, differences in nutritional compositions of endophytic environments will also affect the community through competition.

In a seed and soil inoculation experiment with *Bacillus* spp., the *Bacillus* inocula failed to establish as endophytes in broccoli roots, as in our study, and the main effects of the Bacillus inoculation were a reduction in *Lysobacter* and *Acidovorax* and an increase in *Acinetobacter*, as analyzed by metagenomic sequencing (Gadhave et al., 2018). The authors also reported that the addition of *B. amyloliquefaciens* influenced the endophytic microbial community: the most common *Pseudomonas* endophytes decreased in abundance, accompanied by an increase in *Dyadobacter*, *Variovorax*, *Tahibacter*, and *Sphingomonas*. In contrast, the inoculation of *B. cereus* and *B. subtilis* did not affect the population of *Pseudomonas* though it changed the endophytic community composition of minor genera. Although the genera affected by the *Bacillus* inoculation were different from those in our study, the results obtained by culture-dependent and -independent studies suggest that a microbial inoculation can change an endophytic microbial community, even if the inoculant cannot establish a population as an endophyte. As many studies have shown the importance of endophytes for plant growth promotion, elucidating the interaction mechanisms is an essential line of research.

Although *Bacillus* spp. have been reported as indigenous endophytes in sweet potato (Marques et al., 2014; Puri et al., 2018a; Puri et al., 2018b) and in other crops such as tomato (Tian et al., 2017), banana (Souza et al., 2013), canola (Germida et al., 1998), and switchgrass (Xia et al., 2013), the inoculated OYK and *Bacillus* spp. strains (Gadhave et al., 2018) could not establish populations as endophytes. On the other hand, the inoculation of endophytic *Bacillus subtilis*, isolated from wheat, could establish a population in wheat root and showed potential as a biological control against plant pathogens (Liu et al., 2009). Changes in the compositions of plant metabolites and root exudates that would be caused by OYK might directly change indigenous rhizospheric and endophytic microbial communities and/or might indirectly prevent the successful colonization of OKY due to competition with microbial communities for compounds. As OYK was isolated from the soil, the endophytic potential of an inoculant, whether it was originally isolated as an endophyte, seems to be important. The Shannon diversity index (H) of the isolated endophytic community increased with OYK inoculation (Figure 2.4). The tendency was the

same as that in the results obtained by Gadhave et al. (2018), who also reported an increase in diversity in both *Bacillus amyloliquefaciens*- and mixed *Bacillus* spp.-treated sprouting broccoli, examined by a culture-independent metagenomic approach. In both studies, using different approaches, the number of genera identified increased with the inoculation; however, the mechanisms are still unclear.

2.5 Conclusions

When the commercial biofertilizer, OYK, consisting of a *Bacillus* sp., was applied to two cultivars of sweet potato, the inoculation changed the culturable indigenous endophytic bacterial communities, differently between the cultivars, and increased the diversity of the bacterial communities, although the inoculated OYK was not detected and significant plant-growth-promoting effects were not observed. Competition of the inoculant with indigenous rhizobacteria and endophytes may determine the fates of the inoculant and the endophytic community. Origin of the inoculant, which was isolated from soil, was expected as the possible reasons for the lack of the endophytic potential.

Table 2.1 Number of isolated endophytic bacterial strains of sweet potato, types of morphologies on the agar plates, and strains selected for sequence analysis.

Sample[a]	CFU[b]	Isolated[c]	Morphology[d]	Selected[e]	Identified[f]
AO-N(+)	32	32	11	14	14
AO-N(-)	42	40	17	17	17
AC-N(+)	22	13	6	10	10
AC-N(-)	24	18	9	12	11
HO-N(+)	50	50	12	15	13
HO-N(-)	46	42	14	15	12
HC-N(+)	31	21	11	13	13
HC-N(-)	22	16	11	13	11
Total	269	232	-	109	101

[a] Endophytic strains were isolated from the sweet potato cultivars, Beniazuma (A) and Beniharuka (H). Sweet potato seedlings were inoculated with OYK (O) as PGPR, or with distilled water as the control (C). The modified MR agar medium was used for isolation with nitrogen supplementation (N (+)) or without a nitrogen (N (-)) source.

[b] Number of colonies that appeared on the original agar plates.

[c] Number of successfully isolated colonies.

[d] Number of morphologies observed.

[e] Number of isolates selected based on the relative abundances of morphologies for sequence analysis.

[f] Number of strains successfully sequenced.

Table 2.2 Relative abundance (%) of endophytes from two cultivars of sweet potato, with and without OYK inoculation as PGPR. Bacteria were cultured using a modified MR medium, with and without a supplemental nitrogen source.

Class/ Genus	Beniazuma (A)				Beniharuka (H)			
	N (+)		N (-)		N (+)		N (-)	
	OYK	CTL	OYK	CTL	OYK	CTL	OYK	CTL
Alphaproteobacteria	**21**	**60**	**29**	**55**	**62**	**69**	**50**	**36**
Novosphingobium	-	50	-	45	38	54	25	36
Rhizobium	-	10	6	-	-	15	-	-
Sphingomonas	21	-	6	-	8	-	8	-
Sphingobium	-	-	12	-	15	-	8	-
Caulobacter	-	-	6	9	-	-	8	-
Betaproteobacteria	**7**	**10**	**-**	**9**	**15**	**8**	**17**	**-**
Methylibium	-	10	-	-	-	-	-	-
Burkholderia	-	-	-	9	-	-	-	-
Variovorax	7	-	-	-	8	8	-	-
Mitsuaria	-	-	-	-	8	-	17	-
Gammaproteobacteria	**7**	**-**	**12**	**9**	**-**	**-**	**8**	**-**
Pseudoxanthomonas	7	-	-	-	-	-	8	-
Stenotrophomonas	-	-	6	-	-	-	-	-
Pseudomonas	-	-	6	-	-	-	-	-
Dyella	-	-	-	9	-	-	-	-
Flavobacteriia	**43**	**-**	**24**	**-**	**-**	**-**	**-**	**-**

Chryseobacterium	21	-	24	-	-	-	-	-
Acinetobacter	21	-	-	-	-	-	-	-
Sphingobacteriia	**21**	**20**	**18**	**9**	**8**	**-**	**-**	**-**
Mucilaginibacter	-	20	-	9	8	-	-	-
Sphingobacterium	21	-	-	-	-	-	-	-
Pedobacter	-	-	18	-	-	-	-	-
Actinobacteria	**-**	**-**	**12**	**-**	**8**	**15**	**8**	**27**
Microbacterium	-	-	-	-	8	15	8	27
Streptomyces	-	-	6	-	-	-	-	-
Lysinimonas	-	-	6	-	-	-	-	-
Cytophagia	**-**	**-**	**6**	**9**	**-**	**-**	**-**	**9**
Dyadobacter	-	-	6	-	-	-	-	9
Chryseolinea	-	-	-	9	-	-	-	-
Bacilli	**-**	**10**	**-**	**9**	**8**	**8**	**8**	**27**
Bacillus	-	10	-	9	8	8	8	27
Chitinophagia	**-**	**-**	**-**	**-**	**-**	**-**	**8**	**-**
Filimonas	-	-	-	-	-	-	8	-

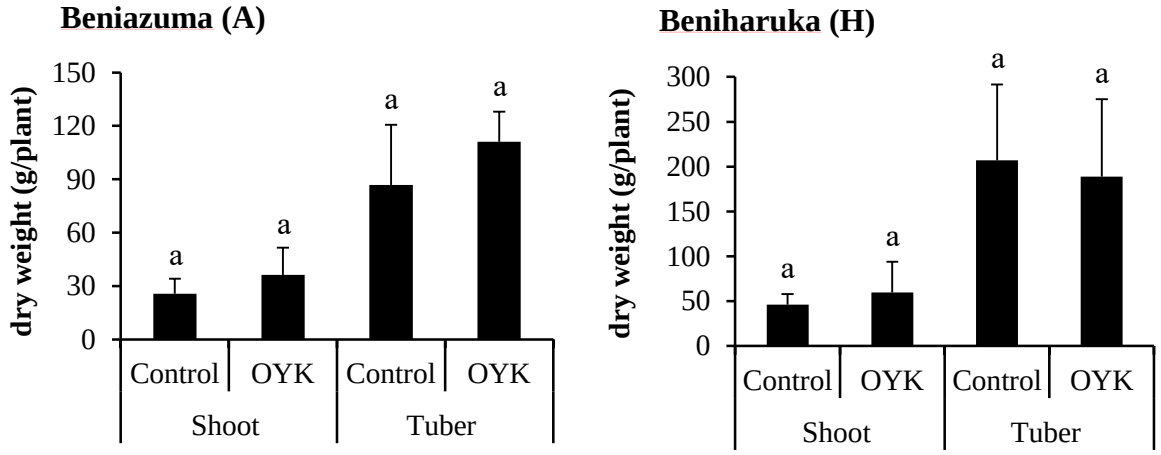

Figure 2.1 Dry weight of sweet potato cultivars, Beniazuma (A) and Beniharuka (H), inoculated with PGPR, OYK compared with control. The bars represent standard deviation (n = 3) and different letters indicate significant differences at $P < 0.05$ by student's *t*-test.

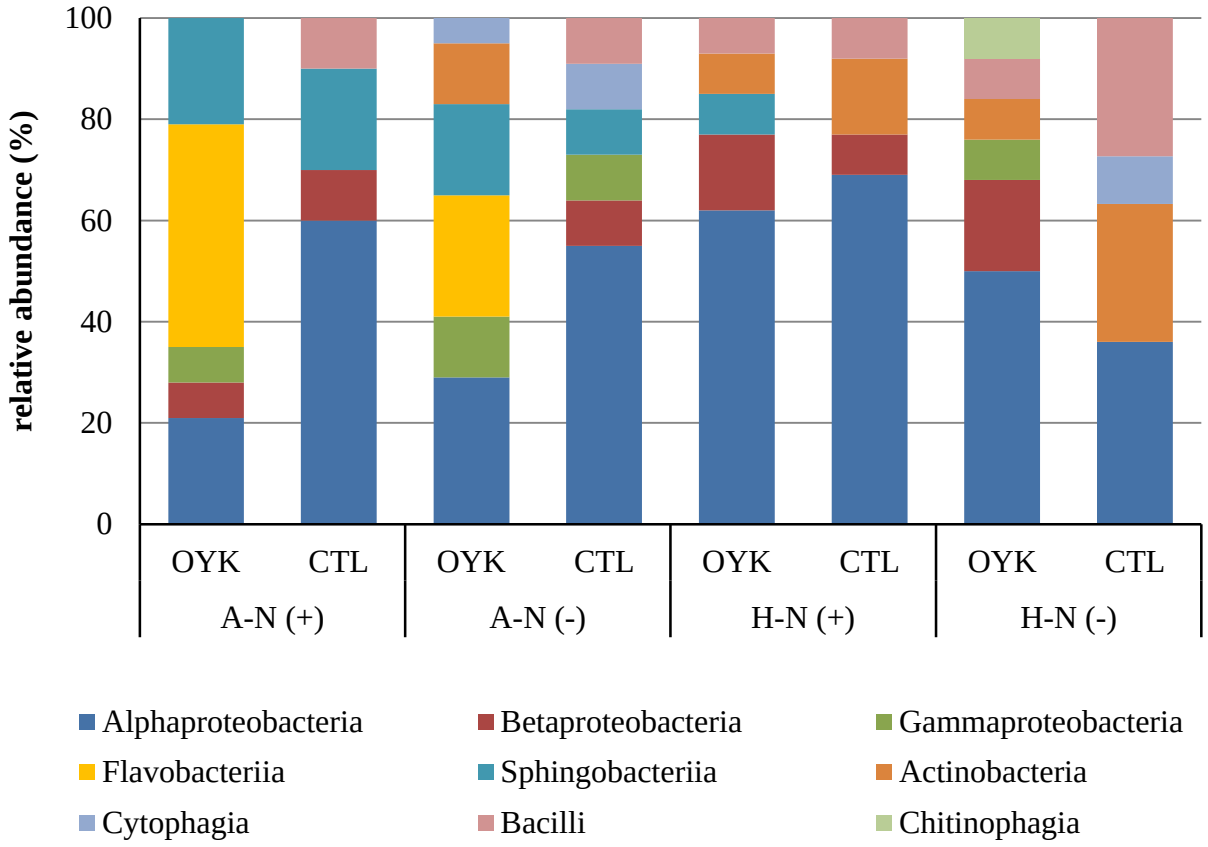

Figure 2.2 Relative class composition of endophytes of sweet potato cultivars, Beniazuma (A) and Beniharuka (H), inoculated with PGPR, OYK compared with control, using a modified MR medium supplemented with and without nitrogen source.

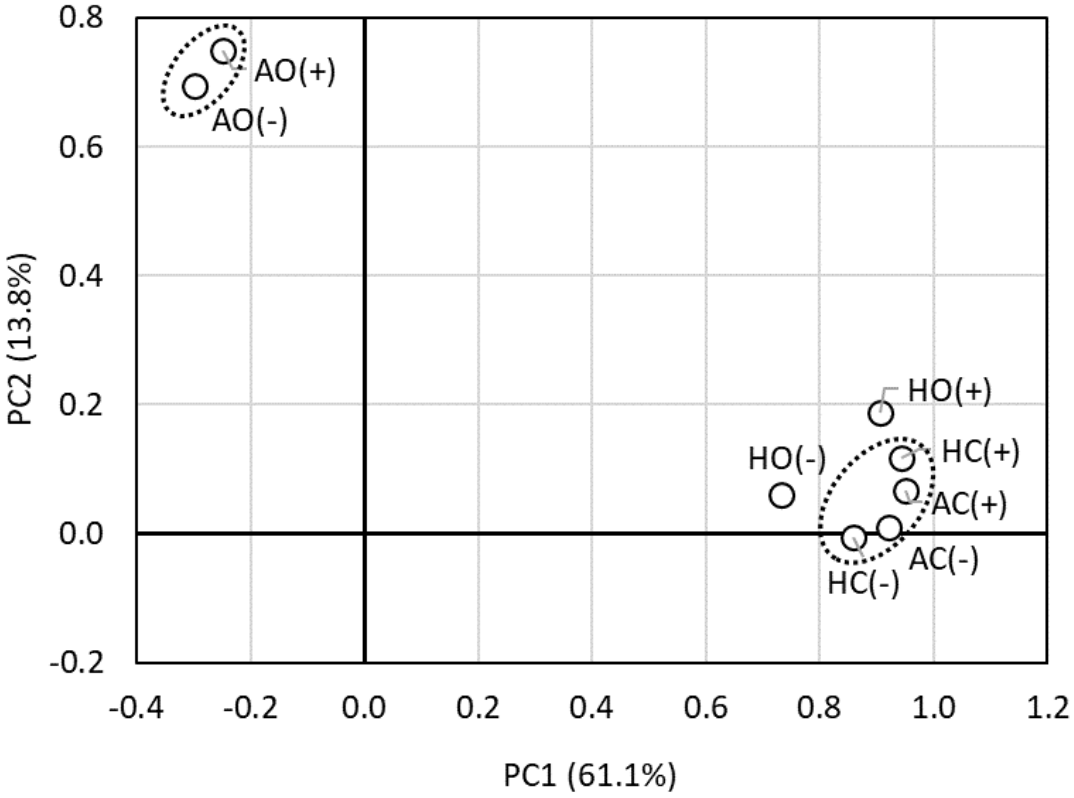

Figure 2.3 Principal component analysis (PCA) of endophytic communities of two sweet potato cultivars, Beniazuma (A) and Beniharuka (H), inoculated with OYK (O) as PGPR compared with the control (C). Bacteria were cultured using a modified MR medium with (+) and without (–) a supplemental nitrogen source. PCA was performed based on the bacterial genera in Table 2.2.

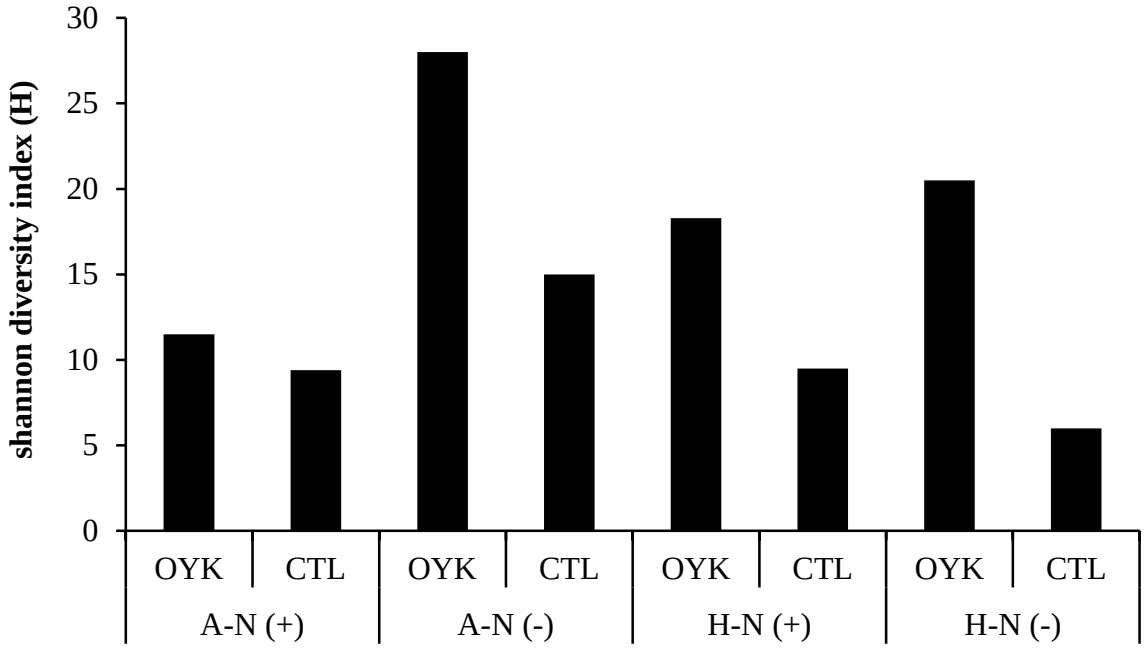

Figure 2.4 Shannon diversity index (H) of endophytic communities of two sweet potato cultivars, Beniazuma (A) and Beniharuka (H), inoculated with OYK as PGPR compared with the control. Bacteria were cultured using a modified MR medium with and without a supplemental nitrogen source.

Supplementary Tables and Figures

Table S2.1 Ingredients of modified MR (N-free MR) agar medium.

Ingredients	**Amount (L^{-1})**
K_2HPO_4	0.8g
KH_2PO_4	0.2g
NaCl	0.1g
$Na_2MoO_4 \cdot 2H_2O$	0.025g
Fe(III)-EDTA	0.012g
yeast extract	0.1g
sucrose	0.5g
mannitol	0.3g
malic acid	0.13g
$MgSO_4 \cdot 7H_2O$	0.2g
$CaCl_2 \cdot 2H_2O$	0.044g
p-aminobenzoic acid	10μg
biotin	5μg
agar	20g
pH	6.8

NH_4NO_3 (0.1g/L) was added for N-plus MR medium.

Table S2.2 PCR ingredients for amplification of 16S rRNA and *nifH* genes.

Ingredients	**Amount (µL)**	
	16S rRNA	***nifH***
Reaction buffer(10X) (BIONEER)	1.0	1.0
dNTPs mixture (2.5mM) (BIONEER)	0.2	0.25
forward primer (12.5µM)[a]	0.4	0.4
reverse primer (12.5µM[a]	0.4	0.4
Taq DNA polymerase (BIONEER)	0.1	0.5
DNA template/Culture	0.5	-[b]
MilliQ water	7.4	7.45
Total	**10**	**10**

[a]fD1 and rP2 (Weisburg *et al*. 1991) and PolF and PolR (Poly *et al*., 2001) for 16S rRNA and *nifH* genes, respectively.

[b]A small amount of culture was directly used as template.

Table S2.3 Closest relative of endophytic bacterial strains from two cultivars of sweet potato with and without inoculation of OYK as PGPR. Bacteria were cultured using a modified MR medium with and without a supplemental nitrogen source.

Strain[a]	Closest relative	Acc. No.	Id. (%)	Class
AO-N(+) 3	*Acinetobacter* sp. BRIO61	KC715854	100	Flavobacteriia
AO-N(+) 5	*Chryseobacterium daecheongense* PICdvs	KF015228	100	Flavobacteriia
AO-N(+) 6	*Chryseobacterium daecheongense* PICdvs	KF015228	100	Flavobacteriia
AO-N(+) 7	*Pseudoxanthomonas mexicana* YU23S MCC3122	MH021678	97	Gammaproteobacteria
AO-N(+) 9	*Acinetobacter* sp. BRIO61	KC715854	100	Flavobacteriia
AO-N(+) 10	*Acinetobacter* sp. BRIO61	KC715854	100	Flavobacteriia
AO-N(+) 12	*Sphingobacterium mucilaginosum* THG-SQA8	KM598234	99	Sphingobacteriia
AO-N(+) 14	*Sphingobacterium siyangense* 9I	KC329836	99	Sphingobacteriia
AO-N(+) 20	*Sphingomonas* sp. M37-VN10-2W	AB299579	96	Alphaproteobacteria
AO-N(+) 22	*Chryseobacterium* sp. CPW406	AJ457206	99	Flavobacteriia
AO-N(+) 23	*Sphingomonas* sp. M37-VN10-2W	AB299579	99	Alphaproteobacteria
AO-N(+) 26	*Variovorax* sp. T529	MG820625	99	Betaproteobacteria
AO-N(+) 29	*Sphingomonas* sp. M37-VN10-2W	AB299579	100	Alphaproteobacteria
AO-N(+) 30	*Sphingobacterium siyangense* 9I	KC329836	99	Sphingobacteriia
AO-N(-) 1	*Sphingobium amiense* D3AT58	JF459959	99	Alphaproteobacteria
AO-N(-) 2	*Stenotrophomonas maltophilia* F3-1-27	KX350012	100	Gammaproteobacteria
AO-N(-) 3	*Sphingobium rhizovicinum* CC-FH12-1	EF465534	98	Alphaproteobacteria
AO-N(-) 5	*Caulobacter* sp. Alpha-64	MH686114	100	Alphaproteobacteria
AO-N(-) 6	*Lysinimonas* sp. LM-2018	MG934617	99	Actinobacteria
AO-N(-) 8	*Chryseobacterium* sp. JCM 28637	LC133668	99	Flavobacteriia
AO-N(-) 9	*Pedobacter* sp. RG53-111M1	KP708597	98	Sphingobacteriia
AO-N(-) 12	*Streptomyces* sp. CR22	MH718844	100	Actinobacteria
AO-N(-) 19	*Sphingomonas* sp. C19	KU323611	99	Alphaproteobacteria
AO-N(-) 22	*Dyadobacter fermentans* PG18	KU350606	99	Cytophagia
AO-N(-) 24	*Chryseobacterium* sp. SAUBS3-1	KC243283	100	Flavobacteriia
AO-N(-) 28	*Chryseobacterium* sp. SAUBS3-1	KC243283	99	Flavobacteriia
AO-N(-) 32	*Chryseobacterium* sp. SAUBS3-1	KC243283	100	Flavobacteriia
AO-N(-) 33	*Rhizobium* sp. 5A2	MG763166	98	Alphaproteobacteria
AO-N(-) 34	*Pseudomonas* sp. NCCP-566	AB740384	99	Gammaproteobacteria

AO-N(-) 40	*Pedobacter humicola* C7	MH348785	100	Sphingobacteriia
AO-N(-) 41	*Pedobacter humicola* C7	MH348785	99	Sphingobacteriia
AC-N(+) 1	*Rhizobium cauense* UAGB147	MH537754	99	Alphaproteobacteria
AC-N(+) 2	*Novosphingobium rhizosphaerae* JM-1	KM365125	98	Alphaproteobacteria
AC-N(+) 3	*Mucilaginibacter* sp. Aws5	JQ977404	98	Sphingobacteriia
AC-N(+) 4	*Novosphingobium rhizosphaerae* JM-1	KM365125	99	Alphaproteobacteria
AC-N(+) 6	*Novosphingobium aromaticivorans* 16J	KF381499	99	Alphaproteobacteria
AC-N(+) 9	*Novosphingobium rhizosphaerae* JM-1	KM365125	99	Alphaproteobacteria
AC-N(+) 15	*Mucilaginibacter* sp. Aws5	JQ977404	97	Sphingobacteriia
AC-N(+) 16	*Methylibium* sp. UTPF84a	AB769202	99	Betaproteobacteria
AC-N(+) 17	*Novosphingobium rhizosphaerae* JM-1	KM365125	99	Alphaproteobacteria
AC-N(+) 19	*Bacillus aryabhattai* B39	LN890215	100	Bacilli
AC-N(-) 1	*Novosphingobium rhizosphaerae* BF-R16	KY292432	100	Alphaproteobacteria
AC-N(-) 2	*Novosphingobium aromaticivorans* 16J	KF381499	99	Alphaproteobacteria
AC-N(-) 3	*Novosphingobium aromaticivorans* 16J	KF381499	97	Alphaproteobacteria
AC-N(-) 8	*Chryseolinea serpens* RYG	NR_108511	99	Cytophagia
AC-N(-) 9	*Burkholderia* sp. KN-28	AB911063	99	Betaproteobacteria
AC-N(-) 10	*Novosphingobium aromaticivorans* 16J	KF381499	99	Alphaproteobacteria
AC-N(-) 11	*Novosphingobium rhizosphaerae* BF-R16	KY292432	97	Alphaproteobacteria
AC-N(-) 12	*Mucilaginibacter Polysacchareus* MRP-14	AB908085	98	Sphingobacteriia
AC-N(-) 14	*Dyella* sp. B12	MF093194	99	Gammaproteobacteria
AC-N(-) 17	*Bacillus altitudinis* MGB3034	MH261049	100	Bacilli
AC-N(-) 20	*Caulobacter* sp. NS11A2	MH899441	98	Alphaproteobacteria
HO-N(+) 11	*Sphingomonas* sp. DhA-33	AJ011505	99	Alphaproteobacteria
HO-N(+) 13	*Mucilaginibacter* sp. QM49	HM204922	98	Sphingobacteriia
HO-N(+) 14	*Novosphingobium* sp. GR 3-02	KM253064	99	Alphaproteobacteria
HO-N(+) 20	*Microbacterium binotii* R6-367	JQ659823	100	Actinobacteria
HO-N(+) 21	*Bacillus megaterium* SP1	KU529280	100	Bacilli
HO-N(+) 25	*Variovorax* sp. Beta-43	MH698893	99	Betaproteobacteria
HO-N(+) 29	*Novosphingobium* sp. GR 3-02	KM253064	99	Alphaproteobacteria
HO-N(+) 34	*Sphingobium* sp. DR 1-12	KM252997	99	Alphaproteobacteria
HO-N(+) 36	*Novosphingobium* sp. GR 3-02	KM253064	99	Alphaproteobacteria
HO-N(+) 41	*Sphingobium* sp. CAP-1	MG966444	99	Alphaproteobacteria
HO-N(+) 43	*Novosphingobium* sp. GR 3-02	KM253064	99	Alphaproteobacteria
HO-N(+) 44	*Mitsuaria* sp. BFE1N	KM187028	98	Betaproteobacteria

HO-N(+) 46	*Novosphingobium* sp. GR 3-02	KM253064	99	Alphaproteobacteria
HO-N(-) 02	*Novosphingobium* sp. GR 3-02	KM253064	99	Alphaproteobacteria
HO-N(-) 03	*Novosphingobium rhizosphaerae* JM-1	KM365125	99	Alphaproteobacteria
HO-N(-) 04	*Caulobacter* sp. Alpha-64	MH686114	99	Alphaproteobacteria
HO-N(-) 06	*Filimonas aquilariae* CC-YHH650	NR157994.1	100	Chitinophagia
HO-N(-) 21	*Bacillus megaterium* YJB3	KU291378	100	Bacilli
HO-N(-) 22	*Pseudoxanthomonas mexicana* LCG70	KY643721	99	Gammaproteobacteria
HO-N(-) 26	*Mitsuaria* sp. CR 6-14	KM252975	100	Betaproteobacteria
HO-N(-) 27	*Sphingomonas* sp. NBRC 101705	AB681531	99	Alphaproteobacteria
HO-N(-) 29	*Microbacterium* sp. M15S1	KX673839	99	Actinobacteria
HO-N(-) 31	*Sphingobium* sp. DR 1-12	KM252997	99	Alphaproteobacteria
HO-N(-) 38	*Novosphingobium* sp. GR 3-02	KM253064	99	Alphaproteobacteria
HO-N(-) 45	*Mitsuaria* sp. BFE1N	KM187028	100	Betaproteobacteria
HC-N(+) 1	*Novosphingobium rhizosphaerae* JM-1	KM365125	99	Alphaproteobacteria
HC-N(+) 2	*Novosphingobium rhizosphaerae* JM-1	KM365125	99	Alphaproteobacteria
HC-N(+) 3	*Novosphingobium rhizosphaerae* JM-1	KM365125	99	Alphaproteobacteria
HC-N(+) 5	*Rhizobium miluonense* UFLA03-466	MF495774	99	Alphaproteobacteria
HC-N(+) 11	*Bacillus megaterium* DC4	MF576262	99	Bacilli
HC-N(+) 15	*Microbacterium binotii* R6-367	JQ659823	100	Actinobacteria
HC-N(+) 18	*Microbacterium* sp. 2318	JX174195	98	Actinobacteria
HC-N(+) 19	*Rhizobium pusense* VAF1243	LC106994	100	Alphaproteobacteria
HC-N(+) 20	*Variovorax* sp. Beta-76	MH698926	99	Betaproteobacteria
HC-N(+) 25	*Novosphingobium rhizosphaerae* JM-1	KM365125	100	Alphaproteobacteria
HC-N(+) 26	*Novosphingobium rhizosphaerae* JM-1	KM365125	100	Alphaproteobacteria
HC-N(+) 27	*Novosphingobium rhizosphaerae* JM-1	KM365125	100	Alphaproteobacteria
HC-N(+) 31	*Novosphingobium rhizosphaerae* BF-R16	KY292432	100	Alphaproteobacteria
HC-N(-) 1	*Novosphingobium rhizosphaerae* JM-1	KM365125	100	Alphaproteobacteria
HC-N(-) 4	*Novosphingobium rhizosphaerae* JM-1	KM365125	100	Alphaproteobacteria
HC-N(-) 5	*Microbacterium* sp. 2318	JX174195	99	Actinobacteria
HC-N(-) 7	*Microbacterium* sp. 2318	JX174195	100	Actinobacteria
HC-N(-) 10	*Dyadobacter fermentans* HJX4	KP979535	98	Cytophagia
HC-N(-) 11	*Microbacterium binotii* R6-367	JQ659823	99	Actinobacteria
HC-N(-) 12	*Bacillus* sp. QS16-25	MH769452	100	Bacilli
HC-N(-) 15	*Bacillus* sp. S1M4	LC099946	100	Bacilli
HC-N(-) 16	*Novosphingobium rhizosphaerae* JM-1	KM365125	99	Alphaproteobacteria

HC-N(-) 17	*Novosphingobium rhizosphaerae* JM-1	KM365125	98	Alphaproteobacteria
HC-N(-) 21	*Bacillus aerophilus* M102	LN997933	100	Bacilli

[a]Endophytic bacteria were isolated from the sweet potato cultivars Beniazuma (A) and Beniharuka (H). Sweet potato seedlings were inoculated with OYK (O) as PGPR, or with distilled water as the control (C). The modified MR agar medium was used for isolation with the supplementation of nitrogen (N(+)) or without a nitrogen (N(–)) source.

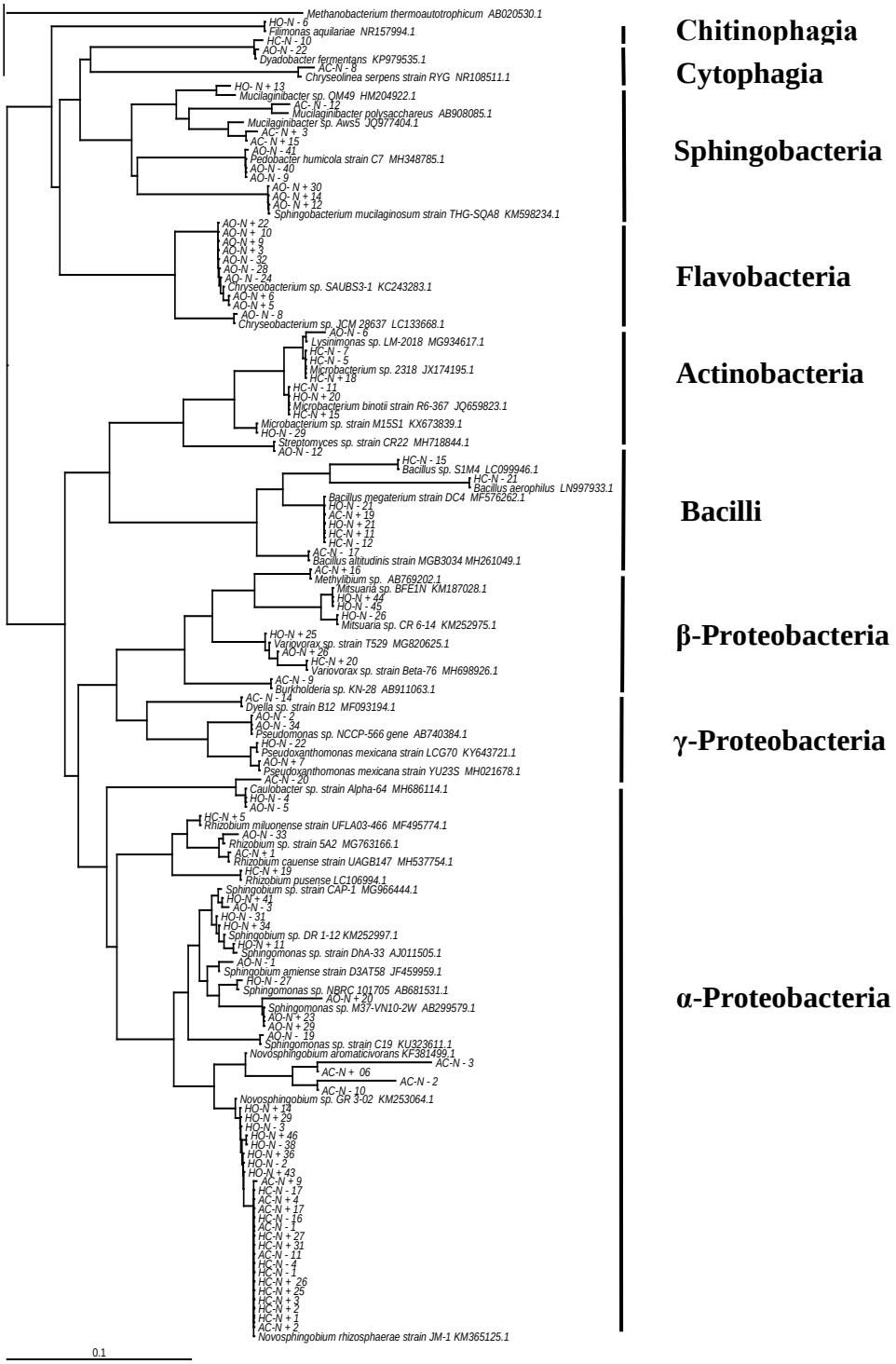

Figure S2.1 Phylogenetic tree of endophytes of two sweet potato cultivars, Beniazuma (A) and Beniharuka (H), inoculated with OYK as PGPR compared with the control, using modified MR medium with and without a supplemental nitrogen source. Phylogenetic analysis is based on partial 16S rRNA gene sequences. The sequence of *Methanobacterium thermoautotrophicum* (AB020530) served as an outgroup. The scale bar indicates the number of substitutions per site.

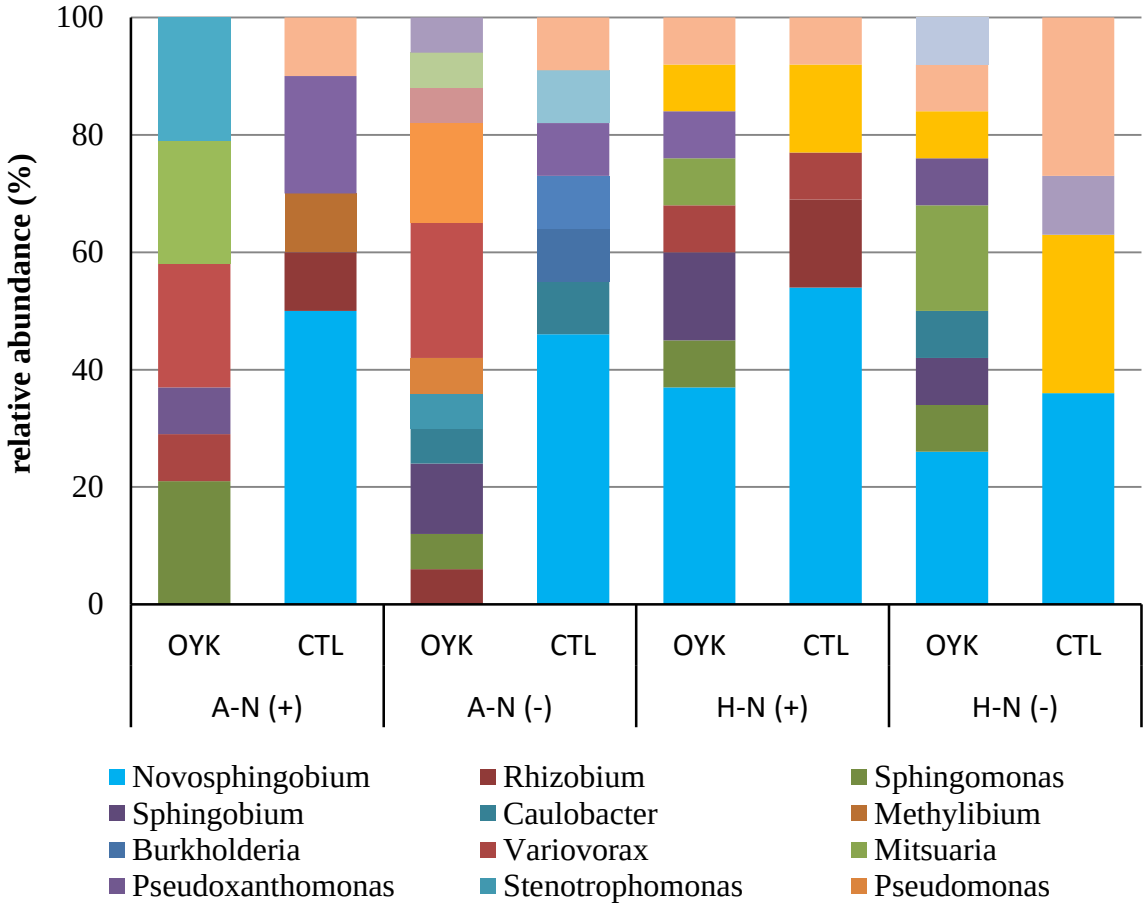

Figure S2.2 Relative genus composition of endophytes of two sweet potato cultivars, Beniazuma (A) and Beniharuka (H), inoculated with OYK as PGPR compared with the control, using modified MR medium with and without a supplemental nitrogen source.

CHAPTER THREE

Effect of Co-Inoculation of *Bacillus* sp. Strain with Bacterial Endophytes on Plant Growth and Colonization in Tomato Plant (*Solanum lycopersicum*)

3.1 Introduction

Plant growth-promoting rhizobacteria (PGPR) are becoming more widely accepted in intensive agriculture to enhance sustainable agricultural production in various parts of the world (Kaymak, 2010). PGPR contain a diverse range of bacteria and several mechanisms have been proposed though they are not fully understood (Glick, 1995). In sustainable agricultural practices using PGPR, inoculation techniques for their colonization at the rhizosphere is critical (Zakria et al., 2008); therefore, a further understanding of the interactions of PGPR with plant and indigenous rhizobacteria is essential.

Bacillus spp. have been recognized as one of the most important PGPR and widely used for sustainable agriculture as biofertilizers and/or antagonists against plant diseases (Vessey, 2003; Miljaković et al., 2020; Kumar et al., 2011; Govindasamy et al., 2010; Govindasamy et al., 2008). *Bacillus* spp. have also received considerable attention because of their benefits over other PGPR in producing stable formulations (Kumar et al., 2011; Emmert & Handelsman, 1999) and stability in rhizosphere soil in semi-arid deserts (Nain et al., 2012). In addition, *Bacillus* spp. exhibit a significant reduction in disease incidence on various crops by inducing systemic resistance (Kloepper et al., 2004; Choudhary & Johri, 2009) and by forming biofilm on root surfaces (Chen et al., 2013).

In our previous study, when the commercial biofertilizer OYK consisting of the *Bacillus* sp. strain was applied to sweet potato, no significant plant growth-promoting effect was observed, and the inoculated *Bacillus* sp. strain was not detected in the plant tubers. The possible reasons were due to competition of the inoculant against indigenous rhizobacteria and endophytes, and a lack of endophytic potential of the inoculant, which was originally isolated from soil (Salehin et al., 2020). As many endophytic *Bacillus* strains have been reported in several plants (Puri et al., 2018a, 2018b; Marques et al., 2014; Germida et al.,

1998; Tian et al., 2017; Souza et al., 2013; Xia et al., 2013; Liu et al., 2009), it is assumed that endophytic bacteria have some colonization strategies in interaction with plants.

In addition to the individual colonizing ability of PGPR, interactions with other co-existing bacteria would be important to determine the colonization and plant growth-promoting potential. Synergetic effects of the inoculation with the other PGPR have been reported in maize (Molina-Romero et al., 2017), cotton (Marimuthu et al., 2002), ryegrass (Castanheira et al., 2017), strawberry (Vestberg et al., 2004), and cucumber (Raupach and Kloepper, 1998). On the other hand, negative interactions with co-existing bacteria should also be considered. They inhibited the colonization of inoculants in sugarcane (Oliveira et al., 2008), and reduced the plant growth-promoting effects in tomato plant (Felci et al., 2008; Dhungana et al., 2019).

For efficient and practical use of PGPR, it is essential to understand its colonizing behavior and abilities to compete with co-existing bacteria. Though several studies have been reported on the effects of co-inoculation with multiple bacteria on plant growth, their effects on colonization have not been extensively studied yet. The aim of this study was to evaluate the colonization properties of *Bacillus* sp. OYK, which was isolated from a soil, in relation to its origin by comparing it with those of the other *Bacillus* sp. strains isolated from plant endosphere and rhizosphere, and then to elucidate the effects of co-inoculation of the endophytic *Bacillus* sp. strain with the other endophytes on their colonization and plant growth-promoting activities.

3.2 Materials and Methods

3.2.1. *Bacterial Strains*

In addition to *Bacillus* sp. OYK, three strains of *Bacillus* sp.: two strains (*Bacillus* sp. RF-12 and RF-37) isolated from the rhizosphere of sweet potato and another one (*Bacillus* sp. F-33) as an endophyte of the same plant cultivated in Japan (Puri et al., 2018b), and three strains of endophytes: *Herbaspirillum* sp. Sal 6, *Klebsiella* sp. Sal 1, and *Enterobacter* sp. Sal 3, isolated from Nepalese sweet potato (Puri et al., 2018a), were used in this study (Table 3.1).

3.2.2. *Plant Growth Promotion and Colonization of Bacillus sp. Strains in Tomato Plant*

To prepare the bacterial inoculum, each *Bacillus* sp. strain was cultivated in Modified Rennie (MR) (Elbeltagy et al., 2001) liquid medium with shaking at 150 rpm at 26 °C for 3 days. The culture was washed twice with sterilized distilled water by centrifugation at 10000× *g* at 4 °C for 10 min, and the cell pellet was resuspended with sterilized distilled water at 10^8 colony forming units (CFU)/mL to prepare an inoculum based on OD–CFU/mL correlated linear equations prepared for each strain.

In this study, we used tomato as a test plant due to the difficulty in preparing bacteria-free plants in sweet potato. Tomato seeds (*Solanum lycopersicum* L. cv. Chika F1 hybrid, Takii & Co., Ltd., Kyoto, Japan) were surface sterilized with 70% ethanol for 1 min followed by 1% sodium hypochlorite with 3–4 drops of Tween-20 for 13 min and washed 7–8 times with sterilized distilled water. The seeds were sown in the sterilized vermiculite in a Leonard jar (Leonard, 1943) supplied with the sterilized Hoagland solution (Hoagland and Arnon, 1950), and 1 mL of the inoculum was added onto the seed zone. The jar was put in a ventilated (<0.2 mm pore size) transparent plastic bag (Sun bag, Sigma-Aldrich, Tokyo, Japan), and after thinning out to one plant per jar, the tomato plant was aseptically cultivated in a phytotron (Model- LH 220S, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) at 28/25 °C (16h/8h, day/night) for 24 days. An autoclaved culture was used as a control, and the experiment was conducted twice, using three plants for each treatment.

After cultivation, the tomato crop was harvested, and the fresh weight and length of the root and shoot were measured. Then, the population of the inoculated strains in the root, shoot, and rhizosphere was determined using two plants for each treatment. A rhizosphere sample was prepared by dipping and gently shaking the roots in sterilized distilled water. After washing the plant surface 6–7 times with sterilized distilled water, the root and shoot samples were separated and macerated with sterilized distilled water using a sterilized motor and pestle, and the samples were subjected to dilution plating for the determination of CFU/g. At the same time, an aliquot of the final washing solution was directly plated, and no colony was observed. The inoculation experiment was conducted twice.

3.2.3. *Effect of Co-Inoculation on Plant Growth Promotion and Colonization of Bacillus sp. F-33 with the Other Endophytic Strains in Tomato Plant*

Bacillus sp. F-33 was used as a representative of the *Bacillus* sp. strains with the other endophytic strains, *Klebsiella* sp. Sal 1, *Enterobacter* sp. Sal 3, and *Herbaspirillum* sp. Sal 6, to examine the effect of co-inoculation on their plant growth promotion and colonization in the tomato plant.

Each bacterial strain was cultivated under the same conditions as described in Section 2.2 to prepare the inoculum at ca. 10^8 CFU/mL. In case of co-inoculation, the same volume of individual cell suspension was mixed. The sterilized seeds were sown in the sterilized vermiculite in a capped glass tube (12 cm × 3 cm) supplied with the sterilized Hoagland solution, and 1 mL of the inoculum was added onto the seed zone. The other procedures were the same as those described in Section 2.2 except that the cultivation period was 14 days, and that the plant samples were macerated using a BioMasher (Nippi, Tokyo, Japan). The morphologies of the colonies of the co-inoculated strains were clearly different for counting separately. The inoculation experiment was conducted twice.

3.2.4. *Effect of Time Interval Inoculation on Plant Growth Promotion and Colonization of Bacillus sp. F-33 and Klebsiella sp. Sal 1 in Tomato Plant*

Bacillus sp. F-33 and *Klebsiella* sp. Sal 1 were used as representatives of the *Bacillus* sp. and the endophytic strains, respectively, to examine the effect of time interval of

inoculation on their plant growth promotion and colonization in the tomato plant. The experimental procedures were the same as those described in Section 2.3 except that *Bacillus* sp. F-33 was inoculated first, and then *Klebsiella* sp. Sal 1 was separately inoculated 7 days after the first inoculation. The tomato plants were harvested at 14 days after the first inoculation. An experiment with a different order of inoculation, *Klebsiella* sp. Sal 1 first and *Bacillus* sp. F-33 second, was also conducted in the same way. The inoculation experiment was conducted twice, but one experiment was done using two plants and one of the plants was used to determine the population.

3.2.5. *Statistical Analysis*

Statistical analysis of the data on the plant growth and population of the inoculant obtained in each twice-repeated experiment was performed using the MSTAT-C 6.1.4 (Freed, 2007) software package. Data were subjected to Tukey's test after one-way ANOVA.

3.3 Results

3.3.1 *Plant Growth Promotion and Colonization of Bacillus sp. Strains in Tomato plant*

The effects of inoculation of the *Bacillus* sp. strains on the growth of the tomato plant are presented in Figure 3.1. All *Bacillus* sp. strains except for *Bacillus* sp. RF-37 showed plant growth promotion. The root and shoot weights, and the shoot lengths of the inoculated tomato plant were significantly larger than the control while the root lengths were not affected. More lateral root development was observed in the inoculated tomato plant compared with the control.

The populations of the inoculated *Bacillus* sp. strains in the rhizosphere, root, and shoot of the tomato plants are presented in Figure 3.2. All *Bacillus* sp. strains were detected in the rhizosphere, root, and shoot, and the populations of *Bacillus* sp. RF-12 and RF-37, which were originally isolated from the rhizosphere of sweet potato, and that of *Bacillus* sp. F-33, which was originally isolated as an endophyte of sweet potato, were higher than that of *Bacillus* sp. OYK, which was originally isolated from soil. The populations of the three *Bacillus* sp. strains were 0.9–2.2, 2.1–2.8, and 1.4–2.2 orders higher than those of *Bacillus* sp. OYK in the rhizosphere, root, and shoot, respectively. The populations were 2.4–4.0 and 3.1–5.2 orders higher in the rhizosphere than those in the root and shoot, respectively. No colony appeared in the control samples.

3.3.2. *Effect of Co-Inoculation on Plant Growth Promotion and Colonization of Bacillus sp. F-33 with the Other Endophytic Strains in Tomato Plant*

The effects of co-inoculation of *Bacillus* sp. F-33 with the other endophytic strains are presented in Figure 3.3. The plant growth tended to be promoted by *Bacillus* sp. F-33 but not significantly. The reduction tendencies of the effects were observed by co-inoculation of *Enterobacter* sp. Sal 3 and *Herbaspirillum* sp. Sal 6. In shoot weight and root length, the effects of the co-inoculation seemed to be negative in most cases.

All strains colonized tomato plants, resulting in a large population, in which those of the endophytic strains were 1.5–1.7, 1.7–2.6, and 1.2–2.3 orders higher than those of *Bacillus*

sp. F-33 in the rhizosphere, root, and shoot, respectively (Figure 3.4). Among the endophytic strains, the populations were not different in the rhizosphere, but the populations of *Herbaspirillum* sp. Sal 6 were about one order of magnitude higher than *Klebsiella* sp. Sal 1 in the plant parts. The populations were 1.8–2.7 and 2.3–3.3 orders higher at the rhizosphere than those in the root and shoot, respectively. No colony appeared in the control samples.

In case of the co-inoculation, no apparent change in the population was observed in most cases. In co-inoculation of *Bacillus* sp. F-33 and *Herbaspirillum* sp. Sal 6, however, the population in the shoot tended to decrease by 0.8 and 1.8 orders in *Bacillus* sp. F-33 and *Herbaspirillum* sp. Sal 6, respectively. In addition, one example of a positive tendency in the co-inoculation was observed in the population of *Klebsiella* sp. Sal 1 in the shoot, in which a 1.4-order increase was observed.

3.3.3. *Effect of Time Interval Inoculation on Plant Growth Promotion and Colonization of Bacillus sp. F-33 and Klebsiella sp. Sal 1 in Tomato Plant*

The effects of the time interval of inoculation of *Bacillus* sp. F-33 and *Klebsiella* sp. Sal 1 are presented in Figure 3.5. The plant growth seemed to be promoted by *Bacillus* sp. F-33 but not by *Klebsiella* sp. Sal 1. When *Klebsiella* sp. Sal 1 was inoculated after *Bacillus* sp. F-33, the plant growth-promoting effects tended to be reduced in root weight. On the other hand, when *Bacillus* sp. F-33 was inoculated after *Klebsiella* sp. Sal 1, the effects seemed to be increased compared with the single inoculation of *Klebsiella* sp. Sal 1.

In individual inoculation, populations of *Klebsiella* sp. Sal 1 were 1.9, 1.7, and 3.0 orders higher than those of *Bacillus* sp. F-33 in the rhizosphere, root, and shoot, respectively, and the populations were 2.7–2.8 and 2.5–3.7 orders higher in the rhizosphere than those in the root and shoot, respectively (Figure 3.6). When *Klebsiella* sp. Sal 1 was inoculated after *Bacillus* sp. F-33, the populations of *Bacillus* sp. F-33 were similar to those in the individual inoculation. When *Bacillus* sp. F-33 was inoculated after *Klebsiella* sp. Sal 1, those were 1.3–2.4 orders lower than those in individual inoculation. The populations of

Klebsiella sp. Sal 1 showed similar levels under any conditions. No colony appeared in the control samples.

3.4 Discussion

Significant plant growth-promoting properties were observed in the *Bacillus* sp. strains except for *Bacillus* sp. RF-37 (Figure 3.1). Similar PGPR properties in *Bacillus* spp. have been previously reported (Saharan and Nehra, 2011; Shen et al., 2012; Xu et al., 2014; Batista et al., 2018). In this study, the inoculants stimulated lateral root growth, resulting in greater root weight, which could explain the inconsistent results on root weight and root length in the inoculated plants. As indole-3-acetic acid (IAA) is known to have similar effects on plants (Egamberdieva, 2012), the plant growth promotion might be caused by IAA production by the inoculants. In another experiment, *Bacillus* sp. RF-12 and F-33 showed an IAA-producing ability while *Bacillus* sp. RF-37 did not (data not shown). However, since *Bacillus* sp. OYK also showed no activity, the reason for the plant growth promotion is unclear.

In addition to the IAA production, other tomato plant growth-promoting mechanisms by *Bacillus* spp. strains have been reported as follows: gibberellic acid (GA3) as well as IAA production (Xu et al., 2014; Chowdappa et al., 2013; Bahadir et al., 2018), organic acid production and phosphate-solubilizing abilities (Xu et al., 2014; Chowdappa et al., 2013; Bahadir et al., 2018), siderophores production (Xu et al., 2014; Abbamondi et al., 2016), nitrogen fixation (Xu et al., 2014), and 1-aminocyclopropane-1-carboxylate (ACC) deaminase production (Xu et al., 2014; Abbamondi et al., 2016).

In our previous study, the inoculated *Bacillus* sp. OYK strain could not establish its population as an endophyte in sweet potato (Salehin et al., 2020), although *Bacillus* spp. strains have been reported as indigenous endophytes in sweet potato (Puri et al., 2018a, 2018b), tomato (Tian et al., 2017), banana (Souza et al., 2013), and switchgrass (Xia et al., 2013). We attributed it to the competition with indigenous rhizobacteria and endophytes, as well as the endophytic ability of the inoculant.

In this study, all *Bacillus* strains colonized in the rhizosphere and endosphere of the tomato plants cultivated using sterilized vermiculite (Figure 3.2), suggesting that *Bacillus* sp. OYK has endophytic potential, and that the presence of indigenous microorganisms inhibited its

colonization. However, the 1.4–2.8 orders lower populations of *Bacillus* sp. OYK in the plants compared with the other *Bacillus* sp. strains, which were isolated from the rhizosphere or as an endophyte (Figure 3.2), suggests decreased competitiveness of *Bacillus* sp. OYK against indigenous plant-associated microbes. Some genes and functions may be involved in the plant colonization ability, and PGPR strains from different habitats may have different interactions with plants. The use of originally plant-associated PGPR could establish their populations at the rhizosphere and/or endosphere of plants.

The plant growth-promoting effects of *Bacillus* sp. F-33 were reduced in the presence of the other endophytes, though the population of *Bacillus* sp. F-33 was maintained (*Klebsiella* sp. Sal 1 and *Enterobacter* sp. Sal 3) or slightly decreased (*Herbaspirillum* sp. Sal 6) (Figures 3.3 and 3.4), suggesting that its phyto-stimulating ability was neutralized by the other strains. As the three co-inoculated strains have IAA-degrading ability (Dhungana et al., 2019), they might degrade IAA produced by *Bacillus* sp. F-33 below the effective level.

Synergetic effects of co-inoculation have been reported (Vestberg et al., 2004; Marimuthu et al., 2002; Castanheira et al., 2017; Molina-Romero et al., 2017), while cancelation of the positive effects (Schmidt et al., 2004; Dandurand and Knudsen, 1993; Garcia et al., 2004), and negative effects of co-inoculation have also been reported (Felci et al., 2008, Dhungana et al., 2019). The effects of the co-inoculation seemed to be dependent on the combination of the strains. In most studies that examined the effects of co-inoculation of PGPR, changes in populations of the PGPR by co-inoculation were not measured. In the limited examples of the study using *Azospirillum brasilense* Sp245 and *Bacillus subtilis* 101 (Felici et al., 2008), and *Klebsiella* sp. Sal 1 and *Herbaspirillum* sp. Sal 6 (Dhungana et al., 2019), their plant growth promotions were reduced even though the populations of the PGPR were maintained, as observed in this study. In our previous study, diverse endophytic bacterial communities were observed in sweet potato, and some components of the communities disappeared by inoculation of *Bacillus* sp. OYK (Salehin et al., 2020). It is crucial to elucidate the mechanisms of the microbial interactions; however, it might be complex given the actual environment.

After the establishment of *Bacillus* sp. F-33 in the rhizosphere and in the tomato plant, *Klebsiella* sp. Sal 1 could colonize the same population as the strain was individually inoculated (Figure 3.6) and inhibited the plant growth-promoting ability of *Bacillus* sp. F-33 without reducing its population (Figure 3.5), as in the co-inoculation experiment. The high colonizing potential of *Klebsiella* sp. Sal 1 seemed not to be affected by the about 2-orders lower population of the previously established *Bacillus* sp. F-33.

On the other hand, after the establishment of *Klebsiella* sp. Sal 1, the colonization of *Bacillus* sp. F-33 was reduced by 1.3–2.4 orders than those in the individual inoculation (Figure 3.6). The relatively lower potential for colonization of *Bacillus* sp. F-33 might be the reason. The microbial community structure might be a crucial factor to determine the fate of allochthonous microorganisms, such as a PGPR inoculant. Pre-inoculation of PGPR prior to transplantation could be one practical method to enhance higher colonization in plants.

In spite of the reduced population of *Bacillus* sp. F-33, the plant growth promotion was increased when the strain was inoculated after *Klebsiella* sp. Sal 1 (Figure 3.5). It was suggested that the level of the population is not a determinant of the potential of the strain. Although the population of *Bacillus* sp. F-33 was maintained both in the co-inoculation and in the inoculation of *Klebsiella* sp. Sal 1 after *Bacillus* sp. F-33, the PGPR potential of *Bacillus* sp. F-33 was reduced in the presence of *Klebsiella* sp. Sal 1, so unknown factors might be involved in plant growth promotion. In addition, the ratio between the populations might not be constant when plants developed, and the kinetic of the different bacterial populations might not be reflected by one sampling time. Time course analysis after inoculation could reveal the progress of colonization in the plant. The results of this study also indicate that there are different niches for the different strains and the colonization of these niches may not have the same impact on plant growth. It may mean that bacteria are competing for some niche colonization.

In addition to plant growth-promoting properties, the colonization potential should be considered as important criteria when assessing their suitability for commercial

development. The lower population of *Bacillus* sp. OYK, which was isolated from soil, than the other *Bacillus* sp. strains, which were isolated from either the rhizosphere or endosphere of plant samples, suggests the importance of the origin of the strains for their colonization. The plant growth promotion and colonization potentials were independently affected by the co-existing microorganisms. Further studies are necessary to evaluate the colonization potential of PGPR under field conditions where diverse microorganisms exist.

3.5 Conclusions

In this study, the higher population of rhizospheric and endophytic *Bacillus* sp. in the plant suggests the importance of the origin of the strains for their colonization. The plant growth promotion and colonization potentials were independently affected by the co-existing microorganisms.

Table 3.1 Bacterial isolates used in this study

Strain	Most Similar Genus [a]	Class	Origin	Accession Number
OYK	*Bacillus* sp.	Bacilli	Soil	LC590219
RF-12	*Bacillus* sp.	Bacilli	Rhizosphere	LC593252
RF-37	*Bacillus* sp.	Bacilli	Rhizosphere	LC593253
F-33	*Bacillus* sp.	Bacilli	Endophytic	LC430058
Sal 1	*Klebsiella* sp.	γ-Proteobacteria	Endophytic	LC389410
Sal 3	*Enterobacter* sp.	γ-Proteobacteria	Endophytic	LC389433
Sal 6	*Herbaspirillum* sp.	β-Proteobacteria	Endophytic	LC389442

[a] Based on the 16S rRNA gene sequence in the database.

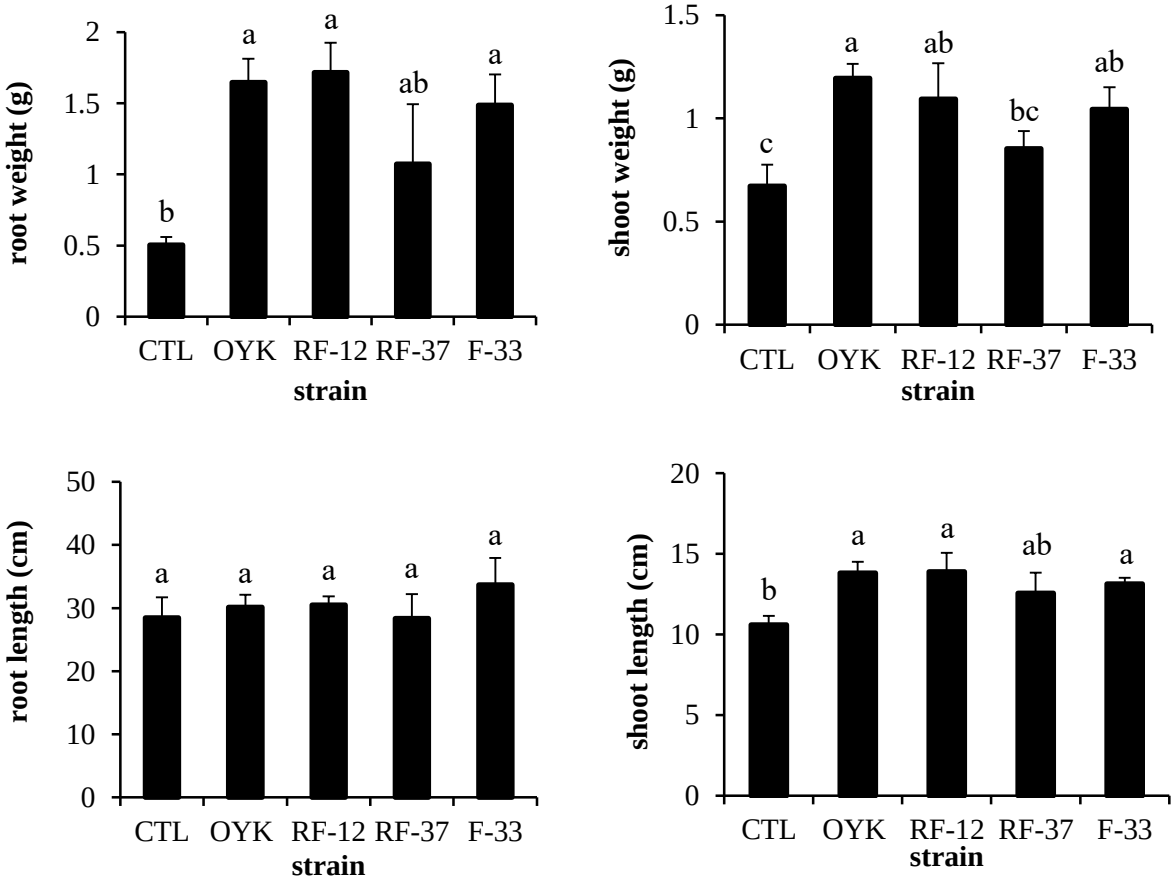

Figure 3.1 The effects of inoculation of *Bacillus* sp. strains on the growth of the tomato plant. The tomato plant was cultivated using sterilized vermiculite, and the parameters were measured at 24 days after seed inoculation. CTL represents the control samples inoculated with autoclaved cultures. The bars represent the standard deviation (n = 6), and different letters indicate significant differences at $p < 0.05$ by Tukey's test.

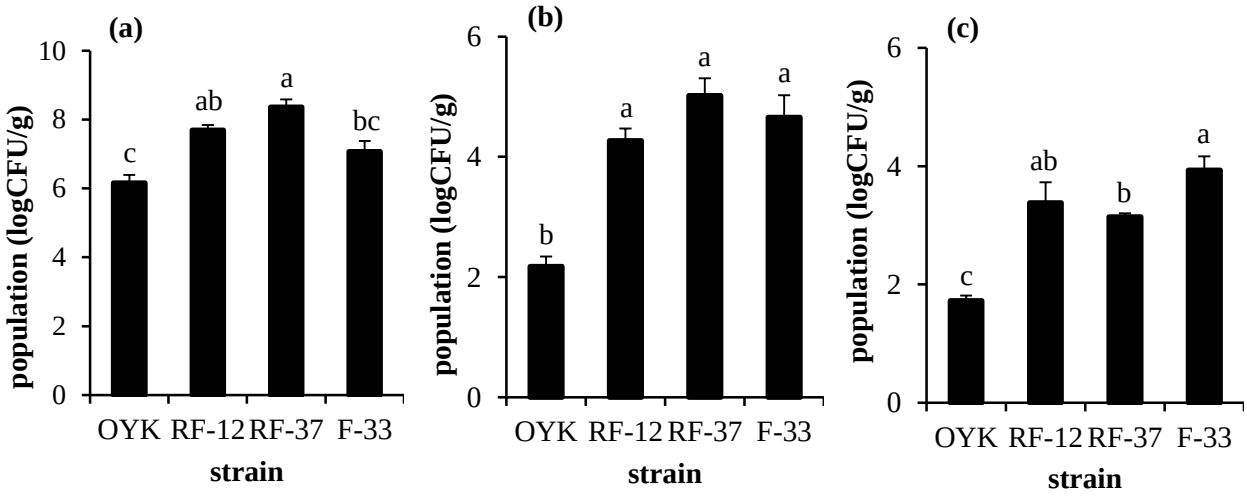

Figure 3.2 Colonization of seed-inoculated *Bacillus* sp. strains in the rhizosphere (**a**), root (**b**), and shoot (**c**) of the tomato plant. The tomato plant was cultivated using sterilized vermiculite, and colonization was examined at 24 days after seed inoculation. No colony appeared in the control samples. The bars represent the standard deviation (n = 4), and different letters indicate significant differences at $p < 0.05$ by Tukey's test.

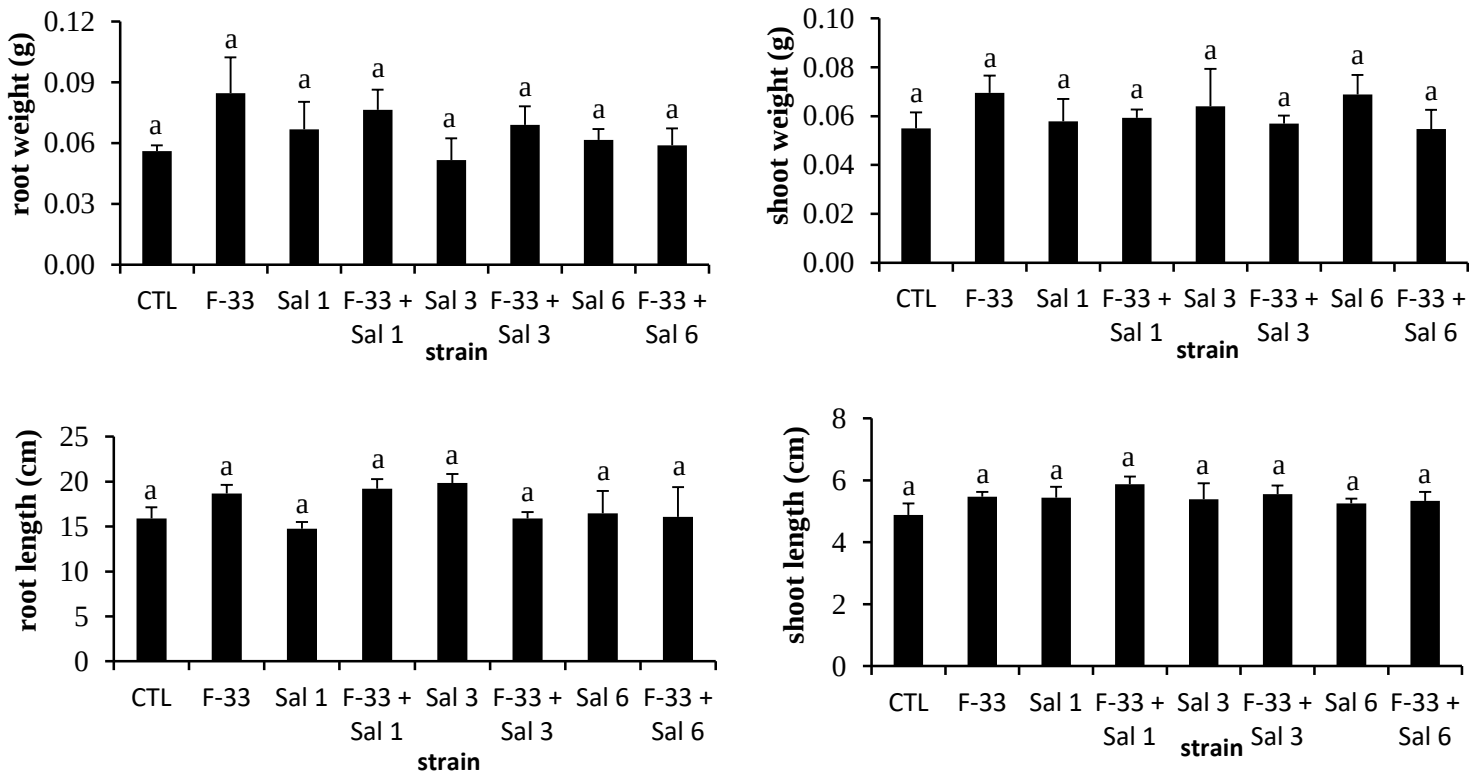

Figure 3.3 The effects of co-inoculation of *Bacillus* sp. F-33 with the other endophytic strains, *Klebsiella* sp. Sal 1, *Enterobacter* sp. Sal 3, and *Herbaspirillum* sp. Sal 6, on the growth of the tomato plant. The tomato plant was cultivated using sterilized vermiculite, and the parameters were measured at 14 days after seed inoculation. CTL represents the control samples inoculated with autoclaved cultures. The bars represent the standard deviation (n = 6), and different letters indicate significant differences at $p < 0.05$ by Tukey's test.

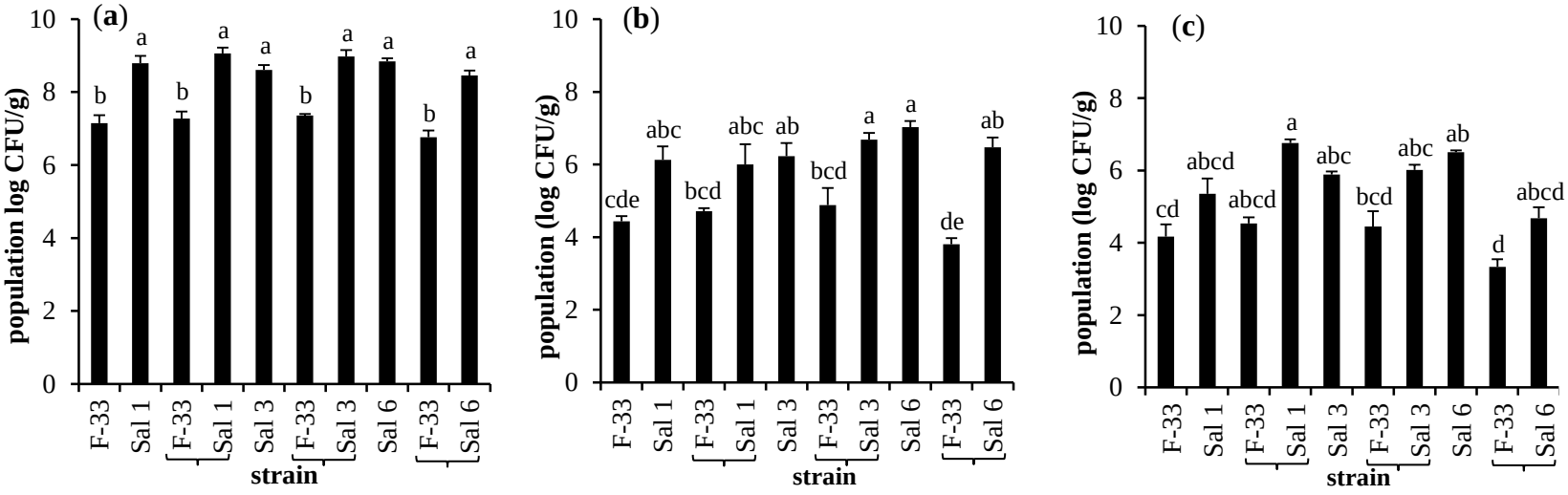

Figure 3.4 The effect of the seed-co-inoculated *Bacillus* sp. F-33 with the other endophytic strains, *Klebsiella* sp. Sal 1, *Enterobacter* sp. Sal 3, and *Herbaspirillum* sp. Sal 6, on colonization in the rhizosphere (**a**), root (**b**), and shoot (**c**) of the tomato plant. The tomato plant was cultivated using sterilized vermiculite, and colonization was examined at 14 days after seed-co-inoculation. The bracket on the x-axis indicates each population in co-inoculation, and no bracket indicates single inoculation. No colony appeared in the control samples. The bars represent the standard deviation (n = 4), and different letters indicate significant differences at $p < 0.05$ by Tukey's test.

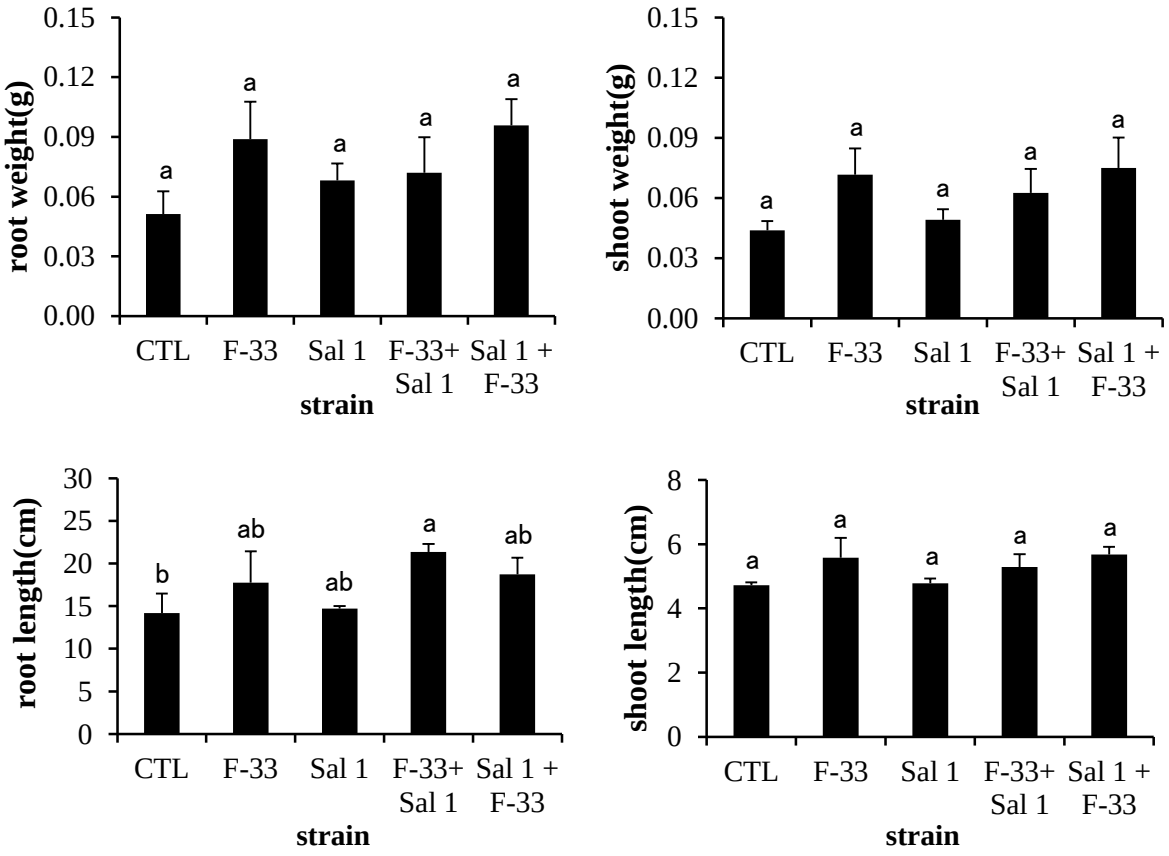

Figure 3.5 The effects of the time interval of inoculation on plant growth promotion and colonization of *Bacillus* sp. F-33 and *Klebsiella* sp. Sal 1 in the tomato plant. The tomato plant was cultivated using sterilized vermiculite, and the parameters were measured at 14 days after seed inoculation. In the time interval of inoculation, F-33 + Sal 1 and Sal 1 + F-33, the second inoculation was conducted 7 days after the first inoculation and analyzed 7 days after the second inoculation. CTL represents the control samples inoculated with autoclaved cultures. The bars represent the standard deviation (n = 5), and different letters indicate significant differences at $p < 0.05$ by Tukey's test.

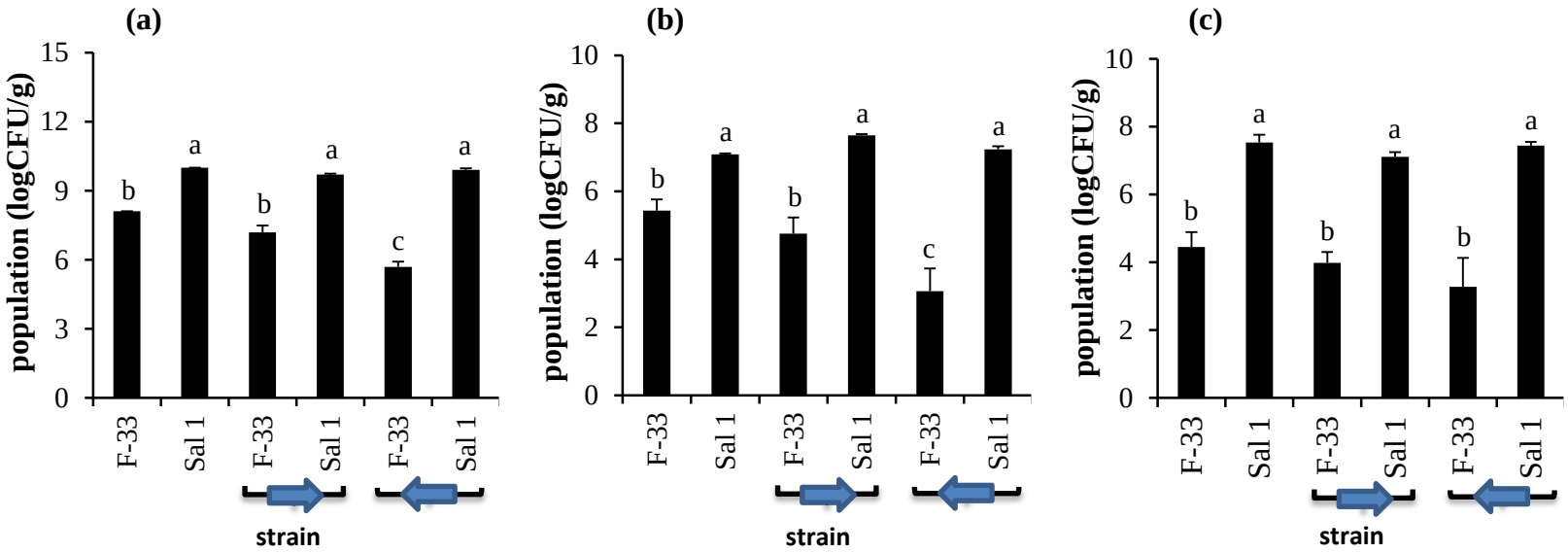

Figure 3.6 The effect of the time interval of inoculation of *Bacillus* sp. F-33 and *Klebsiella* sp. Sal 1 on colonization in the rhizosphere (**a**), root (**b**), and shoot (**c**) of the tomato plant. The tomato plant was cultivated using sterilized vermiculite, and colonization was examined at 14 days after seed inoculation. In the time interval of inoculation, F-33 + Sal 1 and Sal 1 + F-33, the second inoculation was conducted 7 days after the first inoculation and analyzed 7 days after the second inoculation. The bracket on the x-axis indicates each population in the time interval of inoculation, and the arrows on the bracket indicate the order of inoculation. No bracket indicates a single inoculation. No colony appeared in the control samples. The bars represent the standard deviation (n = 3), and different letters indicate significant differences at $p < 0.05$ by Tukey's test.

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Summary

Sweet potato (*Ipomoea batatas* L.) grows well even in infertile and nitrogen-limited fields, and endophytic bacterial communities have been proposed to be responsible for this ability. Plant-growth-promoting bacteria are considered eco-friendly and are used in agriculture, but their application can interact with endophytic communities in many ways. In this study, a commercial biofertilizer, OYK, consisting of a *Bacillus* sp., was applied to two cultivars of sweet potato, and the effects on indigenous endophytic bacterial communities in field conditions were examined. A total of 101 bacteria belonging to 25 genera in 9 classes were isolated. Although the inoculated OYK was not detected and significant plant-growth-promoting effects were not observed, the inoculation changed the endophytic bacterial composition, and the changes differed between the cultivars, as follows: *Novosphingobium* in α-Proteobacteria was dominant; it remained dominant in Beniharuka after the inoculation of OYK, while it disappeared in Beniazuma, with an increase in *Sphingomonas* and *Sphingobium* in α-Proteobacteria as well as *Chryseobacterium* and *Acinetobacter* in Flavobacteria. The behavior of Bacilli and Actinobacteria also differed between the cultivars. The Shannon diversity index (H) increased after inoculation in all conditions, and the values were similar between the cultivars. Competition of the inoculant with indigenous rhizobacteria and endophytes may determine the fates of the inoculant and the endophytic community. Origin of the inoculant, which was isolated from soil, was expected as the possible reasons for the lack of the endophytic potential.

Colonization of a biofertilizer *Bacillus* sp. OYK strain, which was isolated from a soil, was compared with three rhizospheric and endophytic *Bacillus* sp. strains to evaluate the colonization potential of the *Bacillus* sp. strains with a different origin. Surface-sterilized seeds of tomato (*Solanum lycopersicum* L. cv. Chika) were sown in the sterilized vermiculite, and four *Bacillus* sp. strains were each inoculated onto the seed zone. After cultivation in a phytotron, plant growth parameters and populations of the inoculants in the root, shoot, and rhizosphere were determined. In addition, effects of co-inoculation and time interval inoculation of *Bacillus* sp. F-33 with the other endophytes were examined.

All *Bacillus* sp. strains promoted plant growth except for *Bacillus* sp. RF-37, and populations of the rhizospheric and endophytic *Bacillus* sp. strains were 1.4–2.8 orders higher in the tomato plant than that of *Bacillus* sp. OYK. The plant growth promotion by *Bacillus* sp. F-33 was reduced by co-inoculation with the other endophytic strains: *Klebsiella* sp. Sal 1, *Enterobacter* sp. Sal 3, and *Herbaspirillum* sp. Sal 6., though the population of *Bacillus* sp. F-33 maintained or slightly decreased. When *Klebsiella* sp. Sal 1 was inoculated after *Bacillus* sp. F-33, the plant growth-promoting effects by *Bacillus* sp. F-33 were reduced without a reduction of its population, while when *Bacillus* sp. F-33 was inoculated after *Klebsiella* sp. Sal 1, the effects were increased in spite of the reduction of its population. *Klebsiella* sp. Sal 1 colonized dominantly under both conditions. In addition to plant growth-promoting properties, the colonization potential should be considered as important criteria when assessing their suitability for commercial development. The lower population of *Bacillus* sp. OYK, which was isolated from soil, than the other *Bacillus* sp. strains, which were isolated from either the rhizosphere or endosphere of plant samples, suggests the importance of the origin of the strains for their colonization. The plant growth promotion and colonization potentials were independently affected by the co-existing microorganisms. Further studies are necessary to evaluate the colonization potential of PGPR under field conditions where diverse microorganisms exist.

要約

サツマイモ（*Ipomoea batatas* L.）は、窒素含量が十分でない畑でもよく育ち、植物内生細菌群集の存在がこの能力に寄与していると考えられている。植物の成長を促進する細菌は環境に負荷が少なく、農業で使用されていますが、それらの使用は植物内生菌群集に影響を及ぼすと考えられる。この研究では、*Bacillus* sp. を植物成長促進細菌として含む市販のバイオ肥料 OYK をベニハルカおよびベニアズマの 2 品種のサツマイモに接種し、野外で栽培をした時のサツマイモ内生細菌群集への影響を調べた。その結果、全体で 9 綱 25 属に属する合計 101 の細菌が分離された。接種された OYK は検出されず、有意な植物成長促進効果は観察されなかったが、接種によって植物内生細菌の組成が次のように品種間で異なって変化した。優占していた α-Proteobacteria の *Novosphingobium* は、OYK の接種後、ベニハルカでは継続して優占したが、ベニアズマでは消失し、α-Proteobacteria の *Sphingomonas* と *Sphingobium*、Flavobacteria の *Chryseobacterium* と *Acinetobacter* が増加した。Bacilli と Actinobacteria の挙動も品種間で異なっていた。Shannon 多様性指数は、すべての条件で接種後に増加した。接種された菌と土着の根圏細菌および植物内生菌との競争が、接種菌および植物内生菌群集の挙動を決定すると考えられた。接種した OYK（ *Bacillus* sp.）が土壌由来であることが、植物内生菌として定着できなかった理由として推定された。

土壌由来のバイオ肥料 OYK（ *Bacillus* sp.）菌株の植物内定着について、根圏および内生菌由来の 3 種類の *Bacillus* sp. 菌株と比較することで、*Bacillus* sp. 菌株の植物内定着における微生物の起源との関係を調べた。表面滅菌したトマト（*Lycopersicum esculentum* Mill、千果）の種子を、滅菌したバーミキュライトに播種し、各 *Bacillus* sp. 菌株を種子接種した。人工気象器で培養した後、植物の根および地上部の長さと重量、根圏、根内および茎葉内の微生物数を測定した。さらに、*Bacillus* sp. F-33 菌株と他の植物内生細菌との同時接種および時間を空けての

接種の影響について調べた。すべての *Bacillus* sp. 菌株は、*Bacillus* sp. RF-37 株を除いて、植物の成長を促進した。根圏および内生菌由来の *Bacillus* sp. 菌株は、土壌由来の *Bacillus* sp. OYK よりも 1.4–2.8 桁高い菌密度でトマト内に定着した。*Bacillus* sp. F-33 株を各植物内生菌（*Klebsiella* sp. Sal 1 株、*Enterobacter* sp. Sal 3 株、*Herbaspirillum* sp. Sal 6 株）と同時接種すると、*Bacillus* sp. F-33 株の菌密度は維持されたか、わずかに減少しただけであったが、植物成長促進効果は減少した。*Klebsiella* sp. Sal 1 株を *Bacillus* sp. F-33 株の後に接種すると、*Bacillus* sp. F-33 株の菌密度を減少させることなく、植物成長促進効果を減少させた。一方、*Bacillus* sp. F-33 株を *Klebsiella* sp. Sal 1 株の後に接種すると、*Bacillus* sp. F-33 株の菌密度は減少したが、植物成長促進効果は増加した。*Klebsiella* sp. Sal 1 株は、いずれの条件でも、植物内で優占した。根圏および内生菌由来の *Bacillus* sp. 菌株が、土壌由来の *Bacillus* sp. OYK 株よりも植物内定着性が高かったことから、微生物の起源が根圏および植物内での定着性に関係することが示唆された、植物の成長促進効果と植物内定着性は、同時接種および時間を空けての接種した他の細菌の存在により、プラスまたはマイナスの影響を受けた。植物の成長促進効果と植物内定着性の間には関連性がないことが示唆された。

List of Publications

Effects of the biofertilizer OYK (*Bacillus* sp.) inoculation on endophytic microbial community in sweet potato

Salehin, A., Hafiz, M.H.R., Hayashi, S., Adachi, F. and Itoh, K.

Horticulturae, **2020**, 6 (4): 1-12 (DOI: 10.3390/horticulturae6040081)

(Chapter 2)

Effect of co-inoculation of *Bacillus* sp. strain with bacterial endophytes on plant growth and colonization in tomato plant (*Solanum lycopersicum*)

Salehin, A., Puri, R.R., Hafiz, M.H.R. and Itoh, K.

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(Chapter 3)