Compatible bioinoculants for improved plant growth-promotion

植物生育促進効果のある微生物資材

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ABSTRACT

The compatibility of strains is crucial in formulating bioinoculants for plant growth promotion. Here, we assessed the capacity of four potential bioinoculants isolated from potato roots and tubers (*Sphingomonas* sp. T168, *Streptomyces* sp. R170, *Streptomyces* sp. R181, and *Methylibium* sp. R182) in promoting plant growth, which revealed R170 to have the highest ability to produce biochemical substances and tolerance against NaCl (2%) and $AlCl_3$ (0.01%), and growth in a wider range of pH values (5.0–10.0) compared with the other three strains. Because of this, the compatibility of R170 with other strains was tested in combined inoculations, and the results showed that the co-inoculation of R170 with T168 or R182 synergistically increased the plant weight over un-inoculated controls, indicating the compatibility of strains manifested by the increased production of plant growth promoters such as indole-3-acetic acid (IAA) and siderophores, and also by co-localization on roots. However, a parallel test using strain R181, which is the same *Streptomyces* genus as R170, showed incompatibility with T168 and R182 as revealed by the lower plant growth promotion along with a lack of co-localization. Moreover, the strains were screened for promoting mycorrhizal activities, and the results showed that R170 can be potential mycorrhization helper bacteria (MHB) evidenced by the significantly higher acid phosphatase (AP) (32%) and siderophores (134%) produced after co-inoculating PGPB with mycorrhiza compared with the activities of mycorrhiza alone. R170 was also effective in promoting mycorrhizal root infection and sporulation, as well as in enhancing the capacity of mycorrhiza to biologically control *Rhizoctonia solani.* Furthermore, the effect of co-inoculating R170 with mycorrhiza to plant growth revealed a synergistic increase in the weights of potato seedlings over un-inoculated controls. Together our findings suggest that compatibility among microbial inoculants is important for efficient plant growth promotion, and that R170 could be a useful bioinoculant, especially in combined inoculations that contain compatible strains.

ABSTRACT
(Japanese)

植物生育促進のために使用される微生物資材において菌株間の適合性は重要である。今回の研究では、ジャガイモの根と塊茎から単離された 4 種の微生物 *Sphingomonas* sp. T168、*Streptomyces* sp. R170、*Streptomyces* sp. R181、および *Methylibium* sp. R182 の植物生育促進の能力を評価した。このうち、R170 は他の 3 株と比較して NaCl（2%）と $AlCl_3$（0.01%）に対して最も高い耐性を有すること、および広い pH 範囲（5.0〜10.0）での生育が可能であることがわかった。このため、R170 と他の株との適合性を共接種試験で評価した。その結果、R170 と T168 または R182 との共接種は、それらを接種していない対照と比較して植物体生育重量が相乗的に増加し、インドール-3-酢酸（IAA）およびシデロフォアのような植物生育促進物質の生産量の増加、および根においてそれらが共存していることがわかった。一方、R170 と同じ *Streptomyces* 属である R181 株を用いた同様の接種試験では、T168 および R182 との組み合わせにおいて植物生育促進の効果が低いことが明らかになり、植物生育促進物質の相乗的な増加がなく、菌株が共存できないことが原因あると考えられた。さらに、菌根活性を促進する株をスクリーニングした結果、R170 が他の菌株と比較して有意に高い酸性ホスファターゼ活性（32%）およびシデロフォア生産（134%）を示した。R170 は菌根菌の感染および胞子形成の促進に有効なだけでなく、菌根が *Rhizoctonia solani* の増殖を抑制する能力を強化することにおいても有効であった。また、菌根と R170 を混合すると未接種の対照よりもジャガイモ実生の植物体重量の相乗的な増加がみられた。これらの結果は、微生物資材として使用される微生物間の適合性が効率的な植物の生育促進に重要であることを明らかにするとともに、R170 は微生物資材として有用性が高いとこを示した。

INTRODUCTION

The rising concern on environmental conservation has encouraged farmers to introduce agricultural practices that ensure minimal impacts to the environment in order to achieve high yields in crop production. To achieve and maintain a high level of crop productivity, sufficient amount of nutrients should be available for plant absorption, however, the nutrients in soil are usually not bioavailable for plant absorption, thus, resulting in nutrient deficiency. In this regard, biological inoculants containing symbiotic microorganisms are being supplemented to plants to ensure that essential nutrients are provided without imposing any harm to the environment.

The soil is home to diverse microorganisms that naturally interact with each other as well as with plants for survival. In the formulation of bioinoculants, diversity is important because a single strain may not be enough to effectively deliver positive effects to plant growth especially in the presence of competition. Thus, microbial strains should positively complement with each other in terms of their infection to the host plant and in the execution of biochemical activities in order to ensure a higher chance of plant growth promotion.

Plant growth promoting bacteria and mycorrhizal fungi are among the symbiotic microorganisms that are continuously being explored for the development of bioinoculants. These microbes, which usually reside in the rhizosphere, are capable of interacting with host plants by colonizing the surfaces, inter- and intracellular spaces of the roots while acquiring their nutrition from the exudates in the form of photosynthetically-fixed carbon (Pieterse *et al.* 2016). Symbiotic microbes work as plant growth promoters by the production of phytohormones (*i.e.* indole-3-acetic acid), iron-chelating substances (*i.e.* siderophores), lytic enzymes (1-aminocyclopropane-1-

carboxylic acid, β-1,3-glucanase, cellulase, chitinase, protease and lipase), and protective substances (*i.e.* biofilms) that aid in the improved acquisition of nutrients in the soil, as well as in the induction of systemic resistance of plants against biotic and abiotic stressors (Goudjal *et al.* 2013).

Potato (*Solanum tuberosum*), one of the most important crops in the world, serves as food for a billion of people. In 2010, its global production has been reported to reach up to more than 300 million tons (Bradshaw and Borniebale, 2010). In Hokkaido, Japan, the extensive application of fertilizers from inorganic sources has been practiced to secure a continuous supply of necessary nutrients for the growth and development of potatoes and maintenance of their productivity. Because of this, the soil of most potato farms in Hokkaido were reported to have low pH and strong aluminum toxicity which may be attributed to the over-use of chemical fertilizers, eventually resulting in the accumulation of heavy metals and excess nutrients (Shoji and Takahashi, 2002).

To help reduce the undesirable environmental impacts, bioinoculants are used to serve either as a substitute or a supplement to chemical fertilizers to ensure that plants are well nourished, thus, eventually leading to high crop productivity. However, the large scale utilization of some bioinoculants might be hindered by the lack of compatible strain formulations (Malusa *et al.* 2012). In this regard, a comprehensive screening of compatible microbial strains will provide a stronger basis for the selection of more efficient bioinoculants. This study expects to contribute to the development of reliable and efficient bioinoculants for the ultimate improvement of the growth of potato seedlings.

OBJECTIVES OF THIS STUDY

The general objective of this study is to develop potential formulations of compatible PGPB inoculants for the improvement of plant growth.

Specifically this study aimed to:

1) determine the efficiency of bacterial strains isolated from potato in promoting plant growth in terms of biochemical characteristics, stress tolerance, and localization;
2) assess the plant growth-promoting ability of mycorrhiza isolated from a commercial inoculant by its biochemical activities and infection to the roots;
3) test the synergistic effects of combined microbial inoculants in plant growth-promotion based on biochemical activities and infection to the host plant; and
4) identify the most potential PGPB strain based on compatibility with other strains and capacity to assist mycorrhizal functions in the host plant.

Chapter 1

Review of Literature

1.1 Plant-microbe interaction

Plants inherently live with a large number of interacting beneficial and harmful microorganisms (microbes) in their natural habitats. These microbes infect the roots and establish a close relationship with the host plants. They get nutrition from the photosynthetically fixed carbon, released by plants as exudates, and use it for energy production (Saito *et al.* 2007; Karthikeyan *et al.* 2010; Someya *et al.* 2013). Subsequently, microbes assist in the plant growth and development by the provision of water and nutrients, through the by-products of biochemical processes associated with the microbial consumption of plant exudates. Specifically, these biochemical substances help promote the plants' nutrient absorption capacity through one or a combination of the following means: root elongation; formation of many lateral roots and root hairs; expansion of the root surface area; solubilization of insoluble nutrients; and protection against biotic (*i.e.* pathogens) and abiotic stressors (*i.e.* pH, temperature, and heavy metals) (Goudjal *et al*. 2013).

Microbial infection to the host plants is evidenced by the formation and attachment of colonies on the root surface, while some microbes have been noted to advance their infection by penetrating inside the plant tissues. Microbes that infect on plant surfaces are called epiphytes, whereas, those that penetrate inside the plant tissues are referred to as "endophytes". Some endophytes invade plant tissues by entering through cracks and openings on the root epidermis usually in areas where emerging lateral roots are formed (Zachow *et al.* 2010). In the case of mycorrhiza and actinobacteria, the

development of mycelium is a requisite for penetration into the plant cells and tissues (Peterson *et al.* 2004; Toumatia *et al.* 2016).

A diversity of microbes that live in the rhizosphere, either beneficial or harmful, compete for available nutrients and space for infection to the host plant. Generally, if the microbes are compatible with each other, then their infection to the plants might bring synergistic effects as a result of their complementing biochemical productivity. Thus, an improved plant growth accompanied by an induced systemic resistance against pathogens may take place. However, if the microbes are not compatible, then negative impacts such as plant growth inhibition, susceptibility to diseases, or even death may occur (Schenck *et al.* 2012). A general overview of the interactions between plants and microbes is shown in Figure 1.

1.2 Plant-associated beneficial microbes

1.2.1 Plant growth-promoting bacteria (PGPB)

A group of soil microbes that form symbiotic relationship with most species of plants are the free-living soil bacteria, which are generally referred to as PGPB. These PGPB represent an extensive and diverse population of bacteria that grow in close association with the plants (Bhattacharyya and Jha, 2012). These PGPB promote plant growth by boosting plants' physiological activities related with nutrient acquisition and utilization (Hardoim *et al.* 2008). In fact, PGPB has also been reported to protect plants from the possible infection of pathogens (Bloemberg and Lugtenberg, 2001).

The effectiveness of PGPB in plant growth promotion has been extensively reported (Sessitch *et al.* 2004; Siddikee *et al*. 2010; Alam *et al.* 2012; Aly *et al.* 2012; Mostafa *et al.* 2012; Park *et al.* 2012; Sadeghi *et al.* 2012; Kim *et al.* 2013; Glick, 2014; Ittisupornrat *et al.* 2014; Yang *et al.* 2014). Nevertheless, PGPB's efficiency as a plant

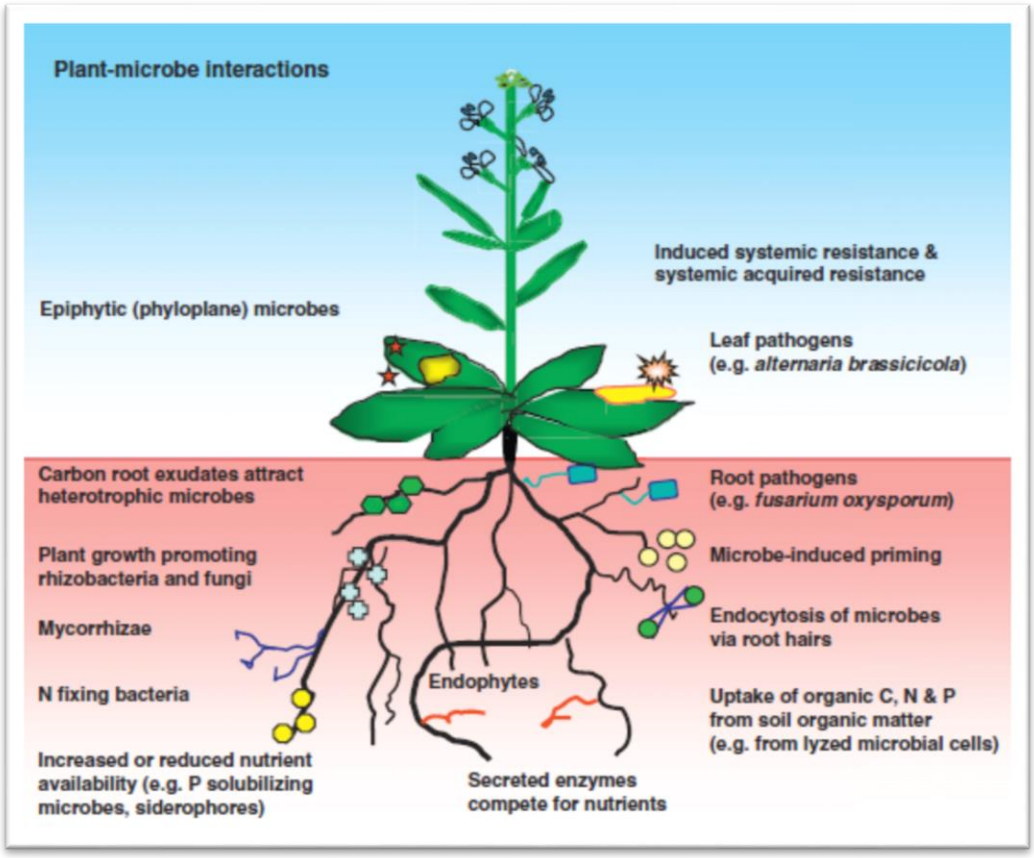

Figure 1. An overview of interactions between microorganisms and host plants (Schenck *et al.* 2012).

growth promoter might be secured with its successful infection and localization to the host (Compant *et al.* 2010).

1.2.2 Mycorrhiza

Mycorrhiza is another group of plant-associated microbes that forms symbiotic association with the roots of plants and facilitates nutrient and water absorption, particularly phosphorus (Afek *et al.* 1990; Allen et al. 1981; Haystead et al. 1988; Young et al. 1988; Sanders and Fitter, 1992; Clark and Zeto, 2000). Many plant species greatly depend on mycorrhiza for plant growth (Ingleby et al. 1997) as they assist in nutrient absorption through the hyphae connecting zone where the plant roots and mycorrhiza are intimately interacting (Rani and Mukerji, 1990; Rani et al. 1999; Gill and Singh, 2002). It has been noted that plants that are naturally associated with mycorrhiza may experience a more improved nutrient absorption through the mycorrhizal roots leading to a more vigorous plant having an enhanced resistance against harmful pathogens (Zambolim and Schenck, 1983; Trotta et al. 1996).

Mycorrhizas have been noted to thrive in degraded habitats and soils with little organic matter (Alloush et al. 2000). Their manifested tolerance to stress conditions could be mainly attributed to their thick multi-layered walls that enable them to stay and survive in a highly disturbed soil for long periods of time (Khalil et al. 1992; Smith and Read, 1997; Castillo, 2004; Aggangan et al. 2011). Because of this, mycorrhizal plants have become more tolerant to stress compared to non-mycorrhizal plants. Thus, mycorrhizal application and benefits to plants have been extensively reported (Mohankumar *et al.* 1988; Husband *et al.* 2002; Muthukumar *et al.* 2004; Renuka, 2012).

1.3 Mechanisms of symbiotic microbes related with plant growth promotion

Symbiotic microbes help enhance the growth of plants by providing the necessary nutrients for development. The provision of nutrients occurs through the production of plant growth promoting substances brought about by biochemical processes that take place as microbes synthesize the carbon sourced from plant exudates (Saito *et al.* 2007; Karthikeyan *et al.* 2010; Someya *et al.* 2013). As cited by Gaeiro *et al.* (2013) plant growth can be promoted by either one or a combination of these processes: phytostimulation, biofertilization, and biological control (biocontrol).

The production of hormones (*e.g.* IAA) (Shahab *et al.* 2009) and important enzymes (*e.g.* ACC deaminase) facilitate the process of phytostimulation (Glick, 2014). Whereas, the advanced accessibility of nutrients through nitrogen fixation, phosphorus solubilization, and siderophore production aid in biofertilization (Bashan, 1998; Bloemberg and Lugtenberg, 2001; Kpomblekou-A and Tabatabai, 2003; Radzki *et al.* 2013). Nevertheless, the biocontrol or the improvement of plant defense against harmful pathogens is made possible by the production of siderophores and antibiosis (Schelkle and Peterson, 1997).

The biocontrol of pathogens through siderophore production is attributed to the specificity of its products, particularly iron, to the microbes that produce them, which results in a larger portion of nutrients allocated to the siderophore-producing microbes compared with the non-siderophore-producing microbes (*i.e.* pathogens) (Schelkle and Peterson, 1997). Antibiosis, on the other hand, works by inhibiting the further growth and infection of pathogens to plants through the release of antifungal metabolites (Li *et al*. 2009). Dhanasekaran *et al*. (2013) and Hozzein *et al* (2011) noted that majority of *Streptomyces* strains can produce bioactive compounds and secondary metabolites that cause anti-microbial activities.

Some of the extensively reported symbiotic microbes that aid in plant growth promotion includes *Azospirillum* sp., *Bacillus* sp., *Methylibium* sp., *Pseudomonas* sp., *Rhizobium* sp., *Sphingomonas* sp., *Streptomyces* sp. (Cassan *et al.* 2014; Fatnassi *et al.* 2015; Gopalakrishnan *et al.* 2014; Khan *et al.* 2017; Pisa *et al.* 2011; Przemieniecki *et al.* 2015; Schwartz *et al.* 2013), and a wide range of mycorrhizal species belonging to the genera of *Glomus*, *Gigaspora*, *Acaulospora*, *Funneliformis*, and *Rhizophagus* (Bona *et al.* 2016; Bonfante, 2003; Ceballos *et al.* 2013; Cordier *et al.* 1998; Gamalero *et al.* 2008). Additionally, Actinobacteria has been associated with the production of IAA (Alam *et al.* 2012), ACC-deaminase (Siddikee *et al*. 2010), siderophores (Sadeghi *et al.* 2012; Wang *et al.* 2014) and hydrolytic enzymes (Alam *et al.* 2012; Aly *et al.* 2012). Whereas, Proteobacteria was reported for nitrogen fixation, IAA (Yang *et al.* 2014), siderophores (Sessitch *et al.* 2004), biofilms (Kim *et al.* 2013), and hydrolytic enzymes (Ittisupornrat *et al.* 2014). Moreover, mycorrhizas have been known to enable the plants' capacity to efficiently acquire insoluble P and iron from the rhizosphere by converting them to bioavailable forms through the production of phosphatases and siderophores, respectively (Aliasgharzad *et al.* 2009; Smith *et al.* 2011).

Efforts to develop efficient bioinoculants containing biochemically equipped plant growth-promoting microbes (PGPM) have been widely studied (Gopalakrishnan *et al.* 2012; Yuttavanichakul *et al.* 2012; Zakry *et al.* 2012). However, the need for testing their efficiency as single or mixed inoculations to a particular crop must be recognized because beneficial effects will not take place if microbes fail to infect the host plant or if the strains contained in an inoculant are not compatible.

1.4 Synergistic interactions among symbiotic microbes

Microbial inoculants containing a diversity of different strains would be more likely to improve plant growth because of a greater variety of combined traits compared to a sole microbial strain (Pandey *et al.* 2012). It has been previously noted that microbial combinations encourage the synergistic interaction among microbes resulting in the stimulation of the activities of one another, and eventually enhancing their capacity of promoting plant growth (Seneviratne, 2003). However, this is not always the case. Some microbes contained in an inoculant may be strongly inhibitory to the one another (Pierson and Weller, 1994). This lack of synergistic interaction among microbes may eventually result in negative impacts to the host plant, which includes a decreased biomass, and the possible attack of pathogens. In contrast, if there is a cooperation among the microbes, then it would be beneficial for the host plants and, thus, it is necessary to screen the compatibility between microbial inoculants. The promising effects of mixed strain inoculants on plant growth have been demonstrated for several bacterial combinations (Bai *et al.* 2002; Wasule *et al.* 2007; Aamir *et al.* 2013; Sanchez *et al.* 2014). Likewise, the combination of mycorrhiza with bacteria has also been confirmed in previous reports to have beneficial effects to plant growth (Artursson *et al.* 2006).

1.5 Potato as a source of potential bioinoculants

Potato (*Solanum tuberosum* L.) is one of the most indispensable food crops in the world (Bradshaw and Bonierbale, 2010). In the province of Tokachi, Japan, potato is regarded as one of major crops along with wheat, sugar beet, and beans (Koga, 2008). Previous studies noted that the rhizosphere of potato houses a wide range of plant-associated microbes which include mycorrhiza, PGPB, and pathogens (Mehrotra and

Barjal, 1992; Berg *et al.* 2005; Someya *et al.*2013; and Lehtonen *et al.* 2008). Particularly, Someya *et al.* (2013) reported that a high diversity of bacterial populations is found on the roots and tubers of potato (cv. Matilda), which can be potential plant growth promoters. In fact, representative strains from these isolates (Someya *et al.* 2013) have been proven to promote the growth of potato seedlings (cv. Hokkaikogane) as reported by the Ministry of Agriculture, Forestry, and Fisheries Research Council (Kenkyuseika, vol. 539. 2015). The list of representative strains isolated from potato is presented in Table 1, whereas the effect of inoculating each strain on the growth of potato seedlings is shown in Figure 2. The greatest number of bacterial strains which have been classified to the nearest known species belongs to the group of Proteobacteria followed by Actinobacteria, whereas the least number fall under Bacteroidetes. The representative strains (R170, R181, R182, and T168) showing the highest relative plant weight (Figure 2) were used for all the conducted experiments in this thesis.

Table 1. List of representative bacterial strains isolated from the phytosphere of potato (cv. Matilda) (Someya *et al.* 2013).

	Phylum	Closest known species	Strain no.
1	Proteobacteria	*Pelomonas soli*	T27
2	Bacteroidetes	*Chitonophaga soli*	T40
3	Proteobacteria	*Polaromonas ginsengsoli*	T90
4	Proteobacteria	*Caulobacter lleidyi*	T92
5	Actinobacteria	*Mycobacterium llatzerense*	T136
6	Proteobacteria	*Devosia insulae*	T153
7	Proteobacteria	*Sphingomonas asaccharolytica*	T168
8	Proteobacteria	*Sphingomonas paucimobilis*	T170
9	Actinobacteria	*Kitasatospora saccharophila*	T172
10	Proteobacteria	*Mitsuaria chitosanitabida*	T191
11	Actinobacteria	*Pimelobacter simplex*	T194
12	Proteobacteria	*Phyllobacterium myrsinacearum*	T224
13	Bacteroidetes	*Chryseobacterium soldamellicola*	R008
14	Proteobacteria	*Mesorhizobium huakuii*	R065
15	Proteobacteria	*Bradyrhizobium japonicum*	R105
16	Proteobacteria	*Rhizobium sullae*	R139
17	Actinobacteria	*Streptomyces cinnamonensis*	R170
18	Actinobacteria	*Streptomyces ryensis*	R181
19	Proteobacteria	*Methylibium petroleiphilum*	R182

The starting letter of the designated strain numbers refers to the area on potato where each bacteria was isolated (*i.e.* T… strain isolated from tuber; R…strain isolated from roots). The strains have been tentatively identified to the closest known species.

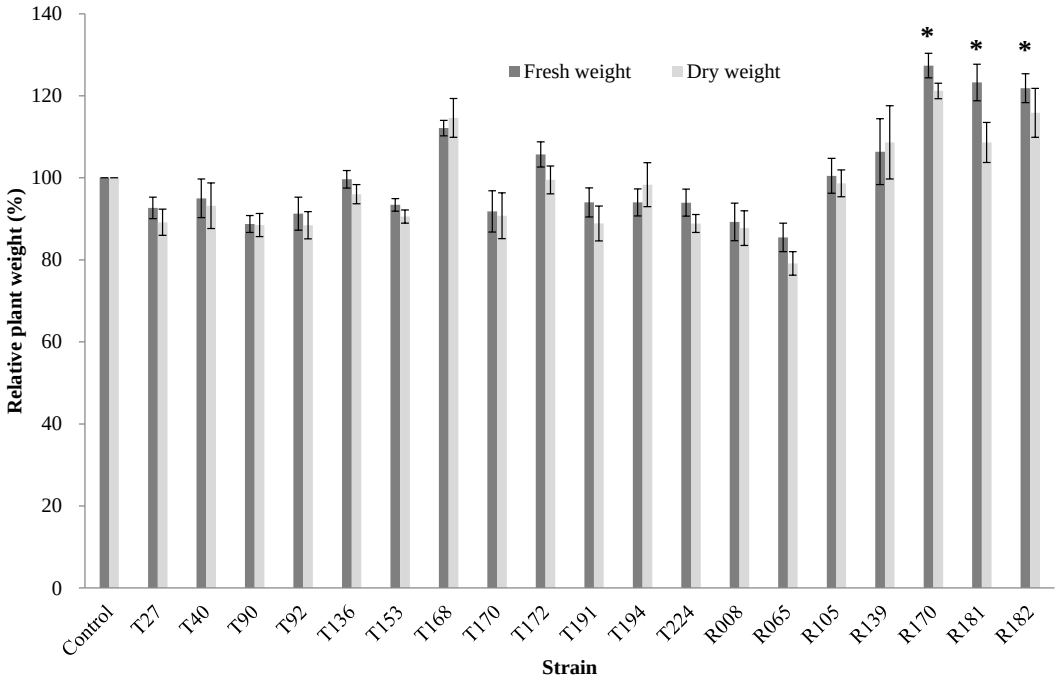

Figure 2. Inoculation effect of isolated bacterial strains on the initial growth of potatoes. Relative plant weight is the growth ratio of inoculated plants compared to control (100%). * statistically significant at $p<0.05$. (Kenkyuseika, vol. 539. 2015)

1.6 Integration of this chapter to this research

This chapter initially showed relevant information about the natural interaction between plants and microbes living in the rhizosphere. After that, the beneficial effects of plant-associated microbes such as the PGPB and mycorrhiza to plant growth has been discussed. Particularly, the benefits of these microbes to plants have been related with their successful infection followed by the efficient execution of biochemical activities. Then, the importance of synergy between interacting microbes was illustrated. Despite the voluminous reports about the effects of single and combined inoculations of PGPM to plant growth, little is known about how strains in a mixed inoculant cause improvement to plant growth. In this regard, this thesis presents some possible mechanisms that elucidate the compatibility between microbes in a mixed inoculant. The results presented here hope to contribute to the development of potentially effective formulations of bioinoculants for plant growth promotion. The following chapters provide additional information about the potentials of PGPB and mycorrhiza as bioinoculants for plant growth. Furthermore, the importance of microbial compatibility in formulating efficient bioinoculants is discussed.

Chapter 2

PGPB as potential bioinoculants

2.1 Introduction

For sustaining global food security, a high-yield in agricultural crop production is desired. Hence, in order to achieve an improved crop productivity, plants should be provided with essential nutrients for their growth and development. Plants continuously get nutrients from the soil, and it is thus necessary to maintain the soil's productivity by replenishing the lost nutrients. For this reason, chemical fertilizers that contain basic nutrient requirements such as the macro-elements (N, P, K, S, Mg, Ca) and trace elements (Fe, Mn, Bo, Cl, Zn, Cu, Mo) are being used to ensure plant nourishment. However, the cost of chemical fertilizers is high and their prolonged use can have negative impacts varying from a deteriorated balance of elements and disrupted populations of natural microflora in the soil (Savci, 2012), increased concentrations of nitrates in the ground water (Shamrukh *et al.* 2001), and irreversible impacts to human health (Sutton *et al.* 2012). In this regard, the development of bioinoculants from novel strains of plant growth-promoting bacteria (PGPB) can be a suitable alternative for chemical fertilizers (Gamalero *et al.* 2010).

The efficiency of bioinoculants is guaranteed by the successful infection of PGPB to the host plant. PGPB may assist in plant growth promotion subsequently after infecting the plant by producing phytohormones such as indole-3-acetic acid (IAA) (Gopalakrishnan *et. al.* 2011) and protective substances such as biofilms (Seneviratne *et al.* 2011), and by initiating the formation of siderophores (Radzki *et al.* 2013), the

release of enzymes such as ACC deaminase (Glick, 2012), *β*-1,3-glucanase (Singh *et al.* 1999), cellulase (Saratale *et al.* 2012), chitinase (O'Brien and Colwell, 1987, Singh *et al.* 1999), protease and lipase (Bai *et al.* 2002), and via the biological control of harmful pathogens (Glick, 2014).

In particular, this chapter dwells on the potential ability of four PGPB strains isolated from the roots or tubers of potato (cv. Matilda) in promoting the growth of potato seedlings (cv. Hokkaikogane). Biochemical tests were conducted to identify the strain with the highest potential as a bioinoculant. Environmental stress test was also performed for each strain to determine which of the four may potentially provide stress tolerance to the host plant. PGPB's infectivity to potato seedlings was also examined. Results indicate that the infection of biochemically productive and stress-tolerant PGPB to the host plant encourages the promotion of plant growth.

2.2 Materials and Methods

2.2.1 Bacterial strains and medium

Nineteen representative strains with high affinity for potato roots or tubers (cv. Matilda, one of the common varieties in Hokkaido, Japan) isolated previously (Someya *et al.* 2013), were studied for their plant growth promotion (Kenkyuseika, vol. 539. 2015), and the four best PGPB strains, namely, *Sphingomonas* sp. T168 (accession number AB730532), *Streptomyces* sp. R170 (AB730341), *Streptomyces* sp. R181 (AB730352), and *Methylibium* sp. R182 (AB730353) selected among them were used throughout the experiment. Strains T168, R170, R181, and R182 belong to AP6, AC4, AC1, and BP12 of OTU-group shown in previous study (Someya *et al.* 2013), respectively. All strains were grown in R2A medium (BD, Sparks, MD, USA).

2.2.2 Assessment for biochemical and enzyme activities

Indole-3-acetic acid (IAA) production was determined using the Salkowski assay (Gopalakrishnan *et al.* 2011) with the following modifications: bacterial strains were grown in R2A broth containing 2 mM L-tryptophan for 72 hr, and after centrifugation, the supernatant (400 µL) was poured into Salkowski reagent (composed of 500 µL of 60% $HClO_4$, 17 µL of 0.5M $FeCl_3$, and 350 µL of distilled water) (800 µL) and incubated at 30°C for 30 min in a dark place. IAA production was determined (n=3) by optical density at 530 nm using a spectrophotometer (Ultrospec3100pro, GE Healthcare Life Sciences, Buckinghamshire, UK). At the same time, the number of living cells was measured by plate dilution methods in the usual manner using cell pellets left after removing the supernatant to determine the IAA contents per cell (µg 10^8 colony-forming unit $[CFU]^{-1}$).

Siderophore production was evaluated using the Chrome-Azurol S (CAS) agar diffusion assay (Shin *et al.* 2001) with some modifications: holes (6 mm dia.) made on R2A agar containing 10% CAS (Schwyn and Neilands, 1987) were filled with a final bacterial suspension of 24-hr-old cultures (35 µL). After incubation at 30°C for 7 days, the diameters of the halos formed around the holes containing a bacterial colony were measured. Siderophore production (n=3) is expressed as the ratio of halo diameter (halo dia. minus colony dia.) per colony diameter (Soltani *et al.* 2012).

The biofilm production was determined by microtiter plate assay (Yuttavanichakul *et al.* 2012) with the following modifications: 100 µL of 2-day-old cultures in R2A broth were transferred into a 96-well polystyrene microtiter plate and incubated at 30°C for 12 hr. After loosely associated bacteria were removed, wells were washed with sterilized distilled water, air dried, and then stained with 1% crystal violet solution (150 µL) for 45 min. The wells were washed again with distilled water and

destained with 95% ethanol (200 μL), and then 100 μL from each well was transferred to new microtiter plates. Absorbance at OD_{595} was measured (n=3) using a microplate reader (iMark Microplate Absorbance Reader, Bio-Rad Laboratories, Tokyo).

The 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity was determined by the production of α-ketobutyrate (AKB) generated by the cleavage of ACC according to the method reported by Penrose and Glick (2003) except that the cells were grown in R2A broth. At the end of the assay, absorbance at OD_{540} (n=3) was measured by a spectrophotometer. The activity is expressed as nmol AKB (mg wet weight of cell)$^{-1}$ per hour.

The cellulase, protease, lipase, and chitinase activities were determined by the size of the halo diameter formed from the periphery of the colony at 30°C, 7 days after bacterial spot inoculation (5 μL) on agar medium (n=3). The cellulase activity was estimated according to the method reported by Crabbe *et al.* (1994) using yeast-extract salts (YES) agar medium containing 2% carboxymethyl cellulose. The protease and lipase activities were measured according to the method reported by Bhattacharya *et al.* (2009) using skim milk agar (3% skim milk and 1.5% agar), and R2A agar supplemented with 1% Tween 20 and 0.01% $CaCl_2 \cdot 2H_2O$, respectively. The chitinase activity was evaluated according to the method reported by O'Brien and Colwell (1987) using R2A agar supplemented with 1% colloidal chitin. For lipase and chitinase, only the presence or absence of their activities was noted because of the indistinct boundaries of halos produced.

The β-1,3-glucanase activity was assessed according to the method reported by Singh *et al.* (1999) using R2A broth supplemented with 1% colloidal chitin. The amounts of reducing sugars were determined by measuring the absorbance at OD_{530} (n=3) by a spectrophotometer. One unit of activity was defined as the amount of enzyme

that liberated 1 μmol of glucose per hr.

2.2.3 Evaluation of bacterial strains for stress tolerance

A portion of the 24-hr pre-culture in R2A broth was added to a fresh R2A broth (6 mL) in L-tubes adjusted to different levels of $AlCl_3$ (0.0001%–1%, w/v), NaCl (1.0%–5.0%, w/v), pH (4.0–10.0) and temperature (10°–40°C), and the growth was monitored (n=3) at OD_{660} by a biophotorecorder (TVS062CA, Advantec Toyo Kaisha, Tokyo). For the aluminum, salts, and pH stress test, each culture was incubated with shaking at 30 rpm for 120 hr at 30°C, and for the temperature stress test, each culture was incubated at temperature between 10 and 40°C under the same conditions as those used for the shaking and incubation periods mentioned above.

2.2.4 Bacterial inoculation and evaluation for plant growth promotion

Both the R170 and R181 strains were grown in R2A broth at 30°C for 24 hr with shaking at 130 rpm. After centrifugation at 10,000 rpm, at 4°C for 5 min, the cell suspension was adjusted to 1×10^8 CFU mL^{-1} in sterilized distilled water. The T168 and R182 strains were grown on R2A agar medium for 72 hr under the same temperature condition described above. Cells were collected directly from the agar medium, and the cell suspension was adjusted to the cell density mentioned above.

To verify the plant growth-promoting ability of bacterial strains on potato seedlings, we directly inoculated 1 mL of cell suspensions on potato seeds (n=18) derived from the open pollination of cv. Hokkaikogane sown on pots containing approx. 100 g of sterilized seedling-raising culture soil (PotAce N, Katakura & Co-op Agri Corp., Tokyo). The pots were covered with aluminum foil and placed in a growth chamber under light (23.5°C for 14 hr) and dark (20.0°C for 10 hr) conditions,

respectively. The cover was removed upon the germination of seeds, and 30 days after the inoculation, the plant growth parameters such as the plant weight (mg) and germination rate were measured (*n*=3). The plant dry weight was measured after oven drying at 60°C for 3 days.

2.2.5 Tissue localization of inoculated strains in the plant roots

For the observation of the plant tissue localization of strains T168 and R182, we introduced the *gusA* gene encoding *β*-glucuronidase (GUS) (Wilson *et al.* 1995) into the cells. For strain T168, plasmid pHRGFPGUS (Ramos *et al.* 2002), which expresses the *gusA* and *gfp* genes constitutively under the control of gentamycin resistance gene promoter, was introduced into the cells by electroporation using a MicroPulser™ in accord with its operating instructions (Bio-Rad Laboratories). For strain R182, plasmid pmTn*5*SS*gusA20* (Wilson *et al.* 1995), which expresses the *gusA* gene constitutively under the control of *aph* promoter, was introduced into the cells by bi-parental mating according to the method reported by Simon (1984). Briefly, *E. coli* S17-1 (donor) and R182 strain (recipient) cells in the exponential phase were mixed together and centrifuged for 3 min. After the cell pellets were suspended in 50 μl of 0.85% NaCl, mating was carried out on a mixed cellulose ester membrane filter (pore size 0.45 μm, Advantec Toyo Kaisha) placed on R2A agar medium at 30°C for 2 days. The cell suspensions were spread on R2A agar medium containing spectinomycin (50 μg/mL), streptomycin (50 μg/mL), and fosfomycin (50 μg/mL).

Approximately 14-day-old potato seedlings were grown on plant agar (0.3%) containing a 500-times dilution of HYPONeX® 6-10-5 (HYPONeX Japan, Osaka, Japan) and inoculated bacterial inoculant prepared as described above. We identified the tissue localization of strains in the plant roots by GUS and/or Gram staining. For

the plants inoculated with GUS-marked strains, bacterial cells were stained by immersing plant samples in a GUS-staining solution (16 mL of 125 mM sodium phosphate; 80 µL of 0.5 M Na_2EDTA, pH 8.0; 800 µL of 2% X-Gluc [5-bromo-4-chloro-3-indolyl-*β*-D-glucuronic acid] cyclohexylammonium salt; 80 µL of 10% SDS; and 23.6 mL of distilled water) with continuous deaeration in a desiccator connected to a vacuum pump for 30 min, and then allowed to incubate on plants at 30°C for 3 days.

Gram staining was done according to the instructions of Favor G "*Nissui*" (Nissui Pharmaceutical Co., Tokyo) with some modifications. Briefly, plants inoculated with strain R170 or R181 were soaked in Victoria blue solution for 1 min, washed with distilled water, submerged in a destaining reagent for 5 min, and then washed again with distilled water to remove excess stain.

We examined the tissue localization of the inoculants in the plant roots in 70% glycerol under a light microscope (IX70 Inverted Microscope, Olympus, Tokyo) until 28 days after the inoculation. Photomicrographs were taken using a high-sensitivity CCD camera (VB-7000, Keyence, Osaka, Japan).

2.2.6 Statistical analysis

All experiments were performed with a minimum of three replicates for each treatment. The statistical analysis was carried out using SPSS Statistics for Windows v.22.0. Data were either subjected to an analysis of variance (ANOVA) or Student's t-test. A post-hoc comparison of mean values among treatments was performed using Tukey's honestly significant difference (HSD) test at the 5% confidence level.

2.3 Results

2.3.1 Biochemical and enzyme activities

We screened the four PGPB strains with high affinity for potato roots (cv. Matilda) (*Sphingomonas* sp. T168, *Streptomyces* sp. R170, *Streptomyces* sp. R181, and *Methyllibium* sp. R182) for their biochemical and enzyme activities (Table 2). The results of the biochemical tests showed that even though all four strains produced IAA, R170 exhibited the most efficient production with 1.8 µg IAA per 10^8 CFU (p=0.000) at 72 hr. The production of siderophores (p=0.001) and biofilm (p=0.001) was also observed in all four strains, but the levels were highest for R170.

Regarding the enzyme activities (Table 2), ACC deaminase activity was observed in all four strains ranging from 421.8 to 690.6 nmol AKB mg^{-1} cell per hr (p=0.000), with R170 showing the highest, followed by R182, R181, and T168 in that order. With regard to the production of other enzymes, β-1,3-glucanase (p=0.004) and cellulase (p=0.000) activities were the highest in R182, followed by R170, R181, and T168 in that order. Protease activity (p=0.000) was also observed for all strains except T168, whereas lipase and chitinase activities were observed for only R170 and R182, respectively.

2.3.2. Environmental stress tolerance

Table 3 shows the effects of NaCl and $AlCl_3$ stresses on the growth of the four PGPB strains. Among the strains, R170 showed the highest tolerance to NaCl with approx. 61%, 46%, 7%, and 5% growth compared to the control at 1%, 2%, 3%, and 4% NaCl, respectively. R181 showed a similar trend with R170 having approx. 52%, 46%, and 7% growth at 1 to 3% NaCl, but a lower tolerance to 4% NaCl. T168 and

Table 2. Biochemical activities of the four plant growth-promoting bacteria (PGPB) strains.

Strain	Important biochemical substances for plant growth			Lytic enzymes					
	IAA (µg per 10^8 CFU)	Siderophore (mm dia.)	ACC deaminase (nmol AKB mg^{-1} cell hr^{-1})	β-1,3-glucanase	Cellulase	Protease	Chitinase	Lipase	Biofilm (OD_{595})
T168	0.26 ± 0.02 a	2.23 ± 0.24 a	421.84 ± 9.84 a	+	-	-	-	-	0.20 ± 0.01 a
R170	1.82 ± 0.42 b	3.26 ± 0.16 b	690.60 ± 10.36 d	+	+	+	-	+	0.33 ± 0.03 c
R181	0.50 ± 0.12 a	1.89 ± 0.23 a	494.02 ± 12.54 b	+	+	+	-	-	1.89 ± 0.03 b
R182	0.36 ± 0.15 a	2.51 ± 0.35 a	638.68 ± 17.00 c	+	+	+	+	-	2.51 ± 0.00 ab

Values are expressed as means± SD. One way ANOVA was performed to compare significant differences among means. Mean values in the same column with common letters are not significantly different from each other ($p \leq 0.05$) according to Tukey's HSD test. AKB stands for α-ketobutyrate. Lytic enzymes are presented with + or – for the presence or absence of activity respectively.

Table 3. Environmental stress tolerance of the four plant growth-promoting bacteria (PGPB) strains.

Strain	NaCl (M)	$AlCl_3$ (µM)	pH	Temperature (^oC)
T168	~ 0.17	0.7 ~ 75	5 ~ 9	20 ~ 30
R170	0.17 ~ 0.68	0.7 ~75	5 ~ 10	10 ~ 30*
R181	0.17 ~ 0.51	0.7 ~ 75	5 ~ 10	20 ~ 30
R182	~ 0.17	0.7 ~ 7	5 ~ 9	20 ~ 30

Range values of NaCl and $AlCl_3$ are presented as molar (M) and micromolar (µM) respectively.
*Strain was observed to initiate growth at temperature greater than 10^oC but lower than 20^oC at approx. 96 ~ 120 hrs.

R182 were relatively sensitive to NaCl stress, showing only 17% and 11% growth even at 1% NaCl, respectively.

In terms of $AlCl_3$ stress, the growth of all of the strains was recorded up to 0.01%. However, R170 and R181 showed higher tolerance compared to the other two strains, and the tolerance of R170 tended to be higher than that of R181; at 0.0001% $AlCl_3$, R170 and R181 showed growth similar to that of the control, whereas T168 and R182 showed 90% and 65% growth, respectively. At 0.001% $AlCl_3$, R170 still showed growth similar to that of the control, whereas R181, T168, and R182 showed 96%, 88%, and 64% growth, respectively. At 0.01% $AlCl_3$, R170, R181, T168, and R182 showed 68%, 67%, 65%, and 7% growth, respectively. Aluminum concentrations higher than 0.1% were lethal for all strains.

Table 3 also shows the growth profiles of the four PGPB strains at different ranges of temperature and pH. The most favorable temperature for all strains was approx. 30^{o}C with the growth peak at 24 hr after incubation. At 20°C, the growths of R170, R181, and T168 were delayed with peaks recorded at 48, 48, and 72 hr, respectively, whereas no growth was noted for R182. Among the four strains, R170 started to grow earlier than the other strains. At 10 and 40°C, the growth of all four strains was severely inhibited until at least 96 hr after incubation.

Regarding pH, T168, R170, and R181 showed similar growth profiles at pH 5.0 and 6.0, although T168 exhibited a slight delay (Table 3). An alkaline condition (pH 8.0 and 9.0) caused slightly delayed growth of T168 and R182 compared to R170 and R181. Strains R170 and R181 were observed to survive at high pH (10.0), and the growth of all four strains was inhibited at the low pH of 4.0.

2.3.3 Effect of bacterial inoculation on the growth of potato seedlings

Figure 3 shows the effect of inoculating PGPB strains (T168, R170, R181, and R182) on the growth and germination of potato seedlings and seeds, respectively. Among the four strains, R170 showed the highest value in terms of fresh weights compared with the control followed by T168 and R182. For the dry weight, all strains showed significantly higher values compared to control, but no significant difference between the strains was observed. The inoculation of strains revealed to have no significant effect on the germination of potato seeds in reference to control.

2.3.4 Localization of PGPB to potato seedlings

The progress of localization of each PGPB strain on the seedlings of potato is presented in Figure 4, whereas their characteristic localization in their initial interaction with the roots is shown in Figure 5. All strains exhibited an efficient colonization as demonstrated in the GUS- or Gram-stained plant tissues. Specifically, T168 showed localization at the base of lateral roots (Figure 5c-d), and R182 was noted on the root hairs and root surfaces (Figure 5e-h). Whereas, the two species of *Streptomyces*, R170 (Figure 5i-l) and R181 (Figure 5m-p) showed random infection and partially covered the plant roots.

In particular, T168 seemed to readily localize near the base of root emergence at 3 to 7 days before the formation of new lateral, and it appeared as spots on the roots. Very little infection was shown by T168 at the tips of the root hairs (Figure 5d). However, unlike T168, R182 started to localize near the tips of the root hairs and then spread to the whole root hairs and root surface as shown with a visible heavy GUS stain. In addition, R182 was not observed at the base of lateral roots (Figure 5h). The infection

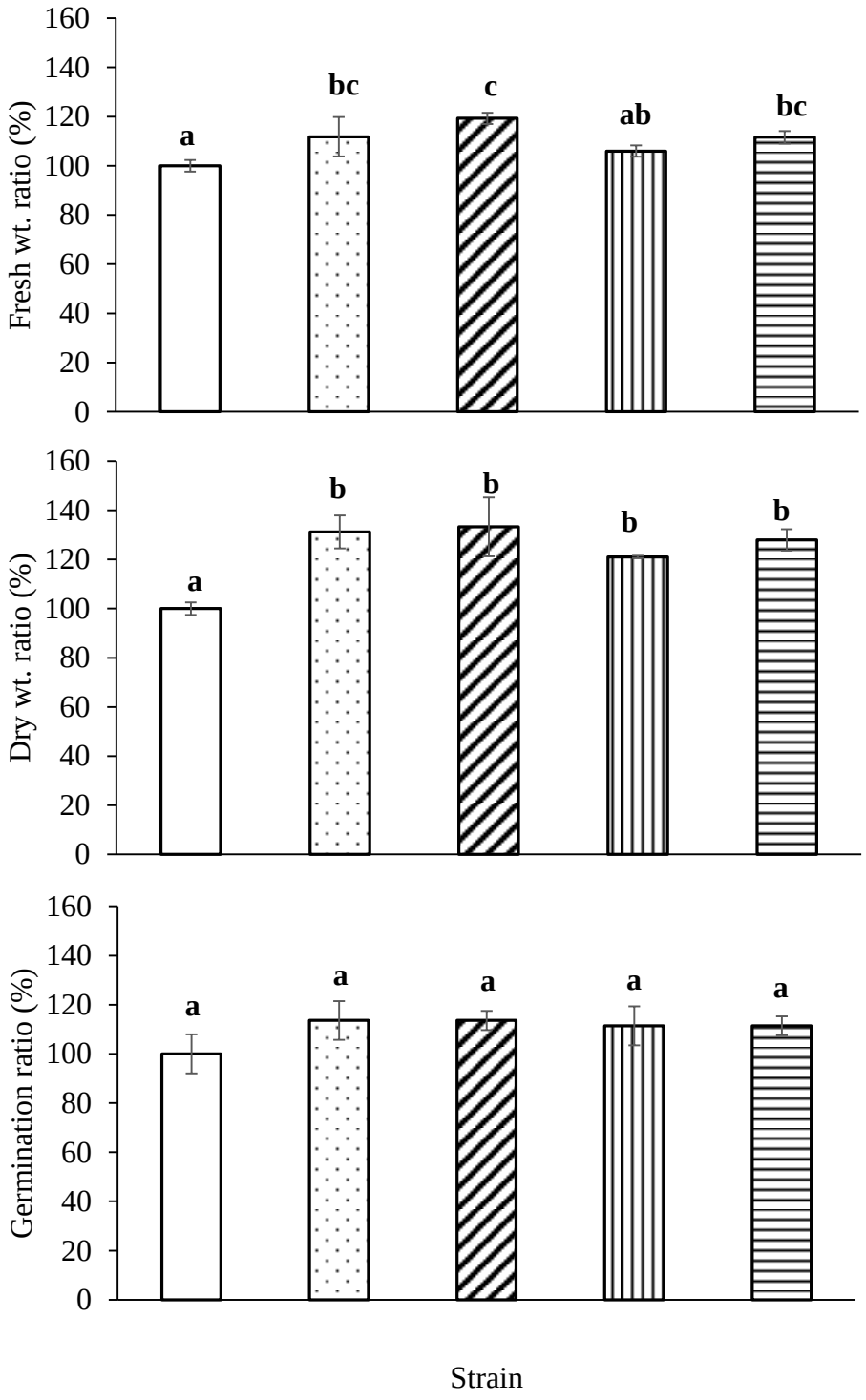

Figure 3. Effects of single strain inoculation on the growth of potato seedlings. The plant weight and germination rate ratios are means ± SD (n=3) against the uninoculated control. One-way ANOVA was performed to compare significant differences among mean ratios. Mean values with common letters are not significantly different from each other ($p \leq 0.05$) according to Tukey's HSD test. . ▭, Uninoculated control; ⁘, T168; ▨, R170; ▥, R181; ▤, R182.

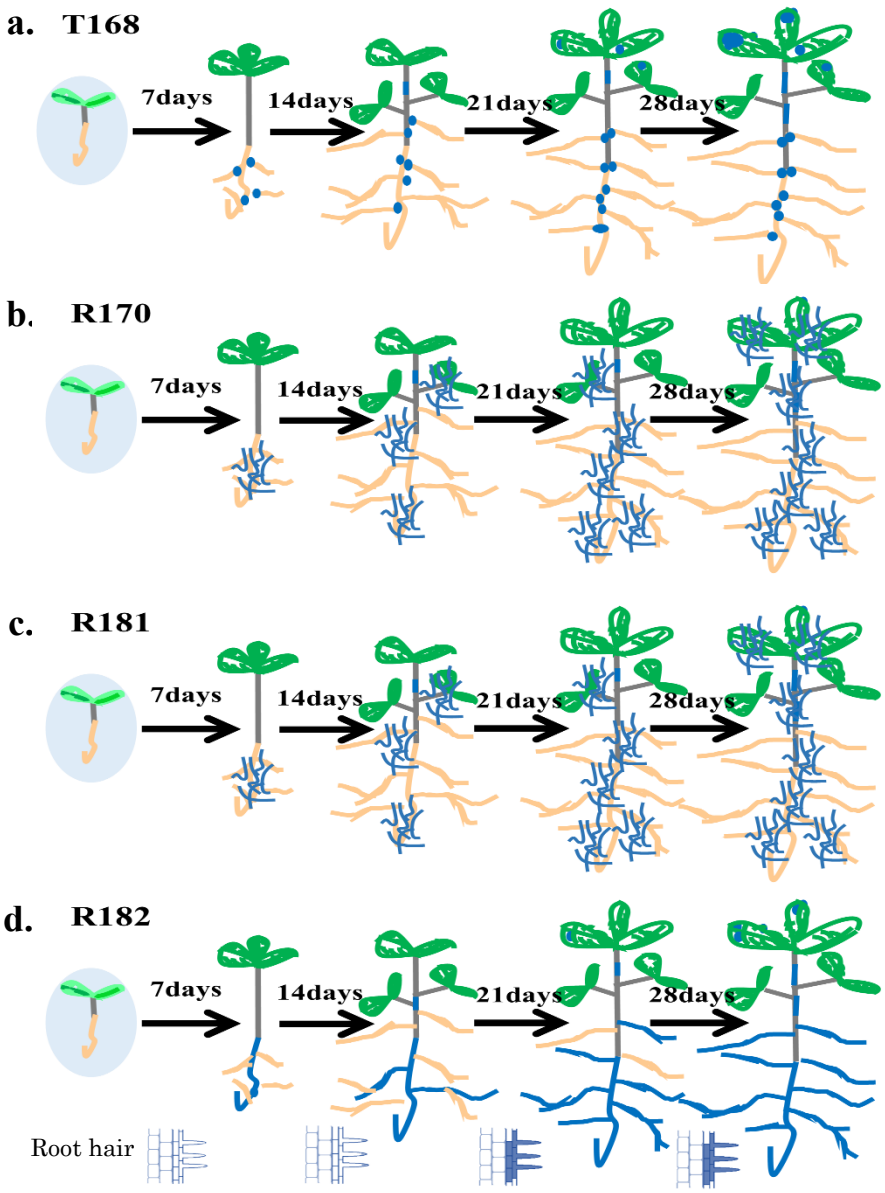

Figure 4. Localization of each strain on potato seedlings. **(a)** T168 initially spotted on the roots gradually localized at the base of lateral roots, and eventually reached the stem and leaves. Massive hyphal growth of **(b)** R170 and **(c)** R181 showing gradual and random localization from the primary and secondary roots to the stem and leaves. **(d)** Infection of R182 to the entire root surface including root hairs, gradually spread from the root hair tips (<1d) to the base. Blue color represents stained parts of plant tissues.

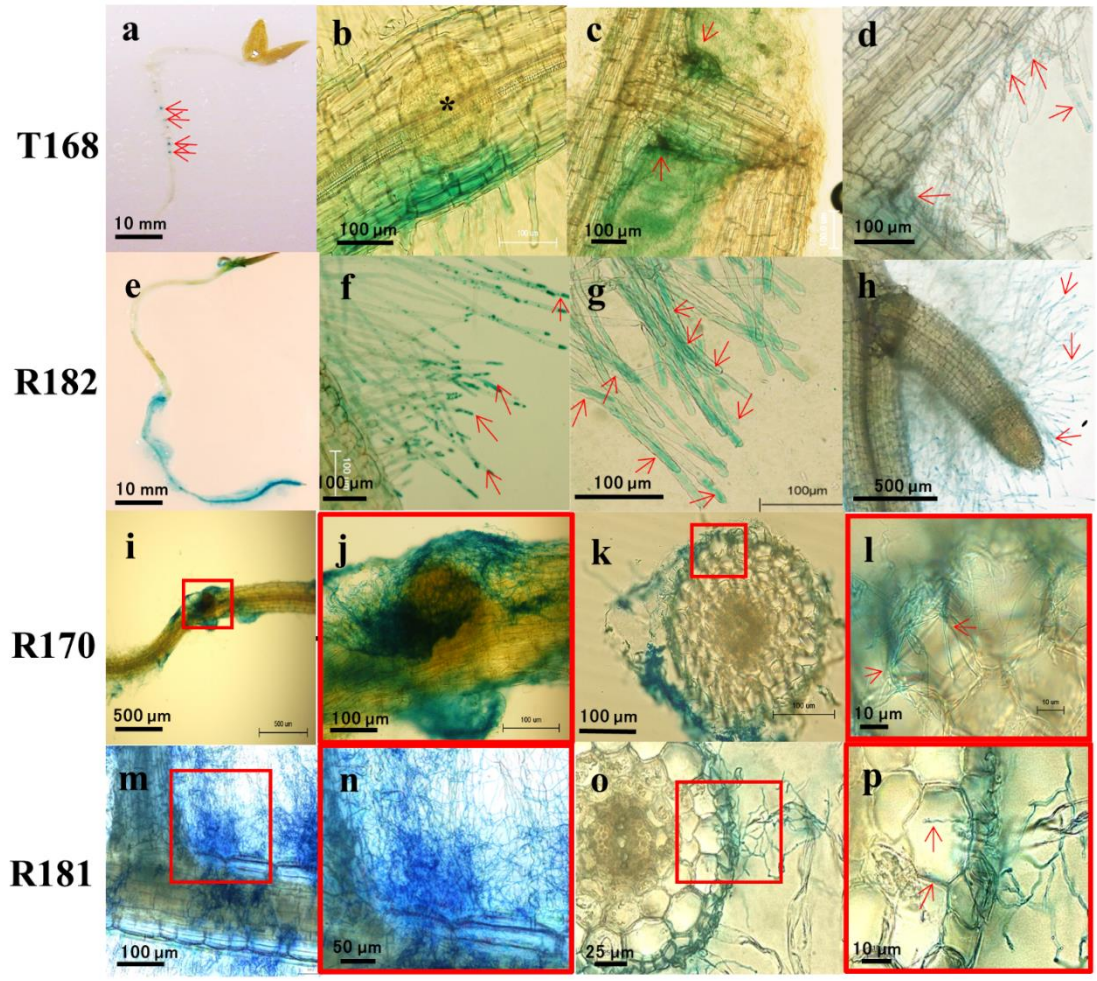

Figure 5. Characteristic localization of the four PGPB strains on the roots of potato seedlings. (**a)** T168 spots on the roots [at 7 days (7d)]. (**b)** Strong infection near the primordium of lateral root (3d). (**c)** Intense stain at the base of lateral root (7d). (**d)** Very little infection at the tips of root hairs (7d). (**e)** Infection of R182 to the entire root surface including root hairs (7d). (**f)** Infection spread from the root hair tips (<1d) (**g)** to the base (7d). (**h)** Absence at the base of lateral roots (7d). (**i,j)** Massive hyphal growth of R170 surrounding the lateral root (2d) and (**k,l**) into the inter- and/or intracellular spaces of the main root (cross-section) (7d). (**m,n)** Mass of R181 hyphae surrounding the main and lateral roots (7d) and (**o**,**p**) inside the cortical layer of an adventitious root (cross-section) (14d). Major infected regions are shown by red arrows and boxes. Pictures of R170 (i,k) and R181 (m,o) are enlarged in boxes j and l, and n and p respectively, to show a clearer view of the hyphae. (Santiago *et. al.* 2017, Microbes and Environ. 32(1), *In Press*).

of R170 started to be randomly scattered on the surfaces of the roots (Figure 5i-j). Endophytic infection was confirmed at 7 days as shown by the extending hyphae into the inter- and/or intracellular spaces of the roots (Figure 5k-l). Similarly, R181 was observed to have an initial random and scattered infection on the surfaces of the roots (Figure 5m-n). Partial infection into the inter- and/or intracellular spaces of the main root was also observed with R181 (Figure 5o-p).

2.4 Discussion

The results of this study confirmed that the four PGPB strains with high affinity for potato roots had both the ability to produce plant growth-promoting substances as well as tolerance to environmental stress, and that these strains could be potential bioinoculants. In fact, the inoculation test with these bacteria (T168, R170, R181, and R182), showed significant increases in the dry weight of potato seedlings compared with the control, supporting a previous report that these strains have plant growth-promoting abilities (Kenkyuseika, vol. 539. 2015. Tsukuba Office, Agriculture, Forestry and Fisheries Research Council Secretariat, Japan).

In the evaluation of potential bioinoculants, strains R170 was identified to be the most suitable candidate due to its ability of producing the highest level of important plant growth promoting substances (IAA, siderophore, biofilm and ACC deaminase) among the four PGPB, and capability of producing hydrolytic enzymes (β-1,3-glucanase, cellulase, protease and lipase). It has been known that IAA-, siderophore-, and ACC deaminase-producing bacteria can improve plants' growth by promoting the root elongation and proliferation of lateral roots (Shahab *et al.* 2009; Radzki *et al.* 2013; Glick, 2014), providing bioavailable forms of iron (Radzki *et al.* 2013), and reducing high levels of ethylene (Glick, 2014), respectively. Whereas, biofilms were noted to

indirectly promote the plant growth by supporting the establishment of bacterial infection to plants (Timmusk *et al.* 2005; Seneviratne *et al.* 2011). Other enzymes such as *β*-1,3-glucanase, cellulase, protease, and lipase have also been implied to indirectly influence plant growth (Singh *et al.* 1999; Bhattacharya *et al.* 2009; Glick, 2012).

The usefulness of R170 as a potential bioinoculant for the plant growth may also be evidenced with its higher tolerance against NaCl and $AlCl_3$, and bacterial growth in a wider range of pH values compared with the other three strains. However, the efficiency of a single-strain bioinoculant could not be confirmed unless it is applied in the field and exposed to many external factors (including stress) and its interaction with other soil microorganisms.

Low soil pH, strong aluminum toxicity and depleted nutrients (e.g., phosphorus), which characterize the volcanic ash soil in the Tokachi area, Hokkaido, Japan, have caused the inhibition of plant growth and development in the area. The application of lime and fertilizers has been reported to overcome these problems, but its effectiveness is limited to the soil surface only (Shoji and Takahashi, 2002). In contrast, bioinoculants containing two or more useful strains that have physiological and biochemical characteristics, were also reported to promote plant growth by ensuring the bioavailability of nutrients, while maintaining the balance of soil pH and preventing negative impacts to the environment (Pandey *et al.* 2012).

Chapter 3

Mycorrhiza as a bioinoculant

3.1 Introduction

Mycorrhizas are naturally occurring beneficial fungi that form symbiotic association with plants and serve as extensions of the root system for the easy access of moisture and insoluble nutrients from the soil (Alloush *et al.* 2000). Phosphorus (P) and iron are crucial nutrients for the proper utilization of energy in plants, as well as in respiration and photosynthesis, respectively (Schachtman *et al.* 1998; Lehmann and Rillig, 2015). Despite the abundance of P in soil, they cannot be easily absorbed by the roots because their forms are not bioavailable for plant consumption. In contrast, mycorrhiza has been known to enable the plants' capacity to efficiently acquire insoluble P and iron from the rhizosphere and convert them to bioavailable forms through the production of phosphatases and siderophores, respectively (Aliasgharzad *et al.* 2009; Smith *et al.* 2011).

The application of mycorrhiza to agriculture has been extensively reported (Duffy and Cassells, 2000; Douds *et al.* 2007; Rotor and Delima, 2010; Anyanwu, 2014). In fact, its efficiency to plant growth promotion has encouraged the on-farm production of mycorrhizal inoculants worldwide (Douds *et al.* 2007). In the Philippines, a mycorrhizal inoculant under the name of *MYKOVAM*® has been developed as a biofertilizer for a wide range of crops with published reports for applications in corn (Rotor and Delima, 2010) and tomato (Anyanwu, 2014).

The efficiency of mycorrhiza is guaranteed through a successful infection to the host plant, which is initiated by the attachment of hyphae to the root epidermis or root hairs (Peterson *et al.* 2004).

In the previous chapter, PGPB has been proven to improve the growth of potato seedlings. Here in the present chapter, the ability of mycorrhiza to produce phosphatases and siderophores was evaluated. Then the infection of mycorrhiza to the roots, mycorrhizal spore germination, and biological control against pathogen was assessed. Ultimately, the capacity of mycorrhiza from a commercial biofertilizer in promoting the growth of potato seedlings was examined. Results of this study suggest that mycorrhiza can be an efficient bioinoculant for the growth promotion of potato seedlings.

3.2 Materials and Methods

3.2.1 Isolation and single spore production of mycorrhiza

Mycorrhizal spores were isolated from a soil inoculant (*MYKOVAM*®) provided by the National Institute of Molecular Biology and Biotechnology of the University of the Philippines Los Baños. The isolation of mycorrhizal spores from *MYKOVAM*® that contains 8 different species, was conducted following the standard methods of wet sieving and decanting as reported by Gerdemann and Nicolson (1963). Among the spores isolated, only a single inoculum, which has been previously identified as *Gigaspora margarita* (Aggangan *et al.* 2013) and verified in this study by morphological characterization following the manual of Schenck and Perez (1990), was cultured in a soil:sand medium using wheat (cv. Kitahonami) as host plant, as well as in MSR agar medium prepared according to the methods reported by Declerck *et al.* (2005). Prior to the *in vitro* propagation of mycorrhiza on MSR agar, spores were

carefully disinfected by chloramine T solution (containing 2–3 drops of Tween 20), streptomycin, and gentamycin following the methods reported by Mertz et al. (1979).

3.2.2 Assessment for the phosphatase activity of mycorrhiza

True potato seeds (cv. Hokkaikogane) (disinfected with 70% ethanol, and a solution containing 10% NaClO and 1% Tween 20) were germinated in 0.3% plant agar inoculated with 5 plugs of mycorrhiza. Thirty five days after inoculation, the acid (pH 5.5) and alkaline (pH 11) phosphatase activities of mycorrhiza in roots were assessed (Tabatabai, 1994). Absorbance values at 450 nm were measured using a spectrophotometer (Ultrospec3100pro, GE Healthcare Life Sciences, Buckinghamshire, UK). Phosphatase activity was calculated based on the standard curve of *p*-nitrophenol.

3.2.3 Assessment for the siderophore production of mycorrhiza

Siderophore production was determined by liquid assay (Schwyn and Neilands, 1987) with the following modifications: fifty microliters of mycorrhizal spore suspension (with approx. 5 spores) were grown in MSR broth (without iron) at 27ºC and 60 rpm for 24 hrs. After incubation, the spore suspension was centrifuged at 3000 rpm at low temperature for 15 min. Nine hundred microliters of supernatant was mixed with 100 µL of CAS solution (Schwyn and Neilands, 1987) and 10 µL of 400 mM sulfosalicylic acid, and incubated for 20 min. Absorbance values of the sample and reference solution (900 µL growth medium, 100 µL CAS solution, and 10 µL 400 mM sulfosalicylic acid) were measured at 630 nm, and the siderophore activity was expressed as ratio of the difference between the reference and the sample against the reference.

3.2.4 Assessment for mycorrhiza root infection

Fourteen days old potato seedlings were inoculated with the spores of mycorrhiza. Mycorrhizal inoculant was prepared by suspending the spores harvested from MSR agar to sterilized distilled water. One hundred microliters of spore suspension containing approx. 10 spores were directly inoculated to the roots. Twenty eight days after inoculation, mycorrhizal root infection was assessed following the grid-line intersection method (Giovannetti and Mosse, 1980). Prior to microscope observation, roots were cleared of cell contents following the manual instructions of SUNJin Lab using *RapiClear*® 1.55 (Funakoshi Co. Ltd., Tokyo), immediately followed by staining using 0.05% trypan blue in glycerol and incubated for 24 hrs at 30°C, and then de-stained with 85% lactic acid. Mycorrhizal root infection was assessed by the presence of either one of the following structures: hyphae, vesicles, arbuscules, and spores which were visible after staining. The number of infected and uninfected roots that intersect gridlines were counted. Percent mycorrhizal infection was calculated by the ratio of the sum of infected roots against the total number of roots.

3.2.5 Assessment for mycorrhiza spore germination

For the assessment of the viability of mycorrhiza, a total of 8 spores were initially grown on MSR agar under dark conditions at 27°C for 14 days. After incubation, the total number of spores on MSR agar was counted. Spore germination was computed by getting the ratio of the final spore density (final spore number – initial spore number) against the initial spore density.

3.2.6 Assessment for biological control activity

The biological control activity (BCA) of mycorrhiza against a pathogen was performed according to the methods reported by Schelkle and Peterson (1997) with the following modifications: mycorrhizal plugs (6 mm dia.), taken from the periphery of growing colonies on MSR agar, were placed at the edge of a petri plate containing a mixture of MSR and PDA (1:1) buffered with 3.3% 2-(N-morpholino)ethanesulfonic acid (MES), and then incubated in the dark at 27ºC for 12 hrs. Subsequently, plugs (6 mm dia.) of *Rhizoctonia solani* (MAFF no. 305250), acquired from the Genebank of the National Agriculture and Food Research Organization (NARO) and maintained on potato dextrose agar (PDA) were placed on the opposite side of a 12-hr culture of mycorrhiza, and then inoculated with 100 µL of 0.1 M $MgSO_4 \bullet 7H_2O$. All treatments were replicated three times. After 14 days of incubation in the dark at 27ºC, inhibition zones between the two growing colonies were recorded.

3.2.7 Inoculation test on potato seedlings

Mycorrhizal spore (1 mL) suspensions, prepared as described above, were directly inoculated on potato seeds (n=18) derived from the open pollination of cv. Hokkaikogane sown on pots containing approx. 100 g of sterilized seedling-raising culture soil (PotAce N, Katakura & Co-op Agri Corp., Tokyo). Controls were either inoculated with 1 ml of mycorrhizal spore suspension or not inoculated. Pots were covered with aluminum foil and placed in a growth chamber under light (23.5°C for 14 hr) and dark (20.0°C for 10 hr) conditions, respectively. The cover was removed upon the germination of seeds, and 30 days after the inoculation, the plant growth parameters such as plant weight (mg) and germination rates were measured (n=3). Plant dry weight was measured after oven drying at 60°C for 3 days.

3.2.8 Statistical analysis

All experiments were performed with a minimum of three replicates for each treatment. The statistical analysis was carried out using SPSS Statistics for Windows v.22.0. All data were subjected to analysis of variance (ANOVA). A post-hoc comparison of mean values among treatments was performed using Tukey's honestly significant difference (HSD) test at the 5% confidence level.

3.3 Results

3.3.1 Phosphatase activity of mycorrhiza

Figure 6 shows the acid phosphatase activity (p=0.000) of mycorrhiza in the roots of potato seedlings expressed as mg p-nitrophenol per g root sample hr^{-1}. The inoculation of mycorrhiza showed a significantly higher acid phosphatase activity in the roots compared with the uninoculated control. In contrast, very minimal alkaline phosphatase activity was manifested in the roots, and no significant difference was observed with the inoculation of mycorrhiza compared with the uninoculated control.

3.3.2 Siderophore production of mycorrhiza

Figure 7 shows the siderophore production of mycorrhiza on MSR agar with CAS manifested by the change in color evidenced by the appearance of yellow orange halo formed around the colonies. In contrast, Figure 8 shows the percent siderophore activity (p=0.000) of mycorrhiza in liquid assay manifested by the change in the color of the medium from blue to purplish orange. Siderophore activity recorded for mycorrhiza was 7% in reference to the control solution containing CAS and sulfosalicylic acid.

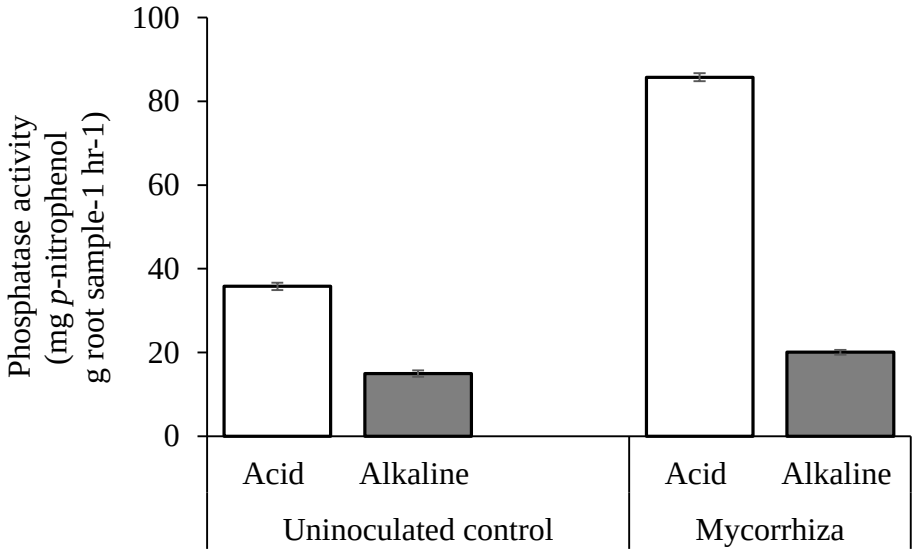

Figure 6. Phosphatase activities of mycorrhiza. Vertical axes display the phosphatase activities expressed as mg *p*-nitrophenol per gram root sample per hr. The data are means ± standard deviation (SD) of three replicates. The SDs of the means were less than the 0.05 significance level. □, acid phosphatase; ■, alkaline phosphatase.

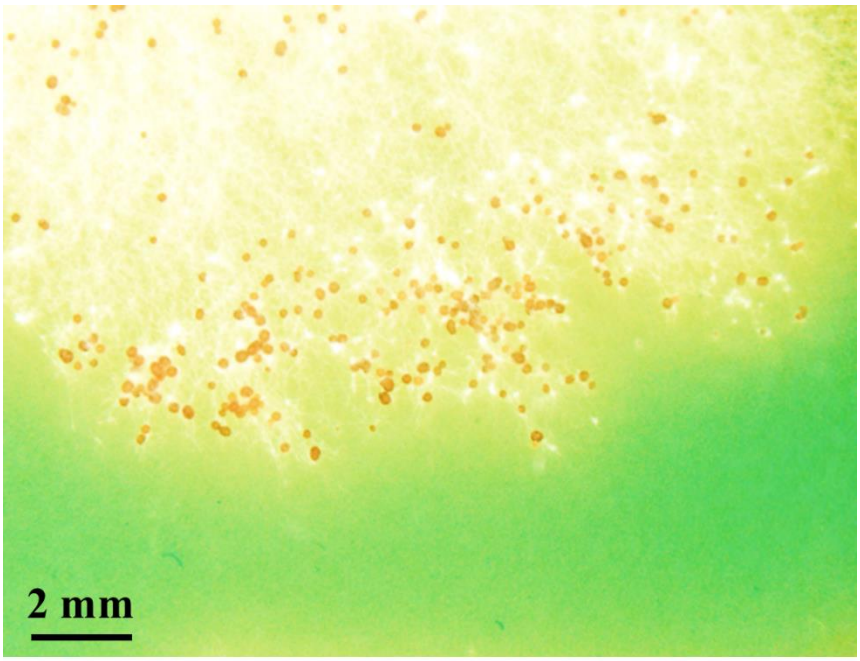

Figure 7. Confirmation of mycorrhizal siderophore activity on a CAS agar. Siderophore production was manifested by the appearance of a yellow-orange halo surrounding the colony of mycorrhiza grown on a blue-colored agar.

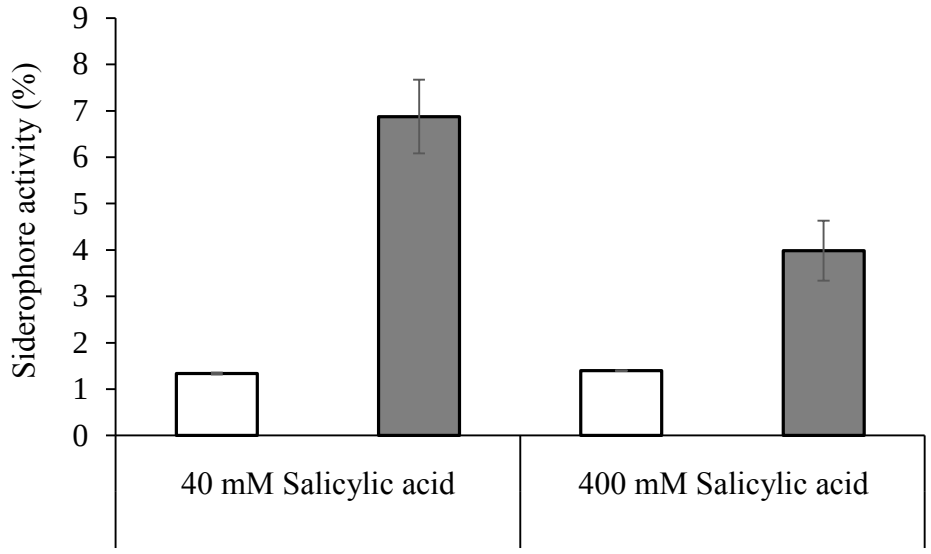

Figure 8. Siderophore activity of mycorrhiza in liquid assay. Salicylic acid having concentrations of 40 and 400 mM were used as shuttling solution for iron. Vertical axes display the siderophore activity expressed as the ratio of absorbance values of the solution containing mycorrhiza (OD_{630} reference solution - OD_{630} mycorrhiza's supernatant) against the reference solution (OD_{630} reference). Data are means ± standard deviation (SD) of three replicates. The SDs of the means were less than the 0.05 significance level. □, Reference; ■, Mycorrhiza.

3.3.3 Root infection of mycorrhiza to potato seedlings

Approximately 60% of the roots of potato seedlings were infected by mycorrhiza manifested by the attachment of spores, hyphae, and in some instances vesicles and arbuscules (Figure 9). Spores were round to be oval in shape, yellow to brown in color, and were connected at the tip of a bulbous sporogenous cell (Figure 10a). Spore diameters were approx. 250-400 µm (n=30) having 2 layers of spore walls (Figure 10b). The hyphae connects the spores to the roots (Figure 9a). Vesicles and arbuscules appeared as round or elongated cells and tree-like structures, respectively, which were observed either in the intra- or intercellular spaces of the roots (Figures 9b). Germination of spores started from 7 days after the inoculation. Whereas, the formation of vesicles and arbuscules inside the roots was noted at 21 days after inoculation.

3.3.4 Biological control activity of mycorrhiza

Figure 11a shows that mycorrhiza significantly inhibited the growth of *Rhizoctonia solani* evidenced by the production of a clear zone between mycorrhiza and the pathogen on MSR+PDA medium. Under optimum conditions without an antagonist, the hyphae of *R. solani* on water agar showed a smooth, healthy-looking and right angled branching (Figure 11b). However, after challenging the pathogen with mycorrhiza, a change in the normal structure of the hyphae was observed with evidences of deformation, degradation, and in some instances leakage (Figure 11c).

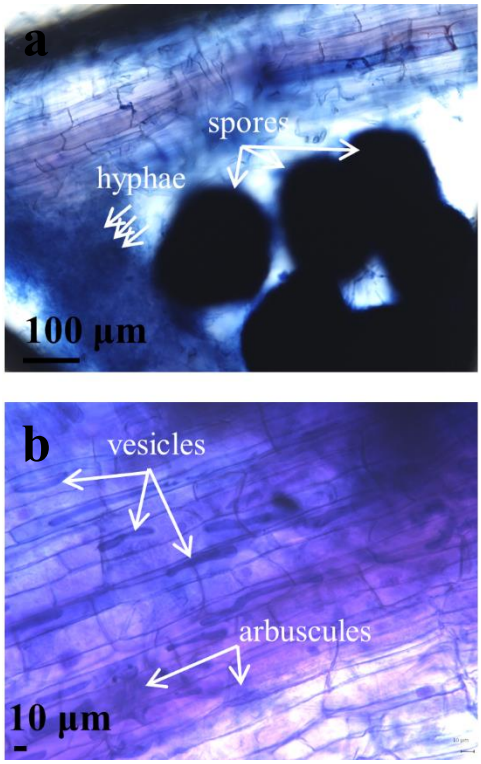

Figure 9. Mycorrhizal infection to the roots of potato seedlings. **(a)** Mass of hyphae surrounded the surface of the lateral root connecting the spores to the root. **(b)** Vesicles and arbuscules formed in between the cortical cells of the root.

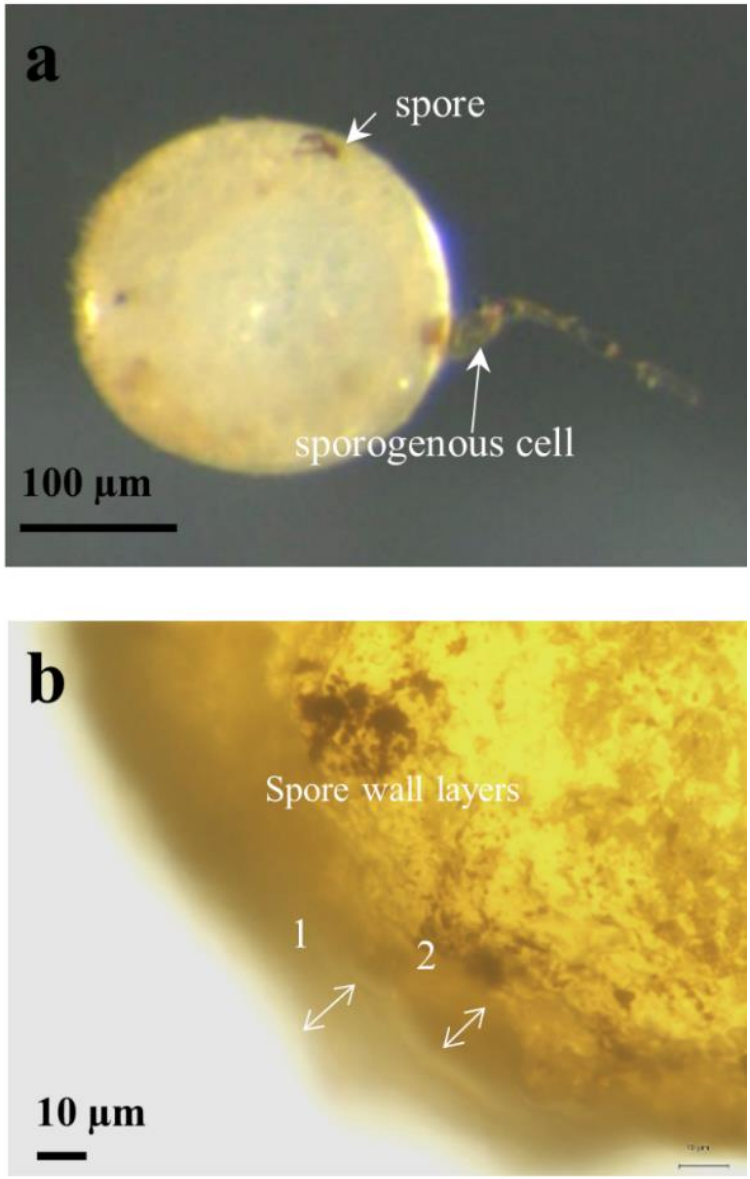

Figure 10. Photomicrographs of the spore of *Gigaspora margarita*. **(a)** Single spore formed at the tip of a bulbous shape and transparent sporogenous cell (20x magnification). **(b)** Enlarged photo of the spore showing two layers of spore wall (100x magnification).

3.3.5 Efficiency of mycorrhiza in plant growth promotion

The effect of inoculating mycorrhiza isolated from a commercial soil inoculant (*MYKOVAM*®) in promoting the growth of potato seedlings compared with the control as well as with PGPB is shown in Figure 12. Mycorrhiza showed a significant improvement in the fresh (p=0.000) and dry weight (p=0.000) of seedlings compared with the uninoculated control. Specifically, in terms of the fresh weight, mycorrhiza demonstrated the highest increase among the PGPB strains. Whereas for the dry weight, the inoculation of mycorrhiza were just comparable with the inoculation of PGPB strains.

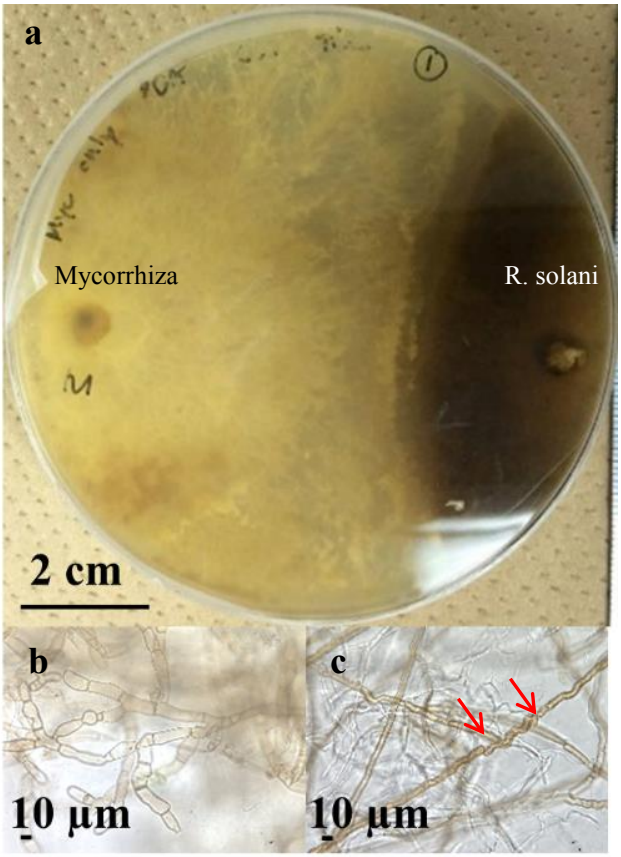

Figure 11. Biological control of mycorrhiza against *R. solani*. **(a)** Dual culture of mycorrhiza and *R. solani*. **(b)** Normal growth of *R. solani* on water agar showing smoothly defined, healthy-looking hyphae with right-angled branching. **(c)** *R. solani* showing crooked formation of mycelium taken from the edge of inhibition zone formed by the inoculation of mycorrhiza.

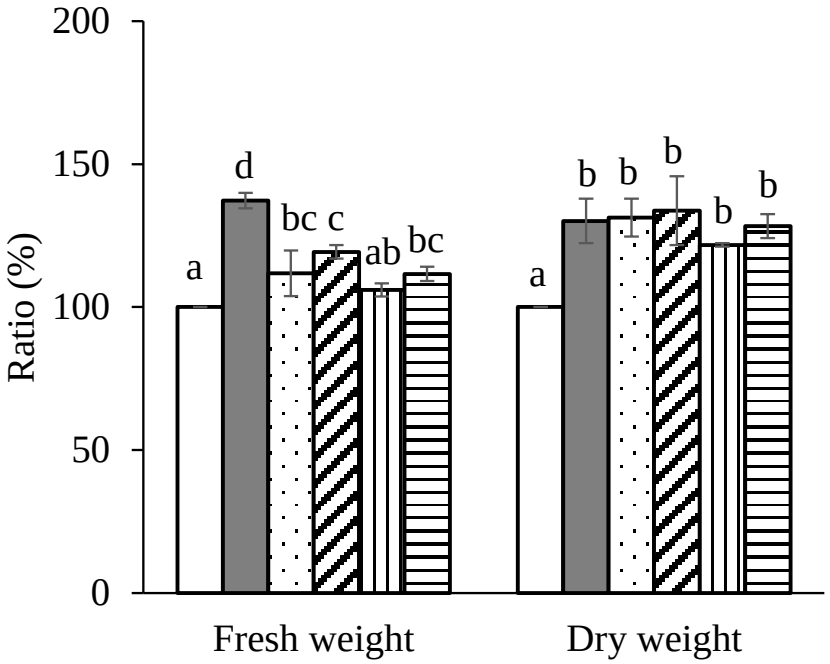

Figure 12. Effects of inoculating mycorrhiza on the growth of potato seedlings compared with PGPB. Vertical axis shows the ratio of plant fresh and dry weight against the uninoculated control. One-way ANOVA was performed to compare significant differences among mean ratios. Mean with common letters were not significantly different from each other ($p \leq 0.05$) according to Tukey's HSD test.
□, Uninoculated control; ■, Mycorrhiza; ⁘, T168; ▨, R170; ▥, R181; ▤, R182.

3.4 Discussion

The results of this study showed that the strain of mycorrhiza (*Gigaspora margarita*) isolated from a commercial soil inoculant from the Philippines (*MYKOVAM*®) can also infect the roots of a local cultivar of potato seedlings grown in Japan (cv. Hokkaikogane) and effective in promoting its growth. The enhanced growth of seedlings may be attributed to the ability of mycorrhiza to solubilize insoluble P in the medium and provide them to plants for absorption which is associated with the production of phosphatases (Schachtman *et al.* 1998, Smith *et al.* 2011, Zheng *et al.* 2011). This was manifested by the higher acid phosphatase (AP) activity of mycorrhiza in the roots compared with the uninoculated control, which can be related with the significantly heavier fresh and dry weight of seedlings inoculated with mycorrhiza in reference to the uninoculated control.

Another evidence that demonstrates the growth promotion of potato seedlings may be the production of mycorrhizal siderophores which might have probably enhanced the iron acquisition in the plants (Aliasgharzad *et al.* 2009). The acquisition of iron by mycorrhiza was clearly manifested by the change in the color of the medium containing CAS upon the administration of sulfosalicylic acid that serves as a shuttling solution for the rapid transport of iron. However, the total amount of iron acquired by the host plant inoculated with mycorrhiza should be examined as well in future research.

Nevertheless, prior to the execution of plant growth promoting activities, microbial inoculants should have a stable infection to the host plant in order to secure an easy exchange of nutrients between the symbionts. Mycorrhizal infection starts from the attachment of hyphae to the root surface or root hairs, which eventually penetrates into the root cells, and form structures (vesicles and arbuscules) that are essential in the storage and transport of nutrients in plants (Peterson *et al*. 2004; Gutjahr and Parniske,

2013). In this regard, the high root infection of mycorrhiza reported in this particular study may also be related with the viability of the spores as revealed by their capacity to germinate. However, sporulation was only confirmed on an artificial medium (MSR agar). Thus, the viability of spores that are already associated with the roots should be examined in further study.

In addition, it has been reported that mycorrhizas can serve as biological control against harmful pathogens (Schelkle and Peterson, 1997; Tahat *et al.* 2012). In the present chapter, the ability of mycorrhiza as a biological control agent of *R. solani* was demonstrated by the inhibition zones between mycorrhiza and the pathogen. In relation to this, the manifested deformation and degradation of the pathogen's hyphae as shown on dual culture on agar indicated that mycorrhiza can effectively suppress the growth of *R. solani.*

Hence, the inhibition of *R. solani* by mycorrhiza may also be attributed to the siderophore activity of mycorrhiza which may have deprived the pathogen's nutrition because mycorrhiza have already efficiently utilized the nutrients from the medium (particularly iron), thus, leaving the pathogen with less nutrients to use. Moreover, the utilization of microbial siderophores is specific only to the microbes that produce them (Schelkle and Peterson, 1997).

The results of this study confirmed that mycorrhiza isolated from *MYKOVAM*® (commercial fertilizer from the Philippines), is an effective bioinoculant for potato seedlings (cv. Hokkaikogane), manifested by its efficient infection to the roots, acid phosphatase and siderophore production activities, resulting in the significant increase in plant growth.

Chapter 4

Compatible strains for plant growth promotion

4.1 Introduction

Previous chapters have demonstrated that symbiotic microbes, which includes bacteria and mycorrhiza, can be potential plant growth promoters manifested by having at least one or an array of biochemical products, including IAA, siderophore, ACC-deaminase, biofilms, hydrolytic enzymes, phosphatases etc., which are responsible for the execution of mineral nutrition in plants. The beneficial effects of using microbes for enhancing plant growth has been extensively reported (Singh *et al.* 1999; Duffy and Cassells, 2000; Douds *et al*. 2007; Shahab *et al.* 2009; Rotor and De Lima, 2010; Seneviratne *et al.* 2011; Glick, 2012; Anyanwu, 2014; Glick, 2014). But several accounts also presented better plant growth promotion with the use of a group of two or more strains of beneficial microbes due to their combined biochemical productivity (Amutha *et al.* 2009; Mahmood *et al.* 2010; Castillo *et al.* 2013).

Pandey *et al.* (2012) reviewed about the complementary effects of a consortium of microbial strains in plant growth promotion. Despite the voluminous reports about the positive effects of combined strain inoculation, it is still unclear how these microbial combinations promote the plant growth in detail. The present chapter demonstrates how compatible microbial strains improve plant growth. Initially, plant growth-promoting bacteria (PGPB) (*Sphingomonas* sp. T168, *Streptomyces* sp. R170, *Streptomyces* sp. R181, and *Methylibium* sp. R182) were tested for compatibility. We focused on the co-inoculation of R170 with T168 or R182, which demonstrated positive effects on plant growth, and compared them with a parallel combination of R181 in

place of R170. Subsequently, the compatibility of these PGPB strains with mycorrhiza was also tested for efficiency in plant growth promotion. The infectivity of each PGPB strain within a combination on plant was also examined. Whereas, for the co-inoculation of PGPB with mycorrhiza, the infection capacity of mycorrhiza was assessed. IAA and siderophore production activities of combined PGPB strains were examined, and the effect of these PGPB strains on the capacity of mycorrhiza to perform plant growth-promoting functions (acid phosphatase, siderophore, biological control) were also determined. This study indicated that bacterial compatibility in combined inoculation is crucial in enhancing plant growth owing to the synergistic effect of compatible PGPB strains, which resulted from the increased production of plant growth-promoting substances, along with the co-existence of strains in the host plant. Likewise, the compatibility of PGPB with mycorrhiza was demonstrated to significantly enhance mycorrhizal functions resulting in plant growth promotion.

4.2 Materials and Methods

4.2.1 Tests for bacterial compatibility

4.2.1.1 Bacterial inoculation and evaluation for plant growth promotion

Both R170 and R181 strains were grown in R2A broth at 30°C for 24 hrs with shaking at 130 rpm. After centrifugation at 10,000 rpm, 4°C for 5 min, the cell suspension was adjusted to 1×10^8 CFU mL^{-1} in sterilized distilled water. The T168 and R182 strains were grown on R2A agar medium for 72 hrs under the same temperature condition described above. Cells were collected directly from the agar medium, and the suspension was adjusted to the cell density mentioned above.

To evaluate for the compatibility of strains in plant growth promotion, we focused on the combination of R170, which exhibited the highest potential as a bioinoculant

with T168 or R182, with a parallel combination using R181 instead of R170. Combined cell suspensions (2 mL; 1 mL from each strain) were directly inoculated on potato seeds (*n*=18) derived from the open pollination of cv. Hokkaikogane sown on pots containing approx. 100 g of sterilized seedling-raising culture soil (PotAce N, Katakura & Co-op Agri Corp., Tokyo). The pots were covered with aluminum foil and placed in a growth chamber under light (23.5°C for 14 hrs) and dark (20.0°C for 10 hrs) conditions. The cover was removed upon the germination of seeds, and 30 days after the inoculation, fresh weight of plants (mg) was measured (*n*=3). Plant dry weight (mg) was measured after oven drying at 60°C for 3 days.

4.2.1.2 Localization of co-inoculated PGPB to plant roots

Approximately 14-days-old potato seedlings were grown on plant agar (0.3%) containing a 500-times dilution of HYPONeX® 6-10-5 (HYPONeX Japan, Osaka, Japan) and inoculated with a single or combined PGPB prepared as described above. The tissue localization of each strain in a combination in the plant roots was examined by GUS and/or Gram staining. For the plants inoculated with GUS-marked strains, bacterial cells were stained by immersing plant samples in a GUS-staining solution (16 mL of 125 mM sodium phosphate; 80 µL of 0.5 M Na_2EDTA, pH 8.0; 800 µL of 2% X-Gluc [5-bromo-4-chloro-3-indolyl-*β*-D-glucuronic acid] cyclohexylammonium salt; 80 µL of 10% SDS; and 23.6 mL of distilled water) with continuous deaeration in a desiccator connected to a vacuum pump for 30 min, and then allowed to incubate on plants at 30°C for 3 days.

Gram staining was done according to the instructions of Favor G "*Nissui*" (Nissui Pharmaceutical Co., Tokyo) with few modifications. Briefly, plants inoculated with strain R170 or R181 were soaked in Victoria blue solution for 1 min, washed with

distilled water, submerged in a destaining reagent for 5 min, and then washed again with distilled water to remove excess stain.

The tissue localization of bacteria in the plant roots was examined in 70% glycerol under a light microscope (IX70 Inverted Microscope, Olympus, Tokyo) until 28 days after the inoculation. Photomicrographs were taken using a high-sensitivity CCD camera (VB-7000, Keyence, Osaka, Japan).

4.2.1.3 Cross-streak test between co-inoculated strains

Each of the co-inoculated PGPB was grown in R2A agar medium at 30°C for at least 3 days and then streaked perpendicularly on freshly prepared R2A agar medium; *i.e.*, after the first strain was allowed to grow at 30°C for 3 days, the second strain was streaked at an angle of approx. 90° going outward from the emerged colonies of the first strain. The second colony was allowed to incubate at 30°C for another 3 days. Strain combinations that showed inhibition zones at the intersection of the paired strains were noted.

4.2.1.4 Assessment for PGPB's biochemical activities in combined inoculation

Indole-3-acetic acid (IAA) production was determined using the Salkowski assay as reported by Gopalakrishnan *et al.* (9) with the following modifications: single and combined PGPB (1:1 of each strain) were grown in R2A broth containing 2 mM L-tryptophan (precursor of IAA) at 30°C for 72 hrs, and after centrifugation, the supernatant (400 µL) was poured into the Salkowski reagent (composed of 500 µL of 60% $HClO_4$, 17 µL of 0.5M $FeCl_3$, and 350 µL of distilled water) (800 µL) and incubated at 30°C for 30 min in a dark place. IAA production was determined (n=3) by

optical density at 530 nm using a spectrophotometer (Ultrospec3100pro, GE Healthcare Life Sciences, Buckinghamshire, UK). At the same time, the number of living cells was measured by plate dilution methods in the usual manner using cell pellets left after removing the supernatant to determine the IAA contents per cell (μg 10^8 colony-forming unit $[CFU]^{-1}$).

Siderophore production was evaluated using the Chrome-Azurol S (CAS) agar diffusion assay (26) with some modifications: holes (6 mm dia.) made on R2A agar containing 10% CAS (22) were filled with a final bacterial suspension of 24-hr-old cultures (35 μL); *i.e.,* single strains were equally mixed with R2A broth to reach a final volume of 35 μL, and the combined strains had equal volumes of each strain in the mixture for a final volume of 35 μL. After incubation at 30°C for 7 days, the diameters of the halos formed around the holes containing a bacterial colony were measured. Siderophore production (*n*=3) is expressed as the ratio of halo diameter (halo dia. minus colony dia.) per colony diameter (30).

4.2.2 Tests for the compatibility of PGPB with mycorrhiza

4.2.2.1 Inoculation test for combined PGPB and mycorrhiza on potato seedlings

Mycorrhiza (1 mL) and bacterial (1 mL) suspensions containing approx. 100 spores and 1 x 10^8 CFU, respectively, were prepared and directly inoculated on potato seeds (*n*=18) derived from the open pollination of cv. Hokkaikogane sown on pots containing approx. 100 g of sterilized seedling-raising culture soil (PotAce N, Katakura & Co-op Agri Corp., Tokyo). Controls were either inoculated with 1 ml of mycorrhizal spore suspension or not inoculated. The pots were covered with aluminum foil and placed in a growth chamber under light (23.5°C for 14 hrs) and dark (20.0°C for 10 hrs) conditions. The cover was removed upon the germination of seeds, and 30 days after

the inoculation, the plant growth parameters such as plant weights (mg) and germination rates were measured (*n*=3). Plant dry weights were measured after oven drying at 60°C for 3 days.

4.2.2.2 Mycorrhizal phosphatase and siderophore production

For phosphatase activity, potato seeds (cv. Hokkaikogane) (disinfected with 70% ethanol, and a solution containing 10% NaClO and 1% Tween 20) were germinated on 0.3% plant agar inoculated with 5 plugs of mycorrhiza. Fourteen days after seed germination, mycorrhized roots of potato seedlings were directly inoculated with bacterial cells containing $1x10^8$ CFU of T168, R170, R181, and R182, respectively. Thirty five days after inoculation, the acid phosphatase activity (pH 5.5) in roots was assessed (Tabatabai, 1994). Alkaline phosphatase activity (Tabatabai, 1994) (pH 11) was also assessed, but, it was very weak in the roots and was unaffected by the inoculation of mycorrhiza combined with PGPB. Thus, data on alkaline phosphatase activity were not presented. Absorbance values at 450 nm were measured using a spectrophotometer (Ultrospec3100pro, GE Healthcare Life Sciences, Buckinghamshire, UK). Phosphatase activity was calculated based on the standard curve of *p*-nitrophenol.

Siderophore production was determined by liquid assay (Schwyn and Neilands. 1987) with the following modifications: fifty microliters of mycorrhizal spore suspension (with approx. 5 spores) were grown in combined R2A and MSR broth (without iron) at 27^{o}C and 60 rpm for 24 hrs., then 50 µL each of the 1-day old bacterial strain was mixed together with the spores and incubated for 7 days following the same growth conditions. After incubation, the mixture of mycorrhiza and bacteria was centrifuged at 3,000 rpm at low temperature for 15 min. Nine hundred microliters of supernatant were mixed with 100 µL of CAS solution (Schwyn and Neilands. 1987)

and 10 µL of 400 mM sulfosalicylic acid, and incubated for 20 min. Absorbance values of the samples (supernatant from PGPB and/or mycorrhiza) and reference solution (900 µL growth medium, 100 µL CAS solution, and 10 µL 400 mM sulfosalicylic acid) were measured at 630 nm, and the siderophore activity was expressed as ratio of the difference between the reference and the sample against the reference.

4.2.2.3 Mycorrhiza root infection and spore germination

Fourteen days old potato seedlings were inoculated with either mycorrhiza alone or with PGPB. Mycorrhizal inoculant was prepared by suspending the spores harvested from MSR agar to sterilized distilled water. For mycorrhiza, 100 µL of spore suspension containing approx. 10 spores were directly inoculated to the roots, whereas for mycorrhiza with PGPB, 200 µL (1:1 of mycorrhiza and PGPB) were inoculated. The concentration of bacteria used was $1x10^8$ CFU per ml. Twenty eight days after inoculation, mycorrhiza root infection was assessed following the grid-line intersection method (Giovannetti and Mosse. 1980). Prior to microscope observation, roots were cleared of cell contents following the manual instructions of SUNJin Lab using *RapiClear*® 1.55 (Funakoshi Co. Ltd., Tokyo), immediately followed by staining using 0.05% trypan blue in glycerol and incubated for 24 hrs at 30°C, and then de-stained with 85% lactic acid. Root infection was assessed by the presence of either one of the following structures: hyphae, vesicles, arbuscules, and spores which were visible after staining. The number of infected and uninfected roots that intersect gridlines were counted. Percent mycorrhizal infection was calculated by the ratio of the total number of roots infected against the total number of roots.

For the assessment of spore germination, mycorrhizal cultures on MSR agar were treated with the 4 PGPB strains each having cell concentrations of $1x10^8$ CFU. From

an initial spore density of 8, the total number of spores on MSR agar was counted after 14 days of incubation. Percent spore germination was calculated by getting the ratio of the final spore density (final number of spores – initial number of spores) against the initial spore density.

4.2.2.4 Biological control of pathogen

The biological control activity (BCA) of mycorrhiza co-inoculated with PGPB against a pathogen was performed according to the methods reported by Schelkle and Peterson (1997) with the following modifications: mycorrhizal plugs (6 mm dia.), taken from the periphery of growing colonies on MSR agar, were placed at the edge of a petri plate containing a mixture of MSR and PDA (1:1) buffered with 3.3% 2-(N-morpholino)ethanesulfonic acid (MES), and then incubated in the dark at 27ºC for 12 hrs. Subsequently, plugs (6 mm dia.) of *Rhizoctonia solani* (MAFF no. 305250), acquired from the Genebank of the National Agriculture and Food Research Organization (NARO) and maintained on potato dextrose agar (PDA) were placed on the opposite side of a 12-hr culture of mycorrhiza concomitantly inoculated with 100 µL of bacterial solution. For the control, 100 µL of 0.1 M $MgSO_4 \bullet 7H_2O$ was inoculated to the mycorrhiza alone. All treatments were replicated three times. After 14 days of incubation in the dark at 27ºC, straight line measurements of inhibition zones between the two growing colonies were performed.

4.2.3 Statistical analysis

All experiments were performed with a minimum of three replicates for each treatment. The statistical analysis was carried out using SPSS Statistics for Windows v.22.0. Data were either subjected to an analysis of variance (ANOVA) or Student's t-

test. A post-hoc comparison of mean values among treatments was performed using Tukey's honestly significant difference (HSD) test at the 5% confidence level.

4.3 Results

4.3.1 Effect of co-inoculated PGPB on the growth of potato seedlings

Table 4 shows the effect of co-inoculating PGPB strains on the fresh and dry weights of potato seedlings. Results indicated that the combined inoculation of R170 with T168 or R182 significantly improved the plant growth compared with the un-inoculated control in terms of both fresh (p=0.000) and dry weights (p=0.000). Accordingly, the increased levels of dry weight (fresh weight) over the control were more than the sum total of those by each strain at approx. 80% (59%) for the combination of R170 with T168, whereas only approx. 65% (31%) was observed for the sum total of each strain. Moreover, an approx. 80% (62%) increase over the control was recorded for the combination of R170 and R182, whereas only approx. 62% (31%) was noted for the sum total of each strain.

On the other hand, a parallel inoculation test using R181 with R182, showed no significant difference in the plant fresh weight in reference to control. An increase was observed with the fresh weight of R181 with T168 compared with the uninoculated control, but the level was not significantly different with the single strain inoculation of T168. Likewise, plant dry weights brought about by the co-inoculation of R181 with T168 or R182, showed no significant difference compared with their single strain inoculations. Hence, the increased levels for the R181 combinations tended to be lower than the sum total of those by each strain; in particular, the levels were close to those achieved by R181 single inoculation. Thus, approx. 25% was recorded for the combination of R181 and T168 or R182, whereas approx. 22% was noted for the R181

Table 4. Effect of combined bacterial strain inoculation on the growth of potato seedlings compared with the control and single strain inoculation.

Strain	Plant wt. (mg)	
	Fresh	Dry
Uninoculated control	226.1 ± 5.4 a	16.6 ± 0.4 a
Single		
Sphingomonas sp. T168	252.7 ± 18.0 bc	21.8 ± 1.1 d
Streptomyces sp. R170	269.8 ± 5.3 d	22.2 ± 2.0 d
Streptomyces sp. R181	239.7 ± 5.2 b	20.0 ± 0.1 c
Methylibium sp. R182	252.3 ± 5.6 c	21.3 ± 0.7 d
Combined		
Sphingomonas sp. T168		
+ *Streptomyces* sp. R170	**359.8 ± 6.6 e**	**29.9 ± 1.8 e**
+ *Streptomyces* sp. R181	261.0 ± 1.3 cd	20.8 ± 3.4 bcd
+ *Methylibium* sp. R182	263.7 ± 4.8 cd	21.6 ± 1.3 d
Methylibium sp. R182		
+ *Streptomyces* sp. R170	**366.3 ± 1.1 e**	**29.9 ± 1.3 e**
+ *Streptomyces* sp. R181	248.6 ± 28.7 abc	20.8 ± 3.4 bcd
Streptomyces sp. R170		
+ *Streptomyces* sp. R181	239.8 ± 4.3 b	18.5 ± 0.1 b

Values are expressed as means±SD. One way ANOVA was performed to compare significant differences among means. Mean values in the same column with common letters are not significantly different from each other ($p \leq 0.05$) according to Tukey's HSD test.

single inoculation, based on dry weight over control. The co-inoculation of strains T168 with R182, as well as R170 with R181 showed significant increases in the plant dry weights compared with the uninoculated control, but the levels seemed to be lower than the sum total of each strain in the combined inoculations (T168 and R182 or R170 and R181).

4.3.2 Localization of co-inoculated PGPB to potato seedlings

Table 5 summarizes the characteristic localization of T168 and R182 co-inoculated with either R170 or R181 in comparison with their single strain inoculation. Figure 13, on the other hand, shows the visual presentation of the localization of compatible and incompatible strains. For the compatible strains (T168 and R170), the colonization of T168 at the base of lateral roots was the same as the T168 single inoculation after GUS staining (Figure 13a-b). Using the same seedling, subsequent Gram staining showed that R170 also localized around the base of lateral roots, indicating that each strain could coexist. For the incompatible strain combination (T168 and R181), however, a very weak infection of T168 was observed at the base of lateral roots after GUS staining (Figure 13c-d). Whereas, R181 was noted to partially cover the base of lateral roots after Gram staining, indicating that each strain could not coexist because the infection of T168 was inhibited by the presence of R181.

For another compatible strain combination (R182 and R170), the localization of R182 to the root hairs was observed in the same way as in the R182 single inoculation after GUS staining (Figure 13e-f). Subsequently, Gram staining showed that R170 also localized on the primary root of the same seedling co-inoculated with R182. For the incompatible strain combination (R182 and R181), the infection of R182 to the root hairs was clearly weak, with no sign of advanced infection during cultivation after GUS

Table 5. Characteristic localization of single strains and the effect of co-inoculation on the infection of bacteria on the roots of potato seedlings.

Strain	Primary root	Lateral root (base)	Root hairs
Single			
Sphingomonas sp. T168	++	+++	-
Streptomyces sp. R170	+++	+++	+++
Streptomyces sp. R181	+++	+++	+++
Methylibium sp. R182	++	-	+++
Combined			
***Sphingomonas* sp. T168**			
+ *Streptomyces* sp. R170	++	+++	-
+ *Streptomyces* sp. R181	-	-	-
***Methylibium* sp. R182**			
+ *Streptomyces* sp. R170	++	+	+++
+ *Streptomyces* sp. R181	-	-	-
***Streptomyces* sp. R170**			
+ *Sphingomonas* sp. T168	+++	+	+++
+ *Methylibium* sp. R182	+++	+++	+++
***Streptomyces* sp. R181**			
+ *Sphingomonas* sp. T168	+++	++	+++
+ *Methylibium* sp. R182	+++	+++	+++

Symbols such as +, -, ++, and +++ represents presence, absence, strong, and very strong infection on specific areas on the roots respectively. The infection of strains written in bold letters were noted for each of their combined inoculations.

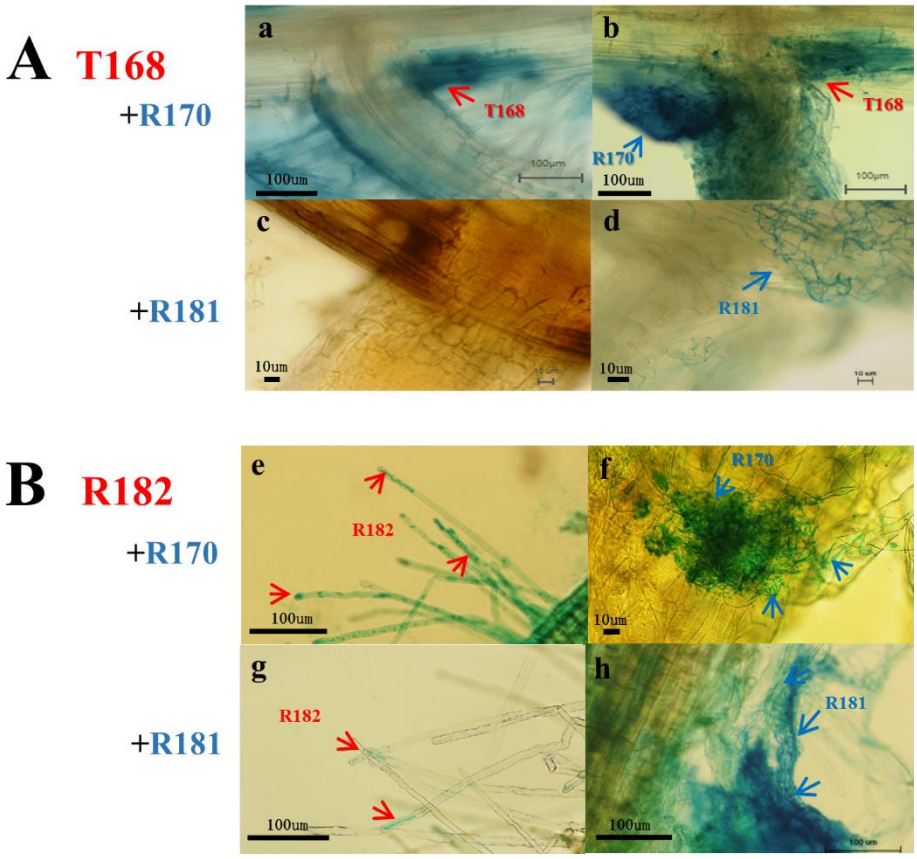

Figure 13. Tissue localization of co-inoculated PGPB strains on the roots of potato seedlings. **A:** (**a**) Localization of T168 at the base of lateral root in the combined inoculation (T168+R170) that resulted in higher plant growth promotion confirmed by (**b**) the co-existence of both strains (7d after inoculation); and (**c**) the absence of T168, and localization of R181 at the base of lateral root (7d after inoculation) in the combined inoculation (T168+R181) which showed lower plant growth promotion. **B:** (**e**) Localization of R182 that resulted in higher plant growth promotion (R182+R170) confirmed by intense infection to root hairs by R182, and (**f**) R170 on the same primary root (7d after inoculation). **g:** Localization of R182 in lower plant growth promotion (R182+R181) brought about by weak infection of R182 at root hairs. **h:** Localization of R181 that resulted in lower plant growth promotion (R182+R181) revealed by the weak infection of R182 on root hairs. (Santiago *et. al.* 2017, Microbes and Environ. 32(1), *In Press*).

staining (Figure 13g-h). Whereas, R181, demonstrated a very aggressive growth which covered the root hairs where R182 was supposed to localize after Gram staining.

4.3.3 Co-culture of compatible and incompatible PGPB strains

Table 6 and Figure 14 show the presence or absence of competition between two compatible (R170 and T168 or R182) and incompatible (R181 and T168 or R182) strains examined by dual culture on agar plates. The compatible strains (T168 and R170), which showed coexistence on the roots, was reflected by the co-culture of both strains on the same plate with no trace of growth inhibition (Figure 14A).

R182 and R170 also showed no growth inhibition between strains even though R170 or R182 was streaked prior to the other strain (Figure 14A). However, a clear evidence of growth inhibition was exhibited when R181 was first streaked instead of T168 or R182 (Figure 14B). Moreover, a sufficient inhibition zone was created by R181, which suppressed the growth of T168, whereas the growth of R182 was slightly inhibited by R181. The growth of R181 was not suppressed when T168 or R182 was streaked on plates prior to R181.

4.3.4 Additive effect of co-inoculating PGPB on their biochemical productivity

The effect of combined PGPB strains on the biochemical productivity is shown in Table 7. Results showed that IAA and siderophore production were improved by the combination of two compatible strains (R170 and T168 or R182), and the levels were increased more than those of the sum total produced by each strain as follows. The IAA level was increased by approx. 53% (p=0.002) (R170 and T168) and 13% (p=0.005) (R170 and R182), and the siderophore level was increased by 70% (p=0.000) (R170

Table 6. Presence or absence of competition between two strains in a combined inoculation demonstrated by the inhibition of colonies.

Strain	+ *Streptomyces* sp. R170	+ *Streptomyces* sp. R181
Sphingomonas sp. T168	-	++
Methylibium sp.R182	-	+

Symbols such as + or - represent the presence or absence of competition between two strains evidenced by the appearance of inhibition zones formed at the intersection of two colonies after R170 or R181 was streaked prior to R168 or R182 respectively. ++ represents a wide inhibition zone produced.

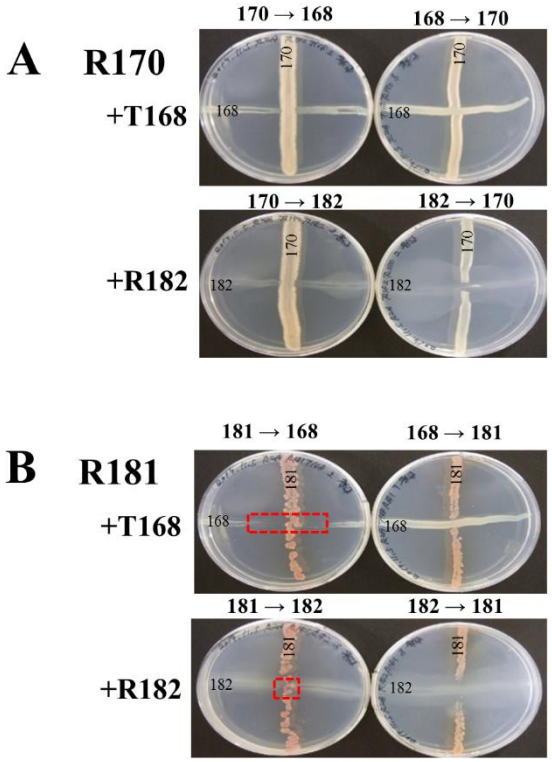

Figure 14. Cross-streak test between co-inoculated strains. Each of the co-inoculated strains was streaked perpendicularly in the order shown by the arrows. **A:** Cross-streak test between combined strains that resulted in higher plant growth promotion (R170 and T168 or R182). No inhibition zone was observed at the intersection between strains. **B:** Cross-streak test between combined strains that resulted in lower plant growth promotion (R181 and T168 or R182). When R181 was streaked first before either T168 or R182, an inhibition zone was produced at the intersection as shown by red boxes. (Santiago *et. al.* 2017, Microbes and Environ. 32(1), *In Press*).

Table 7. Compatibility of bacteria after co-inoculation in terms of biochemical activities related with plant growth promotion.

Strain	IAA (µg per 10^8 CFU)	Siderophore (mm dia.)
Single		
Sphingomonas sp. T168	0.26 ± 0.02 a	2.23 ± 0.24 ab
Streptomyces sp. R170	1.82 ± 0.42 c	3.26 ± 0.16 c
Streptomyces sp. R181	0.50 ± 0.12 b	1.89 ± 0.23 a
Methylibium sp. R182	0.36 ± 0.15 ab	2.51 ± 0.35 b
Combined		
Sphingomonas sp. T168		
+ *Streptomyces* sp. R170	**3.19 ± 0.20 e**	**3.80 ± 0.19 d**
+ *Streptomyces* sp. R181	0.52 ± 0.13 b	2.47 ± 0.19 b
Methylibium sp. R182		
+ *Streptomyces* sp. R170	**2.47 ± 0.24 d**	**3.33 ± 0.18 c**
+ *Streptomyces* sp. R181	0.59 ± 0.07 b	2.58 ± 0.32 b

R170, selected to be the most efficient strain tested for compatibility with Proteobacteria (T168 or R182), and compared with a parallel co-inoculation with R181. Values are expressed as means±SD. One way ANOVA was performed to compare significant differences among means. Mean values in the same column with common letters are not significantly different from each other ($p \leq 0.05$) according to Tukey's HSD test.

and T168) and 76% (p=0.001) (R170 and R182), over the sum total produced by each strain. However, the parallel test, using R181 with T168 or R182, showed no significant increases in IAA and siderophore production ($p \leq 0.05$).

4.3.5 Effect of co-inoculating PGPB with mycorrhiza on the growth of potato seedlings

The compatibility of mycorrhiza with PGPB in promoting plant growth is presented in Table 8. Results indicated that the combined inoculation of mycorrhiza with PGPB strains R170, R182, and T168 significantly promoted the growth of potato seedlings in reference to the un-inoculated control in terms of the fresh (p=0.000) and dry weights (p=0.000). Particularly, the dry (fresh) weights of seedlings increased by 172 % (268%), 100% (92%) and 72% (90%), with the co-inoculation of mycorrhiza with R170, R182, or T168, respectively, compared with the uninoculated control, which were higher than the single inoculation of mycorrhiza. However, among the 4 PGPB, only the co-inoculation of R170 with mycorrhiza showed a significantly higher increase in bothfresh and dry weights in comparison with the sum total of each strain (R170 or mycorrhiza). In contrast, the co-inoculation of R181 with mycorrhiza did not show improvement in the fresh and dry weights of potato seedlings in reference to the uninoculated control. In fact, a significant decrease in dry weight was noted for the co-inoculation of R181 with mycorrhiza compared with each of their single strain inoculations (mycorrhiza or R181).

4.3.6 Effect of co-inoculating PGPB on the infection capacity of mycorrhiza

Table 9 shows the effect of PGPB on the infection of mycorrhiza (p=0.000) to the roots of potato seedlings and spore germination on MSR agar. Approximately 60%

Table 8. Effect of co-inoculating PGPB with mycorrhiza on the growth of potato seedlings compared with the control and single strain inoculation.

Strain	Plant wt. (mg)	
	Fresh	Dry
Uninoculated control	93.3 ± 13.0 a	14.0 ± 2.0 a
Single		
Mycorrhiza (*Gigaspora* sp.)	128.1 ± 27.9 b	18.2 ± 1.1 c
Sphingomonas sp. T168	104.4 ± 6.4 ab	18.4 ± 0.6 c
Streptomyces sp. R170	111.4 ± 3.7 b	18.7 ± 0.3 c
Streptomyces sp. R181	98.9 ± 10.7 ab	17.0 ± 0.3 b
Methylibium sp. R182	104.2 ± 26.4 ab	18.0 ± 4.5 abc
Combined		
Mycorrhiza (*Gigaspora* sp.)		
+ *Sphingomonas* sp. T168	177.1 ± 14.0 c	24.1 ± 1.9 de
+ *Streptomyces* sp. R170	**343.3 ± 8.4 d**	**38.2 ± 0.5 f**
+ *Streptomyces* sp. R181	93.9 ± 5.8 a	15.2 ± 0.8 a
+ *Methylibium* sp. R182	179.2 ± 39.4 c	28.0 ± 5.7 e

Values are expressed as means±SD. One way ANOVA was performed to compare significant differences among means. Mean values in the same column with common letters are not significantly different from each other ($p \leq 0.05$) according to Tukey's HSD test.

Table 9. Effect of PGPB inoculation on mycorrhiza root infection and spore germination.

Strain	Root infection (%)	Spore germination (%)
Single		
Mycorrhiza (*Gigaspora* sp.)	59.7 ± 0.7 c	100.0 ± 8.0 b
Combined		
Mycorrhiza (*Gigaspora* sp.)		
+ *Sphingomonas* sp.T168	44.6 ± 2.7 b	0.4 ± 0.6 a
+ *Streptomyces* sp. R170	**80.1 ± 2.5 e**	**152.2 ± 7.2 c**
+ *Streptomyces* sp. R181	36.6 ± 4.5 a	1.5 ± 0.8 a
+ *Methylibium* sp. R182	74.7 ± 1.0 d	99.8 ± 2.9 b

Values are expressed as means±SD. One way ANOVA was performed to compare significant differences among means. Mean values in the same column with common letters are not significantly different from each other ($p \leq 0.05$) according to Tukey's HSD test.

of the roots of potato seedlings were infected by mycorrhiza manifested by the formation of external spores, extra- and intraradical hyphae, vesicles and arbuscules located in the inter- and intracellular spaces of the root cells. Without the inoculation of PGPB, vesicles were occasionally observed and barely visible.

However, upon the inoculation of PGPB, particularly, R170 and R182, vesicles were more frequent. The infection of mycorrhiza to the roots was significantly promoted by 34% and 25% with R170 or R182 inoculations, respectively. In contrast, the inoculation of T168 or R181 did not promote mycorrhizal infection to the roots.

In terms of mycorrhizal spore germination, only R170 significantly promoted the germination of mycorrhizal spores (p=0.000) by 52%. R182 inoculation showed a slight improvement in spore germination, however it was not significantly different with the number of spores produced by mycorrhiza alone. No spore germination was observed with the inoculation of T168 and R181 to mycorrhiza, thus, the production of massive hyphae was noted on the plates.

4.3.7 Effect of co-inoculating PGPB on the biochemical productivity of mycorrhiza

Table 10 shows the effect of PGPB on the acid phosphatase (AP) activity (p=0.000) of mycorrhiza in the roots of potato seedlings which ranged from 79 to 113 mg *p*-nitrophenol per g root sample hr^{-1} compared with the single strain inoculation of mycorrhiza with approx. 86 mg *p*-nitrophenol per g root sample hr^{-1} . The AP activity recorded for the roots without inoculation was approx. 37 mg *p*-nitrophenol per g root sample hr^{-1}, whereas, the 4 PGPB strains were noted to have no AP activity. But, after co-inoculating PGPB with mycorrhiza, a significant improvement in the AP activity of mycorrhiza was observed specifically with the co-inoculation of R182 (32%) or R170 (7%) compared with mycorrhiza alone. The co-inoculation of T168 with mycorrhiza

Table 10. Effect of PGPB on the mycorrhizal acid phosphatase and siderophore production activities.

Strain	AP activity (mg *p*-nitrophenol g root sample^{-1} hr^{-1})	Siderophore production (%)
Single		
Mycorrhiza (*Gigaspora* sp.)	85.8 ± 1.0 c	6.9 ± 0.6 b
Sphingomonas sp. T168	0.0 ± 0.0 a	2.1 ± 0.1 a
Streptomyces sp. R170	0.0 ± 0.0 a	3.2 ± 0.1 b
Streptomyces sp. R181	0.0 ± 0.0 a	1.8 ± 0.1 a
Methylibium sp. R182	0.0 ± 0.0 a	2.4 ± 0.1 a
Combined		
Mycorrhiza (*Gigaspora* sp.)		
+ *Sphingomonas* sp. T168	86.7 ± 2.2 c	8.3 ± 0.2 d c
+ *Streptomyces* sp. R170	**92.2 ± 1.7 d**	**16.1 ± 0.3 d**
+ *Streptomyces* sp. R181	78.7 ± 0.3 b	5.6 ± 0.6 a
+ *Methylibium* sp. R182	**113.1 ± 1.7 e**	6.4 ± 0.2 b

Values are expressed as means±SD. One way ANOVA was performed to compare significant differences among means. Mean values in the same column with common letters are not significantly different from each other ($p \leq 0.05$) according to Tukey's HSD test. Acid P activity in the roots without inoculation, and absorbance value of the reference solution (R2A+MSR broth, CAS, sulfosalicylic acid) without inoculation represent the control values.

showed no improvement, whereas with R182, a significant decrease in the AP activity was observed compared with the single inoculation of mycorrhiza.

Percent siderophore activity (p=0.000) in liquid assay was manifested by the change in the color of the medium from blue to purplish orange. The siderophore activity recorded for mycorrhiza alone was approx. 7% in reference to the uninoculated control solution containing CAS and sulfosalicylic acid. The co-inoculation of PGPB strains with mycorrhiza, particularly T168 and R170, significantly promoted the levels of siderophore production in the solution by 8% and 16%, respectively, compared with mycorrhiza alone. On the contrary, the co-inoculation of R182 showed no improvement in the siderophore levels, whereas with the co-inoculation of R181, a significant decrease was observed compared with mycorrhiza alone.

4.3.8 Effect of PGPB on the biological control activity of mycorrhiza

Figure 15 shows a visual representation of the co-inoculation of PGPB with mycorrhiza which significantly inhibited the growth of *Rhizoctonia solani* based on the results of dual culture evidenced by the production of inhibition zones between mycorrhiza and the pathogen on MSR+PDA medium. On the other hand, Figure 16 shows the biological control activities of mycorrhiza, and mycorrhiza with PGPB against the pathogen represented by the width of inhibition zones produced between the two colonies (mycorrhiza and *R. solani*) (p=0.000). Particularly, the co-inoculation of R170, R181, or R182 with mycorrhiza significantly increased the biological control activity by 246%, 220%, and 345%, respectively, manifested by wider inhibition zones compared with the inoculation of mycorrhiza only.

Under optimum conditions without an antagonist, the hyphae of *R. solani* on water agar showed a smooth, healthy-looking and right angled branching. However, after

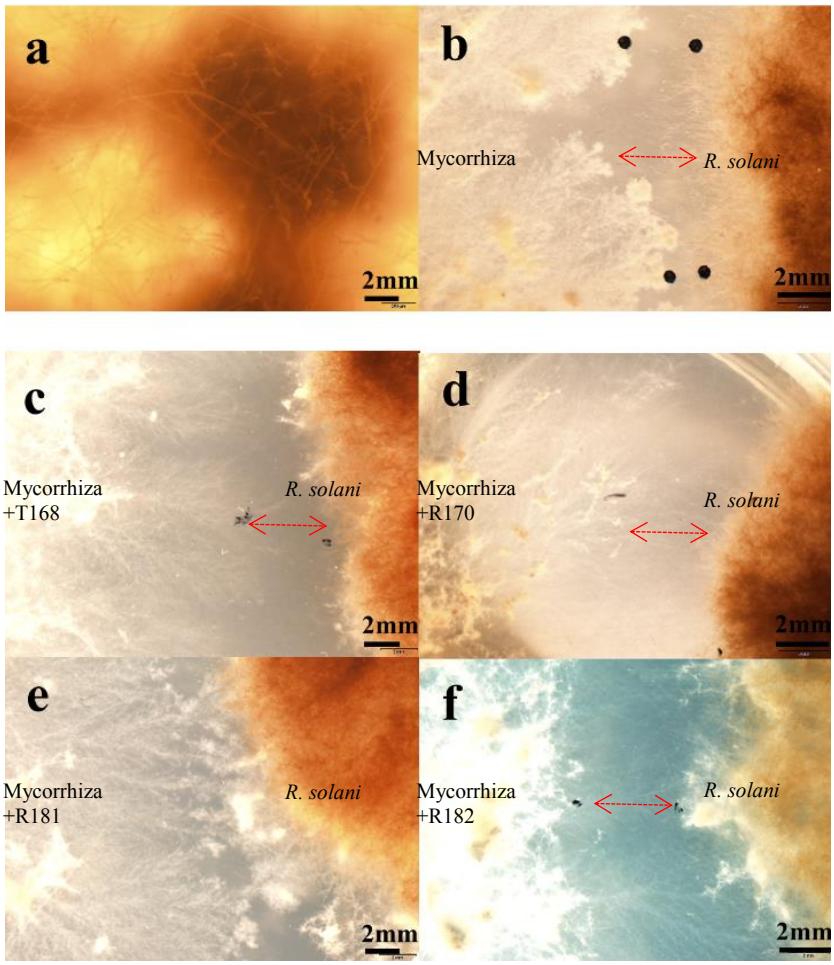

Figure 15. Biological control activity of mycorrhiza with bacteria against *R. solani* manifested by inhibition zones between colonies. (**a**) Pure culture of *R. solani* on PDA; (**b**) Dual culture of mycorrhiza and *R. solani* on MSR+PDA; (**c-f**) Mycorrhiza supplemented with PGPB (T168, R170, R181, and R182) against *R. solani.*

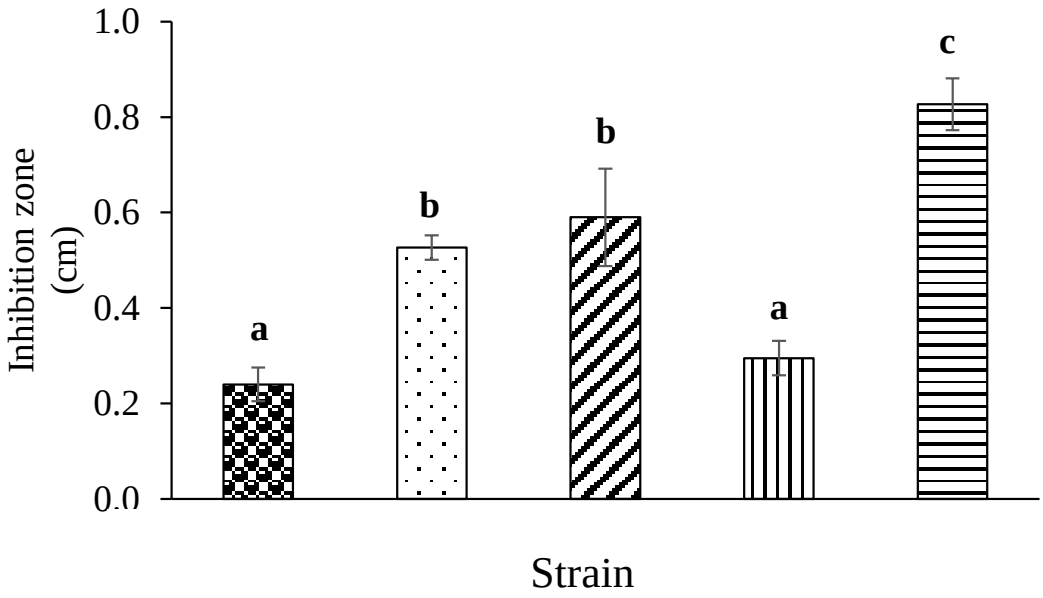

Figure 16. Biological control activity of mycorrhiza with bacteria. Vertical axis displays the width of inhibition zones created by mycorrhiza with bacteria against the pathogen. Data are means ± standard deviation (SD) of three replicates. The SDs of the means are less than the 0.05 significance level. Means with different letters are significantly different from each other. , Mycorrhiza; , +T168; , +R170; , +R181; , +R182.

challenging the pathogen with mycorrhiza and/or with the co-inoculation of mycorrhiza and PGPB, the normal structure of the hyphae was modified with evidences of deformation, degradation, and leakage. Thus, among the PGPB strains co-inoculated with mycorrhiza, R182 inflicted the greatest hyphal inhibition of *R. solani* which showed rupture and leakage of contents.

4.4 Discussion

The results presented in this chapter proved that the compatibility between strain inoculants is crucial for enhancing plant growth. A previous report showed that bioinoculants, containing a variety of useful strains having diverse physiological and biochemical characteristics, can increase plant productivity by ensuring the bioavailability of nutrients, while maintaining the balance of soil pH and preventing negative impacts to the environment (Pandey *et al.* 2012).

In the present study, a synergistic interaction was observed between compatible PGPB strains, as well as with the co-inoculation of PGPB with mycorrhiza which was primarily evidenced by the significant increase in the weights of potato seedlings (R170 with T168 or R182; R170 with mycorrhiza) compared with the uninoculated control and with the single strain inoculation. This improvement in plant growth can be related with the enhanced biochemical productivity of co-inoculated strains which may be attributed to the ability of strains to complement each other's plant growth-promoting activities. This improved plant growth promoting activities of co-inoculants were made possible by the efficient infection and co-existence of strains to the host plants which were specifically observed in the combination of R170 with T168 or R182 showing co-localization of strains to the host plant. Likewise, R170 was proven to advance the infection of mycorrhiza to the roots of potato seedlings which may be related with the

enhanced spore germination on MSR agar upon the co-inoculation of R170. Whereas, the low mycorrhizal root infection for T168 or R181 co-inoculations may be due to the decreased capacity of mycorrhizal sporulation on MSR agar. Thus, the localization of PGPB with mycorrhiza was not examined. Further investigations are necessary.

Synergistic interaction between co-inoculants has also been evidenced by the improved IAA and siderophore productions noted for the inoculation of R170 with T168 or R182. Probably, the improved production of IAA and siderophores in combined strains inoculation could be due to the increased cell number and/or producing activity of either or both bacteria. Thus, further investigations are needed.

Similarly, with the co-inoculation of R170 and mycorrhiza, a significant improvement in the mycorrhizal AP activity was recorded, whereas a synergistic increase on the siderophore production was observed. In agreement with previous reports (Schachtman *et al.* 1998; Smith *et la.* 2011; Xun *et al.* 2015), the enhanced plant growth promotion may be brought about by the increased AP activity of mycorrhiza. Thus, AP activity may be attributed to the improved root infection of mycorrhiza after the co-inoculation of PGPB. In contrast, the improved production of microbial siderophores may be because of the synergistic effect of co-inoculating R170 with mycorrhiza, and hence, may imply an enhanced iron acquisition in plants resulting in improved plant growth. Thus, the total amount of iron acquired by the host plant co-inoculated with mycorrhiza and PGPB should be examined as well in future research.

While the compatibility of R170 with T168 or R182 was verified by the lack of inhibition on dual culture, which resulted in the co-localization of strains on the roots, the incompatibility of R181 with T168 or R182, on the other hand, could be attributed to the growth competition of R181 against other bacteria, manifested by the inhibition of T168 at the base of lateral roots and a weakened infection of R182 to the root hairs.

Furthermore, the dominance of R181 over T168 or R182 was manifested by the presence of inhibition zones at the intersection of two colonies, which may be related with the production of toxins in the hyphae of R181. This phenomenon was reported in majority of *Streptomyces* strains producing toxins that inhibit the growth of other bacteria and yeasts (Hozzein *et al.* 2011). Thus, a thorough assessment on this assumption for R181 is recommended for further study.

It has been reported that mycorrhizas can serve as biological control agents against harmful pathogens (Schelkle and Peterson, 1997; Tahat *et al.* 2012). In the present study, mycorrhiza showed the ability to suppress the growth of *R. solani* which was enhanced by the co-inoculation of R182, R170, and T168. Biological control was demonstrated by the inhibition zones produced between mycorrhiza and *R. solani*. In this regard, the hydrolytic enzymes present in PGPB, which include β-1,3-glucanase (T168, R170, R181, R182), cellulase (R170, R181, R182), protease (R170, R181, R182), chitinase (R182), and lipase (R170) that are known for cell wall degrading properties (Kubicek *et al.* 2014), may have improved the biological control activity of mycorrhiza. Additionally, Schelkle and Peterson (1997) cited that the suppression of pathogens may also be due to the competition for nutrients and space in the rhizosphere which is also related with the siderophore production of PGPB and mycorrhiza as well.

The results of this study suggest that the compatibility of strains in combined inoculation is important in promoting plant growth. Strain R170 proves to be a promising strain (particularly in combination with other compatible strains such as T168, R182, or mycorrhiza) for the formulation of efficient bioinoculants for potato seedlings. Furthermore, the use of R170 with compatible strains may eventually reduce the utilization of chemical fertilizers.

Chapter 5

Overall Summary, Conclusions and Recommendations

Crop productivity is an answer to the increasing demand for food to feed the inevitably rising population. But, in order to reach and maintain high yields, plants should be provided with the necessary nutrients for their growth and development. In this regard, artificial sources of nutrients commercially packed as fertilizers that are specific for plant growth promotion have been extensively utilized, which usually results in the excessive nutrient accumulation in soil, thus, leading to environmental pollution. Because of this, farmers have resorted to alternative agricultural practices such as the application of bioinoculants containing beneficial microorganisms (*i.e.* plant growth promoting bacteria and mycorrhiza) in order to provide the hardly acquired moisture, minerals, and nutrients for plant nutrition without imposing harm to the environment.

Bioinoculants may be formulated with only one or a variety of strains that are proven to deliver positive results to plant growth. However, the efficiency of bioinoculants in plant growth promotion greatly depends on factors such as host specificity, biochemical productivity, stress tolerance, and compatibility especially for those containing a consortium of different strains.

Thus, this study confirmed that the four new bacterial strains isolated from potato roots (R170, R181, R182) or tubers (T168) are efficient plant growth promoters either as single inoculants, in co-inoculation with each other (R170 with T168 or R182) or with mycorrhiza (+R170 or +R182). Particularly, R170 strain was revealed to be the most potential plant growth promoter essentially because of its biochemical

productivity (IAA, siderophore, ACC-deaminase, β-1,3-glucanase, cellulase, and protease), and tolerance to stress (NaCl, $AlCl_3$, and pH). Moreover, R170 revealed to have synergistic effect with T168, R182, or mycorrhiza on the growth of potato seedlings. This synergistic effect of the co-inoculation of R170 with another compatible PGPB or mycorrhiza has been related with the harmonious interaction of strains revealed by the compatible localization of R170 with T168 or R182 to the roots of potato seedlings, which has led to the improved production of IAA and siderophores, and the enhanced siderophore production with mycorrhiza, respectively, eventually resulting in plant growth promotion. Thus, the increased acid phosphatase activity in roots manifested by the co-inoculation of R170 with mycorrhiza has been attributed with the improved mycorrhizal infection. The efficiency of R170 in enhancing mycorrhiza infection was verified by the increase in mycorrhizal spore germination. However, the infection of PGPB with mycorrhiza to the roots, and the germination of mycorrhizal spores associated with the roots were not examined. In this regard, PGPB's co-localization with mycorrhiza to the host plant, and the assessment of mycorrhizal sporulation associated with the roots are recommended for further investigations.

On the contrary, strain incompatibility was shown in the parallel co-inoculation of R181 with T168 or R182, as well as with mycorrhiza manifested by the significant decrease (R181 with mycorrhiza) or insignificant increase (R181 with T168, R182, or R170) in the dry weight of potato seedlings. These results has been related with the lack of synergistic interaction of R181 with other PGPB strains and was attributed to the competitive characteristic of R181 causing the growth inhibition and absence of T168 or R182 localization on agar and to the roots of potato seedlings, respectively. Additionally, the incompatibility of R181 with other PGPB was revealed by the decreased IAA and siderophore productivity of the combined strains (R181 with T168

or R182) compared with the sum total of the IAA and siderophores produced by the single inoculants. Moreover, the co-inoculation of R181 with mycorrhiza also revealed a decrease in the acid phosphatase and siderophore production activities compared with the performance of mycorrhiza alone, though a significant increase in both acid phosphatase and siderophores were recorded in reference to the uninoculated control. Although R181 can be an effective bioinoculant in single inoculations, its incompatibility with other microorganisms may not ensure plant growth promotion especially in field applications.

Furthermore, PGPB strains have been confirmed to aid in the improvement of the biological control activity of mycorrhiza against *Rhizoctonia solani.* In particular, the biological control activity was significantly increased by the co-inoculation of R182, R170, or T168 with mycorrhiza evidenced by the wider inhibition zones recorded compared with the single inoculation of mycorrhiza. This capacity of mycorrhiza and PGPB to control the growth of a pathogen has been attributed to their ability of producing siderophores. Thus, a thorough assessment on the biological control activity of the PGPB strains is recommended for further study.

The results of this study suggest that R170 might be a promising strain that can be a potential bioinoculant for the growth promotion of potato seedlings as evidenced by its biochemical productivity, tolerance to environmental stresses, and synergistic effect with compatible PGPB and mycorrhiza strains. Moreover, our results hope to provide additional information that will contribute to the development of potential bioinoculants containing effective formulations of compatible strains for plant growth promotion which might possibly reduce the utilization of chemical fertilizers.

JAPANESE SUMMARY

日本語要約

本研究では、*Sphingomonas* sp. T168 株（以降 T168 株）、*Methylibium* sp. R182 株（以降 R182 株）、および *Streptomyces* sp.R170 株（以降 R170 株）とのそれぞれの組み合わせによる共接種において植物の生育促進を確認した。R170 株、*Streptomyces* sp. R181 株（以降 R181 株）、および R182 株は、ジャガイモの根から分離され、T168 株はジャガイモの塊茎から単離された新規の細菌であり、それぞれ単独でも植物生育促進作用がみられたが共接種ではさらに高い効果がみられた。特に、R170 株と T168 株または R182 株との組み合わせ、菌根菌と R170 株または R182 株による共接種では植物の生長を促進する効果がみられた。特に R170 株は、植物の生育促進効果のある IAA、シデロフォア、ACC デアミナーゼ、β-1,3-グルカナーゼ、セルラーゼ、およびプロテアーゼの高い生産量、さらに、pH、 $AlCl_3$、塩化ナトリウムのストレスに対する高い耐性が示しされた。T168 株、または R182 株と R170 株の組み合わせによる共接種の相乗効果は、インドール酢酸 （IAA）とシデロフォア生産の強化と、菌根菌との相互作用に関係していることが示された。

R170 株を適合性のある Plant Growth Promoting Bacteria （PGPB）または菌根菌と共接種することによるこの相乗効果は、R170 株と T168 株または R182 株の組み合わせにおけるジャガイモ実生への局在化および相互作用に関連しており、 IAA とシデロフォア産生の増強を示し、最終的に植物の生育促進を引き起こした。また、R170 株と菌根菌との共接種によって根における酸性ホスファターゼ活性の増加がみられ、菌根感染の改善に起因することが明らかになった。菌根菌の感染時における R170 株の効果は、菌根胞子発芽率の増加であることが確認された。しかしながら、他の PGPB では菌根への感染および根に関連する菌根胞子の発芽は調べることはできなかった。よって、PGPB の宿主植物への菌根との共存、および根に関連する菌根胞子形成についてさらに調査する必要があると考えられた。

一方、R181 株は T168 株または R182 株の組み合わせでは適合性を示さなかった。R181 株と菌根菌の組み合わせでは、ジャガイモ実生の乾燥重量の有意な減少がみられたが、T168 株、R182 株、または R170 株との組み合わせではわずかな増加がみられた。これらの結果は、R181 株と他の PGPB との相乗的相互作用の欠如に関連しており、寒天上で栽培したジャガイモ実生の観察において T168 株、または R182 株との組み合わせではこれらの菌株の局在化が抑制され、R181 株とは共存できないことが原因であると考えられた。R181 株と他の PGPB との不適合は、単接種の場合と比較して IAA およびシデロフォアの生産性の低下によって明らかとなった。R181 株と菌根菌との共接種は、無接種の対照と比較して酸性ホスファターゼおよびシデロフォアの両方の有意な増加がみられたが、菌根菌の単独の場合と比較して、酸性ホスファターゼおよびシデロフォア生成活性の低下が明らかになった。

さらに、PGPB 株は、*Rhizoctonia solani* に対する菌根の生物学的防除活性を改善することが確認された。特に、菌根菌の単接種と比較して R182 株、R170 株または T168 株と共接種した場合は、生物学的防除活性を示す阻止円の面積が有意に増加することが示された。病原体の増殖を抑制する菌根菌および PGPB の能力は、シデロフォアを産生する能力に起因していると考えられたが、PGPB 株の生物学的制御活性に関するより詳細な評価が必要である。

以上の結果は、R170 株のすぐれた生化学的特性、環境ストレス耐性、および共存可能な PGPB と菌根株との相乗効果によりジャガイモ実生の生育促進のための有望な微生物資材であることを示した。本研究の結果によって、化学肥料の使用を減らし、植物の生育を促進する有望な微生物資材の開発が期待される。

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APPENDICES

Appendix 1. Analysis of variance (ANOVA) table for the comparison of means of the IAA production of single PGPB strain inoculants.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.767	3	1.589	29.940	.000
Within Groups	.425	8	.053		
Total	5.191	11			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df; F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value.

Appendix 2. Analysis of variance (ANOVA) table for the comparison of means of the siderophore production of single PGPB strain inoculants.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.057	3	1.019	15.587	.001
Within Groups	.523	8	.065		
Total	3.580	11			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df. F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value

Appendix 3. Analysis of variance (ANOVA) table for the comparison of means of the biofilm production of single PGPB strain inoculants.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.025	3	.008	18.478	.001
Within Groups	.004	8	.000		
Total	.029	11			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df; F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value

Appendix 4. Analysis of variance (ANOVA) table for the comparison of means of the production of ACC-deaminase activity of single PGPB strain inoculants.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	420146.678	3	140048.893	861.127	.000
Within Groups	5204.302	32	162.634		
Total	425350.980	35			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df; F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value

Appendix 5. Analysis of variance (ANOVA) table for the comparison of means of the plant growth promotion of single PGPB strain inoculants.

		Sum of Squares	df	Mean Square	F	Sig.
Fresh wt.	Between Groups	3203.178	4	800.794	9.069	.002
	Within Groups	883.045	10	88.304		
	Total	4086.222	14			
Dry wt.	Between Groups	60.671	4	15.168	12.689	.001
	Within Groups	11.953	10	1.195		
	Total	72.624	14			
Germination rate	Between Groups	259.259	4	64.815	2.250	.136
	Within Groups	288.066	10	28.807		
	Total	547.325	14			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df; F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value

Appendix 6. Analysis of variance (ANOVA) table for the comparison of means of the plant growth promotion of combined PGPB strain inoculants.

		Sum of Squares	df	Mean Square	F	Sig.
Fresh wt.	Between Groups	59855.201	6	9975.867	74.303	.000
	Within Groups	1879.629	14	134.259		
	Total	61734.830	20			
Dry wt.	Between Groups	496.923	6	82.821	19.587	.000
	Within Groups	59.196	14	4.228		
	Total	556.120	20			
Germination rate	Between Groups	286.613	6	47.769	.699	.655
	Within Groups	956.485	14	68.320		
	Total	1243.098	20			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df; F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value

Appendix 7. Analysis of variance (ANOVA) table for the comparison of means of the plant growth promotion of mycorrhiza in single and combined inoculation with PGPB strains.

		Sum of Squares	df	Mean Square	F	Sig.
Fresh wt.	Between Groups	24116.029	5	90014.68052	179.16319	.000
	Within Groups	474189.431	48	502.417		
	Total	180065.354	53			
Dry wt.	Between Groups	8882.392	5	36013.07077	194.61282	.000
	Within Groups	188947.746	48	185.050		
	Total	24116.029	53			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df. F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value

Appendix 8. Analysis of variance (ANOVA) table for the comparison of means of the siderophore production activity, using 40 and 400mM SA (salicylic acid) shuttling solution for iron transport, by mycorrhiza and/or mycorrhiza co-inoculated with PGPB.

		Sum of Squares	df	Mean Square	F	Sig.
40mM SA	Between Groups	470.812	5	94.162	663.975	.000
	Within Groups	2.553	18	.142		
	Total	473.365	23			
40mM SA	Between Groups	292.057	4	73.014	429.208	.000
	Within Groups	2.552	15	.170		
	Total	294.609	19			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df; F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value

Appendix 9. Analysis of variance (ANOVA) table for the comparison of means of the phosphatase (acid and alkaline) activities of mycorrhiza and/or mycorrhiza co-inoculated with PGPB.

		Sum of Squares	df	Mean Square	F	Sig.
AcidP	Between Groups	.010	5	.002	947.362	.000
	Within Groups	.000	12	.000		
	Total	.010	17			
AlkP	Between Groups	.000	5	.000	23.358	.000
	Within Groups	.000	12	.000		
	Total	.000	17			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df; F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value

Appendix 10. Analysis of variance (ANOVA) table for the comparison of means of the percent root infection and spore density of mycorrhiza in single and combined inoculation with PGPB.

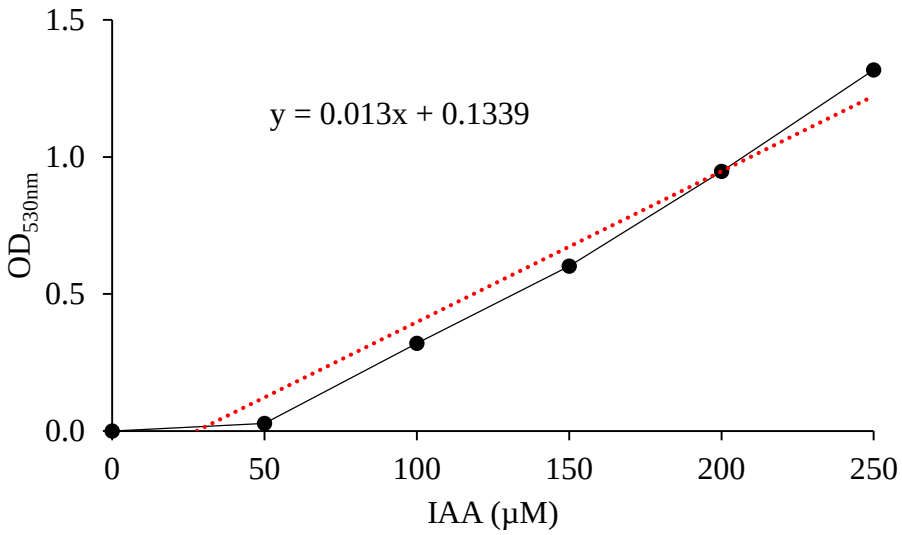
		Sum of Squares	df	Mean Square	F	Sig.
Root infection	Between Groups	12613.746	4	3153.436	456.496	.000
	Within Groups	276.317	40	6.908		
	Total	12890.062	44			
Spore density	Between Groups	43388.766	4	10847.191	1165.229	.000
	Within Groups	93.091	10	9.309		
	Total	43481.857	14			

Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df; F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value

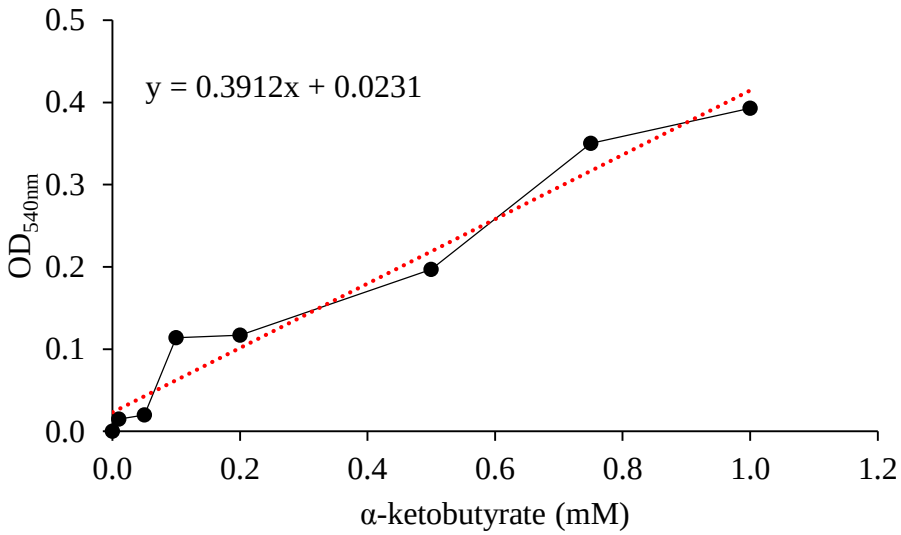
Appendix 11. Analysis of variance (ANOVA) table for the comparison of means of the biological control activity of mycorrhiza and/or mycorrhiza co-inoculated with PGPB against *R. solani*.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	206.142	4	51.535	9.195	.000
Within Groups	224.200	40	5.605		
Total	430.342	44			

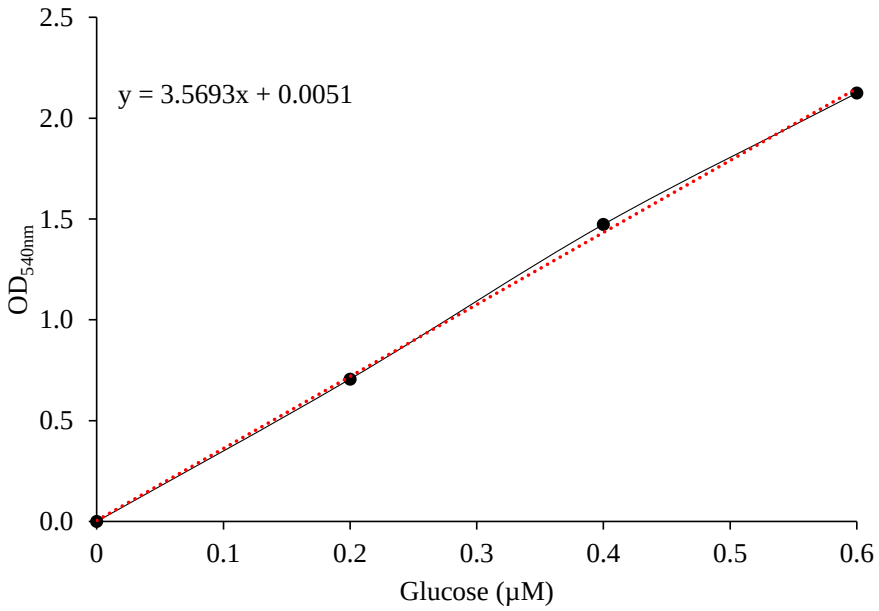
Sum of squares corresponds to the numerator of the variance ratio; df stands for degrees of freedom associated with each estimate of variance; Mean Square calculated by dividing the sum of square by its df. F calculated by dividing mean square between-groups by mean square within groups; and Sig. stands for the significance of the F ratio which is the p-value



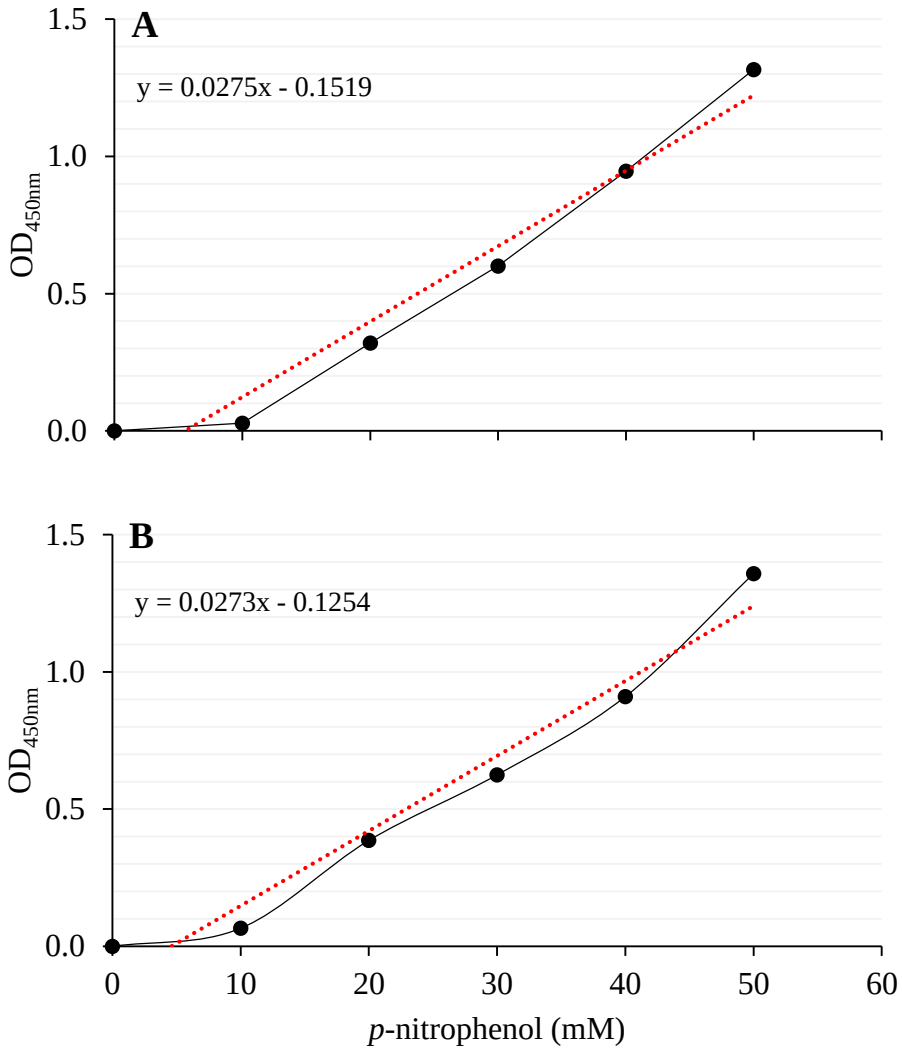
Appendix 12. Standard curve of indole-3-acetic acid (IAA). Vertical axes show absorbance values of standard solution at different concentrations of IAA. IAA production activity (x) per ml solution was computed following the linear equation (y=mx+b) displayed in the chart, wherein y is the absorbance, b is the slope, and m is the y-intercept.



Appendix 13. Standard curve of α-ketobutyrate. Vertical axes show absorbance values of standard solution at different concentrations of α-ketobutyrate. ACC-deaminase activity (x) was computed following the linear equation (y=mx+b) displayed in the chart, wherein y is the absorbance, b is the slope, and m is the y-intercept.



Appendix 14. Standard curve of glucose. Vertical axes show absorbance values of standard solution at different concentrations of glucose. *β*-1,3-glucanase activity (x) was computed following the linear equation (y=mx+b) displayed in the chart, wherein y is the absorbance, b is the slope, and m is the y-intercept.



Appendix 15. Standard curve of *p*-nitrophenol at (**A)** acid (pH 5.5); and (**B)** alkaline (pH 11.0.) conditions. Vertical axes show absorbance values of standard solution at different concentrations of *p*-nitrophenol. Acid and alkaline phosphatase activities (x) were computed following the linear equation (y=mx+b) displayed in the chart, wherein y is the absorbance, b is the slope, and m is the y-intercept.