ANALYSIS OF MICROBIAL COMMUNITIES ASSOCIATED WITH THE PIONEER PLANT ON THE VOLCANIC DEPOSITS OF THE 2000 ERUPTION IN MIYAKE-JIMA

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学 位 論 文 要 旨

ANALYSIS OF MICROBIAL COMMUNITIES ASSOCIATED WITH THE PIONEER PLANT ON THE VOLCANIC DEPOSITS OF THE 2000 ERUPTION IN MIYAKE-JIMA

三宅島 2000 年噴火火山灰堆積物におけるパイオニア植物に関連する微生物群集の解析

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Pioneer plants are the first to colonize raw mineral soils or disturbed sites, which can occur naturally or as a result of human activity. Microbial interactions with roots play a crucial role in the establishment and colonization of pioneer plant species, especially in harsh conditions. Root-inhabiting microbes, also known as rhizosphere or endosphere microorganisms, are specifically adapted to their hosts and local soil conditions. They contribute to the adaptation and survival of their plant hosts under abiotic stress conditions, particularly in extreme environments like volcanic ecosystems. One such volcanic ecosystem is Miyake Island, located in the Izu archipelago of Japan in the Pacific Ocean. It is a volcano known for its volcanic activity, with an area of 55.5 km^2 and an altitude of 775 m. The physicochemical parameters (such as nutrient limitation, acidity or alkalinity) and geographical factors (intense volcanic activity) in such extreme environments pose challenges for the survival of aboveground plants and belowground microorganisms. Understanding how plant-associated microorganisms enhance plant health and productivity is therefore essential. However, the processes and mechanisms underlying plant growth on volcanic deposits are not well understood.

In this study, the objectives of this research were as follows: 1. To compare the structure and diversity of

rhizosphere and endophytic microorganisms in *M. condensatus*, which grows in extreme volcanic deposits. We characterized the root microbial communities associated with *Miscanthus condensatus*, which is a pioneer colonizing vegetation found near the crater of Miyake Island. 2. To identify endophytic bacteria and fungi associated with *M. condensatus* using both culture-dependent and culture-independent methods. 3. To evaluate the feasibility and effectiveness of using core Dark Septate Endophytic (DSE) isolates in promoting plant (rice as proxy) growth under abiotic stress conditions, such as soil acidity, through inoculation. We hypothesized that *M. condensatus* hosts a specific root-associated microbial community that contributes to its adaptability to harsh soil conditions.

The main findings of this study are as follows: 1. By comparing the structure and diversity of rhizoplane and endophytic microorganisms in *M. condensatus* growing in extreme volcanic deposits, we isolated a total of 339 putative fungal isolates and 301 bacterial strains from the roots and rhizoplane of *M. condensatus.* 2. By combining culture-dependent and culture-independent methods, we identified endophytic bacteria and fungi associated with *M. condensatus* colonizing the volcanic deposits near the crater of Oyama. The most abundant fungal community identified by both methods belonged to the Helotiales order. This combined approach provided a comprehensive understanding of microbial diversity, as well as the physiological properties and metabolisms of individual isolates. 3. Through inoculation with DSE isolates, we assessed the feasibility of using core isolates to promote rice growth in acidic soil conditions. The shoot biomass of rice inoculated with DSE isolates increased up to 7.6 times compared to non-inoculated controls, demonstrating their potential as a management strategy for mitigating the adverse effects of acidity on crops.

In conclusion, this study provides a comprehensive understanding of the characteristics and functions of associated microorganisms, particularly DSE fungi, in pioneer plants colonizing volcanic deposits. These DSE taxa such as Helotiales play a key role in plant growth and stress tolerance. The data obtained in this study can serve as a basis for future research on the application of microbial inoculants in enhancing plant growth under stressed soil conditions. Studying and utilizing these core isolates have significant implications for volcano ecosystem recovery and potentially sustainable agriculture.

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Contents

CHAPTER 1

General Introduction

The current world population of 7.6 billion and is expected to increase beyond 9.8 billion by 2050 (United Nations, 2017). Accompanying this dramatic growth in population is the anticipated increase in the demand for agricultural food. The production and quality of agricultural crops are decreased by i) destructive human activities, such as deforestation and the overuse of chemical fertilizers, and ii) a wide range of abiotic stresses, including extreme temperatures, drought, salinity, nutrient deficiency and acidic soil. It is estimated that more than 50% of global yield loss for major agricultural crops is due to abiotic stress. The combination above poses a serious threat to global food security and stability of ecology.

Pioneer plants are those that first colonize raw mineral soils or disturbed sites, which are created by natural or anthropogenic origin. Microbial interactions with roots are crucial for the establishment and colonization of pioneer plant species under stressed conditions. The rhizosphere is the area that recognized as the most relevant active zone between roots and soil. The microbes in the rhizosphere is intensive and their interactions with root play a crucial role in element biogeochemical cycling, such as nutrient acquisition and carbon turnover. Root-inhabiting microbes, known as rhizosphere microorganisms or endosphere microorganisms, are specifically adapted to their host and local soil conditions and thereby contribute to the adaptation and survival of their plants host to abiotic stress conditions, particularly in extreme environments, such as volcanic ecosystems.

After a volcanic eruption, and for the soil formation process, microbes aid to restore soil structure

to the creation of new horizons. At the beginning of soil formation process, the environment is colonized by primary vegetation (pioneer plants) that subsequently support the colonization of other plants. In the early stages of ecological succession, associative nitrogen-fixing bacteria can promote the growth of pioneer species, such as by proving nitrogen source from mineral and secreting plant growth hormones, such as IAA. Similarly, arbuscular mycorrhizal fungi can significantly promote phosphorus uptake by plants and then increase plant biomass. Furthermore, the diversity of soil microbial community is also a key factor in nutrient cycling, such as enhancing the bioavalibilty of nutriments via minerals mining. Therefore, the pioneer plant associated microorganisms may help plant growth and adaptation under diverse harsh environments.

Here, we characterized the root microbial communities associated to root of *Miscanthus condensatus*, which is the pioneer colonizing vegetation at the volcanic deposits near the crater of Miyake-jima. The present study hypothesized that *M. condensatus* hosts a specific root-associated microbial community that being responsible for promoting its high adaptability of plant to the soil conditions at this harsh environment. The data can also serve as a baseline for future studies on application of microbial inoculants in enhancing plant growth at stressed soil condition.

1.1 Extreme and volcanic ecosystems

The extreme ecosystem (terrestrial, marine and aquatic) includes many natural biotopes and anthropopenic such as geothermal areas, volcanic soils, the Artctic and Antarctic, deserts, salt plains, and acid mines. The physicochemical (such as nutrient limitation, acidity or alkalinity), environmental (aridity, or hyper-salinity) or geographical (permafrost, elevation gradients, or intense

volcanic/geothermal activity) parameters in the extreme environments are restrictive for survival of most upper organisms forms (Rothschild and Mancinelli, 2001).

Terrestrial volcanic areas are extreme environments characterized by their harsh physical and chemical conditions. The terrestrial ecosystem in volcanic areas undergoes succession. After a volcanic eruption disturbance in the very beginning, the newly formed or exposed land surfaces comprise new parent materials (e.g., tephra, ash, lava), rather than developed soils in the primary succession stage (Guo *et al*., 2014). Volcanic material may cover large areas, such as hundreds of km^2 for pyroclastic flows (Muñoz *et al*., 2021). Severral reduced gases are emitted in variable concentrations, including hydrogen sulfide (H_2S), hydrogen (H_2), carbon monoxide (CO), ammonia (NH_3), and the potent greenhouse gas methane (CH_4) (Picone *et al*., 2020). The material from volcanic eruptions, such as lava flows, ash and tephra deposits, creates clean land surfaces on which ecosystem development processes such as plant colonization and soil formation begin. Volcanic ash could severely affect the ecosystem development, which is critical for agricultural areas and is particularly important in areas such as Indonesia, Philippines, Japan, Havaii and Pacific Islands. In this scenario, the soil substrates are formed maily of sulfide-rich minerals generating sulfuric acid through oxidation processes. Thus, the soil usually shows very low pH (Maki *et al*., 2008). Also, due to less plant derived carbon input, there is a lack of soil organic matter formed and bioavailable nutrients. As a result, the limited resources and stressed condition in volcanic zones at the primary succession stage shape and limit the ecosystem development (Rincón-Molina *et al*., 2022). The volcanic ecosystem, yet, provides unique site for studying the limits of life (plant and microorganisms, for examples) and their adaptations to thrive in extreme conditions.

1.2 Description of Miyake-jima and the latest eruption in 2000

The island of Miyake (with Miyake-jima; 55.5 km^2 in area; and 775 m of altitude) is a volcano located in the Izu archipelago of Japan in Pacific Ocean (34°05′N, 139°11′E). This island is part of the Fuji volcanic southern zone in the East Japan volcanic belt (Fig. 1.2.1), and known for its volcanic activity. One of the most notable eruptions in recent history occurred in 2000, which had a significant impact on the island and its ecosystem. The latest eruption occurred at Mount Oyama, the summit of the island, from July to September in 2000. After the crater formation, large amounts of volcanic ash and gas containing SO_2 and H_2S were exposed. The deposition of these ash formed acidic volcanic deposits (pH 3-4), and the total volume of volcanic deposits in the 2000 eruption is about 2.3×10^{10} kg (Nakada *et al*., 2005). The monthly average emission rate of SO_2 peaked at 54 kt d^{-1} in December 2000, and gradually decreased to 7 kt d^{-1} in the end of 2002 (Guo, 2014). About 60% of vegetation on the island was initially damaged by the heavy deposition of acidic volcanic ash which lack carbon and nitrogen, and almost all vegetation that covered around the crater defoliation and lost caused by the toxic gas (Guo et al., 2014; Arsyadi et al., 2023). The volcanic activity on Miyake-jima has made it a unique and scientifically valuable location for the study of the recovery of vegetation on the land affected by volcanic eruption.

1.3 Ecosystem development after the 2000 eruption of Miyake-jima

With the decreasing of SO_2 emission after 2001, the ecological recovery process has occurred on Miyake-jima. According to a chronosequence study on lava flow of different ages, the process of

primary succession on Miyake-jima Island can be summarized as follows: successful colonization of pioneer species, such as *M*iscanthus *condensatus* (a perennial grass) on bare lava flows, and colonization of several deciduous species such as *Prunus speciosa* and climax evergreen species such as *Machilus thunbergia* and *Castanopsis sieboldii*, with the decreasing of pioneer species (*M. condensatus*) (Kamijo *et al*., 2002; Zhang *et al*., 2020).

Volcanic soils are typically nutrient-poor and often lack organic matter, making them stressed environments for plant growth. However, some grass species have adapted to these harsh conditions and have developed mechanisms to establish themselves as pioneer species. Pioneer grasses play a crucial role in the early stages of ecological succession and recovery on volcanic deposits. For instance, Miscanthus genus, owing to their C4 photosynthesis and low-nutrient requirement (Stewart *et al*., 2009), is usually the first invader that tolerate a wide range of environmental stresses and frequently found in disturbed habitats including lahar deposited by volcanic eruptions as primary vegetation (Yoshii, 1937; Inoue, 2003; Watanabe *et al*., 2006; Hirata *et al*., 2007; An *et al*., 2008; Ezaki *et al*., 2008). In Miyake-jima, the site of focus in this study, *M. condnsatus* was the most dominant species in the ecological recovery process on the new bare land near the crater following the volcanic eruption in 2000.

1.4 Microbial communities and their interaction with pioneer plant

Microorganisms are often the earliest colonizers of newly exposed or formed terrestrial surfaces, and have important roles in the early development of ecosystems. These early microbial colonizers including oligotrophs, autotrophs, chemotrophs, can survive in nutrient-limited environments by

fixing carbon from atmosphere and exploit nitrogen and other nutrients from mineral or ash (Dragone *et al*., 2023). The early microbial colonizers provide organic carbon (usually produced via anabolism) and nutrients for the subsequent plant and microorganisms during succession. Previous work described the pioneer microorganisms are chemotrophs near the mountaintop of Oyama, Miyake-jima, and then transitioned to heterotrophs when the pioneer plant colonization in unvegetated volcanic ash deposits (Fujimura *et al*., 2016; Sato *et al*., 2009). The pioneer plants, which establish symbiotic interactions with microbial communities, colonize the newly formed soil. To achieve sustainability, the early interactions between microbial and pioneer plants play crucial roles in i) being able to avoid or tolerate extreme environmental conditions and nutrient limitations, and ii) modifying the physical and chemical characteristics of the substrates or new formed soil. Microorganisms associated to pioneer plants promote plant growth through direct or indirect biochemical mechanisms, such as N_2 fixation, inorganic phosphate solubilization, auxin synthesis, and production of siderophores and other metabolites. For example, chemoautotrophic bacteria such as nitrifiers (*Nitrosomonas* and *Nitrobacter*) are important for nutrient cycling since they oxidize ammonium to nitrate in nitrification processes. Symbiotic (*Rhizobium*) and non-symbiotic free living (*Azotobacter*, *Clostridium*) nitrogen-fixing bacteria greatly increase N supply to the system. These plant-microbe interaction may occur in the rhizosphere, and endophytic. However, microbial endophytes may access nutrients and water more easily than those on the rhizosphere or rhizoplane. On the other hand, the beneficial effects of endophytes to their host plants are in general greater than those of rhizosphere, which might be intensified when the plant is growing under either biotic or abiotic stress conditions (Compant *et al*., 2010; Ma *et al*., 2011). Hence, in addition to their beneficial effects on plant growth,

endophytes have a great biotechnological potential to improve the efficiency and applicability of phytoremediation techniques.

An understanding of how plant and soil factors manipulate and reshape the soil microbiome, and how the plant associated microorganisms, in turn, improve the plant health and productivity is essential. Most of the microbiological research in extreme ecosystem that has been carried out to date has focused on glacier forefront, desert, and heavy metal polluted site (Cázares *et al*., 2005; Jumpponen *et al*., 2002; Op De Beeck *et al*., 2015; Ortiz *et al*., 2015). Yet, the processes and mechanisms underpinning plant growth on volcanic deposit are not well understood. Furthermore, the knowledge of the composition and distribution of *M*. *condensatus*-related microbial communities is still limited, which inhibits the application of potential beneficial microbial communities in volcanic ecosystem or agroecosystem.

1.5 Culture-dependent and culture-independent method to identify core microorganisms

For decades, microbial diversity analysis was carried out by the traditional culture-dependent method. However, this method has several disadvantages (Narsing Rao *et al*., 2021). Most of the microorganisms in this method remain hidden or difficult to grow. Next-generation sequencing allows for culture-free microbial diversity detection. In the past few years, this method has played an important role in understanding the microbial diversity of various ecological niches. However, these techniques also have serious limitations in identifying the majority of unknown taxa into species level, since many sequences deposited in GenBank are associated with erroneous taxon names and many species groups cannot be discriminated by using ITS or 16S rDNA (Dissanayake *et al*., 2018).

Therefore, the combination of culture-dependent and culture-independent methods can effectively reveal the microbial diversity in environmental samples and the roles of different microorganisms in the environment. Bai *et al*. (2015) established Arabidopsis root-derived bacterial culture collections representing the majority species that are reproducibly detectable by culture-independent community sequencing. Valérie *et al*. (2021) investigated the fungal and bacterial community in soils receiving wheat and oilseed rape residues and confirmed the feasibility of the combined culture-unculture approaches that revealed consistent community profiles. The role of keystone taxa revealed by the sequencing data-based co-occurrence network can be further validated by culturing. For example, isolation was used to test whether the interaction between micro-organisms predicted by metagenomics sequencing actually occurs (Laval *et al*., 2021). By isolation and inoculation, (Zheng *et al*., 2021) identified the strong decomposition ability of keystone taxa such as Chryseobacterium (bacteria) and Fusarium, Aspergillus, and Penicillium (fungi), which are consistent with the keystone taxa revealed by the co-occurrence network. The combination of sequencing and culturing methods, therefore, are powerful in the identification of putative taxa (either individually or creation of synthetic communities). Yet, studies on microorganisms in volcanic ecosystem by culture-unculture approaches are lacking. Therefore, both culture-dependent, culture-independent Illumina sequencing were used for the in-depth investigation of microbial communities to identify the core microbial groups in soils of the volcanic ecosystems. Further, the inoculation to validate function in plant growth were adopted, to comprehensively evaluate the functions of Miscanthus-associated microbial communities, in plant growth and adaptation to stressed volcanic soils.

1.6 The role of Dark septate endophyic fungi in promoting plant growth and acidic stress tolerance

Dark septate endophytes (DSE) is one of the most common groups of monocotyledonous root endophytes, which are usually found to colonize in more than 600 herbaceous and woody plant species (Jumpponen and Trappe, 1998). The increasing evidence found that DSE gradually becomes the most prevailing root colonizers (Deram *et al*., 2008; Regvar *et al*., 2010). Dark septate endophytic fungi are able to enhance nutrients availability and subsequent plant uptake in pine seedling by mineralizing organic substances such as proteins and peptides, or breaking down of complex carbohydrates into simple sugars (Upson *et al*., 2009). Several studies suggest that DSE fungi is able to produce phytohormone substances for host plants growth (Wu and Guo, 2008). Also, root-associated fungi use their hyphae to reach, mobilize and transport nutrients, and thus facilitate plant nutrients uptake (Behie and Bidochka, 2014; Souza *et al*., 2015). DSE fungus, *Heteroconium chaetospira* was reported to play a role in promoting Chinese cabbage growth via mutualistic symbiosis (Usuki and Narisawa, 2007).

Abiotic stress is the major limiting factors for plant productivity. Recent studies have explored and evaluated the capability of root associated DSE in increasing plant tolerance to a variety of environmental stresses such as low pH. Acid soil gained a global attention over the last decades. These soils occupy around 70% of the world's potentially arable land. Plants commonly encounter deficient and toxic levels of mineral elements when grown in acidic (pH<5) soil. Soil pH is a highly sensitive factor to determine plant survival, distribution, and interactions with microorganisms, which are rather vital for the availability of essential nutrients (Luo *et al*., 2013). Fungi are probably common

in the acidic soil, as they are able to maintain a relatively neutral pH by establishing a low proton membrane permeability and pumping protons out of the cell (Nicolay *et al*., 1987). Although AMF are reported to play a key role in the protection of plants in acidic soils, there is still a lack of reports of DSEs improving host plant growth under acidic condition, especially ultra-acidic conditions (pH 3.0).

Research Objectives

Based on the above-mentioned research, to obtain a comprehensive understanding of the development of pioneer plants colonized on volcanic deposits, and their associated microorganisms, the objects of this research were：

1. To compare the structure and diversity of rhizosphere and endophytic microorganisms from pioneer grass *M*. *condensatus* that grow at the extreme volcanic deposits.
2. By using culture-dependent and culture-independent method, to identify endophytic bacteria and fungi associated with pioneer grass *M*. *condensatus* colonizing on the volcanic deposits near the crater of Oyama.
3. To evaluate and validate the feasibility of core DSE in promoting plant growth under abiotic stress such as soil acidity via isolates-inoculation.

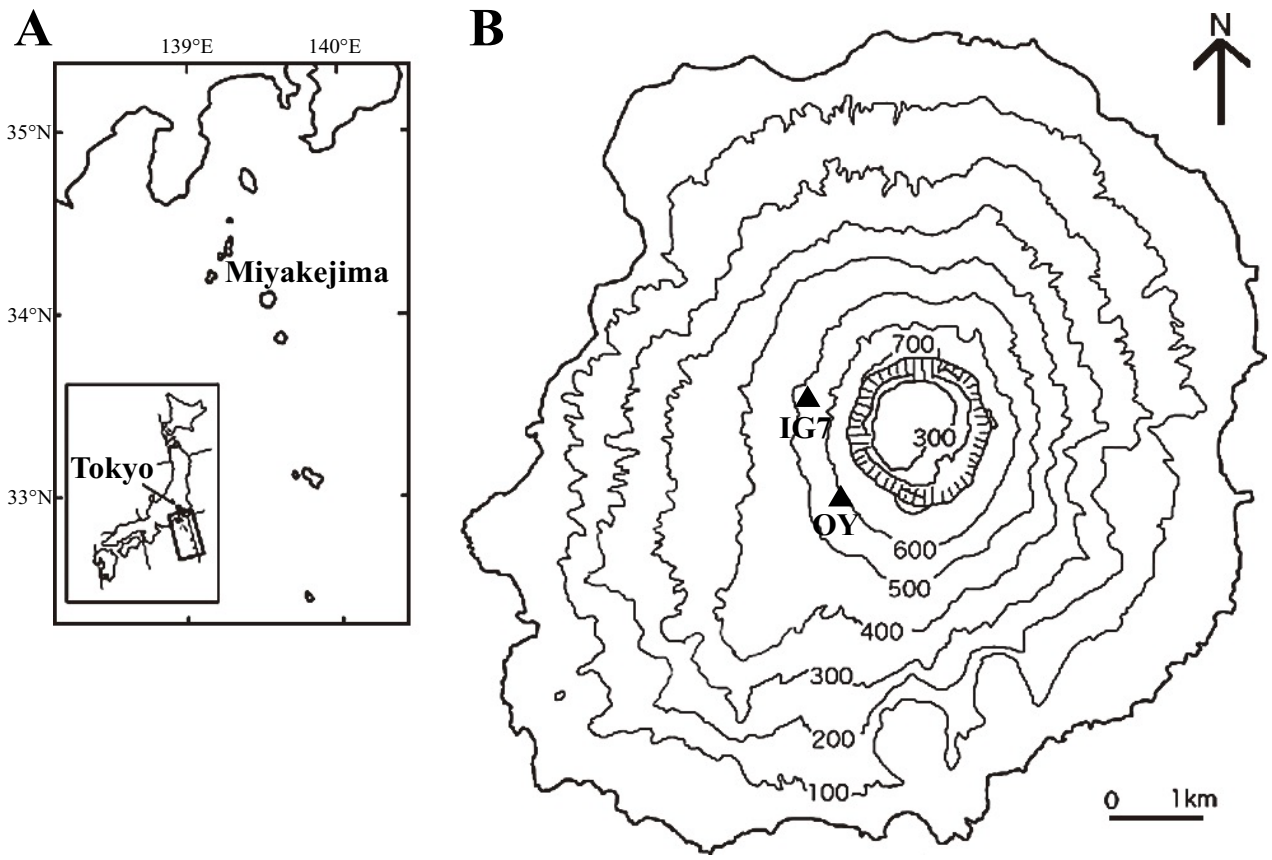


Fig. 1.2.1 Maps showing the location of the Island of Miyake (Miyake-jima) in the western rim of the Pacific Ocean (A) and the sampling site OY and site IG7 this research (B).

Chapter 2

Isolation and Identification of endophytic microbial communities associated with the pioneer plant on the volcanic deposits of the 2000 eruption in Miyake-jima

2.1 Introduction

Volcanic ecosystem appears to be sterile and hostile to life. However, a number of living organisms, including plants and microorganisms have evolved adaptive mechanisms for adjusting to these extreme conditions in volcanic ecosystem. One important strategy by which volcanic pioneer plants adapt to these harsh conditions is through their association with microbial communities. Microbes associated with the roots of pioneer plants are capable of promoting growth and stress tolerance in a number of ecosystems (Li *et al*., 2022; Mahmood *et al*., 2016; Marasco *et al*., 2012).

Plants accommodate and interact with different microbes within their tissues (endosphere), the narrow region surrounding the root soil interface (rhizosphere) and the area around the stems and leaves (phylosphere). Among the plant compartments, roots are the primary sites for plant nutrition acquisition from soil and organic molecular exudation into soil, and therefore the hotspot for plant-soil interactions (Sun *et al*., 2021). Particularly, bacterial endophytes may access nutrients and water more easily than those on the rhizosphere or the rhizoplane (Beattie, 2006). In addition, root endophytes are ecologically adapted to the target niche and therefore can better overcome defensive reactions than microorganisms originating from the rhizosphere. Therefore, root endophytes, due to its colonization on the interior of the plant root, can have a bigger impact on the host plant than rhizosphere microbes. On the other hand, in addition to their beneficial effects on plant growth, using endophytic microbes can better help plant to be resistant from biotic and abiotic threats from the

plant's surroundings (Sharma *et al*., 2023). Also, endophytes have a great biotechnological potential to improve the efficiency and applicability of phytoremediation. Considering the rhizosphere microbiome associated with various host plants have been extensively characterized (Berendsen *et al*., 2012; Kolton *et al*., 2017; Zhalnina *et al*., 2018), the endosphere microbiome of pioneer plant in stressed environment is less documented and requires further research to understand its function.

Miscanthus genera is a pioneer perennial grass plant native to eastern Asia and often dominates in the extreme ecosystems. Their ability of water efficiency, low-nutrient requirement, ensures either moderately or highly tolerant of extreme heat, drought, flooding, salinity in the environment. Moreover, root-associated microbiome are found to contribute to the growth of Miscanthus under stressed condition in several ecosystems. The understanding of the isolation, classification, characterization of the root endophytic microbiome can clarify mechanisms underpining their capabilities of promoting Miscanthus survive/growth in volcanic ecosystem and provide valuable knowledge for future agricultural practices. The current chapter, therefore, focuses on the isolation, classification, characterization, and sequencing of bacterial and fungal endophytes from root of pioneer grass *Miscanthus condensatus* colonizing on the volcanic deposits in Miyake, Japan.

2.2 Materials and methods

2.2.1 Sampling site description and sample collection

Miyake-jima is an island situated in the western rim of the Pacific Ocean, about 180 km south of Tokyo. Mt. Oyama in Miyake-jima is an active basaltic volcano. The climate type in this region is humid subtropical, with a mean annual rainfall of 3024.7 mm and a mean air temperature of 18.0 ºC

from 1991-2020 (Arsyadi *et al*., 2023). Geologically recent eruptions were recorded in 1874, 1940, 1962, 1983, and 2000. The last eruption in 2000 ejecting large quantity of volcanic ash and eventually forming a large collapsed crater (more than 1 km in diameter and more than 400 m in depth). Since the formation of the crater, a large amount of volcanic gas (mainly SO_2) has been emitted. According to satellite remote sensing studies, almost all vegetation around the crater was severely damaged by the deposition of ash, exposure to volcanic gas, mudflows triggered by rainfall and the acidification of ash by SO_2 gas (Sato *et al*., 2009). The fresh volcanic ash had been characterized by high contents of fine sand (36-76%), acidity [pH (H_2O), 3.1-4.0], and high amounts of exchangeable Ca^{2+} (33.5-115) cmol kg^{-1}) and Al^{3+} (0.8-10.2 cmol kg^{-1}) (Kato, *et al.*, 2002). An on-site vegetation survey in 2003 showed that vegetation has gradually recovered halfway up the mountaintop, with the coverage <20% in 2009, <45% in 2013, and 90% in 2018. In the course of succession, vegetation changed from devastated land to *Miscanthus condensatus*. The sampling site of interest in this study was chosen near the summit crater (site OY) of Mt. Oyama (Fig. 1.2.1), where a patchy vegetation of a pioneer grass, *Miscanthus condensatus,* was reestablished here recently years. The plants with rhizosphere soils were collected at site OY in November 2017, March and September 2018 (Fig. 2.2.1) From each period, three apparently healthy plants were collected and kept in plastic bags at 4ºC and processed within 48h after collection.

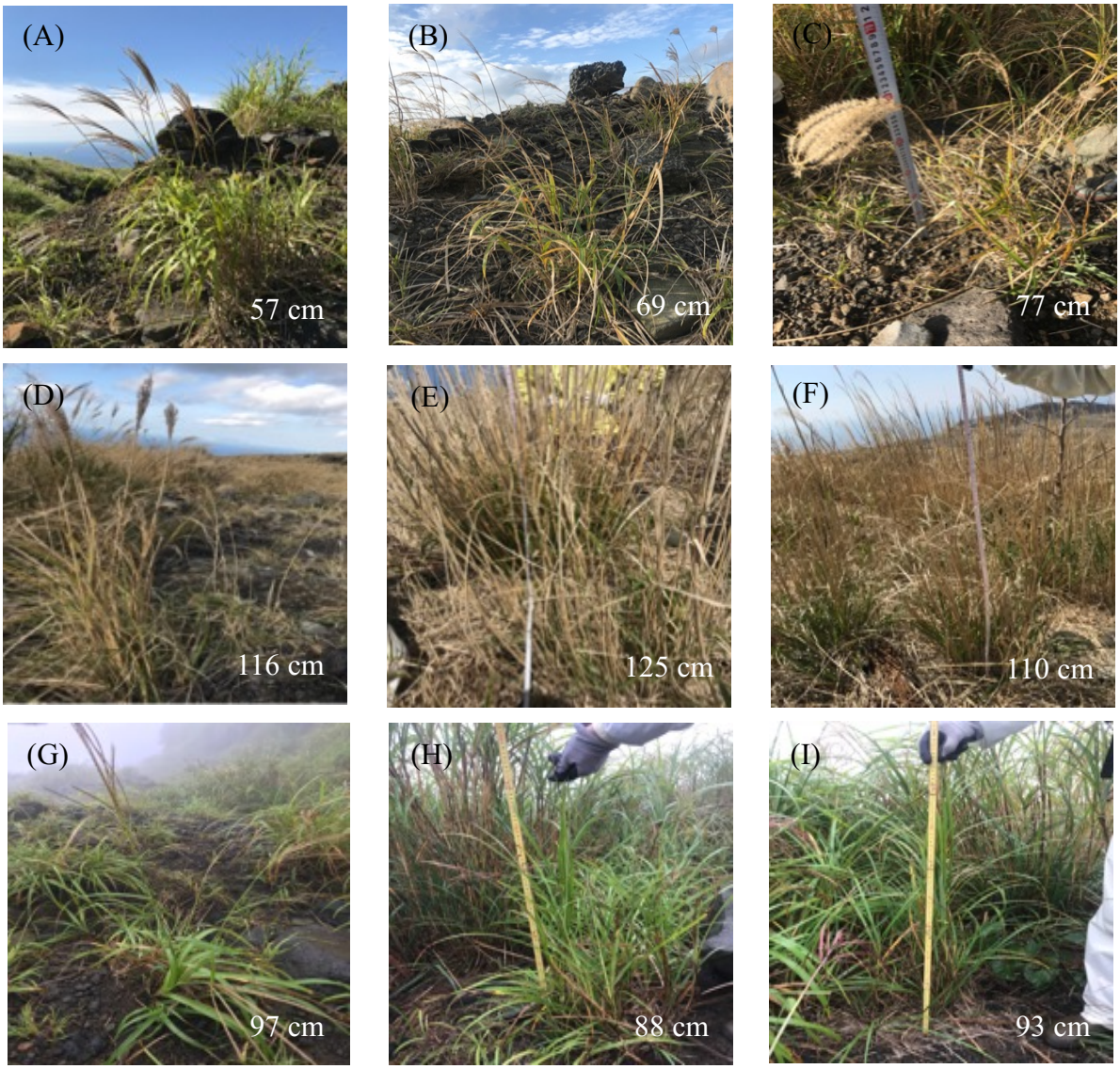


Fig. 2.2.1. Photographs showing the pioneer plants Miscanthus condensatus on site OY in. November (A-C), March (D-F), and September (G-I). Plant height was shown at the bottom.

2.2.2 Surface sterilization test on root of *Miscanthus condensatus*

Requirement of surface sterilization protocol is different according to different host plant. The

sterilant solution, concentration and exposure time could be optimized based on host plant and tissue type. In the present study, the sterilant solution, concentration and exposure time were optimized by a series of gradient experiments (single factor and multilevel design). In order to remove adhering soil and free-living microbes, which are unlikely to interact with the roots of plants, root samples were gently rinsed with tap water. Individual roots were severed aseptically 1 cm long sections with a sterile scalpel, and put into 50ml conical centrifuge tubes. Then they were superficially sterilized with three methods, i) 0.005% Tween 20 for 3 min and then rinsed with sterilized distilled water; ii) 0.005% Tween 20 for 3 min, followed with 70% ethanol for 1 min, and then rinsed with sterilized distilled water; iii) a further step in the above foundation, 1% sodium hypochlorite was added and sterilize for 5 min. Finally, sections were rinsed with sterilized distilled water three times. After surface sterilization, the final wash of each method was spread plated onto R2A agar plate to confirm the disinfection and incubate for 1 week at 30ºC to examine for the presence of growth colony.

2.2.3 Isolation and molecular identification of endophytic isolated bacteria and fungi

Root samples from this fraction were added with 10 ml aliquot of sterile distilled water and macerated with mortar and pestle for bacteriological analysis. Serial dilutions were made and 100 µl of each was plated on R2A agar (Table 2.2.1) for total bacteria. As one of the most important beneficial bacteria groups, Miscanthus-associated diazotrophs assemblages were also examined in this work which was incubated on Nitrogen-free base agar (NFA) (Table 2.2.4), and then they were incubated at 30ºC for 1 week. DNA from each bacterial isolate was extracted using phenol-chloroform procedure as (Wang and Wang, 1997).

Root sections were dried with sterile filter paper overnight for endophytic fungal isolation and then placed onto cornmeal agar medium (Table 2.2.2) containing 0.1 mg kg^{-1} streptomycin and incubated at 23ºC for 2 weeks. After incubation, pure cultures were obtained by transferring single hyphae to half cornmeal malt yeast agar medium (Table 2.2.3). Fresh mycelia were harvested and genomic DNA was extracted using PREPMAN Ultra Sample Preparation Reagent (Applied Biosystems USA) according to the manufacturer's protocol.

Amplification, sequencing, and phylogenetic analysis

PCR amplification was carried out in a 50 µl reaction mixture containing 100 ng of genomic DNA, 0.2 µM concentration of each primer, 0.2 mM of each deoxy nucleoside triphosphate (dNTP), 10× Ex *Taq* buffer, 0.25 U of Ex *Taq* DNA polymerase (TakaRa Bio, Japan), and sterilized MilliQ water. The amplification for the 16S rRNA gene was performed with the universal primer pairs of 10F and 1541R (Table 2.2.7), under thermal conditions of 25 cycles of 96ºC for 5 min, 96ºC for 30 s, 60ºC for 1 min and 72ºC for 1 min on a TaKaRa PCR Thernal Cycler Dice TP600. Polymerase chain reaction (PCR) was conducted to amplify the partial 18S small subunit (SSU), internal transcribed spacer (ITS) 1-5.8S-ITS2 regions, and partial 28S large subunit (LSU) region with universal primers ITS5 F and ITS3 F (White *et al*., 1990), and LR0R F and LR5 R (Vilgalys and Hester, 1990) for fungal isolates under thermal conditions of 4 min at 94ºC, 35 cycles of 94ºC for 35 s, 52ºC for 55 s, and 72ºC for 2 min, and a final extension of 72ºC for 10 min. PCR products were purified suing the mixture of 12 µl of 3 M sodium acetate (pH 4.8), 30 µl of 40% PEG, 1.5 µl of 200 mM $MgCl_2$, and 50 µl of PCR product. PCR for sequencing was carried out in 10 µl reaction mixture containing 1.5

µl of 5× Sequencing buffer, 1.0 µl of each primer (3.2 pM), 1.0 µl of BigDye, 5.5 µl of sterilized MilliQ water, and 1.0 µl of purified DNA. The cycling conditions were as follows: 96℃ for 2 min, 25 cycles at 96℃ for 30 sec, 50℃ for 15 sec, and 60℃ for 3 min. PCR sequencing products were purified using the mixture of 1 µl of EDTA (12.5 mM), 1 µl of 3 M sodium acetate (pH 5.2), and 25 µl of 100% ethanol. PCR sequencing products were re-suspended in 20 µl Hi-Di^{TM} formamide solution (Applied Biosystems, USA) and sequence analysis was carried out using a BigDye Terminator v3.1 DNA sequencer instrument and sequenced using an Applied Biosystems 3130xl DNA sequencer. All the DNA sequences were aligned with the sequences from GenBank using the Basic Local Alignment Search Tool for nucleotides (BLASTN) program to find sequence homology with closely related taxa. Sequence alignment by using the CLUSTAL W and construction of the phylogenetic tree was performed using MEGA version 11.0 with the Neighbor-Joining method. The robustness of the phylogenetic tree was confirmed by using bootstrap analysis based on 1000 replications of the sequences. The genetic distances were calculated using the maximum composite likelihood model.

Table 2.2.1. R2A medium

Peptone	0.5 g
Yeast extract	0.5 g
Casamino acid	0.5 g
Glucose	0.5 g
Soluble Starch	0.5 g
K_2HPO_4	0.3 g
$MgSO_4 \cdot 7H_2O$	0.05 g
Sodium Pyruvate	0.3 g
Distilled water	1000 ml

pH 7.0~7.4

Table 2.2.2. 50% Corn Meal agar medium

Corn Meal	8.5 g
Malt extract	10.0 g
Yeast extract	2.0 g
Agar	15.0 g
Distilled water	1000 ml

Table 2.2.3. 50% Corn Meal Malt Yeast agar medium

Corn Meal	8.5 g
Agar	15.0 g
Distilled water	1000 ml

Table 2.2.4. Nitrogen-free agar medium (NFA)

DL-Malic acid	5.0 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2g
NaCl	0.1 g
$CaCl_2 \cdot 2H_2O$	0.02 g
Micronutrient solution	2.0 ml
Bromthymol blue solution	2.0 ml
Vitamin solution	1.0 ml
Fe-EDTA (1.64%)	4.0 ml
Distilled water	1000 ml
Agar (Wako)	15.0 g

pH 6.8

Table 2.2.5. Micronutrient solution

$CuSO_4 \cdot 5H_2O$	0.4g
$ZnSO_4 \cdot 7H_2O$	0.12g
H_3BO_3	1.4g
$Na_2MoO_4 \cdot 2H_2O$	1.0g
$MnSO_4 \cdot H_2O$	1.5g
Distilled water	1000 ml

Table 2.2.6. Vitamin solution

Biotin	10.0 mg
Pyridoxol HCl	20.0 mg
Distilled water	100 ml

Table 2.2.7. PCR primers set for identification of fungal isolates in this study

Primer name	Primer sequence (5'-3')	Target gene	Reference
10F	AGTTTGATCCTGGCTCAG	16S	Fujimura *et al*. (2012)
1541R	AAGGAGGTGATCCAGCCGCA		
ITS5	GGAAGTAAAAGTCGTAACAAGG	SSU, ITS1-5.8S-ITS2	White *et al*. (1990)
ITS3	GCATCGATGAAAACGCAGC		
LR0R	ACCCGCTGAACTTAAGC	LSU	Vilgalys and Hester (1990)
LR5	TCCTGAGGGAAACTTCG		

2.3 Results and Discussions

2.3.1 Surface sterilization test on root of *Miscanthus condensatus*

The preliminary results of sterilization test were obtained after incubation. The roots sterilized merely by Tween20 still had a lot bacteria remained (3300 ± 560 colonies ml^{-1}), while the root after ethanol addition sterilization had 120 ± 30 colonies ml^{-1}. Only sodium hypochlorite addition was able to totally eliminate contaminating microbes. Therefore, we defined the root sterilized by the second method as rhizoplane fraction while by the third method as endophyte fraction.

2.3.2 Isolation and molecular identification of rhizoplane and endophytic bacteria

To evaluate the populations of potential bacteria in/adhere the root of *M. condensatus*, the density of bacterial populations of both compartment of rhizoplane and endophyte were counted. The results revealed that rhizoplane fraction contained 7.2 $\times 10^6$ CFU g^{-1} (root dry weight) which was approximately 4.2 times higher bacterial density than endophyte fraction (1.7×10^6 CFU g^{-1}). A total of 301 bacterial isolates were obtained, of which 138 from rhizoplane fraction and 163 from endophyte fraction (Table 2.3.1). Nucleotide sequencing of the nearly full length of the 16S rRNA gene from all isolates possessed 97-100% similarity with a species reference in the GenBank database. These bacteria were affiliated with 43 species of 17 genera. In terms of phylum, most isolates belonged to *Proteobacteria* (94.36% of the total number of isolates), followed by *Firmicutes* (4.32%). Isolates from phylum *Bacteroidetes* and *Actinobacteria* comprised only 0.66% of each (Table 2.3.1). Interestingly, the results showed that bacterial diversity was similar between rhizoplane fraction (13 genera) and endophyte fraction (11 genera).

Although Proteobacteria was the predominant bacterial phylum in both rhizoplane and endophyte factions with similar abundance (more than 90%), the bacterial community composition at the class level within the phylum were different (Table 2.3.1). The culturable bacterial community of endophyte fraction was characterized by the most predominance of *Gammaproteobacteria* (77.3%), composed of mainly *Pseudomonas*, and the second predominance of class *Betaproteobacteria* (14.72%) mainly detected by the genera of *Burkholderia*. In contrast, *Betaproteobacteria* groups (54.35%), represented by the genera *Janthinobacterium*, *Duganella*, and *Burkholderia* were predominant class in the rhizoplane bacterial community, while class *Gammaproteobacteria* accounted for 41.3%. Comparing between two fractions, the phylum of *Bacteroidetes*, the class of *Alphaproteobacteria*, the family of *Microbacteriaceae* were only detected in endophyte fraction. At the genus level among these groups, *Chitinophaga*, *Rhizobium*, *Sphingomonas*, *Luteibacter*, and *Stenotrophomonas* were described as plant growth promoting endophytic bacteria (Chimwamurombe *et al*., 2016; Guglielmetti *et al*., 2013; Luo *et al*., 2019; Palaniappan *et al*., 2010; Singh and Jha, 2017). In addition, *Microbacterium testaceum* was described by its effectiveness on protection against plant pathogens, Morohoshi *et al*. (2011) also presented in endophyte fraction merely. Whereas, *Janthinobacterium*, *Collimonas*, *Massilia* and *Curtobacterium* with antifugal activity (Haack *et al*., 2016; Ofek *et al*., 2012; Raupach and Kloepper, 1998; Song *et al*., 2015) existed in rhizoplane fraction only.

Interestingly, *Pseudomonas helmanticensis*, a phosphate-solubilizing bacterium (Ramírez-Bahena *et al*., 2014), was abundant in both fractions and only cultured in samples collected in November. Phosphorus is one of the major macronutrients necessary for biological growth and

development of plants. However, volcanic ash is rich in minerals aluminum and iron which can bind to phosphates making it unavailable to plants. The phosphate-solubilizing bacteria are able to release phosphorus through the process of mineralization and solubilization to be available for plants (Sembiring *et al*., 2015). Besides, well known diazotrophic bacterial *Herbaspirillum frisingense* also been identified in both fractions. In the case of samples collected in September, *Pseudomonas rhodesiae* was abundant in both fractions. *Pseudomonas rhodesiae* was known to antagonize fungal pathogens and able to solubilize inorganic P sources and produce siderophores (Romero *et al*., 2016). Genus *Pseudomonas* were predominant in both fractions in samples from two periods, possibly due to their rapid growth and ability to utilize various substrates as nutrients and produce various compounds to survive under such stressing conditions. The second dominant group in endophyte fraction was genus *Dyella* (sampled in March), a member of the family *Xanthomonadaceae*. Also, members of *Collimonas*, *Duganella* and *Burkholderia* were enriched in the rhizoplane fraction, possibly owing to their versatile abilities to utilize root metabolites, degrade aromatic compounds and produce antimicrobial substances (Yang *et al*., 2017).

Regarding to bacteria capable of fixing nitrogen, 120 bacterial isolates were able to grow and change the NFA medium color from yellowish green to brilliant blue, suggesting that they have the ability to reduce atmospheric nitrogen through nitrogenase reductase enzyme. The nitrogen-fixing bacteria were species of genera *Pseudomonas* (26.3%, 54%), *Burkholderia* (21.1%, 17.9%), *Dyella* (2.6%, 23.2%), *Xanthomonas* (7.9%, 1.8%) in rhizoplane and endophyte fraction, respectively. Besides, *Janthinobacterium*, *Massilia*, *Duganella*, *Collimonas*, *Curtobacterium*, and *Neisseria* were only detected in rhizoplane fraction accounted for 42% while *Staphylococcus* and *Sphingomonas* only

existed in endophyte fraction and accounted for 3.6%. These results supporting the hypothesis that these isolates might be diazotrophic bacteria. In addition, the sequence analysis indicated that there are potential new bacterial species among the isolates which showed less than or equal to 97% identity to published 16S rRNA genes of known species. These novel putative species were related to genera *Collimonas*, *Duganella*, and *Burkholderia*. It is not surprised that there are novel bacteria in *M. condensatus* which colonized on the volcanic deposit, as this plant and its associated bacterial community has been rarely studied previously. Also, it is possible that these bacterial species could support the plant to adapt to the nutrient poor habitat.

Proteobacteria due to its traits of phototrophy, photoheterotrophy, and chemolithotrophy, usually dominates in the early bacterial community of ecosystems with limited nutrient resources such as deglaciated soils, volcanic deposits and other newly exposed minerals (Guo *et al*., 2014). Our results also showed Proteobacteria was the most abundant phylum in the endophytic bacterial community of plant growth on volcanic deposits. Especially the family *Pseudomonadaceae* in *Gammaproteobacteria* and *Oxalobacteraceae* in *Betaproteobacteria*. The findings of these results are consistent with previous studies following Fujimura *et al*. (2012) and Guo *et al*. (2014).

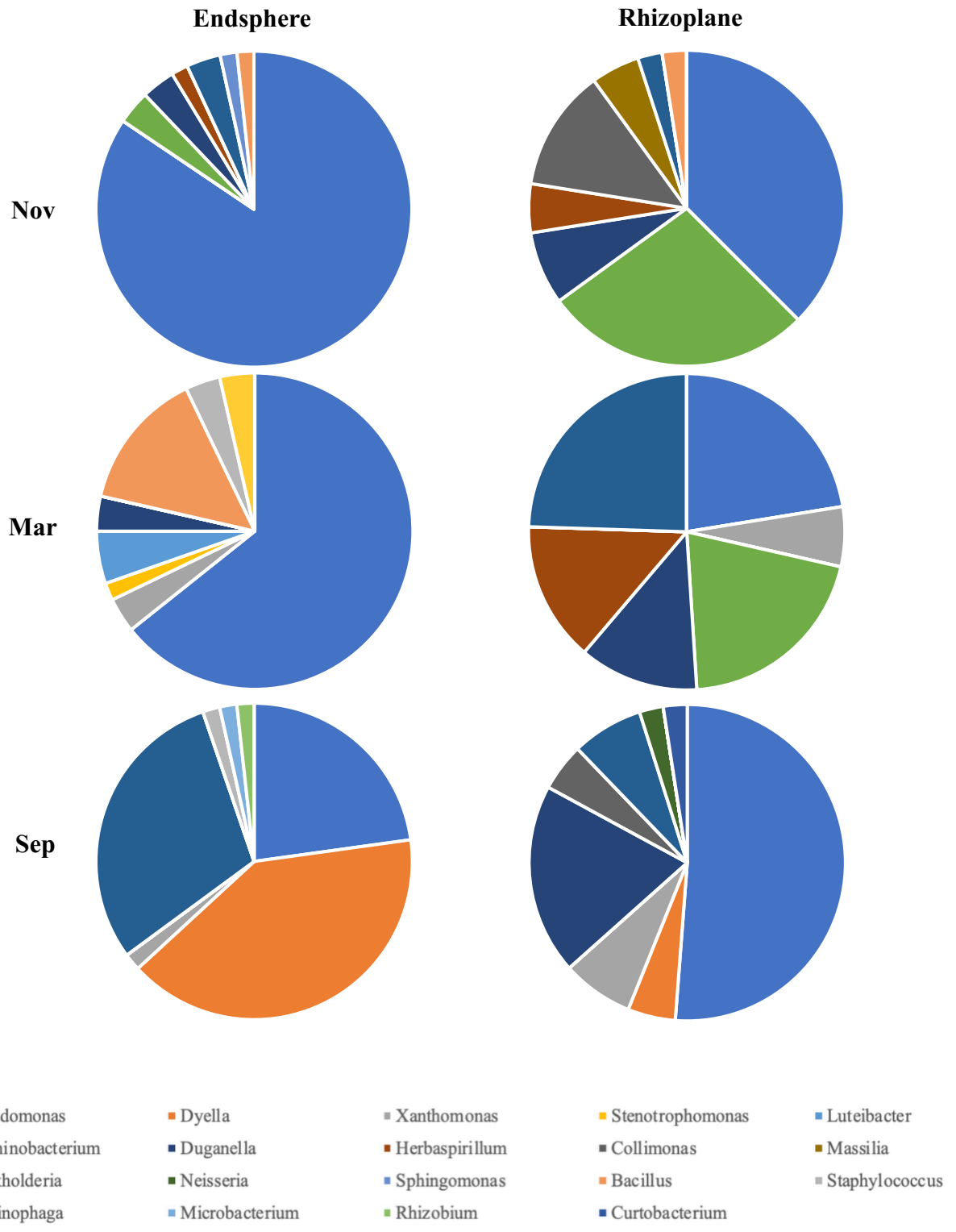


Fig. 2.3.1 Pie diagrams show strains count of endosphere and rhizoplane fraction from three sampling periods at genus level.

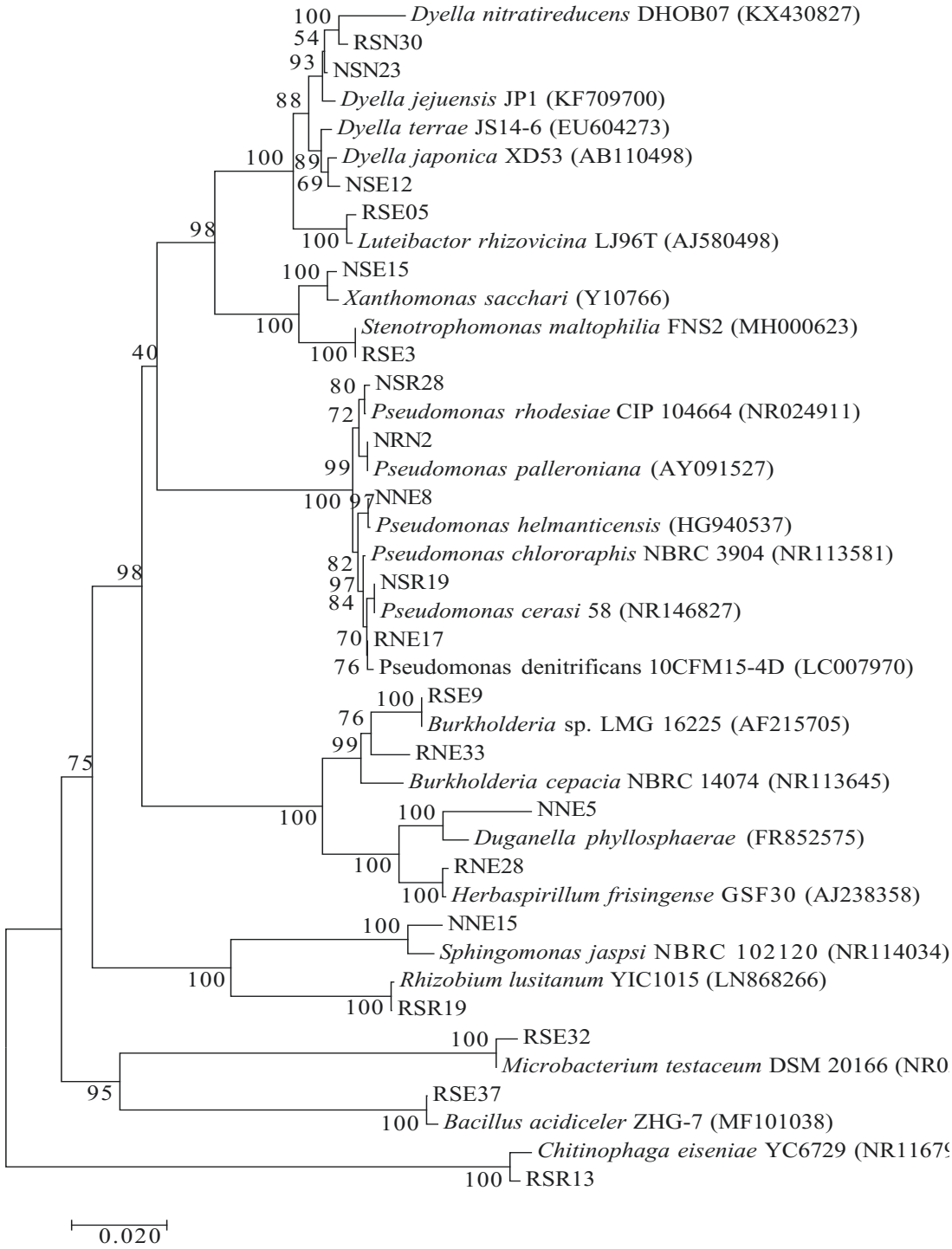


Fig.2.3.2. Neighbor-joining phylogenetic tree of the 16S rRNA gene sequences (1301 nt) of bacterial isolates of pioneer plant, *Miscanthus condensatus.* Isolate names are coded with tripartite code indicating medium (N, NFb medium; R, R2A medium), sampling period (N, Nov sample; M, March sample; S, September sample), and sample fraction (R, Rhizoplane; E, Endosphere), followed by number of isolates in the brackets.

Table 2.3.1. Taxonomic classification of bacterial isolations from two fractions, three sampling periods, and two mediums.

Identified taxum	Fraction		Sampling period			Medium	
	Rhizoplane	Endosphere	Nov	Mar	Sep	R2A	NFA
Proteobacteria	132	152	96	93	95	164	120
Alphaproteobacteria	0	2	1	0	1	1	1
Rhizobium (*R. lusitanum*)	0	1	0	0	1	1	0
Sphingomonas (*S.* sp.)	0	1	1	0	0	0	1
Betaproteobacteria	75	24	31	37	31	42	57
Janthinobacterium (*J.* sp)	23	0	13	10	0	1	22
Duganella (*D. zoogloeoide*; *D. sacchari*; *D.* sp.)	17	4	5	8	8	5	16
Herbaspirillum (*H. frisingense, Paraherbaspirillum soil*)	9	1	3	7	0	7	3
Collimonas (*C. arenae*)	7	0	5	0	2	6	1
Massilia (*M.* sp.)	2	0	2	0	0	0	2
Neisseria (*N. subflava*)	1	0	0	0	1	0	1
Burkholderia (*B. sordidicola; B. cepacia; B. lata B. kirstenboschensis; B. nodosa; B. fungorum; Paraburkhoderia caffeinilytica)*	16	19	3	12	20	23	12
Gammaproteobacteria	57	126	64	56	63	121	62
Pseudomonas (*P. helmanticensis*; *P. fluorescens*; *P.* sp *P. palleroniana*; *P. denitrificans*; *P. chlororaphis*; *P. fuscovaginae*; *P. cerasi*; *P. rhodesiae*; *P. prosekii;*	47	98	64	47	34	101	44
Stenotrophomonas (*S. maltophilia*)	0	1	0	1	0	1	0
Luteibacter (*L. anthropi*; *L* sp.)	0	3	0	3	0	3	0
Dyella (*D. japonica*; *D. koreensis*; *D. nitratireducens*; *D. terrae*)	2	23	0	0	25	11	14
Xanthomonas (*X. sacchari*; *X. oryzae*)	8	1	0	5	4	5	4
Firmicutes	5	8	2	10	1	12	1
Bacillus (*B. amyloliquefaciens*; *B. acidiceler*)	2	8	2	8	0	10	0
Staphylococcus (*S. epidermidis*; *S. sp.*)	3	0	0	2	1	2	1
Bacteroidetes	0	2	0	2	0	2	0
Chitinophaga (*C. eiseniae*; *C. sp.*)	0	2	0	2	0	2	0
Actinobacteria	1	1	0	0	2	1	1
Microbacterium (*M. testaceum*)	0	1	0	0	1	1	0
Curtobacterium (*C. flaccumfaciens*)	1	0	0	0	1	0	1
Total	138	163	98	105	98	179	122

* Nov, sampled in November; Mar, sampled in March; Sep, sampled in September.

2.3.3 Isolation and molecular identification of rhizoplane and endophytic fungi

A total of 339 putative endophytic (249 isolates) and rhizoplane (90 isolates) fungi were isolated at a up to 97% sequence similarity level from the root of *M. condensatus*. Based on the partial ITS sequencing, 95.37% of these isolates belonged to the phylum *Ascomycota* and clustered into groups corresponding four classes: *Leotiomycetes* (79.92%), *Sordariomycetes* (6.56%), *Eurotiomycetes* (6.18%), and *Dothideomycetes* (1.93%). In addition, 3.09% belonged to *Basidiomycota*, 1.16% belonged to the phylum of *Mucoromycota and* 0.38% belonged to the phylum *Dikarya* (Table 2.3.2). Fungi of the class *Leotiomycetes*, order *Helotiales* were the most represented both in rhizoplane and endophyte fractions. This order includes many known dark septated endophytic (DSE) fungi such as *Phialocephala fortinii*, and *Phialocephala heltevica*, described to be capable of promoting plant growth and enhancing of plant stress tolerance in various plant species. The presence of *P. fortinii* associated with plant tissues has been potentially demonstrated that has the ability to produce a variety of extracellular enzymes which break down complex forms of carbon, nitrogen and phosphorus commonly present in soils and thus potentially provide originally unavailable nutrients for plants (Jumpponen *et al*., 1998). Thus, it can be an important mycorrhizal symbiont in the volcanic ecosystems.

However, the dominant isolates were *Talaromyces verruculosus* (27.8% of the whole community) in rhizoplane fraction, which has been recognized as an important source of a variety of structurally novel active secondary metabolites. As an example, the ethyl acetate extract exhibited both antifungal activities against 11 strains of plant pathogens and antibacterial activities against four bacterial strains (Miao *et al*., 2012). Within the *Basidiomycota* phylum, four genera: *Tulasnella*, *Phlebiopsis*, *Phaeophlebiops*, and *Hypochnicium* were all isolated from endophyte fraction. Of the 25 genera, 15 were specific endophytic fungi, and only 2 were specific epiphytic fungi, and 8 shared between them. Besides, the relative abundances of some abundant genus were different between rhizoplane and

endophyte fractions. Chiang *et al*. (2001) isolated and sequenced fungal endophytes of *Miscanthus*, they found that their DNA barcodes from isolates were most similar (percentage sequence similarity in BLAST) to *Cladosporium oxysporum* and *C. cladosporioides*. Of the strains isolated from *Miscanthus sinensis* on the island of Dokdo, 75% were *Penicillium* sp. and 25% were *Aspergillus* sp. (Rhu *et al*., 2011). These species differ from those isolates those found in the current study. These fungi might be utilized in future application to enhance plant growth. Yet it requires further validation study through inoculation to test its feasibility.

Table 2.3.2. Summary of the endophytic fungal isolates among three months of sampling in *Miscanthus condensatus*

Phylum	Class	Blast top-hit	Sequence similarity (%)	Accession number in NCBI	Total number		
					Nov	Mar	Sep
Ascomycota	Sordariomycetes	*Acremonium sp*.	98	KT192555.1	4	2	0
		Sarocladium sp.	99	MG649463.1	3	2	0
		Xylariaceae sp.	97	AB741591.1	1	1	0
		Arthrinium phaeospermum	99	MH857420.1	0	2	2
	Leotiomycetes	*Phialocephala fortinii*	97	KJ817297.1	24	17	16
		Phialocephala helvetica	97	MT107593.1	21	36	37
		Phialocephala sp.	99	KT323172.1	11	14	16
		Pezicula ericae	99	NR155653.1	0	5	2
	Eurotiomycetes	*Talaromyces verruculosus*	97	MG748649.1	9	2	2
		Penicillium funiculosum	97	JQ724527.1	3	0	0
	Dothideomycetes	*Pyrenochaetopsis setosissima*	97	LT623227.1	2	2	1
Basidiomycota	Agaricomycetes	*Tulasnella calospora*	98	JQ713577.1	1	0	0
		Hypochnicium cremicolor	97	KP814161.1	1	0	0
		Phaeophlebiopsis peniophoroides	98	KP135417.1	0	0	3
		Phlebiopsis gigantea	98	MH114867.1	0	0	3
		Polyporus arcularius	99	KP283489.1	0	1	0
Mucoromycota	Mortierellomycotina	*Mortierellales sp.*	97	JQ272348.1	0	2	1
					80	86	83

Chapter 3

A novel bacterial species of *Duganella* sp. isolated from the endosphere of the pioneer plant *Miscanthus condensatus*

3.1 Introduction

The genus Duganella, a member of the Oxalobacteraceae family belong to the class Betaproteobacteria. The type strain *Duganella zoogloeoides* isolated from wastewater was first established by (Hiraishi *et al*., 1997) with the reclassification of *Zoogloea ramigera* IAM 12670T (P. R. Dugan 115) due to their different phenotypic and unique 16S rRNA sequence. Afterwards, *D. violaceinigra*, which was isolated from a forest soil sample collected from Yunnan province, China, was then investigated. However, it was proposed to reclassify into a novel genus *Pseudoduganella* gen. nov. as the novel species *Pseudoduganella violaceinigra* comb. nov. by Kämpfer *et al*. (2012) because of the low 16S rRNA gene sequence similarities and striking differences in chemotaxonomic and other phenotypic features, including the colony pigmentation. Up to now, the members of genus Duganella were still short with five species: *D. zoogloeoides* (Hiraishi *et al*., 1997), *D. phyllosphaerae* isolated from the leaf surface of Trifolium repens in Germany (Kämpfer *et al*., 2012), *D. sacchari*, *D. radices* isolated from rhizosphere soil and rhizoplane of field-grown sugar cane (Madhaiyan *et al*., 2013), and the latest species *D. ginsengisoli* isolated from a ginseng field in Korea (Zhang *et al*., 2016). They are Gram-negative, obligately aerobic, chemo-organotrophic, non-spore-forming, rod-shaped bacterium with flagella. In this paper we describe strain NNE5 isolated from root endophyte of *Miscanthus condensatus* which was the pioneer plant near the summit crater after 18 years of the

2000 eruption of a basaltic volcano on the island of Miyake, Japan, located on the western rim of the Pacific Ocean (34º05'N, 139º31'E).

3.2 Materials and methods

3.2.1 Bacterial isolation

Root pieces of *M. condensatus* were washed with tap water, sterilized with 0.005% Tween 20 for 1 minute 3 times and washing 3 times with sterile-distilled water, followed by 70% Ethanol for 1 minute and 3 times final wash with sterile-distilled water. After that, the sterilized pieces were grinded and 100µl homogenate was spread onto Nitrogen-free base agar (NFA) medium. The plates were then incubated at 30ºC for 7 days. A single colony was purified, subcultured and characterized by comparative 16S rRNA gene sequence analysis. Then preserved in R2A broth containing 10% (v/v) glycerol at -80ºC.

3.2.2 Phenotyping and biochemical identification of the isolated bacteria

For phenotypic tests, chemotaxonomic and molecular systematic characterization, strains were cultured in R2A plate. Gram staining was performed with a Gram stain kit. Bacterial suspensions were examined by phase-contrast microscopy for cell morphology. Motility was tested by culturing the organisms in R2A media that contained 0.3% agar (w/v). Growth test was checked on R2A plate, nutrient agar (NA), trypticase soy agar (TSA), potato dextrose agar (PDA), Luria-Bertani (LB) agar and MacConkey agar medium. The optimum temperature for growth were determined at pH 6.8, unless otherwise stated, for 7 days at 4, 10, 15-40°C (at intervals of 5°C). Tolerance to salinity was

evaluated on R2A broth with 0, 1, 2, 3, 4, 5 and 10% (w/v) NaCl at 30°C. The pH range for growth was examined at pH 3.0, 4.0-10.0 at 0.5 pH unit intervals in R2A broth at 30ºC for 3 days. Growth at different pH and the maximum specific growth rate (h-1) at different temperatures and NaCl concentrations were determined by measuring optical densities (OD600) of broth cultures as described previously. Catalase activity was determined by assessing bubble production in 3% (v/v) H_2O_2, and oxidase activity was determined with 1% (w/v) tetramethyl-p-phenylenediamine according to the manufacturer's instructions (Ohta and Hattori, 1983). Indole production was analysed using Kovács' regent in 1% peptone broth. For the hydrolysis of carboxymethyl-cellulose, the isolate was cultured on R2A plates supplemented with 0.5% (w/v) carboxymethyl-cellulose. After culturing, the plates were stained with 0.2% aqueous Congo red dye solution and washed with 1M NaCl solution in order to observe the zone of clearing. Additional phenotypic characteristics were determined using API Zym and API 20NE, API 50CH (bioMerieux) and to determine the use of different carbon compounds, Biolog GENIII Microplates were used according to the manufacturers' instruction.

Cells biomass for chemotaxonomic studies was obtained by incubating stains in shake flasks at 30°C at 180 rpm for 48h. Cells were collected by centrifugation at 8000 g for 15 min at 4°C for 3 times and then freeze-dried overnight. Cellular fatty acids were analysed as Ohta & Hattori (1983). Cellular fatty acid methyl esters were prepared by heating dried cells in 5% anhydrous methanolic HCl at 100ºC for 3 hours (Ikemoto *et al*., 1978) and then analysed by capillary GLC using the Sherlock Microbial Identification System (Version 6.0) (MIDI, USA) based on the TSBA6 calculation method and TSBA6 library databases. For polar lipid analysis, the cellular lipids were extracted three times from 100 mg of freeze-dried cells, using 100 µg of chloroform/methanol (2:1,

v/v). Total lipids were examined by two-dimensional TLC (HPTLC plates, Silica gel 60, 10×10 cm; Merck, USA) with a solvent system consisting of chloroform, methanol, and water (70:27:4, v/v) in the first direction and chloroform, methanol, acetic acid, water (80:12:12:4, v/v) in the second direction, respectively. Then identified using the method of Minnikin *et al*. (1984). Polar lipids were visualized by spraying the TLC plate with 5% molybdophosphoric acid. To detect spots and their colour reactions, ninhydrin solution, Dittmer-Lester regent, anisaldehyde regent, and Schiff's regent were used for lipids containing amino groups, phospholipids, sugar lipids, and glycolipids, respectively. Isoprenoid quinones were extracted and analysed by reverse-phase TLC. Isoprenoid quinones were extracted using the methods described previously (Bao *et al*., 2014), and analysed as described by (Collins' and Jones, 1981).

3.2.3 Molecular identification and sequencing analysis

Genomic DNA of strain NNE5 was obtained using phenol-chloroform extraction according to the method described by Wang and Wang (1997). The 16S rRNA gene sequenes was obtained by PCR amplification of genomic DNA using a universal primer set, and the nucleotide sequence (nucleotide posations 10-1541) was determined as described by Lu *et al*. (2011). The DNA sequence was aligned with available 16S rRNA gene sequences in GenBank using the program BLAST. Multiple alignment with sequences from related species and clones was performed by using the program CLUSTAL W (Thompson *et al*., 1994) in MEGA11. Evolutionary distances were calculated using Kimura's two-parameter model. Phylogenetic trees were reconstructed using the neighbor-joining (Saitou & Nei, 1987) algorithms in MEGA11. Topologies of the resultant trees were evaluated by bootstrap analyses

(Felsenstein, 1985) based on 1000 replications.

3.3 Results

3.3.1 Phenotyping and biochemical identification of the isolated bacteria

Cells of strain NNE5 were Gram-stain-negative, colonies on R2A agar (Difco) are 0.5-2.0 mm in diameter after 2 days of grown with yellow, circular, tough, dry, and low convex with entire margins and a smooth appearance. Catalase activity was present, whereas oxidase activity was weak. Growth occurred between 4-37ºC (optimum 30ºC) and pH 4.5-10 (optimum pH 6.5) and were sensitive to NaCl 0-0.6 (w/v). It was able to grow on R2A agar but not on NA plates, TSA, LB agar, PDA or MacConkey agar. It can hydrolysis starch, aesculin, but not gelatin or urea. Nitrate reduction was positive, but indole production was negative. The predominant polar lipids of strain NNE5 were phosphatidylethanolamine (PE) and phosphatidyglycerol (PG), found in all strains in Duganella. In addition, strain NNE5 contained diphosphatidylglycerol (DPG), Phosphatidylserine(PS), as well as the unidentified aminolipid (AL) (Fig. 3.3.2). The total cellular fatty acid composition of the strain NNE5 and 5 other Duganella species are shown in (Table 3.3.2). The predominant fatty acids were C12:0, C16:0, Sum in feature 3 (C16:1ω7c and C16:1ω6c) and Sum in feature 8 (C18:1ω7c or C18:1ω6c) in addition to C17:0 cyclo (Table 3.3.2). The major isoprenoid quinone is ubiquinone Q-8.

Table 3.3.1. Phenotypic characteristics of strain NNE5 and the type strains of four phylogenetically related Duganella species. Strains:1, strain NNE5 (this study); 2, *D*. zoogloeoides JCM 20729^T (Zhang *et al*., 2016); 3, *D*. *sacchari* KCTC 22381^T (Zhang *et al*., 2016); 4, *D*. *radices* KCTC 22382^T (Zhang *et al*., 2016); 4, *D*. *ginsengisoli* DCY83^T (Zhang *et al*., 2016). +, positive reaction; -, negative reaction.

	1	2	3	4	5
Reduction of nitrates	+	+	-	+	+
Hydrolysis of Aesculin	+	+	-	-	-
Hydrolysis of gelatin	-	+	-	-	+
Arginine dihydrolase	-	+	-	-	-
Enzyme activity (API ZYM)					
Esterase lipase	+	+	-	+	+
Lipase	+	-	+	-	-
Valine arylamidase	+	+	-	-	+
Trypsin	+	-	-	+	-
a-Chymotrypsin	+	+	-	+	-
b-Galactosidase	+	+	-	+	-
a-Glucosidase	+	+	-	+	+
N-Acetyl-b-glucosaminidase	+	-	+	+	-
Assimilation of:					
D-Mannose	+	+	+	+	-
D-Maltose	+	+	+	+	-
Potassium gluconate	+	-	-	+	-
Malic acid	+	+	-	-	-
Trisodium citrate	-	+	-	-	-
L-Rhamnose	-	+	-	+	-
D-Mannitol	-	-	+	-	-
Salicin	-	-	-	+	-
L-Fucose	-	-	-	+	-
L-Arabinose	+	+	+	+	-
Potassium 5-ketogluconate	-	-	+	-	-
Urease	-	+	-	-	-
Lactic acid	+	-	+	+	+
L-Alanine	+	+	-	+	-
L-Serine	+	+	-	+	+
L-Fucose	-	-	-	+	-
L-Histidine	+	-	+	+	-

Table 3.3.2. Fatty acid composition (%) of NNE5 and 5 related Duganella species. Strains: 1, NNE5 (this study); 2, *D. zoogloeoides* IAM 12670^T (Kämpfer *et al*., 2011); 3, *D. phyllosphaerae* T54^T (Kämpfer *et al*., 2011); 4, *D. sacchari* Sac-22^T (Madhaiyan *et al*., 2013); 5, *D. radices* Sac-41^T (Madhaiyan *et al*., 2013); 4, *D. ginsengisoli* DCY83^T (Zhang *et al*., 2016).

Fatty acid	1	2	3	4	5	6
Saturated						
C10:0	0.17	0.5	0.9	0.75	0.6	ND
C12:0	7.27	3.7	13.5	13.6	8.81	4.7
C14:0	1.63	0.6	0.8	1.5	0.41	0.6
C16:0	36.62	27.9	24.7	25.68	29.22	31.2
C18:0	0.22	ND	ND	1.6	0.19	5.1
C17:0 cyclo	10.55	ND	ND	17.96	11.89	ND
C19:0 iso	1.41	ND	ND	ND	ND	ND
Unsaturated						
C16:1ω5c	0.15	ND	ND	ND	ND	ND
C17:1ω9c	0.19	ND	ND	ND	ND	ND
C17:1ω7c	0.7	ND	ND	ND	ND	ND
C18:1ω7c	ND	15.4	8.6	3.1	7.68	ND
Hydroxy						
C10:0 3OH	4.63	4.5	10.3	14.15	6.49	6.4
C12:0 2OH	2.13	ND	ND	ND	ND	2.4
C12:0 3OH	ND	ND	ND	ND	ND	ND
Summed feature 3	29.5	47.4	41.2	20.47	33.78	43.1
Summed feature 8	4.37	ND	ND	ND	ND	4.4

Sum in feature 3 (C16:1ω7c and C16:1ω6c) and Sum in feature 8 (C18:1ω7c or C18:1ω6c)

GEN III MicroPlate ™

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

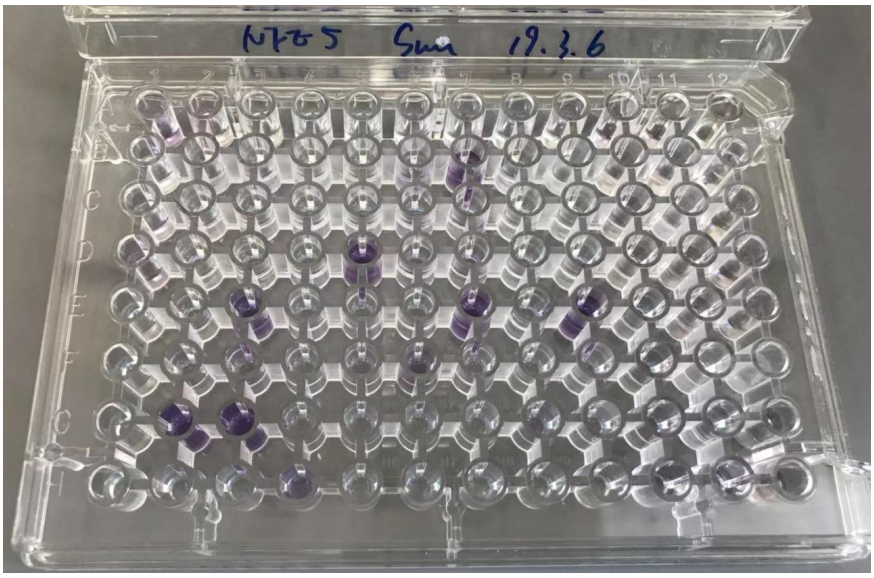


Fig. 3.3.1. 94 biochemical tests (71 carbon source utilization assays and 23 chemical sensitivity assays) to profile and identify the bacteria NNE5.

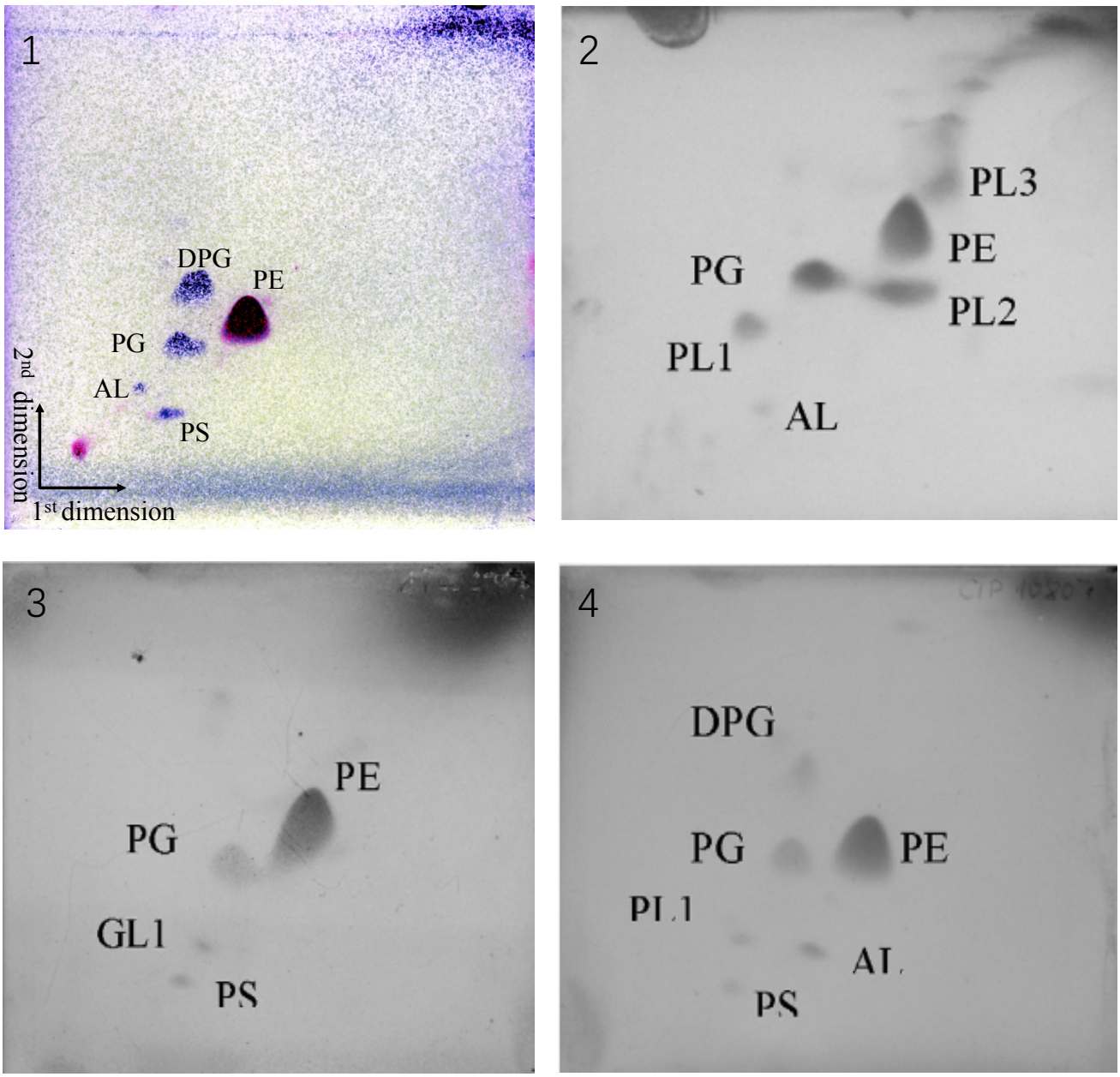


Fig. 3.3.2. Total polar lipid profile of strains: 1, NNE5 (this study); 2, *D. phyllosphaerae* T54; 3, *D. zoogloeoides* IAM 12670T, 4, *P. violaceinigra* YIM 31327T after staining with molybdenum blue. PG, phos- phatidylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine, PL1, PL2, PL3, unidentified phospholipids; AL, unidentified amino lipid; GL1, unidentified glycolipid.

3.3.2 Molecular identification and sequencing analysis

The almost-complete 16S rRNA gene sequence of strain NNE5 (1435 bp) was determined. Sequence comparisons with representative bacteria differentiated this bacterium from all currently described member of the families Duganella (Fig. 3.3.3). Levels of 16S rRNA gene sequence similarity with its closest relatives were 96.7%. As shown in the phylogenetic tree (Fig. 3.3.3), strain NNE5 formed a monophyletic clade with *Duganella phyllosphaerae* and *D. zoogloeoides*.

The major characteristics differentiating strain NNE5 from phylogenetically related genera are summarized in Table 3.3.1. Based on these results and the above-mentioned phylogenetic characterization, we conclude that strain NNE5 should be placed in a novel species of genus Duganella, for which the name *Duganella* NNE5 sp. nov. is proposed.

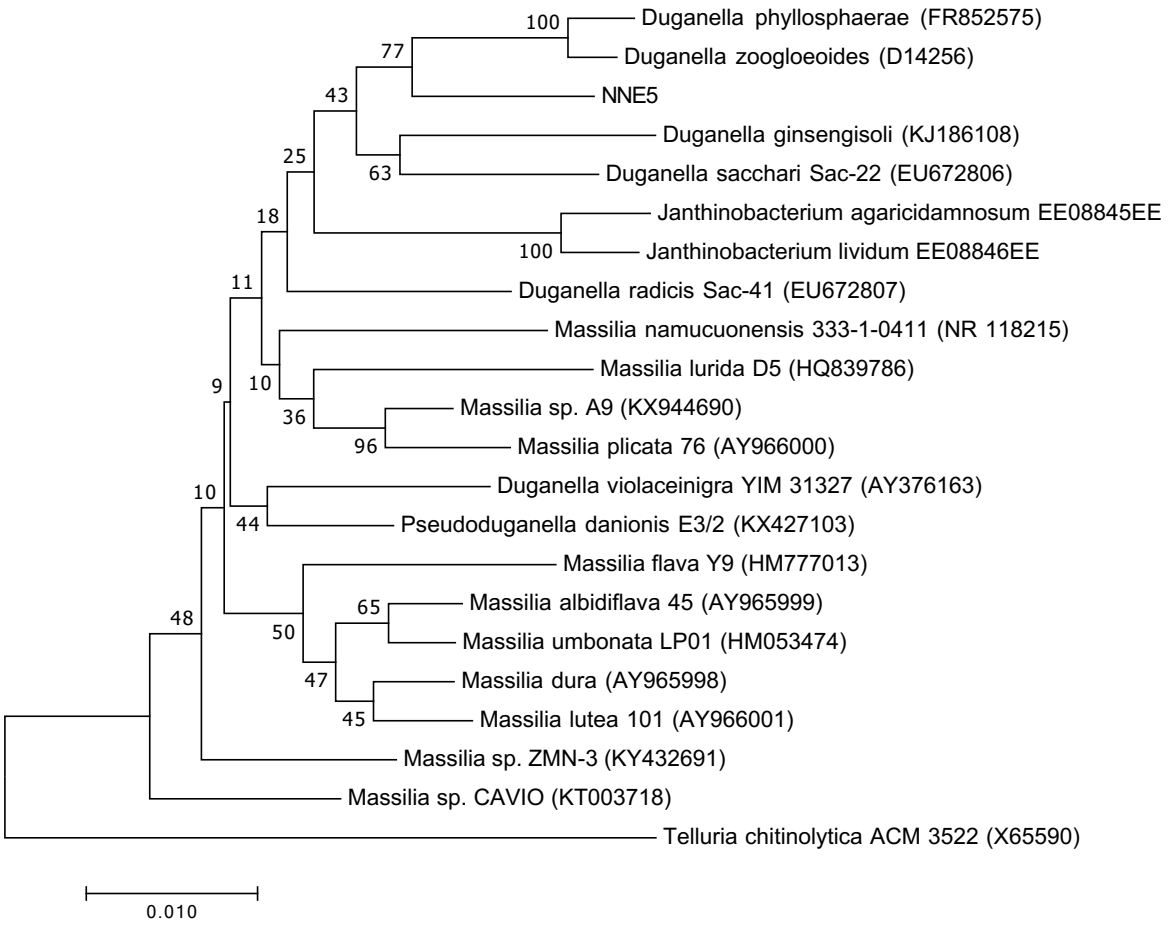


Fig. 3.3.3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain NNE5 and closely related taxa within the family Oxalobacteraceae. Numbers at nodes are bootstrap values (%) based on 1000 replicates, values lower than 50% are not shown. *Telluria chitinolytica* ACM 3522 was used as an outgroup. Bar, 0.01 nt substitutions per position.

3.4 Discussions

An obligately aerobic bacterium, designated strain NNE5, was isolated from root endophyte of pioneer grass *Miscanthus condensatus* at Miyake-jima in Japan. Cells of the strain were Gram-negative, non-spore-forming and motile rods, the strain formed yellow-pigmented colonies on R2A medium. Strain NNE5 grew at pH 4.5-10 (optimal growth at pH 6.5), at 4-37ºC (optimum growth at 30ºC) and at salinities of 0-0.5% (w/v) NaCl. On the basis of 16S rRNA gene sequence analysis, strain NNE5 says shown to belong to the genus Duganella and showed the closest phylogenetic similarity to *Duganella zoogloeoides* (96.7%). The major polar lipids were phosphatidylethanolamine, phosphatidyglycerol, diphosphatidylglycerol, and phosphatidylserine. The predominant ubiquinone and polyamine were Q-8. The major fatty acids were C16:0 and C17:0 cyclo. On the basis of the information described above, from this polyphasic study, strain NNE5 represents a novel species of the genus Duganella, for which the name *Duganella* NNE5 sp. nov. is proposed.

Chapter 4

Characterization of bacterial communities associated with the pioneer plant on the volcanic deposits of the 2000 eruption in Miyake-jima using culture-independent method

4.1 Introduction

It was observed that the specific microbes could associate with root zone through root-microbe symbiosis and exert effects on plants by microbial interfering actions (Lareen *et al*., 2016). Currently, it is widely accepted that the plant rhizo-microbiome contributes directly or indirectly to the growth of plants, providing phytohormones, solubilizing nutrients, fixing nitrogen (N_2), establishing biocontrol of phytopathogens, and chelating metallic ions (De-la-Pena and Loyola-Vargas, 2014). Bacterial community composition can differ substantially amongst rhizo-compartments between rhizosphere soil, rhizoplane, and endosphere (Baudoin *et al*., 2001; Chen *et al*., 2016; Fan *et al*., 2017). The rhizosphere is a complex micro-ecological zone that is enriched in carbon (C), energy, and nutrients (Hinsinger *et al*., 2009). Whilst, bacteria endophytes accommodate within plants tissues (endosphere) and help plant access nutrients and water. Both root associated compartments represent highly dynamic and diverse zones for biochemical interactions between soil components, roots, and microbiota (Hinsinger *et al*., 2006). It was reported that i) bacterial alpha diversity generally decreasing from the rhizosphere soil towards the root, e.g., endosphere (Donn *et al*., 2015; Huang *et al*., 2019); but ii) endosphere exert larger impacts on plant growth via bringing water and nutrients more efficiently and resisting to soil stress strongly. Climatic factors, plant genotype, and especially edaphic variables are the main determinants in shaping the diversity and composition of the root-associated microbiome community in root associated compartments (Bulgarelli *et al*., 2012; Pii *et al*.,

2016; Schreiter *et al*., 2014). Yet, the comparison between both compartments of rhizosphere and endosphere in volcanic ecosystem is still lacking.

As volcanic eruptions have occurred ubiquitously on earth (Cockell *et al*., 2013), the volcano shaped terrestrial environments, i.e., Andisols (Tsai *et al*., 2010). Andisols derived from volcanic ash have many distinctive morphological, physical, and chemical properties which are rarely found in soils derived from other parent materials (Ugolini and Dahlgren, 2002; Tsai *et al*., 2010). The unique properties of Andisols offer numerous opportunities to increase our understanding of volcano shaped ecosystem. The absence of vegetation at many active volcanic sites exacerbates the situation by limiting post-depositional inputs. After years of volcanic eruption, plants start to grow on the Andisols filled with deposits. Volcanic materials can benefit pioneer plant and harbor high diversity of microorganisms within a few years of deposition (Fujimura *et al*., 2016). The role of pioneer bacteria as early colonizers in those new environments contributed to the primary process of soil formation and early ecosystem development on newly exposed volcanic ash. Soil formation and maturity, in turn, modulate microbiome. This is because the microbial colonization and succession are substantially affected by edaphic variables. The availability of suitable substrates and nutrients can be used by microorganisms for energy and biosynthetic metabolism in cells. The spatial and temporal changes of the microbial community and physicochemical properties would be remarkable in Andisols, depending on volcano eruption intensity and distance (with gradient content of ash and nutrients). Investigating into bacterial community dynamics as affected by the variation of volcanic ash content along the distance to volcano is crucial to understand the microbial distributions and functions in the early volcanically derived ecosystems, i.e. regulating the organic matter accumulation,

nutrient availability and plant uptake.

The pioneer bacteria colonizing on early volcanic deposits is able to fix carbon (C) and nitrogen (N) from the atmosphere and mineral, which contribute to the organic matter formation (King and King, 2014; King and Weber, 2008; Oberhofer *et al*., 2019). Various studies on volcanic deposits and other newly exposed minerals have shown that the phylum Proteobacteria usually dominates the early bacterial community (Guo *et al*., 2014; King and Weber, 2008; Oberhofer *et al*., 2019). This is mainly because of their advantageous traits such as phototrophy, photoheterotrophy, and chemolithotrophy in vocanic soils with limited nutrient resources. Additionally, the phylum Chloroflexi is predominant in non-vegetated volcanic soils (Gomez-Alvarez *et al*., 2007; King and Weber, 2008; Weber and King, 2010). Some genera within Chloroflexi have been associated with CO and H_2 consumption in organic matter-poor volcanic deposits. Guo *et al*. (2014) investigated early bacterial communities on the volcanic ash deposit site near the crater and they showed that autotrophs, N_2-fixer, and Fe(II) oxidizers such as *Acidithiobacillus ferrooxidans* and the *Leptospirillum* groups dominate in the bacterial community. Plant-microbe interactions drive primary ecosystem succession on the volcanic deposits. It is unclear how the changes that occurred in volcanic material-derived Andisols can i) influence bacterial community compositions with different distance to volcano, and ii) effect plant associated microorganisms (within different rhizo-compartments).

Relatively few studies have examined the significance of the spatial patterns of bacteria community on the volcanic deposits with different distance to volcanic eruption. A more comprehensive understanding of the microbe-plant interactions in rhizosphere compartments developing on volcanic deposits remains largely unknown. This study, thus, aimed to investigate how

the Miyake-Jima volcano ash (with different distance) induced changes in the composition and diversity of bacterial communities in the rhizosphere and endosphere. We hypothesize that: (i) bacterial community composition will be dependent on sampling site with distance difference to volcano, and (ii) variations in the composition, and the diversity of the bacterial community occurs across rhizosphere and endosphere.

4.2 Materials and methods

4.2.1 Soil sampling for rhizo-compartments

Miyake-Jima is a basaltic volcanic island located on the Izu-Mariana arctic ridge in the Pacific Ocean, approximately 180 km south of Tokyo (34°05′ N, 139°31′E). Mt. Oyama, the active volcano situated in the center of the island, last erupted in 2000. The patchy vegetation of a pioneer grass *Miscanthus condensatus* plant with rhizosphere soil samples were collected from two sites according to volcanic eruptions near the crater site OY (34°04.69′N, 139°31.04′E; 553 m a.s.l.), and less affected by volcanic eruptions IG7 site (34°05.37′N, 139°30.84′E; 547 m a.s.l.) (Fig. 4.2.1). Triplicates were taken at each point, and all soil samples were stored in polyethylene bags at 4 ºC until their transport to the laboratory. The excess soil was manually shaken from the roots leaving approximately 1 mm of soil still attached to the roots. To collect rhizosphere soil, roots of the grass were placed into a sterile flask with 50 ml of sterile phosphate-buffered saline (PBS) solution and stirred vigorously with sterile forceps to separate the soil from the root surfaces. This soil (rhizosphere compartment) was stored at -80°C until DNA extraction. Root surface sterilization was proceeded on the same procedures as previous experiment. The surface sterilized roots were then stored at -80ºC until DNA

extraction.

Fig. 4.3.1 Photographs showing the pioneer plants Miscanthus condensatus on site IG7 (A-C) and site OY (D).

4.2.2 DNA extraction from rhizo-compartments

Total DNA was extracted in duplicates from 0.5-1.0 g of samples using ISOIL for Beads Beating (Nippon Gene, Tokyo, Japan) with skim milk powder (Wako, Osaka, Japan) according to manufacturer's instructions with slight modifications. Additionally, the root designated for endosphere fraction was pre-homogenized before DNA extraction by adding with 10 ml aliquot of sterile distilled water and macerated with mortar and pestle. NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to quantify the DNA concentration. Finally, DNA samples were stored at -80^{o}C before molecular analysis.

4.2.3 Gene amplification and sequencing

The bacterial 16S rRNA gene fragments were amplified using primer sets targeting the V4-V5 variable region (Vandenkoornhuyse *et al*., 2007). The forward primer was 515F (5'-GTGCCAGCMGCCGCGGTAA-3') linked with a specific-sample 5-bp barcode sequence at the 5'end of primer, and 806R (5'- GGACTACHVGGG TWTCTAAT -3') was used as the reverse primer (Bates *et al*. 2011; Caporaso *et al*. 2011). Each sample was amplified in triplicate, and then the three reaction products were pooled and purified using Agencourt Ampure XP beads (USA) and quantified by real-time quantitative PCR (Eva Green TM). All amplicons were pooled across all samples at equimolar concentrations (20 ng μl^{-1}) into a composite sample, and the index sequencing of paired-end 250 bp was performed on an Illumina HiSeq 2000 platform.

4.2.4 Data processing and statistical analysis

The data of bacterial 16S rRNA was processed by the QIIME 1.8.0-dev pipeline (Caporaso *et al*., 2012). Low-quality reads (quality score < 20, read length < 200 bp, and sequence errors) were discarded. Chimeric sequences were identified by UCHIME (Edgar, 2010) and removed. The remaining high-quality sequences were clustered into operational taxonomic units (OTUs) based on a 97% pairwise identity using the UCLUST algorithm (Edgar, 2010). The representative sequences of each OTU were then chosen for subsequent alignment and taxonomic assignment with the RDP classifier. Taxonomy is assigned to bacterial phylotypes of the Green genes database and fungal phylotypes of the UNITE database. All data sets were rarefied to 39,000 sequences per sample for the bacterial α- and β-diversity analyses to prevent potential bias caused by different sequencing depth. For β-diversity analysis, the dissimilarity of bacterial communities was calculated via PCoA.

4.2.5 Differential abundance analysis

To calculate the differential abundance, R package "DESeq2" was used (log2-fold change in the relative abundance of each OTU) between the rhizo-compartments (Love *et al*., 2014). OTUs were independently filtered out that were sparsely represented across samples (*i.e.*, those OTUs for which the DESeq2-normalized count across samples ("base Mean") was less than 0.6). Sparse OTUs did not contain sufficient sequence counts to provide statistically significant results and were removed, thus reducing the number of multiple comparisons performed. We adjusted the *p*-values with the Benjamini and Hochberg (BH) correction method and selected a study-wide false discovery rate (FDR) of 10% to denote statistical significance (Love *et al*., 2014). We defined "responding OTUs" as OTUs with a differential abundance greater than one and an adjusted *P*-value of <0.1.

4.2.6 Co-occurrence network construction

For the co-occurrence network inference, all possible Spearman's rank correlations between OTUs with more than five sequences were calculated (2200 OTUs). This previous- filtering step removed poorly represented OTUs and reduced network complexity, facilitating the determination of the core soil community. We considered a valid co-occurrence event to be a robust correlation if the Spearman's correlation coefficient (r) was both 40.6 and statistically significant (*P*-value <0.01). The nodes in the reconstructed network represent the OTUs at 90% identity, whereas the edges correspond to a significant and robust correlation between nodes. Networks were explored and visualized with the interactive platform Gephi. Cytoscape (v3.5.0) was used for network visualization and topological analysis.

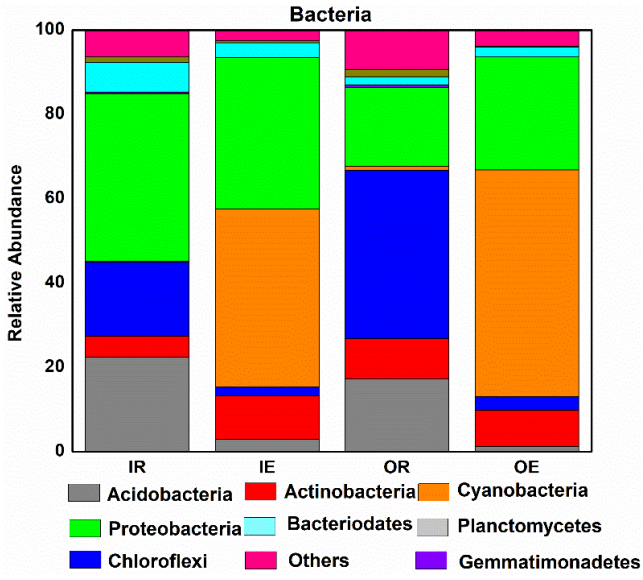
4.3 Results

4.3.1 Taxonomic analysis of the bacterial community

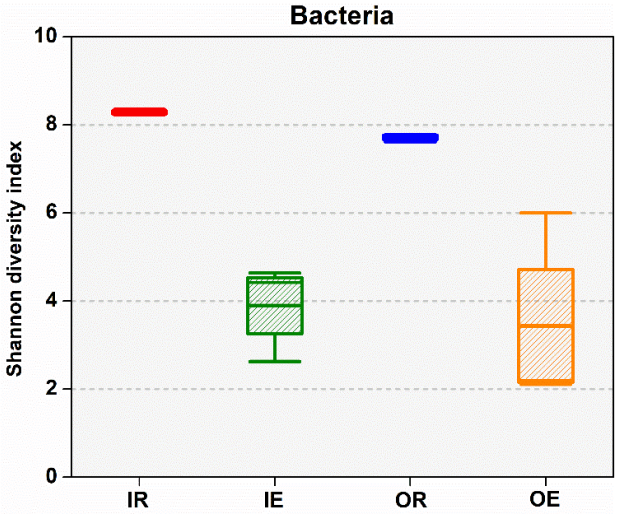
The percentages of relative abundance of rhizosphere bacterial composition at the phylum level varied as affected by ash content derived from a volcanic eruption, showing that Proteobacteria dominated in IG7 site. In contrast, Chloroflexi dominated in OY site (Fig. 4.3.2 a). The relative abundance of the endosphere compartment followed similar bacterial composition with a significantly greater proportion of Cyanobacteria and Proteobacteria regardless of the site differences. Shannon Index of the bacterial community was observed two distinct ranges for rhizosphere (8.3-7.6) and endosphere (4-2.3), respectively. There was no significant difference in α-diversity between the OY and IG7 sites. Nevertheless, the α-diversity of the rhizosphere was considerably higher than that

of the endosphere (Fig. 4.3.2 b). In comparison, bacterial diversities of endosphere had pronounced decrease, which implies the reduction of the complexity of the bacterial community as accompanied by more dominance of phylum Cyanobacteria. The first two axes of PCoA explain 27.9% and 20.5% of the total variation in the microbial community (Fig. 4.3.2 c). Bacterial communities of the endosphere in both IG7 and OY were clustered together and away from those of the rhizosphere. Whereas, the rhizosphere soil samples evident that microbial community was more pronouncedly distinct between two different sites affect by eruption distance.

(A)



(B)



(C)

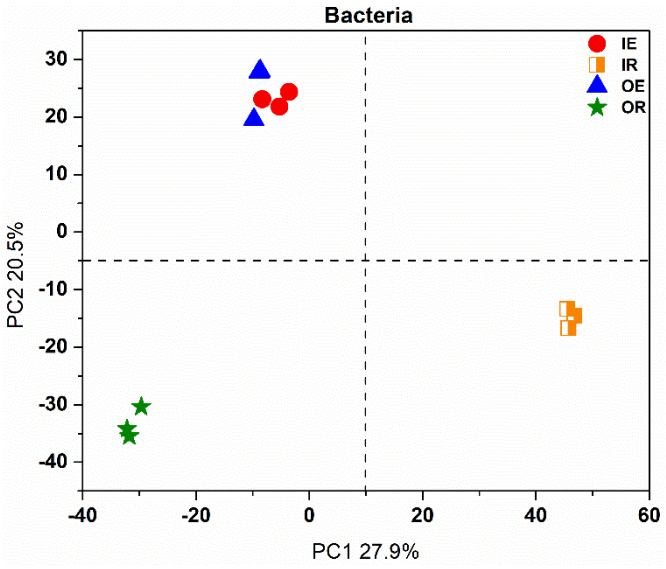
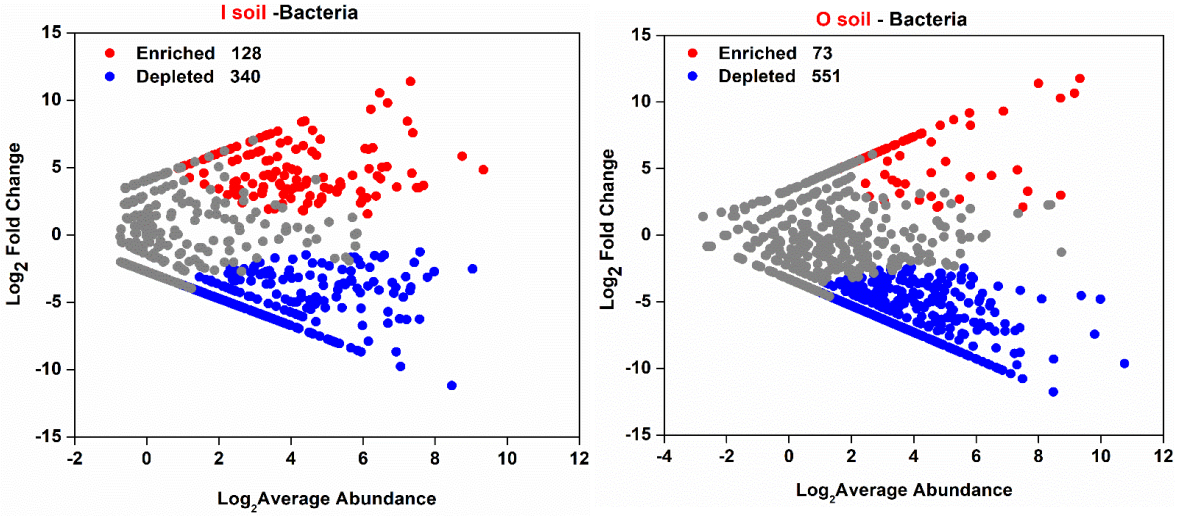


Fig. 4.3.2 Relative abundance of bacterial community composition of rhizosphere and endosphere of both sites. (A), bacterial community (B), alpha diversity (C) and beta diversity across the rhizosphere and endosphere in the differently sites (site OY and IG7) affected by Miyake-Jima volcano eruption.

4.3.2 Association of significantly enriched and depleted OTUs within the rhizosphere compartments

Differential abundance analysis was used to identify enriched OTUs, with community separation between the rhizosphere and the endosphere of two different sites (OY and IG7). The rhizosphere compartment had a high ratio of statistically significant OTUs that were depleted in both soils. The rhizosphere soil of site IG7 was enriched for 128 OTUs, while depleted for 340 OTUs. Whereas, the soil from the site OY was enriched for 73 and depleted for 551 OTUs (Fig. 4.3.3). The majority of the OTUs enriched in the site IG7 were affiliated to order Betaproteobacteriales (phylum Proteobacteria) while order Holophagales belonging to phylum Acidobacteria was depleted. Site OY

was enriched and depleted by Sphingomonadales, and Acetobacterales belongs to phylum Proteobacteria.



Enriched	Depleted
Proteobacteria	Acidobacteria
Actinobacteria	Chloroflexi

Enriched	Depleted
Firmicutes	Proteobacteria
Proteobacteria	Chloroflexi

Fig 4.3.3. Enriched and depleted OTUs of rhizosphere compartments of the site IG7 (A) and site OY (B) compared to the endosphere as determined by differential abundance analysis. Each point represents an individual OTU, and the position along the y-axis represents the abundance fold change.

4.3.3 Co-occurrence network pattern

Network analysis of co-occurrence patterns was conducted to identify coexisting interactions between microbial taxa in the rhizosphere and endosphere. We pooled microbial OTU datasets of each compartment of both sites (IG7 and OY sites) together. Total connections increased in the

rhizosphere (438) compared to the endosphere (28). The percentage of co-presence (bacterial-bacterial) raised from 55.71% to 71.43%, and the drastic reduction occurred for the percentage of mutual exclusion from 44.29% to 28.57% towards endosphere from the rhizosphere. It might indicate that more intensive microbial interactions occurred in the rhizosphere compared to the endosphere. The keystone microbiotas in the rhizosphere were the genera of *Burkholderia, Caballeronia, Asticcacaulis and Paraburkholderia* (affiliated to phylum of Proteobacteria)*, Candidatus_Solibacter* (affiliated to phylum of Acidobacteria), and the corresponding keystone in the endosphere was *Mycobacterium* (affiliated to phylum of Actinobacteria).

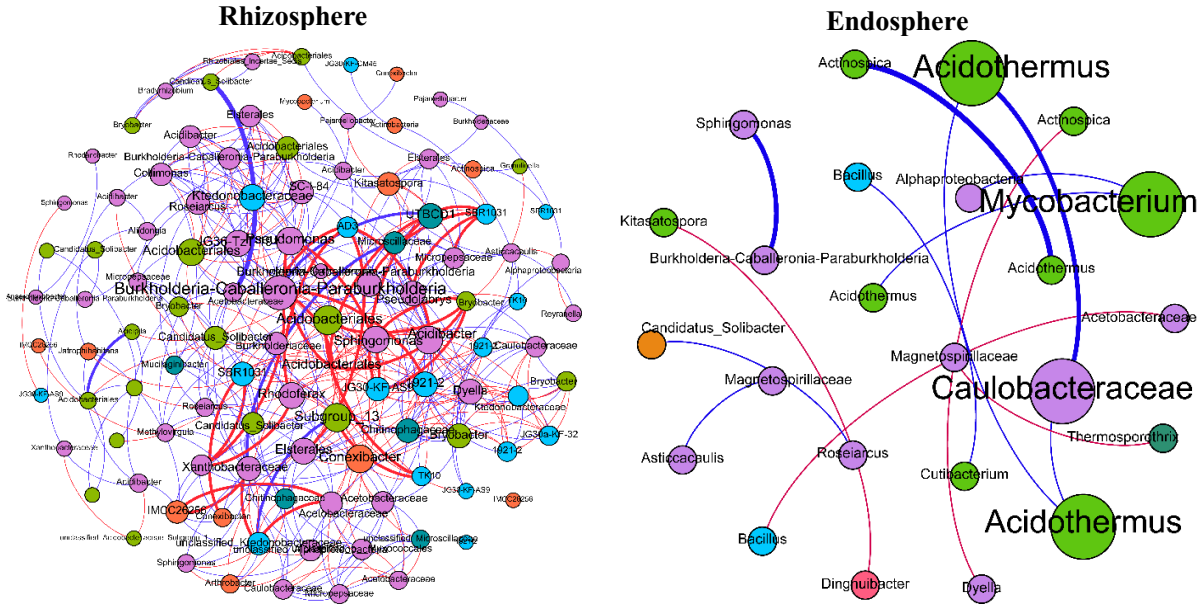


Fig 4.3.4. Microbial co-occurrence patterns of rhizosphere and endosphere microbiomes. In the networks, colored nodes assign if corresponding OTUs are assigned to major genera. The size of each node is proportional to its average relative abundance. The blue and red links indicate significant positive and negative correlations between two nodes.

Table 4.3.1: Topological features of the networks of rhizo-compartments

Topographical properties	Rhizosphere	Endosphere
Nodes	111	42
Edges	438	28
Modularity	0.46	0.25
Clustering coefficient	0.241	0.062
Average path length	2.296	1
Co-presence (%)	55.71	71.43
Mutual-exclusion (%)	44.29	28.57
Keystone genera	*Burkholderia-Caballeronia-Paraburkholderia* *Candidatus_Solirubacter* *Asticcacaulis*	*Mycobacterium* *Acidothermus*

4.4 Discussion

Most studies concerning microbial community inhabiting the rhizosphere compartments have focused on their adaption and responses to the shift of environmental variables (e.g., elevated CO_2), edaphic properties (e.g., soil type), and plant factors (e.g., species) (Chen *et al*., 2016; Jeewani *et al*., 2020). A paucity of information exists on the bacterial communities in the rhizosphere, and endosphere compartments of the soils affected by volcano eruption. The current study investigated the compositional differentiation of bacterial microbiomes inhabiting the rhizosphere and endosphere of *Miscanthus condensatus* on the volcanic ash deposit sites (profoundly affected OY site by short distance to eruption, and less affected IG7 site by long distance to eruption). We found variations in the composition and diversity of bacteria community with varying degrees across root-associated compartments and sites.

4.4.1 Microbial community shift along with sites differently affected by the volcanic eruption

Taxonomic analysis revealed that the bacterial community composition of the rhizosphere compartment of both sites predominantly consisted of the phyla of Proteobacteria, Acidobacteria, Actinobacteria, and Chloroflexi (Fig. 4.3.2 a). It was reported that Acidobacteria and Actinobacteria are stable in the vegetated sites after volcanic eruptions (Gomez-Alvarez *et al*., 2007; Hernández *et al*., 2020). In the current study, it showed that Proteobacteria was the most abundant phylum of the rhizosphere of the *Miscanthus condensatus* on the less affected site by volcanic eruption (the site IG7), while Chloroflexi was the dominant phylum in the site OY near the crater. It was reported that the new substrates derived from volcanic eruptions, e.g., volcanic ash, contains negligible amounts of organic carbon (*Chadwick et al*., 1999). So the filamentous anoxygenic phototrophic Chloroflexi was found to be present at higher levels (about 40%) in the rhizosphere samples of site OY closer to the crater with less vegetation. It was reported that the phylum Chloroflexi is predominant in less vegetated soils after the volcanic eruption (Gomez-Alvarez *et al*., 2007; Hernández *et al*., 2020; Weber and King, 2010), with the ability to consume CO and H_2 in young, organic matter-poor volcanic deposits(Oberhofer *et al*., 2019; Weber and King, 2010). Interestingly, phototrophic genera in the phylum of Proteobacteria were also found to be abundant in young vegetated sites in Miyake-Jima in Japan (Guo *et al*., 2014; Weber and King, 2010). Hydrogen-oxidizing members of Proteobacteria were also isolated from the vegetated volcanic sites in the Miyake-Jima and Kilauea volcanoes (Gomez-Alvarez *et al*., 2007; Weber and King, 2017). Still, the nutrients in the ash, particularly in the site IG7 is abundant for microbial growth. Here, we found the shannon diversity index was higher at the rhizosphere compartment of the site IG7 that was less affected by volcanic

eruptions (Fig. 4.3.2 b). Higher diversity of microbial community in site IG7, was mostly likely due to the adaptation to the larger organic matter and nutrients in soils which had been more developed, compared to the site OY (near the crater with newly deposited volcanic ash acidified by exposure to SO_2 gas. A study by (Gomez-Alvarez *et al*., 2007) showed that bacterial diversity is higher in vegetated deposits than in the youngest soil with depleted nutrients and SOM. It was confirmed that the bacterial diversity was higher in vegetated areas than that of bare volcanic deposits, in which diversity increased as the area became fully vegetated (Byloos *et al*., 2018). It is well-known that root-associated microbial diversity plays a vital role in adapting plants to extreme environments (Redman *et al*., 2002). The benefits to microbe-plant interactions might speed up soil development as well. Overall, these results suggest that the spatial distribution of volcanic deposits along distance to volcano that lead to differences in the community composition.

4.4.2 Microbial community variation along the rhizo-compartments

Although microbes are among the first arrivals on volcanic deposits, relatively little is known about the spatial distributions of microbial colonization patterns along with the rhizo-compartments. The changes of α-diversity within the rhizo-compartments (Fig. 4.3.2 a) likely depends on a "filtration effect" (Dibbern *et al*., 2014). It was reported that the rhizosphere preferentially selected microbiome by recruitment to the vicinity of the root and followed by a transition from the rhizosphere to the endosphere (Edwards *et al*., 2015; Valverde *et al*., 2014). The ordination of beta diversity revealed a clear separation between the different rhizosphere compartments as well as between the two volcanic sites (Fig. 4.3.2 c). The difference in the bacterial community structure in the rhizosphere and

endosphere indicates that selective power acting on bacterial assemblages has likely shaped the well-adapted microbiome along root compartments.

Cyanobacteria dominated the relative abundance of the endosphere compartment compared to the rhizosphere (Fig. 4.3.2 b). Whilst endosphere had a pronounced decrease in bacterial diversities (Shannon index 4-2.3), and that might be accompanied by more dominance of Cyanobacteria (42% and 53% for IG7 and OY soil, respectively) (Fig. 4.3.2 a, b). It is also conceivable that the release of ions and build-up of organic matter from chemotrophic bacterial weathering may play a synergistic effect in promoting the initial build-up of Cyanobacterial, and lichen biomass. The extracellular polysaccharides excreted by phylum Cyanobacteria facilitated adhesion between the ash particles and the changes in their composition. These results suggest an analogy between the processes in the new volcanogenic areas and the biological weathering on volcanic soils (Gerasimenko *et al*., 2013).

The OTUs enriched in the rhizosphere are very successful at colonizing the root, as 128 and 73 OTUs enriched in the rhizosphere of OY, and IG7 sites, respectively. However, a depletion effect in the rhizosphere is implied by the high ratio of significantly depleted OTUs compared with enriched OTUs (340 vs. 128 and 551 vs. 73), implied that the rhizosphere is the most exclusive compartment in both sites (Fig. 4.3.2). The vast majority of microbes enriched in the rhizosphere are depleted in the endosphere (Fig. 4.3.3), suggesting that the selective for colonization of the interior occurs at the rhizosphere and that the rhizosphere may serve an important gating role for limiting microbial penetrance into the endosphere (Edwards *et al*., 2015; Peiffer *et al*., 2013). The co-occurrence network identified complex interactions between the taxa of microbial communities. It is consistent that the enriched genera *Burkholderia, Caballeronia, Paraburkholderia, Asticcacaulis* (Affiliated to

phylum Proteobacteria) was the keystone microorganisms in the rhizosphere co-occurrence network (Fig. 4.3.3 and Fig. 4.3.4). Those taxa are considered as carbon monoxide-oxidizers and N_2 and H_2 fixers that those were symbiotic and non-symbiotic strains from a very wide range of environmental habitats such as volcanic deposits (Weber and King, 2010, Weber and King, 2017; Oberhofer *et al*., 2019). Especially *Burkholderia* and *Paraburkholderia* encode *coxL* genes associated with the CODH enzyme responsible for CO oxidation (King and Weber, 2008; Weber and King, 2017). It was clear that the unique abilities of these bacterial genera cooperate in co-occurrence networks, supply mutual benefits with nutrients for growth and survival, which were considered as playing important roles in the early stages of recovery.

In contrast, the endosphere compartment represents a less complicated microbial network. The most abundant phylum showed higher incidences of co-occurrence in the endosphere was Actinobacteria (Figure 4.3.4). The keystone genera for the endosphere were *Mycobacterium* and *Acidothermus* (affiliated to Actinobacteria). Actinobacteria are known to endophytes through horizontal transmission from rhizosphere, producing a plethora of bioactive compounds (Frank *et al*., 2017), which have diverse biochemical habitats ranges such as extremophile environments (Oberhofer *et al*., 2019; Quintana *et al*., 2013). Thus, Actinobacterial endophytes survive under high selective pressure competing in spatially limited habitat (Caraballo-Rodríguez *et al*., 2017; Oberhofer *et al*., 2019).

Chapter 5

Characterization of fungal communities associated with the pioneer plant using culture-independent method and evaluation effects of Dark Septate Endophytic fungi to promote rice growth under various pH condition

5.1 Introduction

Numerous studies demonstrated that symbiotic fungi play a significant role in the establishment of vegetation in harsh environments such as volcanic soil with limited nutrients, agricultural soils with extremely low pH. The association of these fungal micro-organisms that promote plant colonization or growth is significant in pool conditioned soil. As these fungal symbionts help plant survival mainly by: improving host nutrient uptake (Usuki and Narisawa, 2007; Yadav *et al*., 2009), defending against pathogens (Busby *et al*., 2016), promoting tolerance to abiotic stress (Rodriguez *et al*., 2008; Gill *et al*., 2016), and modifying trophic interactions (Clay, 1996; Omacini *et al*., 2001; Bultman *et al*., 2003).

One of the most common groups of monocotyledonous root endophytes is Dark Septate Endophytes (DSE), which usually colonize in tissues intracellularly and intercellularly of more than 600 living herbaceous and woody plant species (Jumpponen and Trappe, 1998). DSE, which is characterized by their morphology of melanized, septate hyphae and structure like microsclerotia, also confer the ability to improve plant performance through enhanced nutrient uptake, and increased ability to withstand adverse environmental conditions (Khastini and Jannah, 2021). Increasing evidence shows that DSE gradually become the most prevalent root colonizers under extreme

environmental conditions of different ecosystem (Haruma *et al*., 2021; Yu *et al*., 2021). For example, Huusko *et al*. (2017) reported DSE-dominated colonization in *Deschampsia flexuosa* roots along a postglacial land uplift gradient. Gonzalez Mateu *et al*. (2020) found that DSE inoculation *Phragmites australis* had higher aboveground biomass under mesohaline conditions. DSE, e.g., *Phialocephala fortinii*, promote host plant growth and adaptation to the hostile environment by: i) increasing resistance to heavy metal contamination and heat/drought stress via producing melanized cell walls (Haruma *et al*., 2021; Li *et al*., 2018) and, ii) facilitating uptake of nutrients such as nitrogen and phosphorous (Jumpponen *et al*., 1998; Surono and Narisawa, 2017).

Wild plant species living with mycoflora may have been lost their symbiosis ability during breeding of the cultivars used in agriculture (Yuan *et al*., 2010). Whilst, some of symbiotic fungi, which can assist plants to adapt to a given stress in a natural habitat, might increase tolerance of crop species to that stress in an agriculture system. Thus, from an agricultural point of view, the plant symbiotic fungi could be seen as an extended source for crop adaptation and growth in agronomy. In attempts to domesticate “wild” symbiotic fungi (associated with genetically wild type plant), some of these DSE species in natural system have been successfully transferred to agricultural species from their original host, providing benefits to the inoculated crops (Toju *et al*., 2018).

Rice (*Oryza sativa*) is the principal food grain crop (one of the four major food crops) for more than 3 billion people, and its consumption exceeds 100 kg per capita annually in many Asian countries (Yuan *et al*., 2010). During the last several decades, there have been major climatic events, including global warming, soil acidification, etc, that influenced agricultural productivity of rice around the world. Soil pH is a highly sensitive factor to determine plant survival, distribution, and interactions with microorganisms, which are vital for the availability of essential nutrients and plant growth (Luo

et al., 2013). About 13% of the world's rice is produced in acid soil. Compared with other crops, rice has relatively stronger Al toxic resistance (Famoso *et al*., 2010), and is also the most complex cereal crop with Al resistance genes (Ma *et al*., 2002). Nevertheless, as for other crops, heavy metal toxicity in acid soil limits rice growth and nutrients uptake, and subsequently reduces grain yield (Chen *et al*., 2020). The optimal pH range for rice growth is 5.0-8.5, which shows the likely reduction of yield in the soil with the extended pH range (Ma *et al*., 2002). To improve these soil acidity, liming is often used but is practically difficult and unsustainable.

Microorganisms inoculation is a sustainable approach to potentially promote plant resistance to acidic stress. For instance, plant-associated fungi, such as arbuscular mycorrhizal fungi (AM fungi), reportedly play a key role in the protection of plants in acidic soils (Toju *et al*., 2018). Yet, high concentrations of H^+ and Al^{3+} can inhibit hyphal growth and spore germination in AM fungi, thereby decreasing the possibility of colonizing plant roots (Clark, 1997; Van Aarle *et al*., 2002; Postma *et al*., 2007). Comparably, DSE show marked potential to help host plants resist acidity because of their higher H^+ tolerance than other colonizing fungi (Postma *et al*., 2007). Still, there is a lack of reports of DSE improving host crop (e.g., rice) growth under acidic conditions, especially an extremely acidic condition (e.g., pH 3.0).

For this purpose, culture-independent approaches were adopted, to comprehensively reveal the fungal communities of Miscanthus-associated, particularly DSE, from volcanic ecosystems. The core fungal taxa were identified by both culture-dependent and culture-independent methods, and their functions in promoting plant growth (via isolation-inoculation) in different pH soils were further evaluated via isolates-inoculation validation.

5.2 Materials and Methods

5.2.1 Sampling and preliminary treatment

The plant of *Miscanthus condensatus* and its rhizosphere soils were collected at site OY in November 2017. Three healthy specimens of *M. condensatus* were collected, kept in sterile plastic bags, and immediately stored on ice. Samples were kept at -20 ºC until DNA extraction and molecular analysis.

5.2.2 Illumina MiSeq sequencing for culture-independent identification

Roots of sample were added to 10-mL aliquots of sterile distilled water and macerated with a pestle and mortar for DNA extraction with DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA was purified using Ultra Clean DNA Purification Kit (MOBIO, Carlsbad, CA, USA). Then, DNA was eluted in 50 μL of Tris and EDTA buffer. A NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to quantify the DNA concentration. Finally, DNA samples were stored at -80ºC before molecular analysis. The second nuclear ribosomal internal transcribed spacer (ITS2) region of the rRNA operon was targeted using the fungal-specific primer pairs ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Chen *et al*., 2021). PCR amplification was carried out in triplicate with 50-μL reactions containing 25 μL of Premix Taq (TaKaRa, Shiga, Japan), 23 μL of sterilized MilliQ water, 0.5 μL of both forward and reverse primers (125 pmol), and 1 μL of template DNA. The PCR program had the following thermocycling conditions: 35 cycles of denaturation at 94ºC for 30 s, annealing at 54ºC for 30 s, 72ºC for 45 s, and a final extension of 72ºC for 10 min. PCR products were pooled and their relative quantity was estimated by running 5 μL of amplicon

DNA on 1.5% agarose gel, and products were purified with QIA Quick PCR Purification Kit (Qiagen, Shenzhen, China). The purified mixture was diluted and denatured to obtain an 8 pmol amplicon library and mixed with an equal volume of 8 pmol PhiX (Illumina) following the manufacturer's recommendations in the Illumina MiSeq reagent kit preparation guide (Illumina, San Diego, CA, USA). Finally, 600 μL of the amplicon mixtures were loaded with read 1, read 2, and the index sequencing primers. The paired-end sequencing (each 250 bp) was completed on a MiSeq platform (Illumina). The sequencing data were processed using the UPARSE pipeline (http://drive5.com/usearch/manual/uparse_pipeline.html). The raw sequences were subjected to quality control. The singleton and chimeric sequences were removed after dereplication, and the remaining sequences were categorized into operational taxonomic units (OTU) with 97% similarity and then assigned taxonomy using the UNITE database (https://unite.ut.ee/).

5.2.3 Inoculation

To further evaluate the functions of DSE in promoting plant growth, the isolates-inoculation was carried out. The experiment was conducted as a complete randomized factorial design with two factors (isolated species and soil pHs). The treatmeng of isolates-inoculation include: non-inoculation as control, inoculation with three dominant isolates (*Phialocephala fortinii, P. helvetica, and Phialocephala sp.*); and the second factor had three levels: pH 3, pH 4, and pH 5. Each treatment consisted of four replicates, and thus totaling 48 experimental pots in the study. Fungal inoculates were prepared by aseptically growing three dominant DSE isolates on Petri dishes with oatmeal agar medium (10 g L^{-1} oatmeal and 15 g L^{-1} Bacto agar enriched with nutrients: 1 g L^{-1} $MgSO_4.7H_2O$, 1.5

g L^{-1} KH_2PO_4, and 1 g L^{-1} $NaNO_3$).

Rice was chosen as a host plant in this study, considering its important role of rice in consumed cereal in the world particularly in Asia. To use rice as the tested plant is feasible, as rice is from the same family as *Micanthus*, and DSE is featured with its non-specific host character. Each pot was planted with two rice seeds. Rice seeds were surface-sterilized by immersion in 70% ethanol for 2 min, and a solution of 1% sodium hypochlorite for 5 min with agitation. The sterilized seeds were gently rinsed several times with sterilized distilled water, then dried overnight, and plated onto 1% water agar medium in Petri dishes for germination at 30ºC. Following pre-germination, 2-day-old seedlings (two seedlings per plate) were axenically transplanted as growing fungal colonies on the medium at pH 3, pH 4, or pH 5. The adjustment was made with 1M KOH (Wako Chemical Ind., Osaka, Japan) or 1 M HCl (WAKO Chemical Ind., Osaka, Japan). For DSE inoculation, two 5-mm plugs excised from the edge of an actively growing colony on culture medium were inoculated at a 1-cm range close to the rice seedlings. Seedlings transplanted onto non-inoculated medium were used as controls. The whole set was placed into sterile plastic culture bottles and incubated for 3 weeks at room temperature with an 18 h:6 h (L:D) regimen and intensity of 180 μmol $m^{-2}s^{-1}$. Assessed plants were harvested and oven-dried at 40ºC for 72 h. The shoot and root dry weights of treated plants were measured and compared with the control.

5.2.4 DSE root colonization observations

Root colonization by DSE fungal isolates was observed to confirm whether the selected DSE colonized the inner roots endophytically. Roots were harvested from plants after 3 weeks of

cultivation. Root systems were washed thoroughly under running tap water to remove adhering agar, then rinsed with distilled water, and used for root staining. The root samples were cleared with 10% (v/v) potassium hydroxide in a water bath at 80ºC for 20 min. Subsequently, roots were acidified with 1% hydrochloric acid at room temperature for 5 min, then stained with 50% acetic acid solution containing 0.005% cotton blue at room temperature overnight. Root fragments were placed on a slide glass and covered with a cover glass. Fungal colonization was observed using a light microscope equipped with an Olympus DP25 digital camera.

5.2.5 Statistical analyses

All statistical analyses were performed in the R environment (version: V4.1.2). Homoscedasticiy was checked using Levene's test and normality using Shapiro-Wilk's test. The differences of mean dry biomass between the analyzed traits of the seedlings in different treatments in this study were calculated and analyzed statistically with two-way analysis of variance (ANOVA) and Tukey's honestly significant difference test at P-values<0.05.

5.3 Results and Discussions

5.3.1 The core fungal taxa identified by both culture-dependent and culture-independent methods

This study compared the culture-dependent isolates with the fungal taxa revealed by culture-

independent methods. For the culture-independent methods, all reads were clustered into 224 OTUs, based on 97% sequence similarity, and the valid sequences were classified into five phyla, including two major dominant phyla of Ascomycota (71.5%) and Basidiomycota (17.1%), followed by Mortierellomycota, Mucoromycota, and Calcarisporiellomycota, while the cultivable endophytic fungi were classified into two different phyla of Ascomycota (97.5%) and Basidiomycota (2.50%). Fifteen and four classes were detected by culture-independent and culture-dependent approaches, respectively. Specifically, classes Sordariomycetes and Leotiomycetes (both belonging to phylum Ascomycota) were the major classes in terms of the number of OTUs. These data were in agreement with a previous study showing that Leotiomycetes and Sordarimoycetes were the major classes of endophytic fungi associated with plants (regardless of plant species, associated host tissue) in acidic, oligotrophic ecosystems and nutrient-limiting boreal and arctic areas (Arnold *et al*., 2007; Yuan *et al*., 2010; Ghimire *et al*., 2011; Luo *et al*., 2014; Knapp *et al*., 2019).

At the lower level, 27 orders were found by Illumina-based sequencing analysis, and 10 of them had an average abundance over 1%. Among these orders (from sequencing data), seven orders were identified by culture-dependent methods as well (Fig. 5.3.1). Hypocreales (35.6%), Helotiales (21.2%), and Eurotiales (13.2%) were the dominat orders observed by Illumina-based analysis (Fig. 5.1). Through culture-dependent methods, an abundance of Helotiales (70.0%) occupied the whole community, followed by Eurotiales (15.0%) and Hypocreales (8.75%). In general, the abundant orders of fungal isolates also showed abundance in the OTU table generated by high-throughput sequencing. The overlapping of taxa (Hypocreales and Helotiales) identified by both approaches suggests their significance and dominance in *Miscanthus condensatus*-associated fungal communities. Similarly, the key fungal and bacterial community in soils amended with wheat and oilseed residues were identified via culture and non-culture approaches (Laval *et al*., 2021). Several other studies also confirmed the feasibility to reveal major microbial taxa and showed the marked potential of adopting

the combination of both culture and non-culture approaches to identify putative taxa (Bai *et al*., 2015; Laval *et al*., 2021; Zheng *et al*., 2021). A combination of culture-dependent and culture-independent methods might provide a powerful strategy to identify and obtain novel endophytes.

The overlapping order Helotiales that identified by both culture-dependent methods was abundant in the *Miscanthus condensatus*-associated fungal community (Fig. 5.3.1). The isolates including *P. fortinii*, *P. helvetica*, and *Phialocephala* sp. belonged to Helotiales species, which are highly conserved and found to be co-occurring species in the root symbiont communities based on Sanger sequencing (Bruzone *et al*., 2015; Walker *et al*., 2011). This study also found these fungal taxa, *i.e., Phialocephala* sp., *P. helvetica*, and *P. fortinii*, were dominant in all samples irrespective of the sampling period (Table 2.3.2). Previous studies isolated *P. fortinii* from the root of *Pinus resinosa* (Wang and Wilcox, 1985), *Vaccinium vitis-idaea*, *Betula platyphylla* var. *japonica*, *Luetkea pectinate* (Addy *et al*., 2000), *Piceas abies*, *Betula pendula* (Menkis *et al*., 2004), *Rhododendron* sp. (Grünig *et al*., 2008), *Chamaecyparis obtusa*, and *Rubus* sp. (Surono and Narisawa, 2017). Yet, the phylogeny and ecological effects of *P. fortiniii* on plant quality still remain largely unknown (Tedersoo *et al*., 2009). For example, *P. fortiniii* itself is genotypically diverse and composed of at least 21 morphologically indistinguishable but genetically isolated cryptic species (CSP) (Grünig *et al*., 2008). Up to seven isolates belonging to *P. fortinii* have been formally described as CSP (Grünig *et al*., 2008). *Phialocephala helvetica* (sub-species of *P. fortiniii*) associated with the root of *Picea abies* (Stroheker *et al*., 2021) and *Pinus sylvestris* (Landolt *et al*., 2020), is regarded as one of the most common CSP. Although isolating and characterizing microorganisms could provide insights into their phylogenetic identification, physiological properties, and metabolic potentials, the adaptation mechanisms of DSE is required to better understand its colonization (Li *et al*., 2019). Yet, the colonization of these isolates, e.g., *P. fortinii*, on plant root remain largely unknown.

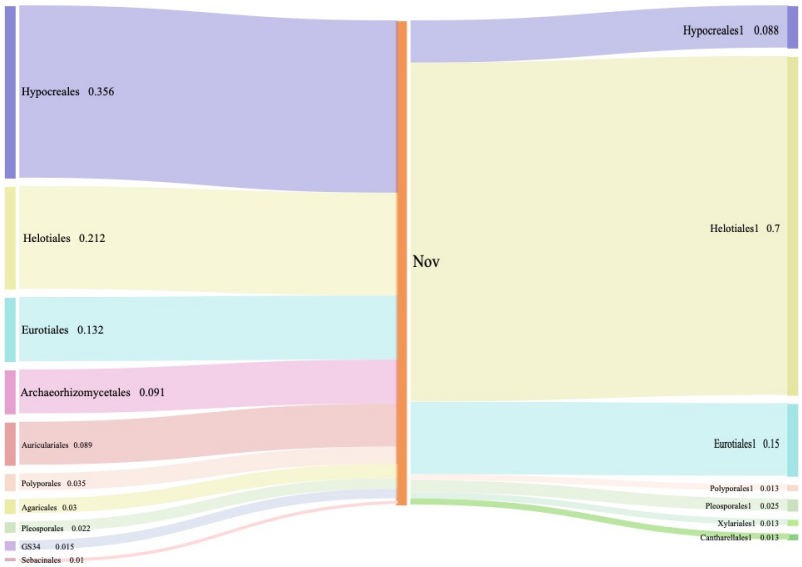


Fig. 5.3.1 Composition and relative abundance of endophytic fungi at order level by culture-independent (left) and culture-dependent methods (right)

5.3.2 Colonization of DSE fungal isolates in rice plant root

As rice is one of the four major food crops, to domesticate these isolated "wild" DSE benefit agriculture production. Thus, we transferred these DSE isolates from their original hosts of *Miscanthus condensatus* to agricultural species (rice). DSE is widely reported to be characterized with non-host specific, but different host (cross family) may have different responses (in terms of morphology) to the inoculated DSE isolate. For example, *P. fortinii* is frequently reported in roots and formed typical ectomycorrhizae with members of the Pinaceae plants (Jumpponen *et al*., 1998). In contrast, for other family plants, *P. fortinii* is often found to be an endophytic fungi. In addition, *C. chaetospira* was reported able to develop and form spiral structures resembling ericoid mycorrhizas within the roots of ericaceous plants (Usuki and Narisawa, 2005). Whilst *C. chaetospira*

colonizing in other host family are characterized by formation of microsleclerotia-aggregations of irregularly lobed hyphae and dark septate hyphae growing inter- and intracellulary. Considering both plants, used in this study as a host (e.g., miscanthus and rice), belong to the same family of grass with similar host responses to DSE (showing non-host specific trait), we tested the colonization of DSE on rice root. Therefore, these three most promising isolates of *Phialocephala* sp., *P. fortinii*, and *P. helvetica*, as typical DSE, were further examined regarding their colonization within plant root. Based on the inoculation test, all rice seedlings exhibited healthy growth throughout the experimental period by fungal isolate × agar pH interaction (Fig. 5.3.3).

After harvesting, the roots were stained with 0.05% cotton blue to determine the endophytism of DSE isolates. Microscopic observation revealed that all DSE isolates successfully colonized hair roots of rice seedlings. The hair roots were coated with loose wefts of fungal hyphae (Fig. 5.3.2). This feature was identical to that previously described for typical DSE, *i.e.,* they are characterized by microsclerotia, thick, and darkly pigmented septate hyphae in the hair roots. Non-inoculated plants as a control showed no DSE colonization. The root colonization pattern was similar in *P. fortinii* and *P. helvetica*, but the degree of fungal colonization of *Phialocephala* sp. was the lowest compared with other two isolates. The images show the dense networks of hyphae of DSE inter- and intra-cellularly colonizing on rice roots (Fig. 5.3.2). Very few studies, however, investigated the role and ecological significance of isolated DSE underlying plant growth.

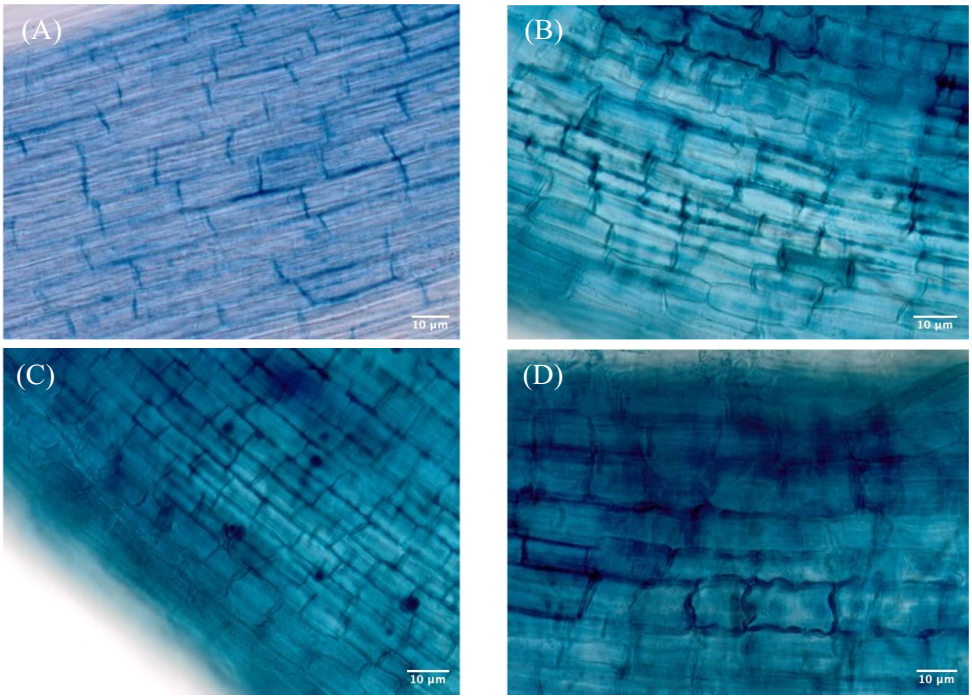


Fig. 5.3.2. (A) Non-treated DSE as control roots. (B) *Phialocephala* sp. (NH1121)-treated roots. (C) *Phialocephala helvetica* (NH1221)-treated roots. (D) *Phialocephala fortinii* (NH1331)-treated roots.

5.3.3 The role of isolated DSE in rice growth promotion

We aimed to test effects of these isolated DSE in crop (rice as a proxy) growth promotion by inoculation. Here, we found that shoot biomass of rice inoculated with DSE isolates increased up to 7.6 times, compared with non-inoculated controls (Fig. 5.3.4). The greatest shoot dry weight was recorded in plants treated with *P. fortinii*, followed by *P. helvetica* and *Phialocephala* sp. The beneficial effects of *P. fortinii* on enhancing plant yield have been reported (Jumpponen *et al*., 1998; Jumpponen and Trappe, 1998). For instance, *P. fortinii* isolates were used to inoculate asparagus plants and promote plant growth, e.g., shoot biomass increased by up to 53.5% (Surono and Narisawa,

2017).

This improvement in plant growth may be related to the ability of these isolates to use organic nitrogen sources under nitrogen-deficient conditions. Low nitrogen uptake by plants is restricted by soil acidity. The presence of *P. fortinii* associated with plant tissues demonstrated its ability to produce a variety of extracellular enzymes that break down complex forms of organic matter containing nitrogen and phosphorus (Jumpponen *et al*., 1998). For example, *Cladophialophora chaetospira* activates soil nitrogen and promotes aboveground nitrogen uptake by Chinese cabbage (Usuki and Narisawa, 2007). Therefore, the most abundant DSE identified by both culture and non-culture approaches, acting as an important mycorrhizal symbiont via melanized septate hyphae formation that removed resource limitation, might promote plant growth. A labeled nitrogen study is required to validate this mechanism.

Rice growth was markedly different depending on the combination of DSE isolates and pH. Differences in dry weight of DSE inoculated rice compared with non-inoculated rice grown at pH 3.0 (as high as 7.6-fold) were significantly greater than for those DSE inoculated rice grown at pH 4.0 and 5.0 (as high as 1.6-fold and 1.2-fold, respectively). In particular, the root dry weight of *P. fortinii*-treated seedlings was the highest at pH 3.0 with respect to that of the control. Also, we observed that inoculated species of *Phialocephala* effectively promoted plant growth, particularly under acidic conditions. The enhanced shoot biomass via DSE isolate inoculation was most marked in acidic environments, *e.g.*, with 7.6, 1.6, and 1.2 times greater shoot biomass at pH 3, pH 4 and pH 5, respectively. Less promotion of plant growth by inoculation with *Phialocephala* at pH 5 compared with 4 and 3 agar indicated that these DSE isolates likely promote plant tolerance to soil acidity.

Many researchers have reported relatively narrow ranges of pH for the presence or activity of mycorrhizal fungi in soils (Clark, 1997; Postma *et al*., 2007). This is consistent with the observation that most colonized isolates associated with plants were found in acidic agar. Similarly, the colonization of investigated plants with DSE significantly decreased with increasing soil pH (Postma *et al*., 2007). The mechanisms underlying the promotion of plant growth by DSE fungal in acidic soils have been addressed previously. Firstly, DSE fungal might help adaptability of crop to acid stress, e.g., the relatively high abundance of DSE supports host survival in stress habitats mainly via high chitin contents and forming melanized septate hyphae and microsclerotia in plant roots (Likar and Regvar, 2013). Secondly, it might increase the concentration of Mg, known to ameliorate Al toxicity, in the roots of *M.sinensis* to decrease Al activity (Haruma *et al*., 2021).

Taken together, this study helps improve our understanding of the community of *Miscanthus condensatus*-associated DSE fungi and their functions. Our findings suggest that DSE have the ability to support rice growth under an extremely acidic conditions, and the formation of melanized septate hyphae and microsclerotia-associated rice tissues might promote increases in rice growth and root biomass via removing stress and resource limitations. Considering acidic soils occupy up to 50% of the arable worldwide, these DSE isolates might be used as a management strategy to reduce acidic harm to crops, showing marked potential in re-vegetation of pioneer plants in post-volcanic ecosystems but also rice growth agriculture.

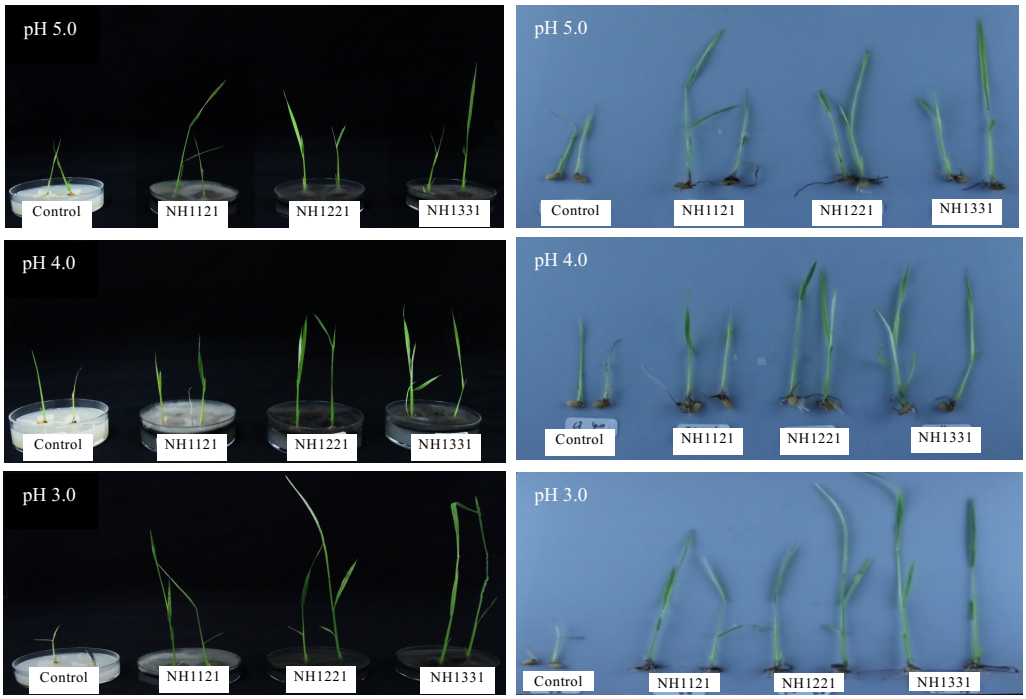


Fig. 5.3.3. Growth and development of rice plants inoculated with DSE fungal isolates under different pH conditions

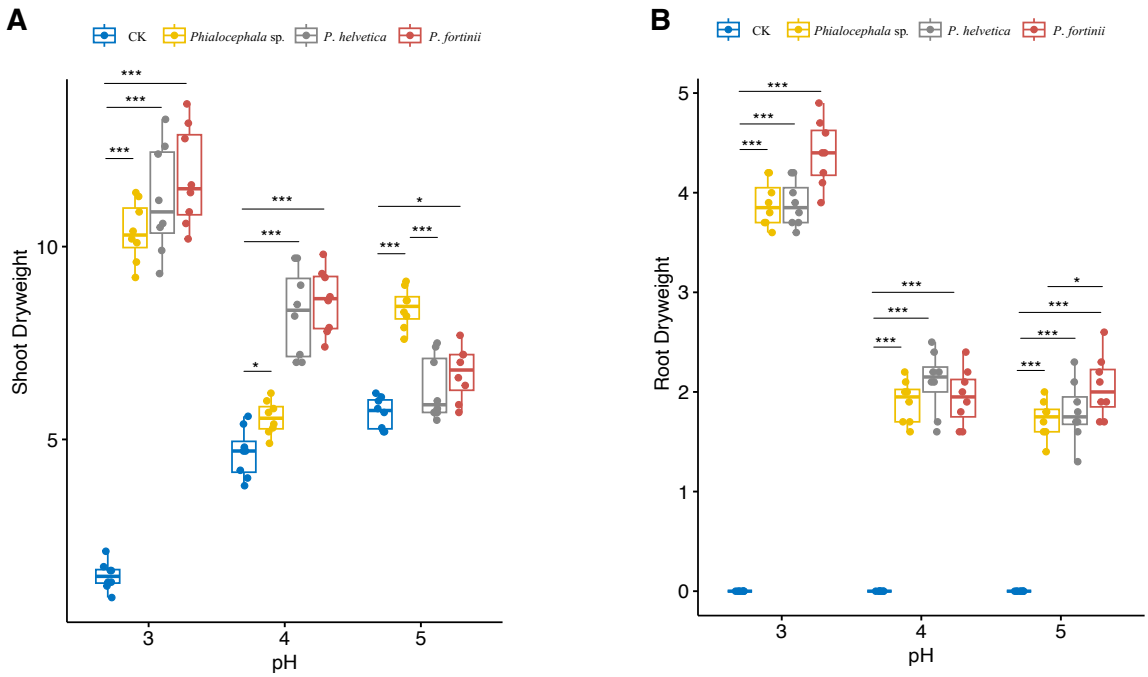


Fig. 5.3.4. Shoot and root dry weights of rice seedlings inoculated with NH1121 (*Phialocephala* sp.), NH1221 (*Phialocephala helvetica*), and NH1331 (*Phialocephala fortinii*) after three weeks of growth on oatmeal agar either at pH 3, pH 4, or pH 5 (acidic conditions). There are biological replicates (n=8). Median values are lines across the box with lower and upper boxes indicating the 25th to 75th percentiles, respectively. Whiskers represent the maximum and minimum values. Significance was determined by ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CHAPTER 6

General Discussion and Conclusion

6.1 Culture-dependent and culture-independent methods to mine the core microbial taxa

Culture-independent methods involve molecular techniques to study microorganisms without the need for cultivation. By directly analyzing DNA or RNA extracted from environmental samples, culture-independent methods provide insights into the diversity and structure of microbial communities. Culture-independent methods have revolutionized our understanding of microbial communities. This method used in this study allowed the identification and characterization of unculturable microorganisms and provide a broader perspective on the diversity and functions of microbial communities in vocanic ecosystem. However, these methods have their own limitations: i) the inability to study the physiology and metabolism of individual microorganisms; and ii) difficult to identify the majority of unknown taxa into species level (Dissanayake *et al*., 2018). Whist, culture-dependent methods involve the cultivation of microorganisms on artificial growth media in the laboratory and the microorganisms are isolated and grown under controlled conditions to observe their characteristics. This method allows for the identification of specific types of microorganisms and their ability to grow under certain conditions.

The combination of both culture-dependent and culture-independent used in this study provides a more comprehensive understanding of microorganisms and their physiological properties. For instance, the overlapping of taxa of Hypocreales and Helotiales were identified by both approaches suggesting their dominance in Miscanthus condensatus-associated fungal communities. Each method

compensate the other's shortage, for instance, the isolating and characterizing microorganisms could provide insights into their physiological properties and metabolisms through culture-dependent methods, while culture-independent method showed much higher diversity of microbial community.

6.2 The role of Dark septate endophytic fungi in promoting plant growth

The rhizosphere is a complex micro-ecological zone that is enriched in carbon, energy, and nutrients (Hinsinger *et al*., 2009). It represents a highly dynamic and diverse zone for biochemical interactions between soil components, roots, and microbiota (Hinsinger *et al*., 2006). Whist, endosphere is a specific ecological niche where fungi establish symbiotic relationships with the host plant. DSE fungi are a group of fungi that form symbiotic associations with plants endosphere. We showed the successful colonizaiton of DSE in rice root mainly due to its hyphal structures that colonize the interior of plant roots. DSE form mutualistic associations with plants, benefiting both the fungus and the plant. In exchange for nutrients, the DSE receive carbon compounds from the plant through photosynthesis. This mutualistic relationship can result in improved plant vigor, resistance to diseases, and increased tolerance to low pH and limited nutrients stresses.

We conducted a validation study to assess the effects of these DSE isolates on rice growth, particularly under extremely low pH conditions. We compared the rice biomass enhancement of an inoculated group with *P. fortinii* to a control group without inoculation. The results showed that rice biomass was enhanced by 7.6 times after inoculation with P. fortinii, indicating the potential of DSE in helping host crops resist acidity and enabling crop cultivation, especially in acidic soil (Postma *et al*., 2007). It is important to note that acidic soils cover approximately 50% of arable land worldwide, with around 13% of paddy fields categorized as acid soil. Soil acidification poses a challenge to crop yield; however, the application of these DSE isolates may serve as a management strategy to mitigate acidic harm to crops. Nevertheless, further investigation is required to evaluate their effectiveness in

field conditions.

DSE have been found to have a positive impact on rice growth, possibly in the following ways.

1) Nutrient uptake. DSE have been shown to enhance nutrient uptake in plants, particularly phosphorus and nitrogen. These fungi can solubilize and mobilize phosphorus in the soil, making it more accessible to plants. They can also facilitate the uptake of other essential nutrients like potassium, and micro-nutrients. This increased nutrient availability can lead to improved plant growth and development.

2) Stress tolerance. DSE help rice cope with environmental stresses such as acidity. It was reported DSE help the host to overcome the drought, salinity, heavy metal toxicity, and pathogens. DSE can produce various substances, including enzymes, antioxidants, and hormones, that help plants in stress adaptation. DSE can also induce systemic resistance in plants, enhancing their defense mechanisms against pathogens. This, yet, awaits further validation.

Overall, the presence of dark septate endophytic fungi in plants has been associated with various beneficial effects on plant growth and stress tolerance. Studying and harnessing these interactions can have significant implications for sustainable agriculture and ecosystem management.

6.3 Conclusion

Based on the aforementioned research, this study achieves a comprehensive understanding of the development of pioneer plants colonized on volcanic deposits and their associated microorganisms.

1) By comparing the structure and diversity of rhizoplane and endophytic microorganisms in pioneer grass M. condensatus that grow in extreme volcanic deposits, a total of 315 putative fungi isolates and 301 bacterial strains were isolated from the roots and rhizoplane of *M. condensatus*, with a sequence similarity level of up to 97%.

2) By using both culture-dependent and culture-independent methods, we identified endophytic bacteria and fungi associated with pioneer grass M. condensatus colonizing the volcanic deposits near the crater of Oyama. The overlapping order Helotiales that identified by both culture-dependent methods was the most abundant in the *Miscanthus condensatus*-associated fungal community.

3) Through isolates-inoculation, we fully assessed the feasibility of using the core DSE isolates to promote rice growth under soil acidity. The shoot biomass of rice inoculated with DSE isolates increased up to 7.6 times, compared with non-inoculated controls, showing great ability to serve as a management strategy for mitigating the harmful effects of acidity on crops. This strategy shows promise not only in the re-vegetation of pioneer plants in post-volcanic ecosystems but also in rice cultivation for agricultural purposes.

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