# Bacterial–fungal interactions between *Paraburkholderia fungorum* GIB024 isolated from a *Rhizopogon roseolus* sporocarp and ectomycorrhizal fungi: Mycelial growth-promoting activity and fungal strain specificity

# (ショウロ子実体から分離した *Paraburkholderia fungorum* GIB024 細菌と外生菌根菌との相互作用：菌糸生育促進活性ときのこ菌株系統特異性)

TOGA PANGIHOTAN NAPITUPULU

2022

# Bacterial–fungal interactions between *Paraburkholderia fungorum* GIB024 isolated from a *Rhizopogon roseolus* sporocarp and ectomycorrhizal fungi: Mycelial growth-promoting activity and fungal strain specificity

# (ショウロ子実体から分離した *Paraburkholderia fungorum* GIB024 細菌と外生菌根菌との相互作用：菌糸生育促進活性ときのこ菌株系統特異性)

By

TOGA PANGIHOTAN NAPITUPULU

A Dissertation for Doctoral Degree

Supervisor:

Prof. Norihiro Shimomura

The United Graduate School of Agricultural Science

Tottori University

2022

# Contents

# Chapter 1

## General introduction

Bacterial-fungal interaction (BFI) is ubiquitous and commonly found in various ecological niche in natural conditions. The interaction can be taken place in the form of mutualism, commensalism, or parasitism. Moreover, the BFI has been also utilized for various bioprospecting purposes, ranging from agriculture, food technology, health and medicine, bioremediation, and revegetation.

Recently, the study of interaction between fungi and their associated-bacteria, particularly in the beneficial interaction, has been subjected to be studied intensively. Some proposed mechanisms of action have been raised, supported by comprehensive findings. Generally, the beneficial interaction between fungi and bacteria rules by physical and chemical interaction. In the physical interactions, fungal mycelia have role as highways for bacterial dispersion throughout soil particles (Steffan et al., 2020), thus allowing associated-bacteria to involved in nutrient cycling (Martin et al., 2012). In the chemical interaction, the contribution of the microbial metabolites in the interkingdom communication plays roles as chemical signaling and nutrients thus affecting morphology and physiology of bacteria as well as their fungal counterparts. For example, *Pseudomonas* sp. P7014 positively promoted the growth of *Pleurotus eryngii* (king trumpet mushroom) via production of indole-3-acetic acid (Kang and Cho, 2014). Auxofuran, an auxin-like metabolite produced by *Streptomyces* AcH 505, specifically promoted the mycelial growth of an ectomycorrhizal fungus, *Amanita muscaria* (Riedlinger et al., 2006). On the other hand, the bacterial growth can be enhanced by the stimulatory effect of fungal exudate excretion, thus facilitating a possibility of metabolite exchange between bacteria and fungi in mutualistic relationship. Trehalose, a distinct fungal disaccharide, produced by *Laccaria bicolor,* stimulate the growth of

*Pseudomonas fluorescens*, as an exchange, the bacterium produced thiamine as pay-off for enhancing mycelial growth of the fungus (Deveau et al., 2010). Moreover, the bacteria are also able to influence their fungal counterparts by modifying the environmental physicochemical conditions such as level of acidity (Rousk et al., 2009) or utilizing the inhibiting by-products (Noble et al., 2009).

In the mycorrhizal relationship, the fungus helps to promote the growth of the plant, as exchange, the photosynthetic products of the plant are provided to the fungus. However, it has been revealed that bacteria were also involved in this symbiotic relation between plant and fungus, thus making a tripartite symbiosis plant-fungus-bacterium. Fruiting body or sporocarp is the most visible part of the fungus on which spore producing structures are born. It harbors unique bacteria that might be have roles in symbiotic relation between plant and ectomycorrhizal fungus, yet relatively unexplored compared with bacteria in rhizospheric or 'hypospheric' soil (Liu et al., 2018).

The roles of bacteria toward development of mycorrhizal fungi have been recognized decades ago (Garbaye, 1994). Further, mycorrhizal helper bacterium (MHB), an ecological classification of mutual mycorrhizal associated-bacteria, has been subjected to intensive research regarding their diversity, mechanism of actions and applications. Taxonomically, the MHBs are diverse, ranging from Gram-positive to Gram-negative, and show a rigid specificity toward their fungal host counterpart (Rigamonte et al., 2010). At least, the MHBs influence and supporting mycorrhization of their fungal counterpart through enhancing pre-symbiotic and extraradical fungal mycelia (Obase, 2019), influencing the root receptivity (Lehr et al., 2007), effects on recognition between root and fungal mycelia (Aspray et al., 2013), rhizospheric soil modification (Barea et al., 2005), and influencing the fungal propagule germination (Panneerselvam et al., 2013).

In ecologically viewpoint, MHBs or ectomycorrhizal-associated bacteria play significant roles on nutrient cycling to support the activity of their ectomycorrhizal fungal counterpart. Interaction between a phosphate solubilizing rhizobacterium, *Pseudomonas* sp., with an arbuscular mycorrhizas fungus (AMF), *Rhizophagus irregularis*, enhanced the available phosphorus for carrot and potato (Ordoñez et al., 2016). Moreover, co-inoculation of nitrogen fixing bacteria (NFB) and AMF increase yield of *Piptadenia gonoacantha*, a legumes crop, although the AMF was not necessary for nodulation of plant root by the NFB (Oliveira et al., 2017). In carbon biogeochemical cycle, oxalotrophic ectomycorhizosphere bacteria play significant role on the carbon fixation of photosynthetic products and fungal exudates, particularly oxalate, through oxalate-carbonate pathway (Sun et al., 2019). Furthermore, bacterial community associated with ascocarp of an ascomycete ectomycorrhiza fungus, *Tuber melanosporum*, enriched with sulfur metabolism encoded genes, hint the role of these bacteria on the sulfur cycling (Antony-Babu et al., 2014).

MHBs were also reported to have specificity trait toward their ectomycorrhizal fungal association. Previous report showed that the spectrum of mutual specificity interaction of MHB toward ectomycorrhizal fungi could be narrow (Garbaye and Duponnois, 1993) or broad (Aspray et al., 2006). Furthermore, some MHBs were reported to not only promote the growth of symbiotic ectomycorrhizal fungi, but also fungal plant pathogens (Lehr et al., 2007). This phenomenon raised the concern related the application of MHBs in field application. Therefore, the study of specificity of MHBs or ectomycorrhizal associated-bacteria is very significant to develop the applicational study, such as bio-fertilizer.

Soil is regarded as the main pool of bacteria associated with extraradical mycelia, mycorrhizal mycelia, or fruiting body, including MHBs. However, a study suggested that mushroom can acquire air-borne bacteria as well, particularly on its surface (Zagriadskaia et al., 2013). The microbial community related to ectomycorrhizal fungi is highly influenced by

soil parameters, such as level of acidity and available nutrients (particularly nitrogen status), and host fungal identity (Pent et al., 2017; Yu et al., 2020). Mycorhizosphere mycelia have selective mechanisms to recruit bacteria from surrounding bulk soil, that might be beneficial for supporting their ecological activities (Uroz et al., 2007), including exudating stimulating (Toljander et al., 2007) or inhibiting compounds (Shirakawa et al., 2019). Furthermore, the filtering mechanism seemingly tighter for the bacteria that associated with fruiting body. The chemistry of fruiting body, including production of specific compound, plays significant role on this selection mechanism (Kumari et al., 2013), resulting a very specific and fungophile bacterial community associated with fruiting body.

Mycelium (vegetative body of fungi) grows primarily by releasing enzymes from the apical tip of the hyphae to digest the surrounding materials and then absorb the necessity nutrients (Money, 2008; Pritsch and Garbaye, 2011). In the process, the hyphal cells will eventually branch and form mycelial network. Furthermore, these enzymes determine and guide how the mycelium grows. If the mycelium is receiving a stimulant compound, the enzymes will respond by proliferating quicker. However, if the inhibitor was received, the mycelium will respond through defense mechanism. In BFI, the mycelial-associated bacteria are able to secret the growth stimulant, thus enhancing the growth of hyphae. Several studies have been identified these bacterial stimulants. Auxofuran, secreted by *Streptomyces* strain AcH 505, enhanced the growth of *Amanita muscaria* (Riedlinger et al., 2006). *Pseudomonas* sp. P7014 produced indole-3-acetic acid (IAA) that has promoting effect toward *Pleurotus eryngii* (Kang and Cho, 2014). During metabolite exchange, thiamine was synthesized by *Pseudomonas fluorescens* after utilizing trehalose secreted by *Laccaria bicolor* (Deveau et al., 2010).

In this research, the bacteria have been isolated from the fruiting body of *Rhizopogon roseolus* that associated with *Pinus thunbergii* by previous researcher and the screening for

their effect on mycelial growth of the fungus have been done as well (Pramoj Na Ayudhya et al., 2019). Six, out of 19 isolated bacteria, were potential to promote mycelium growth of *R. roseolus* according to in vitro confrontation test. All these isolates have been molecularly identified as well. The isolates are GIB024 (*Parabukholderia fungorum*), GIB028 (*Caballeronia sordidicola*), GIB029 (*Janthinobacterium agaricidamnosum*), KN1 (*Parabukholderia caledonica*), ALRC1 (*Novosphingobium rosa*), and ALRC6 (*Rhodobacter azotoformans*).

One of the bacteria, *Paraburkholderia fungorum* strain GIB024, showed a mutual BFI by its ability to promote mycelial growth of *R. roseolus* trough in-vitro direct confrontation screening (Pramoj Na Ayudhya et al., 2019). However, the mycelial promoting mechanism of this bacterium was not revealed yet. In mutual BFI, the bacterium relies on fungal carbonaceous compounds as source of nutrients while extracellularly release mycelial growth-promotor, in the form of volatile or soluble compound. Moreover, the literature studies showed that the bacterium species was ubiquitously presence in various ecological niche of not only as free-living bacterium in soil (de Oliveira Longatti et al., 2013) but also associated with living organisms (Coenye et al., 2001). *P. fungorum* has potency to be utilized for various bioprospecting purposes including crop biofertilizer (Castanheira et al., 2016). *P. fungorum* was also associated with several fungi (Stopnisek et al., 2016; De Felice et al., 2016). Therefore, the aim of this current study was to investigate the potential of the extracellular mycelial growth-promoting metabolite of *P. fungorum* GIB024 isolated from *R. roseolus* sporocarp in *P. thunbergii* forest, identified the possible fungal carbonaceous compounds and its specificity interaction with other ectomycorrhizal mushrooms. The research have been divided into three parts: (1) investigating mycelial growth-promoting potential of extracellular metabolites of *Paraburkholderia* spp. isolated from *R. roseolus* sporocarp, (2) investigating the potential role of the fungal carbonaceous compounds in the

mycelial growth promotion of *R. roseolus* during interaction with the sporocarp bacterium, *P. fungorum* GIB024, (3) investigating the specificity promotion of *P. fungorum* GIB024 toward mycelium growth of other ectomycorrhizal fungi.

# Chapter 2

# Mycelial growth-promoting potential of extracellular metabolites of *Paraburkholderia* spp. isolated from *Rhizopogon roseolus* sporocarp

## 2.1 Introduction

Bacterial-fungal interaction (BFI) is ubiquitous in nature and plays a crucial role in various environmental niches, contributing to the biochemical and physical processes in nature. Moreover, BFI has been exploited for centuries by humans in various sectors, including agriculture, food technology, health, forestry, and environmental protection. The symbiotic relationship between bacteria and fungi can be by mutualism, parasitism, or commensalism. In the mycorrhizal relationship, the fungus helps to promote plant growth through exchange as the photosynthetic products of the plant are provided to the fungus. However, it has been revealed that bacteria, known as mycorrhizal helper bacteria (MHB), are also involved in this symbiotic relationship between plants and fungi, thus making a tripartite symbiosis plant-fungus-bacterium (Frey-Klett et al., 2007; Deveau et al., 2018).

The fruiting body or sporocarp is the most visible part of the fungus on which spore-producing structures are formed. Sporocarp of ectomycorrhizal fungus harbors unique bacteria that might have roles in symbiotic relation between plant and the ectomycorrhizal fungus, yet they are relatively unexplored compared with bacteria in rhizospheric or "hypospheric" soil. In this research, bacteria have been isolated from the fruiting body of *Rhizopogon roseolus* (shouro mushroom in Japanese) and the screening for their effect on mycelial growth of the fungus have been performed (Pramoj Na Ayudhya et al., 2019). *R. roseolus* is an edible ectomycorrhizal fungus that is commonly associated with pine trees. Six out of 19 isolated bacteria had the potential to promote mycelial growth of *R. roseolus*

according to the *in vitro* confrontation test. All these isolates were also molecularly identified. Among these bacteria, we found some bacteria belonging to the genus *Paraburkholderia*.

The genus *Paraburkholderia* has been found ubiquitously with high abundance as mycorrhizosphere bacteria and has fungophilic features (Shirakawa et al., 2019; Pratama et al., 2020), but the roles of these bacteria in their fungal counterparts are not well understood. Some *Paraburkholderia* spp. isolated from *R. roseolus* sporocarp were found to promote mycelial growth of the fungus using an *in vitro* direct confrontation assay. In the mycorrhizal association, the capacity of bacteria to enhance the growth of fungal mycelia is an indication of the role of the bacteria as MHB (Bending, 2007). However, the mode of action or agent responsible for the mycelial growth-promoting activity of these *Paraburkholderia* spp. toward the growth of *R. roseolus* mycelia is still unknown. Some studies have suggested that extracellular metabolites produced by the bacteria are responsible for the mycelial growth effect of their fungal counterparts in a mutualistic symbiosis (Riedlinger et al., 2006; Uehling et al., 2019; Abeysinghe et al., 2020). Therefore, this study aims to evaluate whether the extracellular metabolites produced by two species of *Paraburkholderia* isolates, namely *P. fungorum* and *P. caledonica*, have the potential to promote growth in *R. roseolus* mycelial growth by investigating the volatile and nonvolatile metabolites of the bacteria.

## 2.2 Materials and methods

### 2.2.1 Microorganisms and media

The *R. roseolus* strain TUFC10010 was provided by the Tottori University Fungal Culture Collection, Japan. Two bacterial isolates were employed, *Parabukholderia fungorum* GIB024 and *P. caledonica* KN1, both of which were isolated from the sporocarp of *R. roseolus* associated with *Pinus thunbergii* (Japanese black pine). The bacterial isolation, as

well as molecular identification, have been done in a previous study (Pramoj Na Ayudhya et al., 2019). All bacterial isolates and *R. roseolus* isolates were maintained in potato dextrose agar (PDA) and 1:5 diluted Melin-Norkrans modified medium or 1:5 MMN (with thiamine), respectively, prior to use. In the bioassay experiment, high-sugar medium (hs-dMMN) refers to 1:5 MMN (without thiamine) in the presence of glucose and low-sugar medium (ls-dMMN) refers to 1:5 MMN (without thiamine) in the absence of glucose. The composition of the MMN-based liquid media used is summarized in Table 2.1

### 2.2.2 Dual culture (direct confrontation) between the bacterial isolate and *Rhizopogon roseolus* at various distances

Sterilized hs-dMMN and ls-dMMN with 2% agar were used as media for the dual culture assay. One plug (0.6 cm in diameter) of the shouro mycelium (grown on 1:5 diluted MMN agar plate with 2% agar) from the active part was placed on the edge of the agar medium in the Petri dish. The *R. roseolus* was incubated in a dark incubator at 25 °C for one week prior to the dual culture assay.

One colony was picked from a bacterial plate (7-day-old in PDA) and inoculated in 50 mL of hs-dMMN liquid for 3 days in a dark incubator at 25 °C without agitation. The bacterial cells were then separated from the supernatant by centrifugation at 2200 × *g* for 20 min. The supernatant was discarded, and NaCl 0.9% (w/v) solution was added and mixed with the bacterial cells until the $OD_{600}$ reached approximately 0.2.

One loop of the bacterial suspension was placed at three different distances (5, 25, and 45 mm) from the active edge of one week old *R. roseolus* (Fig. 2.1). A 0.9% (w/v) NaCl solution was used as a control. The plates were then incubated in a dark incubator at 25 °C, and mycelial elongation growth was observed weekly.

### 2.2.3 Investigation of the volatile metabolites of bacterial isolates toward growth of *Rhizopogon roseolus*

The hs-dMMN and ls-dMMN with 2% agar were used as media for growing *R. roseolus*. One plug (0.6 cm in diameter) of *R. roseolus* mycelia (grown on hs-dMMN agar plate) from the active part was placed at the center of the agar medium in a nine-centimeter Petri dish. *R. roseolus* was incubated in a dark incubator at 25 °C for one week prior to the dual culture assay.

Similar to the *R. roseolus* plate, the hs-dMMN and ls-dMMN with 2% agar were used as media to inoculate the bacterial isolate. One colony was picked from the bacterial plate (in PDA) and inoculated in 50 mL of hs-dMMN (without thiamine) liquid medium in the presence of glucose for 3 days in a dark incubator at 25 °C without agitation. Further, 30 µL of each bacterial suspension broth was inoculated into the agar plate (pH before wet sterilization was maintained at 6 ± 0.1). For the control, uninoculated liquid medium was spread onto the agar medium. The bacterial isolates were maintained in a dark chamber at 25 °C for three days.

Indirect contact was applied to investigate the effect of volatile metabolites produced by bacteria on the mycelial growth of *R. roseolus*. The lids of both the bacterial and *R. roseolus* plates were removed, and the *R. roseolus* plate was inverted and placed on top of the bacterial plate (Fig. 2.6C). *R. roseolus* grown in hs-dMMN was placed on top of a bacterial isolate grown in hs-dMMN; *R. roseolus* grown in ls-dMMN was placed on top of the bacterial isolate grown in ls-dMMN. The control of the bacterial plate was also placed beneath the *R. roseolus* plate. The two plate bases were then sealed with a double layer of Parafilm®. The plates were maintained at 25 °C in a dark chamber. A sterile agar plate without bacterial

inoculation was used as a control. The diameter of *R. roseolus* was measured every five days after bacterial inoculation for 35 days of incubation.

### 2.2.4 Investigation of the axenic soluble metabolites of bacterial isolates toward growth of *Rhizopogon roseolus*

The pour plate method was used to investigate the effect of extracellular metabolites produced by bacteria under axenic conditions on the mycelial growth of *R. roseolus*. Initially, one colony was picked from a bacterial plate (7-day-old in PDA) and inoculated in 50 mL of hs-dMMN for 3 days in a dark incubator at 25 °C without agitation. Further, 10 µL of bacterial suspensions of GIB024 and KN1 were inoculated in two different liquid media, hs-dMMN and ls-dMMN (pH before wet sterilization was maintained at 6 ± 0.1). The bacterial isolates were maintained in a dark chamber at 25 °C without agitation (S) and with agitation at 80 rpm (R) for 3 days. Detailed information on the spent broth conditions is presented in Table 2.2. The bacterial cells were then separated from the liquid broth by centrifugation at 2200 × *g* for 40 min. The supernatant was then filtered aseptically through Minisart® 0.45 µm filter paper, followed by Acrodisc® 0.1 µm filter paper and collected in sterile falcon tubes as bacterial spent broth for further assays.

As with bacteria, two different agar media for *R. roseolus*, hs-dMMN and ls-dMMN (pH before wet sterilization was maintained at 6 ± 0.1) were used. Approximately 10%, 20%, and 30% (v/v) of the bacterial spent broth was added to warm hs-dMMN or ls-dMMN agar medium in a 9 cm petri dish until a total volume of 15 mL was reached and then mixed slowly. The bacterial spent broth from the hs-dMMN liquid medium was added to warm hs-dMMN agar medium, while the bacterial spent broth from bacteria grown in ls-dMMN liquid medium was added to warm ls-dMMN agar medium. For control, instead of the bacterial spent broth, uninoculated hs-dMMN or ls-dMMN were added to warm hs-dMMN and ls-

dMMN agar media, respectively. The amount of agar in the Petri dish for each concentration of spent broth was maintained at 2% (w/v). After the agar media solidified, one plug (0.6 cm in diameter) of *R. roseolus* mycelia (grown on hs-dMMN agar plate) from the actively growing edge was placed on the center of the agar medium in the petri dish and sealed with Parafilm®. The petri dish was incubated in a dark chamber at 25 °C, and the mycelial diameter was observed after 14 days. The percentage of mycelial growth was calculated as [(diameter of *R. roseolus* with spent broth - diameter of *R. roseolus* in control)/ diameter of *R. roseolus* in control] × 100%.

### 2.2.5 Statistical analysis

All the procedures for the dual culture assay, pour plate assay, and indirect confrontation assay were repeated in triplicate. Statistical analysis of dual culture data was performed through Dunnett's multiple comparisons test (one-way ANOVA) between control and bacterial test. Statistical analysis of effect of bacterial spent broth (pour plate assay) was performed through unpaired t test between GIB024 and KN1. Statistical analysis of indirect confrontation data was performed through Tukey's multiple comparisons test (two-way ANOVA). For figure that contained statistical analysis data, the asterisk sign, indicates the summary of the comparison test, is stated in each figure legend. Without asterisk sign or ns means not significantly different ($P > 0.05$). All data and illustrations were processed using GraphPad Prism software version 9.2.0.

## 2.3 Results

The interaction between *R. roseolus* and the bacterial isolates in the direct confrontation assay depended on the distance between the tip of *R. roseolus* mycelium and the bacterial line in both hs-dMMN and ls-dMMN agar media (Fig. 2.2; the appearance of the dual culture plate is shown in Fig. 2.3). The period of incubation and post-bacterial inoculation

observation were extended until 35 days because the fungal plug was initially put on the edge of the Petri dish, thus allowing enough space and time for fungal mycelia to reach the farthest edge of the Petri dish (diameter of petri dish is 8.5 cm). Based on our observation, after 35 days of bacterial inoculation, the fungal mycelia were able to elongate maximally 4.5 cm from the initial point of mycelial active edge (day 0 post-bacterial inoculation). Each strain of bacteria promoted *R. roseolus* mycelial growth was observed differently as well. GIB024 promoted the growth of *R. roseolus* at shorter distances, particularly in low sugar medium, whereas KN1 inhibited the growth of the fungus in that medium. Instead, KN1 increased the mycelial growth of *R. roseolus* at long distances in a high sugar medium.

The shortest distance (5 mm) facilitated immediate contact between the bacterial colony and the actively growing mycelial tip of *R. roseolus*. Under these conditions, distinct interactions were observed. In ls-dMMN, GIB024 promoted fungal growth at a distance of 5 mm. However, the promoting effect decreased at a further distance between the bacterium and the fungus (at 25 mm), or even showed an inhibitory effect (at 45 mm). On the other hand, in ls-dMMN, KN1 inhibited the growth of the fungus at the shortest distance (5 mm), thus making the mycelia of *R. roseolus* unable to cross the bacterial colony line (Fig. 2.2). However, the antifungal effect decreased with increasing distance between the bacterium and fungus. These findings suggest that metabolites produced by *R. roseolus* itself might trigger the KN1 of toxic metabolite(s) that inhibit fungal growth in such low sugar environments. In hs-dMMN however, KN1 slightly promoted *R. roseolus* growth at the shortest distance (5 mm), while GIB024 had a neutral effect on fungal growth. At a distance of 25 mm, the promoting effect of KN1 increased, but it decreased at a distance of 45 mm. In hs-dMMN, GIB024 slightly promoted the growth of *R. roseolus* at a distance of 25 mm, but this effect decreased with increasing distance between the bacterium and the fungus.

Extracellular metabolites produced by the bacteria that accumulate in the bacterial spent broth potently promote the mycelial growth of *R. roseolus*. The spent broth mixture with the growth medium increased the diameter of colonies of the fungus mycelia. The spent broth was added to the medium at three different concentrations: 10%, 20%, and 30% (Fig. 2.4A-D). However, various fermentation conditions, such as the presence of additional glucose (sugar) in the fermentation media and agitation during the fermentation period affected the bacteria to produce spent broth with various positive effects toward the growth of *R. roseolus*. Spent broth of GIB024 grown in low sugar medium had a greater ability to promote the growth of *R. roseolus* than that grown in high sugar medium (Fig. 2.4A). On the other hand, the other bacterium, KN1, preferred high sugar medium to produce growth factors for *R. roseolus* mycelial growth (Fig. 2.4B). This tendency agrees with the dual culture assay (Fig. 2.1), where GIB024 promoted *R. roseolus* growth in ls-dMMN and KN1 in hs-dMMN. Agitation conditions during bacterial fermentation also influence the ability of bacteria to produce mycelial growth factors. Bacteria grown under static conditions in both types of media were more effective in producing mycelial growth-promoting metabolites than with agitation.

A lower concentration of the spent broth was more effective in increasing the growth of *R. roseolus* mycelia. According to the bioassay, supplementation of 10% (v/v) of KN1 bacterial spent broth grown in high sugar medium under static conditions provided the highest *R. roseolus*-promoting ability among other bacterial spent broth conditions (Fig. 2.4B, the appearance of the colony is shown in Fig. 2.5). However, the addition of KN1 to *R. roseolus* medium resulted in a decrease in *R. roseolus* growth (Fig. 2.4C and Fig. 2.4D). For most fermentation conditions, particularly in low sugar media, for each bacterium, the spent broth concentration added to the plate was negatively correlated with the growth promoting ability of *R. roseolus* mycelium ($R^2 > 0.8$; negative 1/ slope; see Table 2.3). An exception to

this is GIB024, which when grown in high sugar medium both in static and agitated conditions, showed positive correlation and no correlation, respectively, toward *R. roseolus* mycelium growth.

Volatile compounds produced by the bacteria through indirect confrontation had neutral and inhibitory effects on the growth of *R. roseolus*, depending on the bacterial nutrient medium condition. In high sugar media, the volatile compounds produced by the bacteria had a neutral effect on the growth of *R. roseolus* (Fig. 2.6A). On the other hand, gas metabolites released by the bacteria that grew in low sugar media showed variability in the growth of *R. roseolus* (Fig. 2.6B). GIB024 inoculated in low sugar medium had a neutral effect, whereas KNI in the same medium inhibited the growth of *R. roseolus*. These findings agreed with the direct confrontation assay (Fig. 2.1), in which KN1 inoculated in low sugar media at various distances from the mycelia apical tip inhibited the growth of *R. roseolus*, including the farthest distance of 45 mm, which resembled the indirect confrontation assay condition.

## 2.4 Discussion

The growth of *R. roseolus*, as the impact of confrontation with *Paraburkholderia* spp., depended on the media (presence of excess carbon source, in this case glucose), bacterial strain, and distance between *R. roseolus* and the bacteria. Nutrient availability has been acknowledged as one of the main factors in bacterial-fungal interaction (BFI), determining the inhibiting or promoting effect on the fungal growth (Velez et al., 2018). Among the various macro- and micro-nutrients, carbon and nitrogen sources are the two major sources that play a major role in the effect of nutrient status on the BFI (Lohberger et al., 2019). The quality and quantity of carbon sources have an impact on the interaction between fungi and bacteria, either mutualistically or parasitically. The type of carbon source has been found to determine the selectivity of bacteria toward fungal partners. One example is oxalotrophic

bacteria in the soil ecosystem that uses oxalic acid released by certain soil fungi as chemoattractants and as a carbon source, thus shaping the microbial community around the mycorrhizosphere (Sun et al., 2019). The amount of sugar in the zone of the bacterial-fungal interface can determine whether a positive or negative interaction will occur. The current findings suggest that this phenomenon was also observed during the interaction between *Paraburkholderia* spp. and *R. roseolus*, although it is also strain dependent. For BFIs that prefer low sugar conditions, the stress gradient hypothesis (SGH) condition has been raised, particularly in the marginal soil condition with limited major nutrients and an abundance of recalcitrant compounds (Hammarlund and Harcombe, 2019). Furthermore, antimicrobial agents are sometimes observed to be released by microbes involved in SGH conditions, when carbon sources are abundant.

The interaction between *R. roseolus* and *Paraburkholderia* is also influenced by physical factors, such as the distance between bacteria and mycelial apex of the fungus. In our study, a short distance, that allowed immediate contact between fungal mycelia and bacterial colonies on the surface of the agar, was more beneficial for GIB024 than KN1, suggesting the role of bacterial biofilm or endobacteria feature mechanism of this bacterium toward *R. roseolus* mycelia. The distance between bacteria and the apical tip of the fungal mycelia also influences the distribution of either soluble metabolites released by the bacteria through the agar pores or the volatile compounds emitted in the interface beyond the agar surface. Since the movement of the non-volatile metabolites through the agar relies mostly on the passive diffusion mechanism, which depends on the molecular weight of the metabolites produced by the bacteria, high-molecular-weight biomolecules, for example, will reach the mycelial tip for a longer time than a low molecular weight compound. In addition, the agar pore size can be a significant barrier to the movement of high molecular weight compounds.

The interaction between bacteria and fungi is species-dependent. Although both of bacterial isolates belong to the same genus, *Paraburkholderia*, their characteristics through interaction with *R. roseolus* and utilization of nutrients in the environment media are varied. A taxonomic and biochemical characteristic comparison of these two bacteria, formerly both belonging to the clade of *Burkholderia*, showed that *P. caledonica* has higher sugar-utilization ability than *P. fungorum*, but lacks enzymatic activity compared to *P. fungorum* (Coenye et al., 2001). Consistent with the direct confrontation and pour plate assays, *P. caledonica* KN1 was shown to increase the growth of *R. roseolus* mycelium in high sugar conditions, and inhibited fungal growth in low sugar medium through the production of toxic soluble and volatile metabolites. The phenomenon of species- or strain-dependent interactions with fungi has been widely reported in various environmental niches, as well as for various functional interactions (Carlson, 1983; Jabra-Rizk et al., 1999). In the mushroom field, several strains of *Pseudomonas putida* isolated from soil samples from some mushroom farms showed variability in the mushroom growth-promoting ability toward *Agaricus bisporus* (Zarenejad et al., 2012). The intraspecies variation tendency of not only bacteria but also other kingdoms are caused by genetic and epigenetic factors. From an ecological and evolutionary perspective, this variation strategy is used as a mechanism for maintaining survivability under several environmental conditions that are sometimes unfavorable (Godhe and Rynearson, 2017).

Bacterial extracellular metabolites, particularly non-volatile metabolites, contribute to the growth of *R. roseolus*. Although the fermentation conditions varied, bacterial spent broth of both bacterial strains, GIB024 and KN1, showed a positive mycelial growth-promoting effect. The bioassay of spent broth of these bacteria was correlated with the direct confrontation assay on agar plates under various scenarios. The mechanism by which bacteria promote the growth of fungal counterparts in a mutualistic symbiotic relationship has been

thoroughly studied. Some proposed mechanisms, include direct mechanisms, such as releasing specific or general growth factors and facilitating important and crucial nutrients to the fungi. An indirect mechanism through the inhibition of fungal enemies has also been suggested. Several metabolites produced by bacteria that positively impact the growth of saprophytic as well as mycorrhizal fungi have been identified and characterized. A mycorrhizal helper bacterium (MHB), *Streptomyces* strain Ach 505, was reported to produce the soluble metabolite auxofuran, which specifically increased the growth of the ectomycorrhizal fungus *Amanita muscaria* (Riedlinger et al., 2006). In some studies, several bacteria have been reported to release non-specific compounds such as thiamin (Deveau et al., 2010) or induce the mycosynthesis of growth hormone indole-3-acetic acid (IAA) (Hoffman et al., 2013) during interactions with their fungal counterparts.

The volatile compounds (VCs) produced by our *Paraburkholderia* isolates did not increase the growth of *R. roseolus*. Furthermore, the nutrient conditions, particularly sugar availability, influenced the VCs produced by the bacteria, thus affecting their interaction with the fungal counterpart, and causing a possible shift from neutral or positive interactions to negative interactions. Bacterial VCs have been recognized as one of the modes of action of BFI in various environmental and ecological niches, including soil biogeochemistry. In some studies, the interaction between BFI and VCs provides interkingdom communication without physical contact, but effectively influences the physiological and morphological conditions of both bacteria and fungi depending on the nature of the interaction (parasitic or mutual) (Kai et al., 2007; Schmidt et al., 2016; Schmidt et al., 2017; Weisskopf et al., 2021). Consistent with our results, in the sporocarp or mycorrhizosphere microenvironment, the role of soluble metabolite exchange in close contact between residing bacterial and fungal mycelia is more pronounced than that of long-distance gas signals. Under these conditions, the endophytic bacteria found in the sporocarp have been selected from the corresponding hyposphere or

mycorrhizosphere, suggesting physical contact through the fungal highway mechanism, thus providing a high possibility of interaction between bacteria and fungi via extracellular soluble metabolites during the interface between bacterial cells and fungal mycelium (Liu et al., 2018; Steffan et al., 2020). During mutualistic interactions between the mold *Aspergillus nidulans* and the soil bacterium *Bacillus subtilis*, for example, there was an observed migration of the bacterium along hyphae of *A. nidulans*, followed by the secretion of thiamine as a growth-inducing factor in the apical zone of the fungal hyphae (Abeysinghe et al., 2020).

The quantity of bacterial spent broth affected the growth of *R. roseolus*. The efficacy of the growth-promoting effect of cell-free spent broth of bacteria on the growth of fungal counterparts in a mutualistic symbiosis relationship tends to be low, thus increasing the spent broth concentration, resulting in a decrease in the promoting effect on fungal growth (Aspray et al., 2013). Inversely, for the parasitic interaction between bacteria and fungi, the antifungal activity of bacterial spent broth increased as the concentration of the spent broth increased. Effectivity of spent broth to promote mycelial growth was observed at lower concentrations, indicating that the possible extracellular metabolites contained in the spent broth have roles in molecular signaling and might be extended to interkingdom quorum sensing between bacteria and fungi.

The production of growth promoting extracellular metabolites is influenced by a variety of environmental conditions, such as nutrient status in the growth medium and agitation during the fermentation period. Glucose or sugar availability obviously affected the ability of our bacterial isolates to secrete mycelial growth-promoting compounds. Generally, in conditions with excess major nutrients such as sugar, nitrogen, or soluble phosphorus, bacteria suppress the production of their secondary metabolism products (Martín, 2004; Ruiz et al., 2010). However, in some cases, the abundance of certain nutrients triggers the

formation and secretion of secondary metabolites (Gotoh et al., 1992). Agitation during the fermentation period influences the physiology of bacteria and dissolved oxygen, and thus extracellular metabolites produced by the bacteria (Somerville and Proctor, 2013).

As conclusion, soluble extracellular metabolites, and not non-volatile metabolites, produced by *Paraburkholderia fungorum* and *P. caledonica* can promote the growth of *Rhizopogon roseolus* mycelia. However, the production of these potential metabolites is dependent on various fermentation conditions, such as nutrients (particularly sugar concentration) and agitation during fermentation. *Paraburkholderia* spp. exhibit a species- and nutrient (glucose)-dependent ability to promote the mycelial growth of *R. roseolus*, and the bacterial soluble metabolite(s) play a crucial role in their growth-promoting ability.

Table 2.1 Chemical and quantity composition of Melin-Norkrans modified medium (MMN)-based liquid media for culture maintaining and bioassay in this study.

| **Chemical composition** | **1:5 MMN** | **hs-dMMN** | **ls-dMMN** |
|---|---|---|---|
| Malt extract | 1 g | 1 g | 1 g |
| Glucose | 2 g | 2 g | - |
| $(NH_4)_2HPO_4$ | 0.05 g | 0.05 g | 0.05 g |
| $KH_2PO_4$ | 0.1 g | 0.1 g | 0.1 g |
| $MgSO_4.7H_2O$ | 0.03 g | 0.03 g | 0.03 g |
| NaCl | 5 mg | 5 mg | 5 mg |
| $CaCl_2$ | 10 mg | 10 mg | 10 mg |
| Thiamine hydrochloride | 20 µg | - | - |
| $FeCl_3.6H_2O$ 1% (w/v in deionized water) | 0.24 mL | 0.24 mL | 0.24 mL |
| Deionized water | 999 mL | 999 mL | 999 mL |

Table 2.2 Conditions for investigating the ability of crude bacterial spent broth toward growth of *R. roseolus* via pour plate method. The bacterial spent broth was added to the warm agar medium for *R. roseolus* in three concentrations: 10%, 20%, and 30% (v/v).

| **Condition** | **Agitation** | **Bacterial spent broth medium** | ***R. roseolus* medium** |
|---|---|---|---|
| S ls-dMMN | Static | ls-dMMN | ls-dMMN |
| S hs-dMMN | 80 RPM | hs-dMMN | hs-dMMN |
| R ls-dMMN | Static | ls-dMMN | ls-dMMN |
| R hs-dMMN | 80 RPM | hs-dMMN | hs-dMMN |

Table 2.3 The parameter of the best fit values as well as the goodness of fit as the effect of two bacterial spent broths of *Paraburkholderia* species (GIB024 and KN1) toward the growth of *R. roseolus* through pour plate method.

| **Parameter** | **S ls-dMMN** | | **S hs-dMMN** | | **R ls-dMMN** | | **R hs-dMMN** | |
|---|---|---|---|---|---|---|---|---|
| | GIB024 | KN1 | GIB024 | KN1 | GIB024 | KN1 | GIB024 | KN1 |
| 1/ slope | -0.5215 | -0.2990 | 0.3690 | -0.5485 | -0.2690 | -0.3980 | 0.0545 | -0.3400 |
| $R^2$ | 0.9595 | 0.9904 | 0.9317 | 0.8290 | 0.9895 | 0.9022 | 0.2906 | 0.9939 |

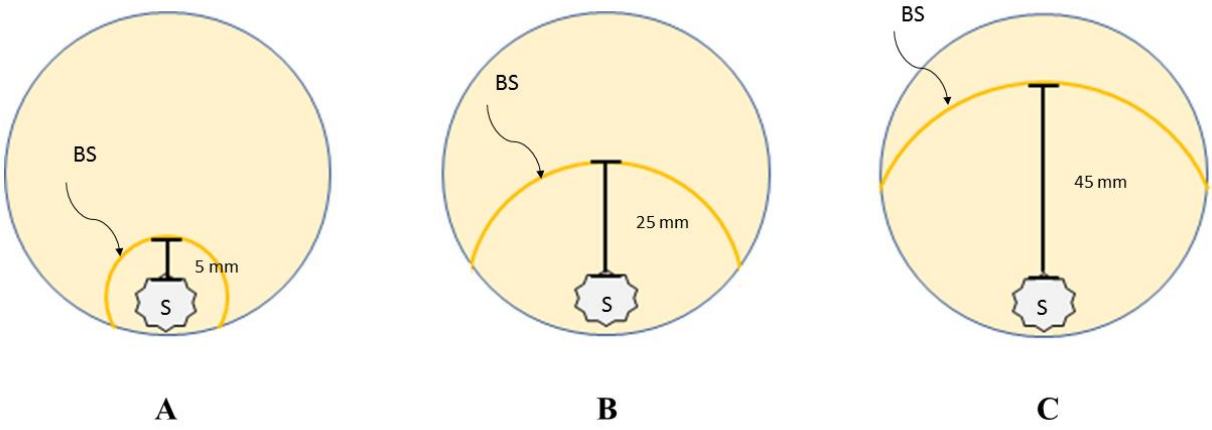



Fig. 2.1 Schematic Figures of the design experiment of dual culture assay between *R. roseolus* and bacterial isolate in a petri dish (ø 8.5 cm). One loop of bacterial suspension (BS) with $OD_{600} \approx 0.2$ was cycled around the edge of actively growing one-week *R. roseolus* mycelial colony (S) in three different distances: 5 (A), 25 (B), and 45 mm (C).

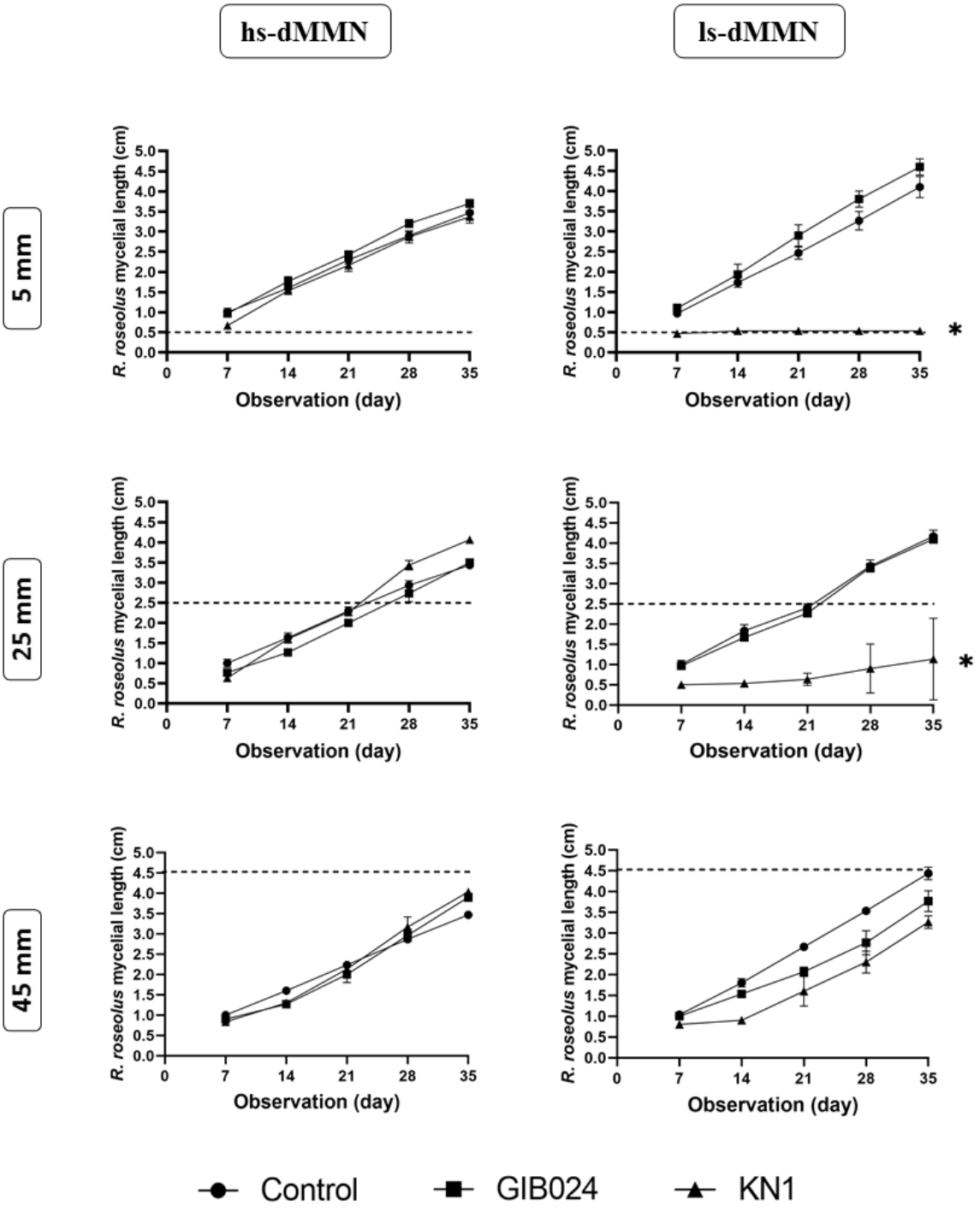



Fig. 2.2 Weekly observation (x-axis in day) of *R. roseolus* mycelial length (y-axis in cm) in dual culture plate with bacterial isolates (GIB024 in square, KN1 in up-pointing triangle, NaCl 0.9% (w/v) solution as control in circle) at various distance (5, 25, and 45 mm) from the edge of one-week actively growing *R. roseolus* mycelium.

The assay was carried out in two agar plate types, 1:5 MMN with glucose (hs-dMMN) and without glucose (ls-dMMN). Dotted line (---) marks the distance between bacterial line and the initial apical tip of one-week *R. roseolus* mycelia (see BS in Fig. 1). Statistical analysis of the data was performed through Dunnett's multiple comparisons test (one-way ANOVA) between control and bacterial test, the asterisk sign indicates the summary of the comparison test, * ($P < 0.05$). Without asterisk sign means not significantly different ($P > 0.05$).

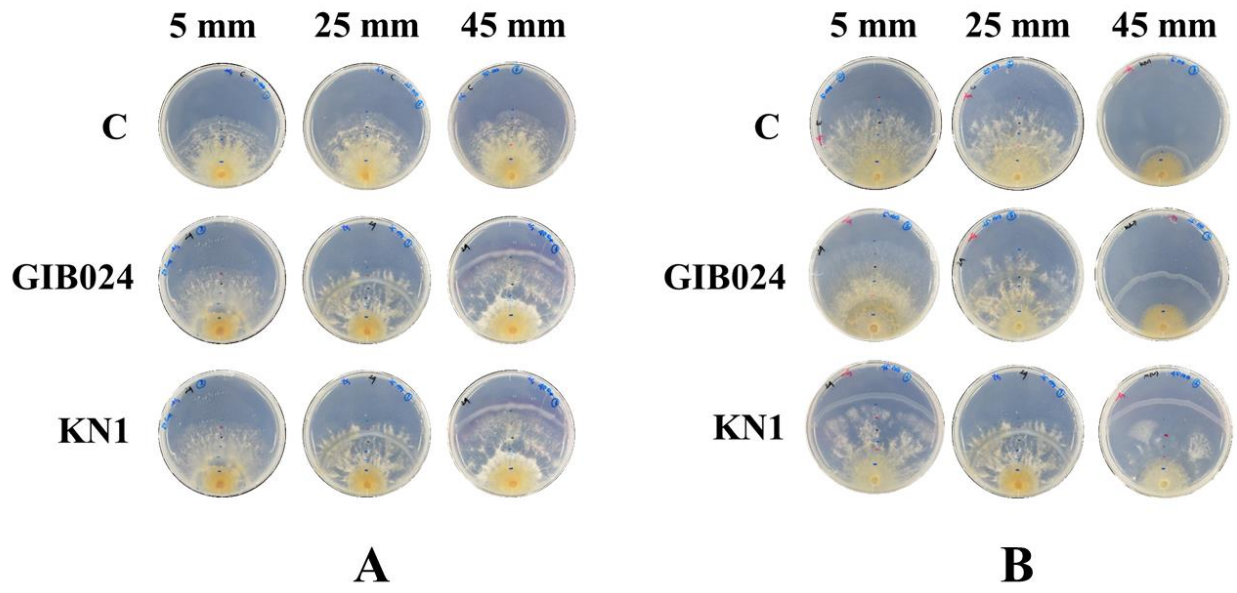



Fig. 2.3 Thirty-five-day-old post-bacterial inoculation of dual culture plates of *R. roseolus* mycelium with two bacterial isolates (GIB024 = *P. fungorum*, KN1 = *P. caledonica*, C = Monoculture of *R. roseolus*) at various distances, 5, 25, and 45 mm from the edge of one-week *R. roseolus* mycelia.

The assay was done in two agar plate types, (A) 1:5 MMN with glucose (hs-dMMN) and (B) without glucose (ls-dMMN).

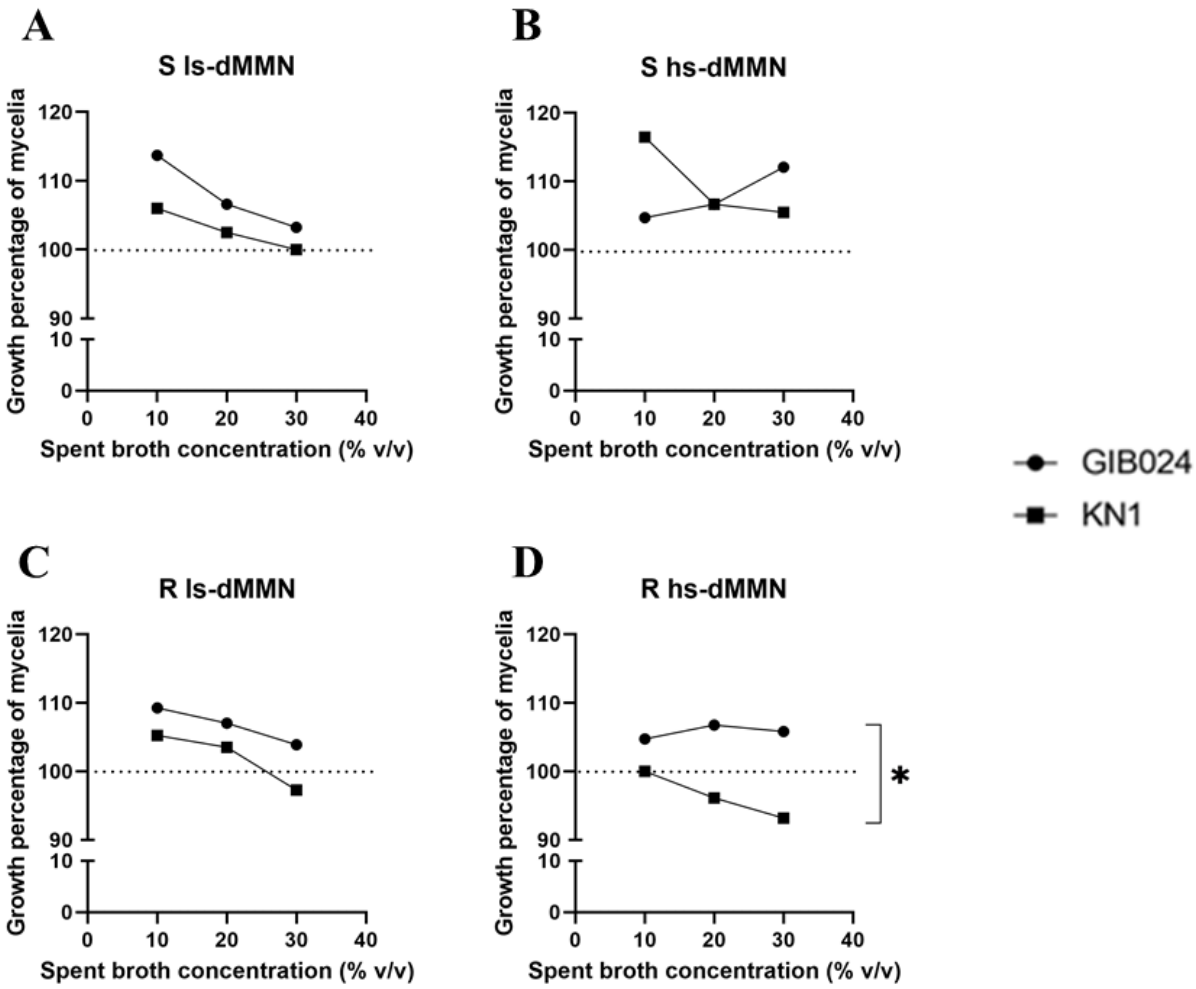



Fig. 2.4 Effect of two bacterial spent broths of *Paraburkholderia* species (GIB024 in circle and KN1 in square) toward the growth of *R. roseolus* through pour plate method.

The spent broth was added to the *R. roseolus* medium in three concentrations: 10%, 20%, and 30%. The spent broth was obtained by growing bacteria in various fermentation conditions by manipulating the sugar concentration (1:5 MMN with glucose [hs-dMMN] and without glucose [ls-dMMN]) and agitation condition (S= static condition, R= with agitation 80 RPM). The influence of bacterial spent broth concentration of GIB024 and KN1 toward growth percentage of *R. roseolus* mycelia through pour plate assay is plotted in **(A)** bacteria grown in S ls-dMMN condition; **(B)** bacteria grown in S hs-dMMN condition; **(C)** bacteria grown in R ls-dMMN condition; **(D)** bacteria grown in R hs-dMMN condition. The dotted line (---)

marks the growth percentage of the control (100%), growth percentage of *R. roseolus* below the line indicates inhibition effect of the bacterial spent broth. Statistical analysis of the data was performed through unpaired t test between GIB024 and KN1, the asterisk sign indicates the summary of the comparison test, * ($P < 0.05$). Without asterisk sign means not significantly different ($P > 0.05$).


Fig. 2.5 The appearance of 14-day-old mycelial colonies of *R. roseolus* grown in hs-dMMN (1:5 MMN with glucose) with addition of 10% and 30% v/v bacterial spent broth that grown in S ls-dMMN (1:5 MMN with glucose and without agitation condition) for 3 days, in dark chamber at 25 ℃.

The control plate contained hs-dMMN only (uninoculated broth), without the addition of bacterial spent broth. The fungal colony size of control plates is smaller than plates with the bacterial spent broth, indicated the presence of mycelial-growth metabolite(s) in the bacterial spent broth of GIB024 as well as KN1.

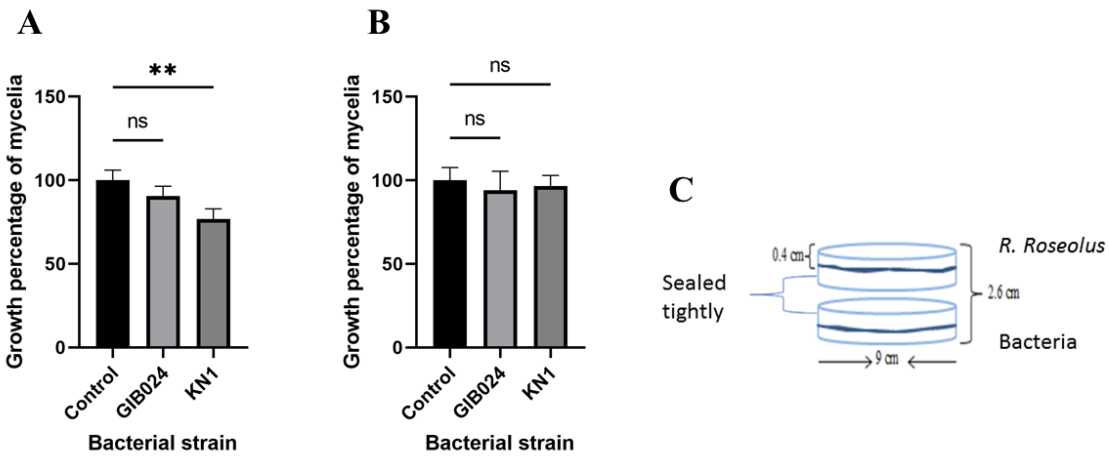



Fig. 2.6 Indirect confrontation of bacterial isolates, GIB024 and KN1, toward the growth of *R. roseolus* mycelia in a closed system 10 days post-confrontation.

The nutrient scenario variation was applied on the assay. (**A**) both *R. roseolus* and bacteria were grown in hs-dMMN. (**B**) both *R. roseolus* and bacteria were grown in ls-dMMN. (**C**) a representative Figure to describe the design of plate petri dish for the indirect confrontation assay, showing the separated position of *R. roseolus* mycelial colony in agar medium (above part) as well as the bacterial colony in agar medium (below part). Statistical analysis of the data was performed through Tukey's multiple comparisons test (two-way ANOVA), the asterisk sign indicates the summary of the comparison test, ** ($P < 0.005$), ns = not significant.

# Chapter 3

# Potential role of organic acids in the mycelial growth promotion of *Rhizopogon roseolus* during interaction with the sporocarp bacterium, *Paraburkholderia fungorum* GIB024

## 3.1 Introduction

Bacterial–fungal interactions (BFIs) are widespread in nature, depending on various abiotic and biotic factors, and can be observed in the form of mutualism, commensalism, or parasitism. In soil ecology, BFIs frequently occur in the rhizosphere, including in mycorrhizal relationships, a form of mutualistic symbiosis between plants and fungi. In mycorrhizal relationships, the fungus colonizes the plant root and promotes plant growth by delivering available water, phosphorus, and minerals; and in exchange, the photosynthetic products of the plant are provided to the fungus (Stuart and Plett, 2020). However, the involvement of surrounding bacteria in this mutualistic symbiotic relationship has led to the use of the term mycorrhizal helper bacteria (MHB) (Garbaye, 1994), indicating a possible tripartite symbiosis plant–fungus–bacterium mechanism (Van Der Heijden et al., 2016).

Mycorrhizal symbiosis influences the bacterial community structure in the mycorrhizosphere, the soil zone that is influenced by the interface-colonized root, and mycorrhizal mycelia. Due to exudation by fungal mycelia, the mycorrhizosphere contains higher amounts of natural energy-rich organic compounds than the bulk soil (Leveau and Preston, 2008) in the form of free sugars, sugar alcohols, organic acids, peptides, and even siderophores (Boer et al., 2005; Winkelmann, 2007). Consequently, these exudates may influence the bacterial community surrounding the fungal mycelium (Duponnois et al., 2008) by acting as a chemical selector for certain mutual microbial groups, thus shaping or shifting

the mycorrhizosphere microbial communities (Sun et al., 2019). Moreover, it is also possible that the fungal mycelium secretes antibiotics as a selection mechanism toward the surrounding beneficial bacteria (Shirakawa et al., 2019).

In mutual interactions, MHB or mycelial-associated bacteria have the capacity to promote the growth of their fungal mycelium counterpart, thus increasing the chance for mycorrhization establishment (Frey-Klett et al., 2007). Previous studies have reported the role of bacterial secondary metabolites in inducing the growth of fungal mycelia. In BFIs, these metabolites seem to determine the specificity of the interactions between bacteria and their fungal counterparts. *Streptomyces* sp. specifically promoted the mycelial growth of an ectomycorrhizal fungus, *Amanita muscaria*, by secreting an auxin-like metabolite identified as auxofuran (Riedlinger et al., 2006).

BFIs, particularly in soil environments, are highly complex processes, and various modes of action of BFIs under natural conditions have been reported. BFIs can occur through physical interactions via direct cell-to-cell interface (Steffan et al., 2020) or chemical interactions via quorum sensing inter-species communication (De Sordi and Mühlschlegel, 2009; Albarracín Orio et al., 2020). The latter mechanism usually involves the use of small molecules for signal communication. However, certain alternative mechanisms are also possible, including modification of environmental pH (Rousk et al., 2009) and utilization of the metabolite by-product (Noble et al., 2009).

In this study, we investigated the interaction between *Rhizopogon roseolus*, an ectomycorrhizal (ECM) mushroom called “shouro” in Japanese, and the bacterium *Paraburkholderia fungorum*. The bacterium was isolated from the sporocarp of *R. roseolus* located in a *Pinus thunbergii* forest. Our previous study (Pramoj Na Ayudhya et al., 2019) showed that the bacterium positively promotes mycelial growth of *R. roseolus* via bacteria

soluble metabolites rather than volatile metabolites. However, the mechanism underlying this mycelial growth promotion of *R. roseolus* by *P. fungorum* remains unknown. Thus, based on our previous findings, we hypothesized the possibility of chemical interactions in interkingdom communication between *R. roseolus* and *P. fungorum*. Because bacteria rely on fungal exudates as the main carbon sources during BFIs, as a first step, we focused on the possible involvement of common carbon compounds associated with fungal mycelia. Therefore, the aim of the current study was to investigate whether specific carbon sources were responsible for *P. fungorum* stimulation of mycelial growth of *R. roseolus*. We designed a two-compartment medium in a non-separated Petri dish to allow the bacterium to grow in a specific sole carbon source and observed whether its metabolite production affected the fungus in another compartment. We also investigated the growth profile of the bacterium in the sole carbon source medium and its influence on environmental acidity.

## 3.2 Materials and Methods

### 3.2.1 Microorganisms and media

The bacterium, *P. fungorum* strain GIB024 (a Gram-negative bacterium belonging to the family *Burkholderiaceae*), was isolated from the fruiting body of a shouro mushroom (*R. roseolus*) located in a Japanese black pine (*P. thunbergii*) dominant forest[16]. The ECM isolate, *R. roseolus* strain TUFC 10010, was provided by Tottori University Fungal Culture Collection, Japan. Prior to use, the bacterium and *R. roseolus* were maintained in potato dextrose agar (PDA; 4 g/L potato extract, 20 g/L dextrose, 15 g/L agar) and diluted 1:5 MMN agar (1 g/L malt extract, 2 g/L glucose, 0.1 g/L $KH_2PO_4$, 0.05 g/L $(NH_4)_2HPO_4$, 0.03 g/L $MgSO_4.7H_2O$, 10 mg/L $CaCl_2$, 5 mg/L NaCl, 20 µg/L thiamine HCl, 1% [w/v] in deionized water 0.24 mL/L $FeCl_3.6H_2O$) with 1.5% w/v agar.

For the direct confrontation assay, we designed a two-compartment media in a non-separated Petri dish (Fig. 3.1). The first compartment medium (FCM) for growing the fungus was diluted 1:5 MMN agar without glucose and thiamine HCl. The second compartment medium (SCM), used as the bacterial medium, was an agar bar with a composition similar to that of FCM without the addition of malt extracts. Instead, the medium was amended with a sole carbon source (1 g/L) that could be grouped as free sugars [D(+)-glucose, D(+)-mannose, D(-)-fructose, D(+)-xylose, L(+)-arabinose, and trehalose], sugar alcohols [D(-)-mannitol, D(-)-sorbitol, and glycerol], organic acids (citric, maleic, malonic, and oxalic acids), biopolymers (chitin and soluble starch), extracts (malt and yeast extracts), and no addition of a carbon source. For simplicity, the chirality notation of free sugars and sugar alcohols is omitted in the following text. Yeast extract was chosen as one of the sole carbon sources because of its rich composition of various complete amino acids (AAs). The AAs were also regarding as a carbon source, beside their nitrogen content. However, instead of using or selecting some AAs, we used yeast extract as a non-selecting source of AAs. For bacterial growth and pH profiling, SCM was used without the addition of agar. The pH of all media was adjusted to 6 ± 0.2 with 1 N NaOH/HCl and wet-sterilized in an autoclave at a temperature of 121 °C for 15 min.

### 3.2.2 Confrontation of fungal mycelium and bacterium in a dual-compartment Petri dish

Approximately 15 mL of warm FCM was solidified in an 8.5-cm Petri dish. A plug (0.6 cm in diameter) of actively growing fungal mycelium (grown for 3–4 weeks) was placed on the fungal initial spot of the first compartment (Fig. 3.1). To activate the mycelium in the compartment, the fungal plug was incubated for three days in a dark chamber at 25°C prior to confrontation with the bacterium.

A colony was picked from a stock bacterial plate (grown on PDA for one week) and inoculated in a 100 mL-conical flask containing 50 mL of sterilized diluted 1:5 MMN for three days in a dark chamber at 25°C without agitation. The bacterial cells were separated from the supernatant by centrifugation at 2200 × g for 20 min. The supernatant was discarded, and sterilized 0.9% w/v NaCl solution was added and mixed with the bacterial cells until the optical density at 600 nm ($OD_{600}$) reached approximately 0.2.

Approximately 15 mL of warm SCM was solidified in a 9-cm Petri dish. The agar was carefully cut into a bar shape with the dimensions 1 × 6 cm. The bar was then placed onto the first compartment in a position that allowed the bacterial line suspension to be streaked 30 mm from the edge of the actively growing fungal mycelium **(**Fig. 3.1**)**. One loop of bacterial suspension was streaked straight (approximately 5 cm) in the middle of the second compartment. As a control, one loop of sterile 0.9% w/v NaCl solution was streaked. The plates were tightly sealed and incubated in a dark chamber at 25°C for 10 days. The increase in mycelial elongation toward and away from the second compartment (bacterial line) from the edge of the initial confrontation was measured as $D_{toward}$ and $D_{away}$, respectively. To express the effect of each sole carbon source on *R. roseolus* mycelial growth in the presence of bacteria or control (without bacteria), the ratio percentage of mycelial growth or $D_{toward}/D_{away}$ (%) was calculated as the ratio percentage of $D_{toward}$ divided by $D_{away}$. $D_{toward}/D_{away}$ (%) above and below 100% indicated the promotion and inhibition effects of the bacteria or control in the tested SCM, respectively. Moreover, an index of mycelial elongation (IME) was calculated by dividing $D_{toward}/D_{away}$ (%) of the bacteria by $D_{toward}/D_{away}$ (%) of the control, to assess the effect of bacterial growth in a specific sole carbon source on *R. roseolus* mycelial growth, whether a promotion outcome (IME > 1) or an inhibition effect (IME < 1).

### 3.2.3 Bacterial growth and pH profiles in various carbon sources

For bacterial growth profile, approximately 40 µL of bacterial suspension ($OD_{600} \approx 0.2$, previously grown in a 100 mL-conical flask containing 50 mL of diluted 1:5 MMN for three days in a dark chamber at 25°C without agitation) was inoculated into a 100 mL-conical flask containing 50 mL of SCM broth. The medium was incubated in a dark chamber at 25°C without agitation. At intervals of 24, 36, 48, 72, 96, and 120 h post-inoculation, CFUs were measured by serial dilution of the liquid medium using 0.9% w/v NaCl solution and subsequent growth in PDA at room temperature for 4–5 days. The CFUs of bacteria were calculated as log CFU/mL of the liquid medium. At every measurement time point, sterile medium was added to the fermentation broth at the same volume as that of the bacterial suspension used for measurement.

For pH profile, first, 100 µL of bacterial suspension ($OD_{600} \approx 0.2$, previously grown in 50 mL of diluted 1:5 MMN for three days in a dark chamber at 25°C without agitation) was inoculated into 100 mL of SCM broth. The medium was incubated in a dark chamber at 25°C without agitation. Every 12 or 24 h post-inoculation, the pH of the fermented broth was measured using a calibrated Horiba PH210 pH-meter (Horiba, Japan). At every measurement time point, sterile medium was added to the fermentation broth at the same volume as that of the bacterial suspension used for measurement.

### 3.2.4 Effect of sterilized bacterial suspension on *R. roseolus* growth

Approximately 100 µL of bacterial suspension ($OD_{600} \approx 0.2$, previously grown in a 100 mL-conical flask containing 50 mL of diluted 1:5 MMN for three days in a dark chamber at 25°C without agitation) was inoculated into a 300 mL-conical flask containing 100 mL of SCM broth (1 g/L maleic acid, pH 6 ± 0.1). The medium was incubated in a dark chamber at

25°C without agitation for 7 days. The bacterial suspension was then autoclaved at 121°C for 15 minutes.

As much as 30, 150, 250, and 400 µL of sterilized bacterial suspension was circularly put around the active edge of the three-day *R. roseolus* (initial diameter of mycelial plug is 0.6 cm) in order to allow immediate contact with the mycelial tip. All the Petri dishes were sealed tightly and then incubated in a dark chamber at 25°C. After 9-day incubation, the mycelial diameter was observed. Percentage of increasing mycelial elongation by sterilized bacterial suspension (%) is the percentage of increasing mycelial elongation in agar with sterilized bacterial suspension (mm) divided by the increasing mycelial elongation in agar with control, namely uninoculated broth (mm). Average percentage of increasing mycelial elongation by control is 100%.

### 3.2.5 Data Analysis

All assay procedures were conducted in triplicate. Statistical analysis of the percentage of mycelial growth or $D_{toward}/D_{away}$ (%) and the percentage of increasing mycelial elongation by application of sterilized bacterial suspension was performed using Šídák's multiple comparison test (two-way analysis of variance [ANOVA]). Statistical analysis of the scattering plot of IME was performed using Tukey's multiple comparison test (ordinary one-way ANOVA). The area under the curve (AUC) of the bacterial growth profile was calculated using GraphPad Prism software, version 9.3.1 (GraphPad Software, Inc.). Statistical analysis of the pH profile was performed using a multiple comparison test (ordinary one-way ANOVA). P-values are provided in each figure containing statistical analysis data. If not stated, the asterisk indicates the P-value summary of the comparison test in each figure legend, while 'ns' indicates no significant difference ($P > 0.05$). All data and illustrations were processed using GraphPad Prism software, version 9.3.1.

## 3.3 Results

The carbon source for the growth of *P. fungorum* GIB024 significantly influenced the bacterial interaction with *R. roseolus* (Fig. 3.2). Therefore, the positive, neutral, and inhibitory outcomes of mycelial growth elongation by the bacterium toward the fungus depend on the available carbon source. A positive interaction was observed when the bacterium grew in all low-molecular-weight organic acid compounds as the sole carbon source, with maleic acid giving a significant promotion outcome (Fig. 3.2C). Besides the organic acid group, a positive bacterial mycelial growth-promoting effect was also observed in the medium containing yeast extract, although the difference was not significant, suggesting that the AAs contained in the yeast extract play a role in the growth support of *R. roseolus* (Fig. 3.2D). The effects of bacteria that grew in free sugars, sugar alcohols, chitin, soluble starch, and malt extract as sole carbon sources on the growth of *R. roseolus* varied from inhibition to neutral, but promoting effects were not observed (Fig. 3.2A–B, 3.2D–E). In the medium without any amendment with a carbon source, an inhibitory effect was observed (Fig. 3.2F). The carbon compound itself, without bacterial inoculation, showed growth supporting properties toward fungal mycelial growth, as indicated by $D_{toward}/D_{away}$ (%) of the control bar being 100%. The highest growth supporting carbon sources for each group were xylose in the free sugar group (Fig. 3.2A), glycerol in the sugar alcohol group (Fig. 3.2B), citric acid in the organic acid group (Fig. 3.2C), yeast extract in the extract group (Fig. 3.2D), and soluble starch in the biopolymer group (Fig. 3.2E).

A comparison between the groups showed that bacterial mycelial growth promotion in the organic acid group was significantly different from that in the free sugar and sugar alcohol groups (P value of 0.0023), but was not significantly different from that of the extracts, biopolymers, and medium without the addition of a carbon source (Fig. 3.3).

Moreover, the calculation of IME showed that the bacteria grown in compounds belonging to the organic acid group as the sole carbon source had promotional effects (average IME < 1), while the other groups, including both the free sugar and sugar alcohol groups, tended to inhibit the mycelial growth of *R. roseolus* (average IME > 1).

As the sole carbon source, organic acid compounds supported the growth of GIB024 better than free sugars and sugar alcohols (Fig. 3.4). Under glucose, mannitol, and citric acid, the bacterium entered the stationary phase after around 36 h of fermentation, with an average bacterial population of 8.3, 8.3, and 8.6 log CFU/mL, respectively. However, when grown in malonic acid and trehalose, the growth exponential phase was extended to 96 h, with an average bacterial population of 8.9 and 8.9 log CFU/mL, respectively. Moreover, the AUC calculation of the bacterial growth profile up to 120 h resulted in organic acids exceeding free sugars and sugar alcohols in the following order: citric acid > malonic acid > mannitol > glucose > trehalose.

Suspension of *P. fungorum* GIB024 was able to alkalinize the medium environment (initial pH was set to 6 ± 0.1) in the presence of organic acids as a carbon source, but acidification occurred when free sugars and sugar alcohols were used as the only carbon sources in the medium (Fig. 3.5). When malonic and oxalic acids were used as the sole carbon sources, the pH of the fermentation medium was relatively stable for the first 24 h, but gradually increased to 8.0 and 8.8, respectively, after 96 h of inoculation, before reaching a plateau. On the other hand, when the bacterium used glucose as the sole carbon source, the pH of the medium was relatively unchanged for the first 12 h, but rapidly plummeted to 4.1 and 3.2 after 24 and 48 h of inoculation, respectively, before reaching a stage where there was no further change. Similarly, in the medium containing mannitol as the sole carbon source, the first 24 h of fermentation exhibited a relatively stable pH, which quickly dropped to 3.6 during the next 12 h, and continuously decreased to 3.2 at 48 h post-inoculation,

without any change in the following hours. In the medium consisting of organic acids (1 g/L) mixed with free sugars/sugar alcohols (1 g/L), the pH of the fermentation medium was increased, but not as high as that in the sole organic acid medium or relatively unchanged compared to the medium with the sole free sugar/sugar alcohol. The pH of the medium containing mannitol supplemented with malonic or oxalic acid showed relatively similar patterns up to the first 36 and 48 h, respectively. In the mixture of mannitol and malonic acid, the pH increased gradually up to the end of observation (168 h), but when malonic acid was replaced by oxalic acid, the pH reached a plateau 36 h post-inoculation. In the mixture of glucose and malonic acid, the pH of the medium was relatively unchanged from the initial pH. However, in the mixture of glucose and oxalic acid, the pH slightly and gradually dropped to 5.0 at 60 h post-inoculation, before reaching a plateau.

The similar pattern tendency of the pH profile of the bacterium in the mixed carbon sources and organic acids only suggests that *P. fungorum* GIB024 prefers organic acids rather than free sugar/sugar alcohol as the main carbon source. This notion was strengthened by calculating the P value between the carbon source and medium composition (Table 3.1). Statistical analysis showed that the P value of the comparison between free sugars/sugar alcohols and the mixture of free sugars/sugar alcohols and organic acids (Table 3.1A) was lower than the P value of organic acids vs. the mixture of free sugars/sugar alcohols and organic acids (Table 3.1B). Moreover, the P value of the comparison between free sugars/sugar alcohols and organic acids was significantly different (Table 3.1C, similar to Table 3.1A). The P-value comparison between free sugars and sugar alcohols (Table 3.1D) and between organic acids (Table 3.1E) was not significantly different. However, the P value of the comparison of organic acids and the mixture of mannitol and organic acids was higher than that of organic acids and a mixture of glucose and organic acids (Table 3.1B), indicating

that glucose might be preferable to mannitol; however, whether the bacterium prefers malonic or oxalic acid remains unclear.

Soluble metabolites produced by *P. fungorum* GIB024 grown in organic acids have roles in the promotion effect toward *R. roseolus* (Fig. 3.6). Based on the direct confrontation assay, the bacterium grown in organic acids, particularly in maleic acid, had a positive impact on the mycelial growth of the fungus (Fig. 3.2C). Therefore, we chose maleic acid as the sole carbon source for the bacterial medium in order to assess its possible mycelial growth-promoting metabolites. The application of sterilized bacterial suspension had a positive impact on the mycelial elongation of *R. roseolus*. However, the positive impact was dependent on the volume of broth suspension added. The average percentage of mycelial elongation of *R. roseolus* after the addition of 30, 150, 250, and 400 µL of sterilized bacterial suspension was 95.3, 96.6, 96.8, and 109.7%, respectively. Statistical analysis showed that the comparison between the average percentage of mycelial elongation by sterilized bacterial suspension and uninoculated broth medium (control) was significantly different ($P < 0.05$) when 400 µL of the bacterial suspension was added.

## 3.4 Discussion

Our data suggest that there is a possible chemical trade-off between *P. fungorum* and *R. roseolus* mycelia mediated by organic acids in the mycorrhizosphere microenvironment. Organic acids exuded by fungal mycelia can be utilized by the bacterial community surrounding the mycelia as a carbon source. Our data showed that *P. fungorum* GIB024 grew better in organic acids than in free sugars or sugar alcohols. Moreover, there is also a tendency for the bacterium to prefer organic acids over free sugars/sugar alcohols. In exchange, the bacterium produces and exudes mycelial-growth-promoting metabolites that increase fungal growth. Such mutualistic interaction patterns mediated by chemical exchange

have been reported in previous studies. In a recent study (Deveau et al., 2010), trehalose, a fungal sugar exuded by an ectomycorrhizal fungus, *Laccaria bicolor*, improved the growth of a fungal-associated bacterium, *Pseudomonas fluorescens*, while the bacterium synthesized thiamine to a concentration that improved the growth of the fungus. Trehalose is a unique sugar that is commonly produced by fungal mycelia as a reserve carbon source, particularly in unfavorable conditions (Fillinger et al., 2001), and plays an important role in mutual BFIs. Another study showed that *Pseudomonas monteilii* grown in a medium supplemented with trehalose produced volatile metabolites that improved the mycelial growth of the ectomycorrhiza *Pisolithus albus* (Duponnois and Kisa, 2006). The role of trehalose in BFIs was also found in arbuscular mycorrhizal symbiotic interactions, particularly under abiotic stress conditions (Sharma et al., 2020). However, based on our data, we found that organic acids, but not trehalose, play important roles as mediators of BFIs, particularly in promoting the mycelial growth of *R. roseolus* by *P. fungorum.*

Organic acids are one of the major groups of carbon exudates released by the mycelia of filamentous fungi to the surrounding microenvironment. However, compared to fungal sugars and sugar alcohols, organic acids are unlikely to be taken up by the mycelium post-exudation (Sun et al., 1999). By elevating the level of acidity, organic acids perform various ecological roles, such as acceleration of soil mineral weathering (Drever and Vance, 1994) and dissolution of insoluble organic and inorganic phosphate (Andrino et al., 2021; Napitupulu et al., 2021). In BFIs, fungal organic acids act as bacterial chemoattractants toward fungal mycelia (Rudnick et al., 2015), shape the bacterial community structure surrounding the mycorrhizosphere zone, and stimulate their activity (Sun et al., 2019; Olsson and Wallander, 1998). However, to the best of our knowledge, no study has been conducted on the utilization of fungal organic acids by fungal-associated bacteria to produce ectomycorrhizal mycelia growth-promoting metabolites. However, recent studies have shown that a wide range of soil

bacteria, including mycelium-associated bacteria, utilize organic acids as carbon and energy sources (Pion et al., 2013; Macias-Benitez et al., 2020). We found that our mycelium-associated bacterium was not only consuming organic acids, but that they were also essential for its positive interaction with its fungal counterpart.

*Burkholderiaceae* members can utilize organic acids that are abundantly present in the exudates of plant roots or fungal mycelia. *Burkholderia* is a major bacterial genus associated with the roots of white lupin, comprising up to 58% of cumulative culturable bacterial species isolates (Weisskopf et al., 2011). Bioassays of these isolates showed that the bacteria were able to utilize organic acids, such as citrate and oxalate, which were abundant as exudates in the white lupin rhizosphere. Another similar study (Kost et al., 2014) showed that oxalic acid secreted by white lupin roots was not only used as a carbon source by *Burkholderia* sp., but also as a signal for root colonization. There may also be a chemical trade-off between *Burkholderia* sp. and plant roots (Li et al., 2018). In that study, after sensing the organic acid exuded by poplar roots, *B. multivorans* secreted a phytohormone, indole-3-acetic acid, which increased the root growth and resulted in greater organic acid exudate. Two poplar-associated microbes, the bacterium *Burkholderia* strain BT03 and the filamentous fungus *Mortierella elongata*, showed a type of metabolite exchange in which the bacterium produced unidentified mycelial-growth-promoting metabolite(s) after sensing the organic acid exuded by the fungal mycelia (Uehling et al., 2019). These findings support our results regarding the characteristics of *Burkholderiaceae* members that utilize organic acids during their interkingdom interactions, particularly with their fungal counterparts.

Exogenous organic acids can stimulate the growth of not only *P. fungorum* but also *R. roseolus*. Similarly, some previous studies showed that supplementation of organic acids improves the mycelial growth of fungi as well as bacteria. Humic acid, a high molecular weight organic acid that is naturally derived from the decomposition of plant debris, has a

positive impact on the growth of two saprophytic mushrooms, *Pleurotus ostreatus* and *Lentinus sajor-caju* (Zahid et al., 2020). Moreover, rhizospheric organic acids also stimulated soil bacteria, determining the bacterial community in the soil (Macias-Benitez et al., 2020). However, to the best of our knowledge, no study has been conducted regarding the co-stimulation of mushrooms, particularly ectomycorrhizal mushrooms, along with their mutualistic surrounding bacteriome after the addition of exogenous organic acids.

*P. fungorum* has an acidity shifting capacity toward its surrounding environment, which might influence its interkingdom interactions, particularly with the fungal mycelia. Environmental pH has a very significant impact on survival and microbial interactions between intra- and inter-species in various ecological niches (Ratzke and Gore, 2018). Our data showed that *P. fungorum* strain GIB024 was able to utilize organic acids as the sole carbon source and produce alkaline compounds, thereby increasing the environmental pH, while the opposite outcome was observed when free sugars and sugar alcohols were used as the sole carbon sources. In a medium containing organic acids supplemented with sugars or sugar alcohols, the environmental pH of the medium was increased or relatively unchanged. These results indicate that the available carbon source affected the acidity of the extracellular environment during bacterial growth. Similar to our findings, Gram-negative bacteria (*Escherichia coli* and *Pseudomonas* spp.) showed acidification with a reduced carbon source (glucose and glycerol) and alkalinization with an oxidized carbon source (fumarate and 2-oxoglutarate) in a previous study (Sánchez-Clemente et al., 2020). Consequently, the modification of environmental pH by *P. fungorum* GIB024 bacteria provides hints of the possible mechanisms for mycelial growth of *R. roseolus*. However, increasing the environmental pH medium surrounding the mycelial tip of *R. roseolus* as a consequence of organic acid utilization by *P. fungorum* GIB024 might be omitted because some previous reports have shown that mycelial growth of *Rhizopogon* spp. was optimized under acidic

conditions (Yamanaka, 2003; Islam and Ohga, 2013; García-Rodríguez et al., 2017). Therefore, there is a possibility of metabolite(s) produced by the bacterium being involved as chemical signals during BFIs between *P. fungorum* and *R. roseolus*.

The specificity of bacterial and fungal co-occurrence in BFIs via diffusible metabolite exchange is suggested to be evolutionarily conserved. The molecules that play roles in this interkingdom signaling were previously considered to be "waste" products, however, several studies have revealed their prominent roles in BFIs. *Collimonas* sp., a fungus-feeding soil bacterium, utilizes oxalic acid that is exuded from the apical tip of the fungal mycelia, thus guiding the bacterium toward its target zone (Rudnick et al., 2015). Moreover, volatile compounds also mediate BFIs. For example, a native mushroom growth-promoting bacterium, *Pseudomonas putida*, initiates primordium formation and promotes the growth of the white button mushroom (*Agaricus bisporus*) by consuming mycelium-inhibiting volatile compounds produced by the fungal mycelia (Noble et al., 2009; Rainey, 1991). Biotin and iron are conserved drivers of BFIs, whereas antibiotics produced by the fungi determine the specificity of these interactions (Pierce et al., 2021).

More advanced biological models are needed to confirm the possible roles of organic acids in *P. fungorum–R. roseolus* interactions. Our current knowledge regarding the possibility of organic acid utilization by *P. fungorum* GIB024 reveals prospective topics for future research, which can contribute to enhancing our understanding of the mechanisms of BFIs for various bioprospecting applications. Designed microcosms in *in planta* model systems are needed to confirm the roles of organic acids in tripartite symbiotic plant–ectomycorrhizal fungus–bacterium systems, with a focus on the mycorrhizosphere microenvironment. Moreover, according to the pH profile of the fermentation broth, *P. fungorum* GIB024 is indicated to prefer organic acids to free sugars/sugar alcohols. Therefore, it is necessary to confirm this selectivity of available fungal-related carbon sources

by bacteria in real-time bacterial–fungal mycelium interfaces. Lastly, determination of the possible metabolites produced by bacteria is necessary to further our understanding of this metabolite exchange mechanism in *P. fungorum–R. roseolus* interaction.

The impact of organic acid utilization by *P. fungorum* and its interaction with *R. roseolus* can be exploited for several bioprospecting purposes, such as soil bioremediation, revegetation of degraded land, and mushroom cultivation. Ectomycorrhizal fungi accumulate organic acids as a coping mechanism in unfavorable conditions, mainly during phosphorus starvation (Zhang et al., 2014) or in the presence of high concentrations of heavy metals (Ahonen-Jonnarth et al., 2000; Ray and Adholeya, 2009). Therefore, there is a possibility of co-application of *P. fungorum* and *R. roseolus* in symbiosis with the plant host under abiotic stress. We hypothesized that under these conditions, the addition of bacteria would benefit the ectomycorrhizal fungi–plant host association by buffering the environmental pH and producing mycelial growth-promoting metabolites, thereby enhancing the survival capacity of the plant hosts and ectomycorrhizal fungi. In mushroom cultivation, owing to the presence of possible growth enhancer metabolites, the application of bacterial spent broth or identified metabolites to the extrametrical hyphae can influence the fungal mycelial growth. It is also possible that these suspected metabolites or the bacterium itself are able to induce primordial formation, as shown by the example of the primordium-inducing effect of *Bradyrhizobium* sp. toward *Laccaria parva* (Obase, 2020). Finally, the co-stimulation effect of both the bacterium as well as its fungal counterpart by additional of exogenous organic acids shows potential in application to mushroom cultivation, to improve the growth of fungal mycelia and increase the population of mycelial growth-promoting bacteria.

Our data suggest that organic acids play important roles in mediating mutual BFIs between *P. fungorum* and *R. roseolus*. Moreover, the trade-off or metabolite exchange may be a possible mechanism of interkingdom communication. Organic acids may be secreted by

fungal mycelia, selected specifically among other carbon compounds by the bacterium, as the main and preferable carbon source. In exchange, the bacterium synthesizes mycelial growth-promoting metabolites that are beneficial for fungal survival. However, further investigations using more advanced biological models are required to support this notion.

Table 3.1 P-values of pH profile comparisons among A (free sugars/sugar alcohols vs. mixture of free sugars/sugar alcohols and organic acids), B (organic acids vs. mixture of free sugars/sugar alcohols and organic acids), C (free sugars/sugar alcohols vs. organic acids), D (free sugars vs. sugar alcohols), and E (between organic acids). Statistical analysis of the data was conducted using Tukey's multiple comparison test (ordinary one-way analysis of variance [ANOVA]). P values less than 0.05 were considered to be statistically significant.

| **Comparison group of carbon source** | **Comparison of carbon source** | **P value** | **Summary** |
|---|---|---|---|
| A | Glucose vs. Glucose + Malonic acid | <0.0001 | significant difference |
| | Glucose vs. Glucose + Oxalic acid | <0.0001 | significant difference |
| | Mannitol vs. Mannitol + Malonic acid | <0.0001 | significant difference |
| | Mannitol vs. Mannitol + Oxalic acid | <0.0001 | significant difference |
| B | Malonic acid vs. Mannitol + Malonic acid | 0.0241 | significant difference |
| | Malonic acid vs. Glucose + Malonic acid | 0.0005 | significant difference |
| | Oxalic acid vs. Mannitol + Oxalic acid | 0.3634 | no significant difference |
| | Oxalic acid vs. Glucose + Oxalic acid | <0.0001 | significant difference |
| C | Glucose vs. Malonic acid | <0.0001 | significant difference |
| | Glucose vs. Oxalic acid | <0.0001 | significant difference |
| | Mannitol vs. Malonic acid | <0.0001 | significant difference |
| | Mannitol vs. Oxalic acid | <0.0001 | significant difference |
| D | Glucose vs. Mannitol | 0.9717 | no significant difference |
| E | Malonic acid vs. Oxalic acid | 0.4895 | no significant difference |

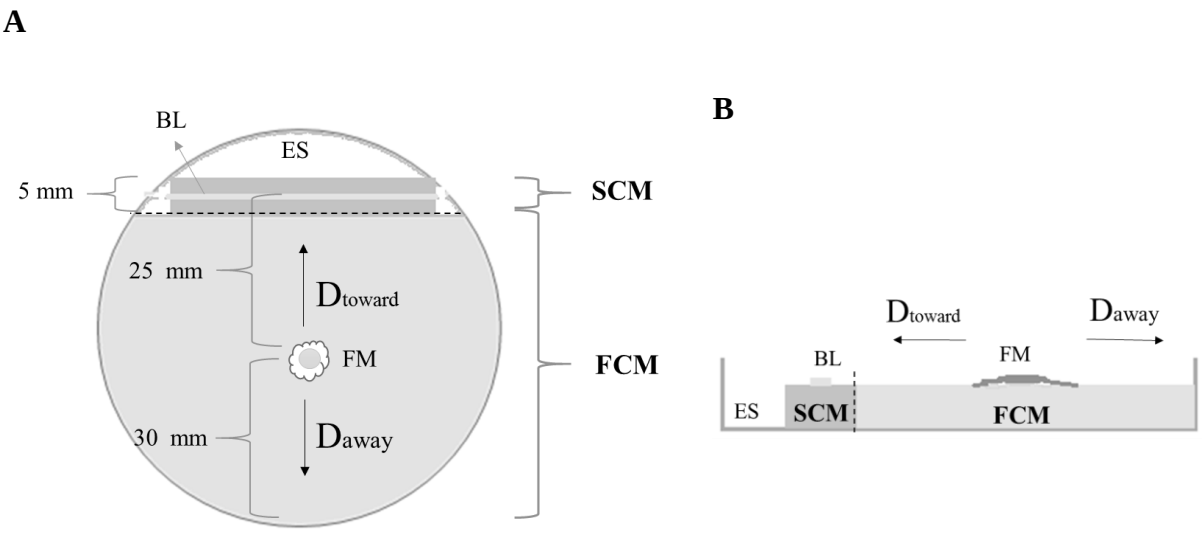



Fig. 3.1 Design of the dual compartment Petri dish for direct confrontation assay between *P. fungorum* GIB024 and *R. roseolus.*

A: top view of the dual compartment; B: side view of the dual compartment. The first compartment medium (FCM) for growing the fungal mycelium (FM) was diluted 1:5 MMN without glucose and thiamine HCl. The second compartment medium (SCM) for growing the bacterial line (BL) was an agar bar of similar composition to FC, without the addition of malt extracts, but amended with a sole carbon source (concentration 1 g/L), leaving an empty space (ES). After 10 days of bacterial inoculation, the increasing mycelial elongation toward and away from the second compartment (BL) from the edge of the initial confrontation was measured as $D_{toward}$ and $D_{away}$, respectively.

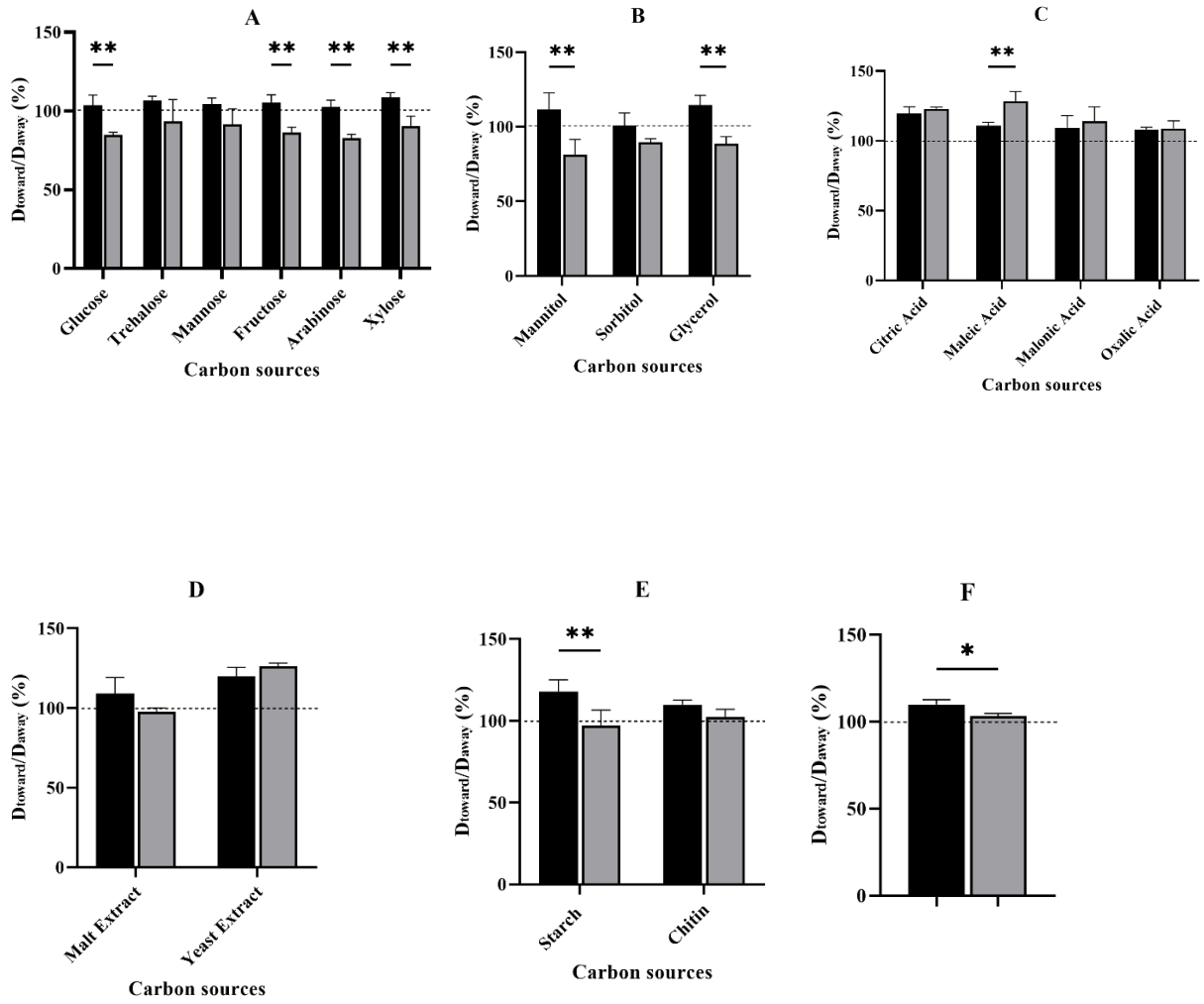



Fig. 3.2 Ratio percentage of mycelial growth or $D_{toward}/D_{away}$ (%) of *R. roseolus* in the first compartment medium (FCM) as the effect of 15-day confrontation with the second compartment medium (SCM) inoculated with *P. fungorum* GIB024 (grey bar) or 0.9% w/v NaCl solution (black bar).

SCM contained a sole carbon source, which was divided into 6 groups; A: free sugars; B: sugar alcohols; C: organic acids; D: extracts; E: biopolymers; and F: no addition of carbon source. Dotted line (---) marked 100% indicates the percentage in the condition that the length of $D_{toward}$ is equal to $D_{away}$; below the mark means inhibition and above the mark means promotion. The asterisk indicates a summary of Šídák's multiple comparison test

(two-way analysis of variance [ANOVA]) that compares the mean of control (black bar) versus the mean of bacterial treatment (grey bar) in each respective column, *($P < 0.05$), **(P: 0.05 – 0.01). No asterisk indicates no significant difference ($P > 0.05$).

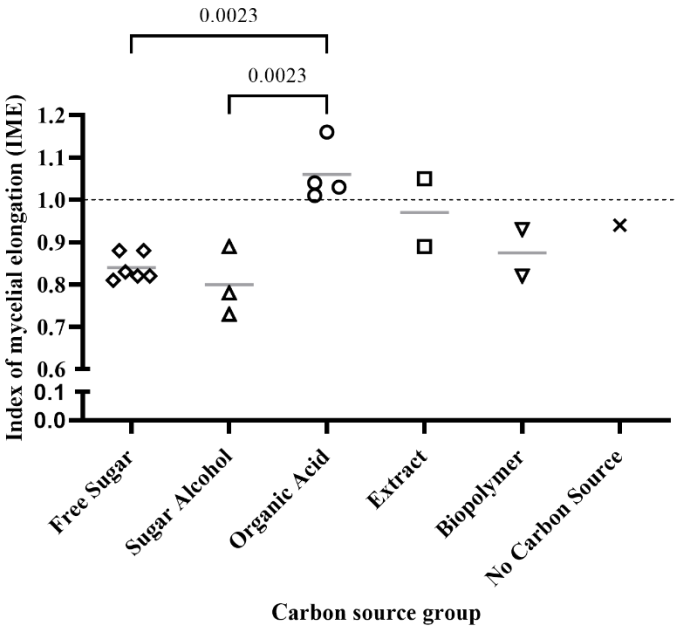
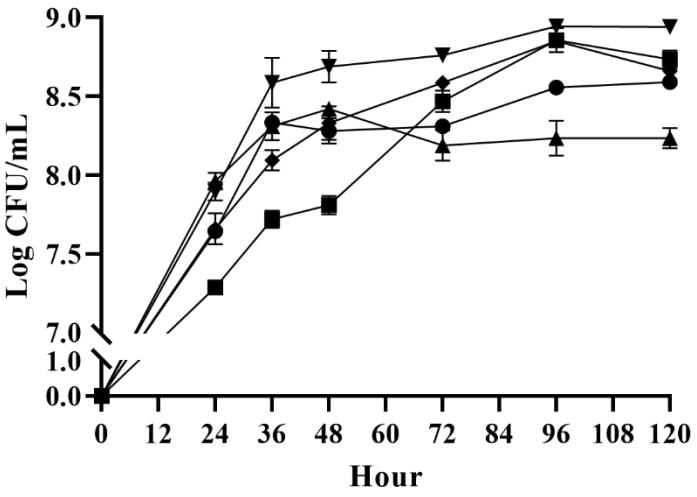



Fig. 3.3 Scatter plot of the index of mycelial elongation (IME) of *R. roseolus* in Petri dishes containing each carbon source, which were clustered into 6 groups of free sugars (rhombus), sugar alcohols (up-pointing triangle), organic acids (circle), extracts (square), biopolymers (down-pointing triangle), and no addition of carbon source (cross-mark).

The IME of each individual carbon source was calculated as the ratio of $D_{toward}/D_{away}$ (%) of GIB024 to 0.9% w/v NaCl solution (control). The average IME of each group is depicted as a grey dash. Dotted line (---) marked IME1 indicates that the $D_{toward}/D_{away}$ (%) of GIB024 is equal to $D_{toward}/D_{away}$ (%) of the control. Statistical analysis of the data was performed using Tukey's multiple comparison test (ordinary one-way ANOVA) by comparing the mean IME of each group. P values greater than 0.05 are not shown (indicating no significant difference).




| Carbon Sources | Area Under Curve (AUC) |
|---|---|
| Mannitol | 894.5 |
| Glucose | 887.5 |
| Trehalose | 885.0 |
| Citric acid | 933.8 |
| Malonic acid | 907.3 |

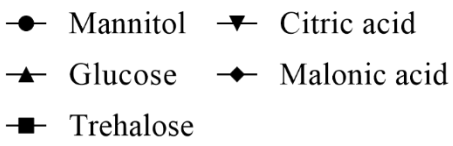



Fig. 3.4 Bacterial growth curves of *P. fungorum* GIB024 in various representative sole carbon source groups: free sugars (glucose in up-pointing triangle and trehalose in square), sugar alcohols (mannitol in circle), and organic acids (citric acid in down-pointing triangle and malonic acid in rhombus).

The bacterium was grown at 25°C in a dark chamber without agitation (static condition). The area under the curve (AUC) was calculated using GraphPad Prism software, version 9.3.1.

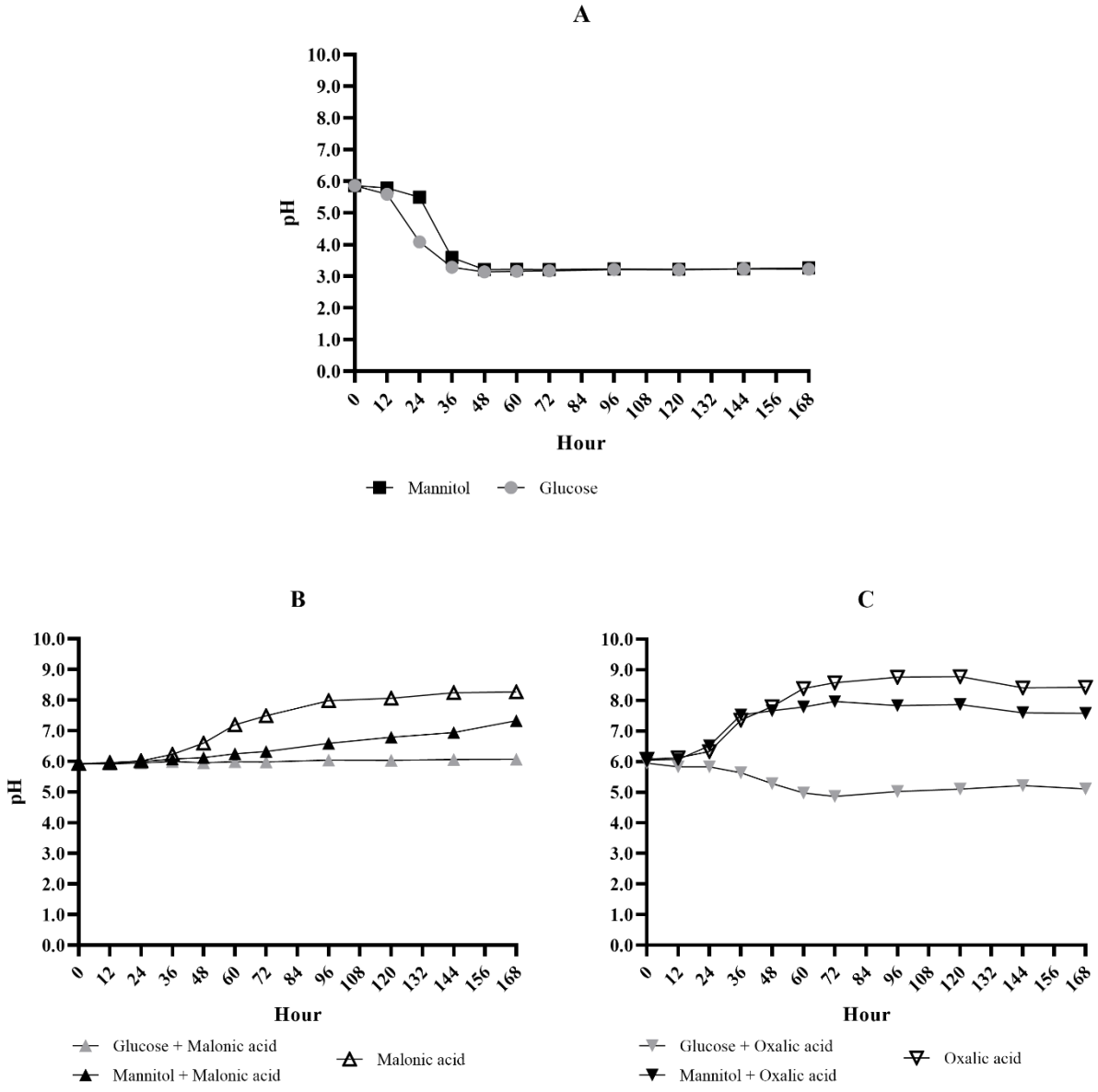



Fig. 3.5 The pH profiles of *P. fungorum* GIB024 during 168 h of fermentation in the presence of various carbon source groups.

A: free sugars/sugar alcohols only (glucose in grey circle and mannitol in black square), B: mixture of free sugars/sugar alcohols and malonic acid (malonic acid in white up-pointing triangle, glucose + malonic acid in grey up-pointing triangle, and mannitol + malonic acid in black up-pointing triangle), C: mixture of free sugars/sugar alcohols and oxalic acids (oxalic acid in white down-pointing triangle, glucose + oxalic acid in grey down-pointing triangle,

and mannitol + oxalic acid in black down-pointing triangle). The bacterium was grown at 25°C in a dark chamber without agitation (static condition).

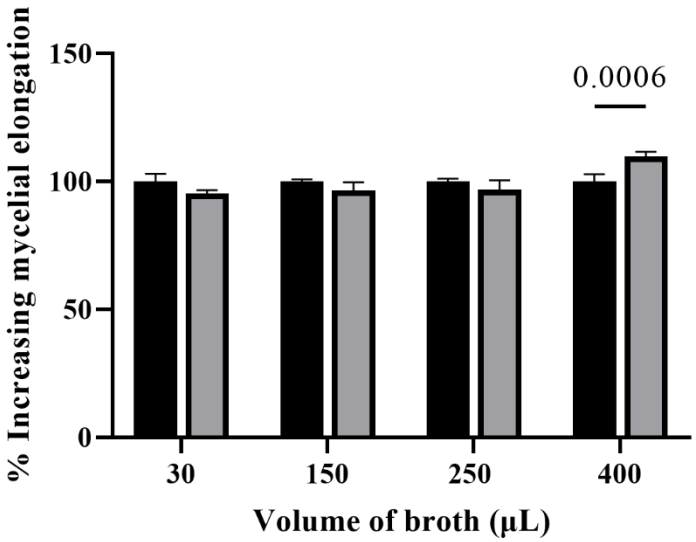



Fig. 3.6 The percentage increasing mycelial elongation of actively growing *R. roseolus* 8 days after application of sterilized bacterial suspension grown in 1 g/L maleic acid with pH 6 ± 0.1 (grey bar) and uninoculated broth as control (black bar) in various volumes of broth.

Statistical analysis of the data was performed using Šídák's multiple comparison test (ordinary two-way ANOVA) by comparing mean % increasing mycelial elongation between grey bar and black bar for each addition volume of broth. P values greater than 0.05 are not shown (indicating no significant difference).

# Chapter 4

# *Paraburkholderia fungorum* GIB024 specifically promotes the mycelium growth of ectomycorrhizal fungi

## 4.1 Introduction

In a mycorrhizal relationship, the fungus helps promote plant growth in exchange for the photosynthetic products of the plant. However, bacteria are also found to be involved in this symbiotic relationship between plants and fungi, and these are termed mycorrhizal helper bacteria (MHB) (Garbaye, 1994; Frey-Klett et al., 2007), thus forming a tripartite symbiosis with plant-fungus-bacterium (Bonfante and Anca, 2009).

The fruiting body or sporocarp is the most visible part of the fungus on which spore-producing structures are formed. It harbors unique bacteria that might be involved in the symbiotic relationship between plants and ECM fungi; however, these remain relatively unexplored compared with bacteria in rhizospheric or 'hypospheric' soil (Liu et al., 2018). In this study, bacteria from the fruiting body of *Rhizopogon roseolus* (an edible mushroom called “shouro” in Japanese) which was associated with *Pinus thunbergii* (Japanese black pine) were isolated and screened for their effect on fungal mycelial growth (Pramoj Na Ayudhya et al. 2019). Six of 19 isolated bacteria demonstrated the potential to promote R. roseolus mycelial growth in the in vitro confrontation test. All these isolates were also identified by molecular analyses, and were found to be Gram-negative and belonged to Burkhorderiales.

*Paraburkholderia fungorum* was found as one the *R. roseolus* sporocarp-associated bacteria that has significant mycelial growth promoting ability (Pramoj Na Ayudhya et al., 2019). Studies have shown that this bacterial species was ubiquitous in various ecological

niches, as free-living bacterium in soil (de Oliveira Longatti et al., 2013) but also associated with living organisms (Coenye et al., 2001) including plant (Rahman et al., 2018), insects (Itoh et al., 2019), mammals (Tan et al., 2020), and human (Loong et al., 2019). *P. fungorum* also has potency to be utilized for various bioprospecting purposes including bioremediation (Andreolli et al., 2011; Liu et al., 2019) and crop biofertilizing (de Oliveira-Longatti et al., 2015; Castanheira et al., 2016), although an indication of its human pathogenicity (Zhang et al., 2014; Nally et al., 2018) might be raise some concerns regarding its field applications. *P. fungorum* was also associated with several fungi. It was been found co-occurance with phytopathogenic molds such as *Alternaria alternata* and *Fusarium solani* (Stopnisek et al., 2016) a white rot fungus *Phanerochaete chrysosporium* (De Felice et al., 2016), as well as an ectomycorrhizal mushroom (Pramoj Na Ayudhya et al., 2019).

However, the ecological roles of *P. fungorum* toward *R. roseolus* in ectomycorrhiza symbiotic system are still remained unknown. Due to the bacterium high versatility and wide pervasive co-occurance with various organisms in diverse ecological niche, specificity of our *P. fungorum* isolate toward host fungi, particularly with other ectomycorrhizal, become important in the ecological perspective of ectomycorrhiza symbiosis. Therefore, the aim of the current study was to investigate the specificity of *P. fungorum* isolated from *R. roseolus* sporocarp in the *Pinus thunbergii* forest on other ECM growth. The parameter of bacterial fitness toward the fungal counterpart was focused on the mycelia growth of the fungus. In the term of mycorrhiza symbiosis, the capacity of bacteria to enhanced the growth of fungal mycelia is an indication of the roles of the bacteria as MHB, since increasing mycelia growth resulting higher chance for promotion of mycorrhiza establishment (Frey-Klett et al., 2007).

## 4.2 Materials and methods

### 4.2.1 Microorganisms and media

In this study, 4 gram-negative bacterial strains that belong to Burkholderiales (Table 1) were employed. One bacterium, GIB024 was used as main bacterium for bioassay. All bacteria were isolated from the sporocarp of *R. roseolus* in the *P. thunbergii* forest (Pramoj Na Ayudhya et al. 2019). The bacterial isolates were maintained in Potato dextrose agar (PDA) plate in dark incubator (25 $^{o}$C) prior to use. The ECM fungal strains for bioassay were provided by Tottori University Fungal Culture Collection (TUFC), Japan, and divided into two groups. The first group consisted of various genera of ECM fungi (Table 2). The second group was consisted of ECM fungi from *Suillus* genus only (Table 3). All the ECM fungal isolates were initially grown in diluted 1:5 MMN agar plate (glucose 2 g/L, malt extract 1 g/L, $(NH_4)_2HPO_4$ 0.05 g/L, $KH_2PO_4$ 0.1 g/L, $MgSO_4.7H_2O$ 0.03 g/L, NaCl 5 mg/L, $CaCl_2$ 10 mg/L, Thiamine HCl 20 µg/L, $FeCl_3.6H_2O$ 1% (w/v in deionized water) 0.24 mL/L, and agar 15 g/L) supplemented with combination of antibiotics (ampicillin 100 µg/mL, kanamycin 50 µg/mL, tetracycline 10 µg/mL, and ciprofloxacin 40 µg/mL) to remove all possible exo- and endo-hyphal bacterial contaminants. After 7 days, one plug (0.6 cm in diameter) of fungal mycelial margin was transferred to diluted 1:5 MMN agar plate without antibiotics and incubated in dark condition (25 $^{o}$C) prior to use for the bioassay experiments. The volume of maintaining agar media in this study was 15 mL

### 4.2.2 Dual culture (Direct confrontation) between bacterial isolate vs fungal isolates

For initial round assay, the diluted 1:5 MMN agar plate in the absence of Thiamine HCl and supplemented with 1.5% agar (w/v) with three variation of glucose concentration: 2 g/L,

1 g/L, and 0 g/L used as media for dual culture assay. For the second assay, the diluted 1:5 MMN agar plate without glucose only was used. One plug (0.6 cm in diameter) of fungal mycelial (grown on diluted 1:5 MMN agar plate for 3-4 weeks) from the active part was put on the edge of agar medium in the petri dish. The fungal strains were incubated in dark incubator in temperature of 25°C for one week prior to the dual culture assay.

One colony was picked from bacterial plate (grown on PDA for 1-2 weeks) and inoculated in 50 mL of diluted 1:5 MMN for 3 days in dark incubator in temperature of 25°C without agitation. The bacterial cells were then separated from the supernatant by centrifugation at 2200 × *g* for 20 minutes. The supernatant was discarded, and sterilized 0.9% w/v NaCl solution was added and mixed with the bacterial cells until the $OD_{600}$ reach around 0.2.

One loop of bacterial suspension was put circularly 25 mm away from the active edge of one-week fungal strain (initial diameter plug of mycelium is 0.6 cm). The sterilized 0.9% w/v NaCl solution was used as control. The plates were then incubated in dark incubator in temperature of 25°C, and the mycelial elongation growth was observed after 15 days. Percentage of increasing mycelial growth is the percentage of increasing mycelial length in agar with bacterial suspension divided by the increasing mycelial diameter in agar without bacterial suspension (control).

### 4.2.3 Investigation of the volatile compounds produced by the bacteria trough indirect confrontation assay

One colony was picked from bacterial plate (grown on PDA for 1-2 weeks) and inoculated in 50 mL of diluted 1:5 MMN for 3 days in dark incubator in temperature of 25°C without agitation. The bacterial cells were then separated from the supernatant by

centrifugation at 2200 × *g* for 20 minutes. The supernatant was discarded, and sterilized 0.9% w/v NaCl solution was added and mixed with the bacterial cells until the $OD_{600}$ reach around 0.2.

A two-compartment petri dish was used for the assay. On one side, the active mycelium of fungus (initial diameter plug of mycelium is 0.6 cm) was put on the center of the compartment and incubated in room temperature without light. After one week, one loop of bacterial suspension was put in zigzag pattern (total length around 9 cm) on the other compartment. The sterilized 0.9% w/v NaCl solution was used as control. The petri dish was sealed tightly with parafilm and was incubated in dark chamber in temperature of 25°C, and the mycelial diameter was observed weekly. Percentage of increasing mycelial growth is the percentage of increasing mycelial length in agar with bacterial suspension divided by the increasing mycelial diameter in agar without bacterial suspension (control).

### 4.2.4 Investigation of the sterilized bacterial suspension on the growth of ECM strain

One colony was picked from bacterial plate (in PDA) and inoculated in 50 mL of 1:5 MMN without glucose for 3 days in dark incubator in temperature of 25°C without agitation. The bacterial suspension was then autoclaved in 121 °C for 15 minutes.

As much 40 µL of sterilized bacterial suspension was put circularly around the active edge of one-week fungal strain (initial diameter plug of mycelium is 0.6 cm) to let the mycelium tip contact immediately with the sterilized bacterial suspension. All the petri dishes were sealed tightly with parafilm and were incubated in dark chamber in temperature of 25°C, and the mycelial diameter was observed after 15 days. Percentage of increasing mycelial growth is the percentage of increasing mycelial length in agar with bacterial extract

divided by the increasing mycelial diameter in agar without sterilized bacterial suspension (control).

### 4.2.5 Effect of *Pinus* spp. on the growth of bacterium

Seeds of *P. thunbergii* and *P. densiflora* were immersed in running tap water for 12 hours. The seeds then surface sterilized by soaking in $H_2O_2$ 30% for 20 min, rinsed 5 times with sterile water. The excess water was removed by put the seeds in laminar air flow until dry completely. The seeds then put in to petri dish on the surface of water agar (2%), sealed tightly for 4 weeks.

As much 1 g fresh seedlings of *Pinus* spp. were put in 300 mL Erlenmeyer flask containing 1:5 MMN liquid medium without glucose, and autoclaved at 121$^{o}$C for 5 minutes. The liquid part than separated from plant remnant by filtering through Minisart® 0.45 µm filter paper. 25 mL of this fortified medium was mixed with 25 mL of fresh 1:5 MMN liquid medium without glucose, autoclaved at 121$^{o}$C for 15 minutes, and cooled. The control medium was 50 mL 1:5 MMN liquid medium without glucose. Further, these media were inoculated with 10 µL of bacterial suspensions of GIB024 ($OD_{600}$ ≈ 0.2) that previously grown in 1:5 MMN liquid medium with glucose for 3 days in dark chamber with temperature of 25$^{o}$C. The bacterium was incubated for 3 days in dark chamber with temperature of 25$^{o}$C. The colony forming unit (CFU) was measured by serial dilution using 0.9% w/v NaCl solution and grown in Potato Dextrose Agar (PDA) for 5 days at room temperature. The CFU of bacterium was calculated as Log CFU/mL of liquid medium.

### 4.2.6 Data Analysis

All the assay procedures were repeated in triplicate. Statistical analysis of the data for dual culture was performed using unpaired t test of the ECM monoculture versus treatment

with the bacterium, where the asterisk indicates the P value of the comparison test, * (P < 0.05), **(P: 0.05 – 0.01), ***(P: 0.01 – 0.001), ****(P > 0.001). Without asterisk sign means not significantly different (P> 0.05). For the correlation analysis of increasing mycelial growth after confrontation with the bacterium in 1:5 MMN liquid medium with various glucose concentration (2, 1, and 0 g/L), a plot was made for each ECM strain (data not shown). The plots were then analyzed for obtaining 1/slope and $R^2$ (R squared) values, representing best fit values as well as the goodness of fit, respectively. For the influence of pine extract on the growth of bacterium, the Dunnett's multiple comparisons test of control vs plant extract was performed, and the P value numbers were shown in the plot. All data and illustrations were processed using GraphPad Prism software version 9.2.0.

## 4.3 Results

On the first-round assay, sugar (in this case glucose) concentration defined the interaction between GIB024 and ECM strain. For the benefit of ECM fungi, the low sugar concentration was preferable than high sugar condition (Fig. 4.1A, B, and C) during the bacterial-fungal interaction. Except in *Rhizopogon roseolus* and *Russula bella*, decreasing sugar concentration alleviated the inhibition effect during direct confrontation of ECM strain with GIB024. For most ectomycorrhizal fungi that confronted with the bacterium, the addition of glucose to the medium was negatively correlated with the mycelium growth of fungi ($R^2$ > 0.7; negative 1/ slope; see Table 4.4). As the bacterium was isolated from the sporocarp of *R. roseolus*, GIB024 did not show significant inhibition effect on high sugar medium (supplemented with 2 g/L of glucose). However, decreasing glucose concentration to 1 g/L, the bacterium then promoted the growth of *R. roseolus*. Furthermore, in the medium without glucose, the effect of bacterium was neutral toward *R. roseolus* growth. In this low sugar condition, GIB024 significantly promoted the mycelial growth of *Suilus bovinus*

TUFC31988 among other genera of ECM strain (Fig. 4.1C), even though it was inhibited in the condition of higher sugar (glucose) concentrations.

Focused on the ECM strain in *Suilus* genus associated with various plant hosts that belong to *Pinaceae*, it is found that the bacterium specifically promoted the mycelium growth of *Suilus bovinus*, but not other *Suilus* spp. GIB024 that isolated from fruiting body of *R. roseolus* in *Pinus thunbergii* forest promoted the mycelium growth of *S. bovinus* associated with *P. thunbergii* as well (Fig. 4.2). However, it is also found that not all mycelium of *S. bovinus* strain was promoted by the bacterium. The mycelial growth of *S. bovinus* TUFC100731 that associated with *P. densiflora* was not promoted by the bacterium. Nevertheless, not all ECM fungal strain associated with *P. thunbergii* are promoted by the bacterium. The growth of *S. granulatus* TUFC31933 inhibited by GIB024, despite its plant host is *P. thunbergii*. Plant host then plays a significant role in determining the specificity interaction of the bacterium toward mycelium growth of the fungal counterpart.

The impact of GIB024 on other *Suilus* spp. beside *S. bovinus* associated with host tree other than *P. thunbergii* showed variability, from neutral to inhibition, but not promotion. The bacterium had a neutral or inhibition effect on the growth of *S. granulatus* in association with *P. luchuensis*, and unidentified *Pinus* spp. In *Suilus* spp. associated with *Larix* spp., the bacterium displayed inhibitory effects on *S. grevillea* and *S. viscidus*, but neutral on *S. cavipes* as well as *S. americanus* in association with *P. koraiensis*.

The mycelial growth of *Suilus* spp. through direct confrontation with other additional bacteria (GIB028, GIB029, and KN1) that isolated from *R. roseolus* fruiting body in *P. thunbergii* forest as well showed similar pattern with the GIB024 (Fig. 4.3). The coefficient of variation percentage (%CV) of the percentage mycelial growth of *Sulius* spp. in confrontation with all bacterial isolates is ranging from 2.3 – 15.9 %. This result suggested

that community of bacteria isolated from similar source of isolation has similar mycelial growth promoting specificity toward ectomycorrhizal mushroom. All bacteria showed promotion effect on *S. bovinus* TUFC31988 and TUFC100826. Moreover, the bacterial isolates have inhibition of mycelial growth on *S. grevillei* TUFC101227. On *S. granulatus* TUFC31933 and TUFC31903 as well as *S. cavipes* TUFC100673, the bacterial isolates showed inhibition to neutral effect.

Non-volatile bacterial metabolites play important role on the mycelial growth promoting of GIB024 toward *Suilus bovinus*. According to indirect confrontation assay of GIB024 and ECM strain (Fig. 4.4A), the growth of *S. bovinus* TUFC31988, TUFC100822, and TUFC100826 were relatively similar with monoculture, although these fungi showed significantly promotion growth through direct confrontation with the bacterium (Fig. 4.2). On the other hand, the application of sterilized bacterial suspension to the actively growing mycelia of these fungi exhibited promotion effect on *S. bovinus* TUFC31988 and TUFC100826 (Fig. 4.4B).

The plant host influences the growth *P. fungorum* GIB024. *P. thunbergii* extract was significantly stimulate the growth of the bacterium ($P < 0.005$), as shown in Fig. 4.5. As comparison, the growth of bacterium in the medium contains extract of *P. densiflora*, another pine host, was shown not significantly different in compare with the medium without pine extract.

## 4.4 Discussion

Nutrient status, particularly carbon (sugar) source concentration, determines the interaction of bacteria and fungi, from antagonism to mutualism. During bacterial-fungal interaction (BFI), low sugar condition is mostly preferable for the benefit of either fungal or

bacterial part. However, this condition can be varied depended on the fungal or bacterial species and natural habitat of the microorganisms. Trough series of in vitro pairwise assays with various nutrient scenarios, bacteria and fungi isolated from oligotrophic desert oasis showed dominant mutualistic interactions in low nutrient media, but mostly antagonistic in rich nutrient media (Velez et al., 2018). Moreover, composition of organic carbon as well as nitrogen also contributed to frame the BFI. In the interaction between *Pseudomonas* spp. and *Morchella* spp, the bacteria were able to enhance fungal proteolytic enzymatic activity to breakdown organic nitrogen source that solely the fungus can degrade (Lohberger et al., 2019). The abundance of nutrient particularly carbon source in the mycorrhizosphere and fruiting body were also determined the bacterial biodiversity and their interaction with mycelial of fungus. Fruiting body with the spore producing structure has richer nutrient environment compared to the mycorrhizosphere zone (Kalač, 2013). *Paraburkholderia fungorum* GIB024 isolated from fruiting body of *Rhizopogon roseolus* showed significant mycelial growth promoting activity toward *R. roseolus* in the medium with additional glucose 1 g/L while exhibited neutral effect in the medium without supplementation of glucose. On the other hand, dual culture with *Suilus bovinus* TUFC31988, the bacterium inhibited the mycelium growth on the medium with glucose 1 g/L, and showed significantly promotion effect on the medium without glucose. This glucose-dependent effect implied that there is possibility that the bacterium promoted the growth of ectomycorrhizal fungi on the specific part of fungus life cycle. Since GIB024 was isolated from fruiting body of *R. roseolus*, it is possible that the mycelial growth promoting activity of the bacterium is specifically occurred on the sugar rich condition such as fruiting body microenvironment as compared to mycorrhizosphere or hyphosphere soil, while the bacterium promoted *Suilus bovinus* growth in nutrient poorer condition such as might be in soil instead of in fruiting body. The growth inhibition effect of bacteria toward most fungal counterpart in the condition with nutrient

abundance such as in the medium with high glucose concentration can be related to stress-gradient hypothesis. In this environment with abundance nutrient available, during cross inter-kingdom interaction, bacteria were reported to more producing antifungal than in nutrient-poorer environment (Sabat and Gupta, 2010).

Mycelial growth promoting of *Paraburkholderia fungorum* GIB024 toward ectomycorrhizal fungi shows species-dependent and plant-host-dependent (Fig. 4.6). In the low sugar medium, the mycelial-growth promoting activity of the bacterium specifically observed in *Suillus bovinus*, but not other *Suillus* spp. It is important to be noted that the selectivity of the bacterium is plant-host-dependent as well, in this case toward *S. bovinus* associated with *Pinus thunbergii*. The bacterial fitness toward mycorrhizal fungi that show species-dependent combined with plant-host-dependent specificity has been previously reported. In the arbuscular mycorrhiza association, an indigenous *Oxalobacteriaceae* bacterium specifically promoted saprophytic growth *Glomus mosseae* as well as its root colonization on the plant host *Medicago truncatula* (Pivato *et al.*, 2009). Furthermore, another example of bacterial specificity toward arbuscular mycorrhiza fungi reported by (Jäderlund et al., 2008) that showed root colonization and growth of wheat was enhanced by certain combination of arbuscular mycorrhiza fungus species and plant growth promoting rhizobacteria, along with the presence of phytopathogenic fungus. For the ectomycorrhiza mushroom, an example of specificity of indigenous bacteria toward the ectomycorrhizal fungus growth and establishment with plant host was shown by (Duponnois et al., 1993). Using microcosm in planta assay, MHB isolated from Douglas fir – *Laccaria laccata* were promoted the ectomycorrhiza formation of *L. laccata* but inhibit other ECM fungi. However, another study showed that some MHB isolated from *Pinus sylvestris* – *Lactarius rufus* exhibited some degree of specificity toward various ECM fungi beside *L. rufus* including *Laccaria bicolor* (Aspray et al., 2006).

The role of plant host in determining the specificity of bacteria toward growth along with formation or mycelial architecture of ECM fungi is still vague and need more investigations. This current study suggesting that the plant host might be improve or stimulate the growth of bacterium, while the plant itself as a host has a specificity role to select the ECM fungi. The specificity of bacteria that inhabiting or associating with plant root is mediated by root exudates (Bais et al., 2006) but the process is characterized by both the plant host (Van Loon, 2007) as well as the bacterium (do Amaral et al., 2020). Possibly, the bacterial fitness in the rhizosphere microenvironment in the term of bacterial growth stimulation is able to pave the way for more intense interaction with suitable ECM fungi, thus improving the fungal mycelial growth. Furthermore, the mechanism of how the fungi recruit the specific bacteria from surrounding environment in order to establish a mutualistic BFI itself is related to their ability to provide suitable nutrient for bacteria (Nazir et al., 2009), releasing bacterial chemoattractant (Emmett et al., 2021), or selection mechanism via secreting antibiotics (Shirakawa et al., 2019). Combining this complex nature of interactions leaded to a hypothesis of tripartite symbiosis plant host – ectomycorrhizal fungus – bacterium. This tripartite interaction has been raised to study more comprehensively in order to understand the roles of bacteria in mycorrhiza symbiosis (Bonfante and Anca, 2009; Van Der Heijden et al., 2016).

According to this bioassay, *P. fungorum* GIB024 is specifically promoted mycelial growth of Suilloid fungi (*Suillus* and *Rhizopogon*). The co-occurance and association of *Burkholderia* bacteria with Suilloid fungi have been reported by several previous investigations. Along with *Pseudomonas* genera, *Burkholderia* has been found as one of the most abundant culturable endobacteria from ECM fungal root tip of *Pinus nigra* - *S. variegatus* association (Izumi and Finlay, 2011). Moreover, *Burkholderia* species isolated from mantel of *R. luteolus* on the root of *P. radiata* has even mycelial growth promoting

ability toward the fungus as well as enhancing the mycorrhizal formation (Garbaye and Bowen, 1989). *Burkholderia* spp. were also reported as primarily culturable bacteria associated with *S. bovinus – P. sylvestris* (Timonen and Hurek, 2006). This bacteria genus was even found as dominance in external mycelia, plant short roots as well as mycorrhizal roots, but interestingly along with other culturable bacterial genera, the bacterial genus is absence in the sporocarp of the ECM fungi. It is then, due to a frequent co-occurrence and association of *Burkholderia* species with Suilloid fungi that have intimate interaction with *Pinaceae*, this is regarding an interesting as well as important part of symbiotic relationship in tripartite interaction, specifically *Pinaceae* - Suilloid fungi - *Burkholderiaceae*.

The indigenous bacterial strains, along with *P. fungorum* GIB024, showed similar mycelial growth specificity pattern on the influence the growth of *Suillus* spp. All these selected bacteria were belonged to gram-negative bacteria. Similarly, an in vitro bioassay in undiluted MMN of bacteria isolated from sporocarp of *S. grevillei* associated with *Larix desidua* showed that the mycelial growth promoting ability was dominance by gram-negative bacteria rather than gram-positive (Varesel et al., 1996). It is also possible that a certain dominance ECM colonizing root associated with distinct and specific bacterial community compared to neighboring ECM colonizing root. In more cutting-edge technique, using culture independent technique, several studies showed that bacterial community associated with ECM colonizing root is depended on fungal species as well as modulated by various environmental factors (Izumi and Finlay, 2011; Rúa et al., 2016; Yang et al., 2019).

Soluble non-volatile metabolite(s) produced by the bacterium during confrontation with the ECM fungi, rather than volatile metabolite(s), plays roles on the promoting or inhibition effect toward the ECM fungal counterpart. The specificity of the interaction between *P. fungorum* GIB024 with ECM fungi is then rely on the soluble metabolite synthesized by the bacterium. The interaction between interkingdom organism such as bacteria and fungi in soil

microenvironment can be occurred by molecular communication or physical interaction or combination by both (Frey-Klett et al., 2011). For the molecular communication, particularly, the mechanism could be mediated through exchanging and conversion metabolites, chemotaxis, signaling, adhesion, antibiosis, or modulation of environmental physicochemical condition (Bonfante and Anca, 2009; Deveau et al., 2018). The possible soluble metabolites produced and released by bacteria can be specific compound that targeted a narrow species of ECM fungi as shown in our study, by combining direct confrontation assay (Fig. 4.2), indirect confrontation assay (Fig. 4.4A), and sterilized bacterial suspension assay (Fig. 4.4B). In the sense of positive interaction, a study conducted by (Riedlinger et al., 2006) showed auxofuran, an auxin-like signaling molecule of *Streptomyces* sp., specifically promoted the mycelial growth of *Amanita muscaria*. Furthermore, fungal inhibition as a selection mechanism by bacteria was also showed by Norway spruce associated *Streptomyces* spp. toward some ECM fungi as well as phytopathogenic fungi, and the bacterial antifungal metabolites were involved in this negative outcome (Schrey et al., 2012). However, it is also showed that non-specific compounds produced by bacteria such as thiamine were involved as growth enhancer toward fungal mycelia (Deveau et al., 2010).

In ecological perspective, specificity of bacteria toward ECM fungal growth might be an indication that bacteria surrounding and associated with ECM fungi and plant hosts play important roles on determining as well as selecting the symbiotic interaction between ECM fungi and plant host. However, the most common understanding is that the dominance ectomycorrhiza fungi shaped the surrounding bacterial communities (Izumi and Finlay, 2011; Li et al., 2017; Yang et al., 2019) through some selection mechanisms including exudating specific nutrient such as fungal sugar or organic acids (Frey et al., 1997; Dutton and Evans, 1996) and antibiosis (Shirakawa et al., 2019). Limitation particularly artificial condition such as agar plate-based assay used in this study which is different with natural condition in soils

with more complex variable was major obstacle to raise a more comprehensive picture regarding this hypothesis. This current study then serves as a preliminary study for the investigation of various factors determining the specificity of bacteria toward ECM fungi, emphasizing on the role of plant host for the specificity pattern.

The study and the derived knowledge of bacterial specificity toward ECM fungi in association with plant host are not without some possible implications to be applied in bioprospecting of not only in mushroom science and biotechnology but also in wood cultivation and forestry. The mycorrhizal helper bacteria (MHB) were reported to have a potency to initiate fruiting body formation (Obase, 2020). Moreover, selectivity of bacteria to promote specific ECM fungi and inhibit growth of other ECM fungi can be taken as soil fumigation for ECM mushroom cultivation application, that considerably more environmentally friendly than using synthetic chemicals. Some MHB were also known to have roles as plant growth promoting rhizobacteria or PGPR (Navarro-Ródenas et al., 2016). Furthermore, a study conducted by (Axelrood et al., 1996) showed that rhizobacteria associated with root of an ectomycorrhizal plant specifically inhibited several fungal plant pathogens, but the specificity of these bacteria toward root associated ECM fungi was not determined.

The recent findings of this study pave the way for some possible further studies including crossed-specificity study of bacteria and elucidation the chemical structure of possible bacterial metabolite(s) that responsible for mycelial growth promoting effect. This study was focused on the bacteria isolated from *R. roseolus* associated with *P. thunbergii* as plant host. The results showed that, the bacteria significantly promoted not only *R. roseolus* but also *S. bovinus* associated with *P. thunbergii* as well. However, we have not investigated the bacteria associated with ECM fungi from other plant hosts. If the similar bioassay of this study was conducted to isolated bacteria from ECM fungi associated with other plant hosts,

for example *S. bovinus* TUFC100731 associated with *P. densiflora* or *S. grevillei* TUFC101227 associated with *Larix* spp., there is a possibility to study of so-called crossed-specificity phenomenon that will enhance our understanding regarding the specificity of bacteria associated with ECM fungi (Fig. 4.6). The phenomenon Furthermore, it is indicated from our results, the bacterial specificity toward ECM fungi relied on the bacterial soluble non-volatile metabolite(s). The identification as well as elucidation of the suspected metabolite will increase our knowledge on the mechanism mycelial-growth promoting ability of the bacteria toward their fungal counterpart during bacterial-fungal interaction.

As conclusion**,** the specificity of mycelial-growth promoting ability of the bacterium is depended on sugar concentration, ECM fungal species, and plant host association of the bacterium as well as the ECM fungi. In low sugar medium (diluted 1:5 MMN without glucose), *P. fungorum* GIB024, isolated from *R. roseolus* sporocarp in *P. thunbergii* forest, specifically promotes the growth of *S. bovinus* associated with *P. thunbergii*, a similar plant host with the bacterium originated isolation, but not promoted *S. bovinus* or other *Suillus* spp. associated with other *Pinaceae* host, and the specificity is depended on the bacterial soluble metabolite produced. However, in diluted 1:5 MMN with addition of glucose 1 g/L, the sporocarp bacterium promote the growth of *R. roseolus* that associated with *P. thunbergii*, but inhibited the growth of *S. bovinus* associated with *P. thunbergii*.

Table 4.1 Bacterial strains used in this study.

| **Bacterial strain code** | **Species name** | **Family** |
|---|---|---|
| GIB024 | *Paraburkholderia fungorum* | *Burkholderiaceae* |
| GIB028 | *Cabaleronia sordidicola* | *Burkholderiaceae* |
| GIB029 | *Janthinobacterium agaricidamnosum* | *Oxalobacteraceae* |
| KN1 | *Paraburkholderia caledonica* | *Burkholderiaceae* |

Table 4.2 Ectomycorrhizal (ECM) fungal strains used for first round dual culture bioassay.

| **EMF strain code** | **Species name** | **Family** | **Order** |
|---|---|---|---|
| TUFC100818 | *Amanita citrina* | *Amanitaceae* | Agaricales |
| TUFC101204 | *Amanita rubescens* | *Amanitaceae* | Agaricales |
| TUFC100672 | *Boletus asiaticus* | *Boletaceae* | Boletales |
| TUFC100970 | *Boletus subtomentosus* | *Boletaceae* | Boletales |
| TUFC100722 | *Laccaria amethystina* | *Hydnangiaceae* | Agaricales |
| TUFC101044 | *Laccaria bicolor* | *Hydnangiaceae* | Agaricales |
| TUFC101211 | *Laccaria vinaceoavellanea* | *Hydnangiaceae* | Agaricales |
| TUFC100632 | *Lyophyllum semitale* | *Lyophyllaceae* | Agaricales |
| TUFC30169 | *Lyophyllum shimeji* | *Lyophyllaceae* | Agaricales |
| TUFC101385 | *Pisolithus arhizus* | *Sclerodermataceae* | Boletales |
| TUFC10010 | *Rhizopogon roseolus* | *Rhizopogonaceae* | Boletales |
| TUFC101232 | *Russulla bella* | *Russulaceae* | Russulales |
| TUFC31988 | *Suillus bovinus* | *Suillaceae* | Boletales |
| TUFC101130 | *Tricholoma bakamatsutake* | *Tricholomataceae* | Agaricales |
| TUFC31964 | *Tricholoma matsutake* | *Tricholomataceae* | Agaricales |

Table 4.3 *Suillus* spp. with various plant host association used for second round dual culture bioassay.

| **EMF strain code** | **Species name** | **Plant host** |
|---|---|---|
| TUFC100731 | *Suillus bovinus* | *Pinus densiflora* |
| TUFC31988 | *Suillus bovinus* | *Pinus thunbergii* |
| TUFC100822 | *Suillus bovinus* | *Pinus thunbergii* |
| TUFC100826 | *Suillus bovinus* | *Pinus thunbergii* |
| TUFC31933 | *Suillus granulatus* | *Pinus thunbergii* |
| TUFC10624 | *Suillus granulatus* | *Pinus luchuensis* |
| TUFC31903 | *Suillus granulatus* | Unidentified *Pinus* spp. |
| TUFC10521 | *Suillus granulatus* | Unidentified *Pinus* spp. |
| TUFC101227 | *Suillus grevillei* | *Larix* spp. |
| TUFC100669 | *Suillus viscidus* | *Larix* spp. |
| TUFC100673 | *Suillus cavipes* | *Larix* spp. |
| TUFC100729 | *Suillus americanus* | *Pinus koraiensis* |

Table 4.4 Correlation analysis of increasing mycelial growth of ECM fungal strains as confronted with *P. fungorum* GIB024 in 1:5 MMN liquid medium with various glucose concentration (2, 1, and 0 g/L). Parameters of analysis were 1/slope and $R^2$ representing best fit values as well as the goodness of fit, respectively.

| Parameter \ TUFC | 100722 | 101044 | 101211 | 100632 | 30169 | 101385 | 101232 | 31988 | 31964 | 10010 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1/slope | -0.033 | -0.047 | -0.036 | -1.592 | -0.038 | -0.065 | 0.094 | -0.023 | -0.084 | -1.279 |
| $R^2$ | 0.7293 | 0.8148 | 0.9596 | 0.0028 | 0.9753 | 0.5241 | 0.8071 | 0.8162 | 0.8057 | 0.0015 |

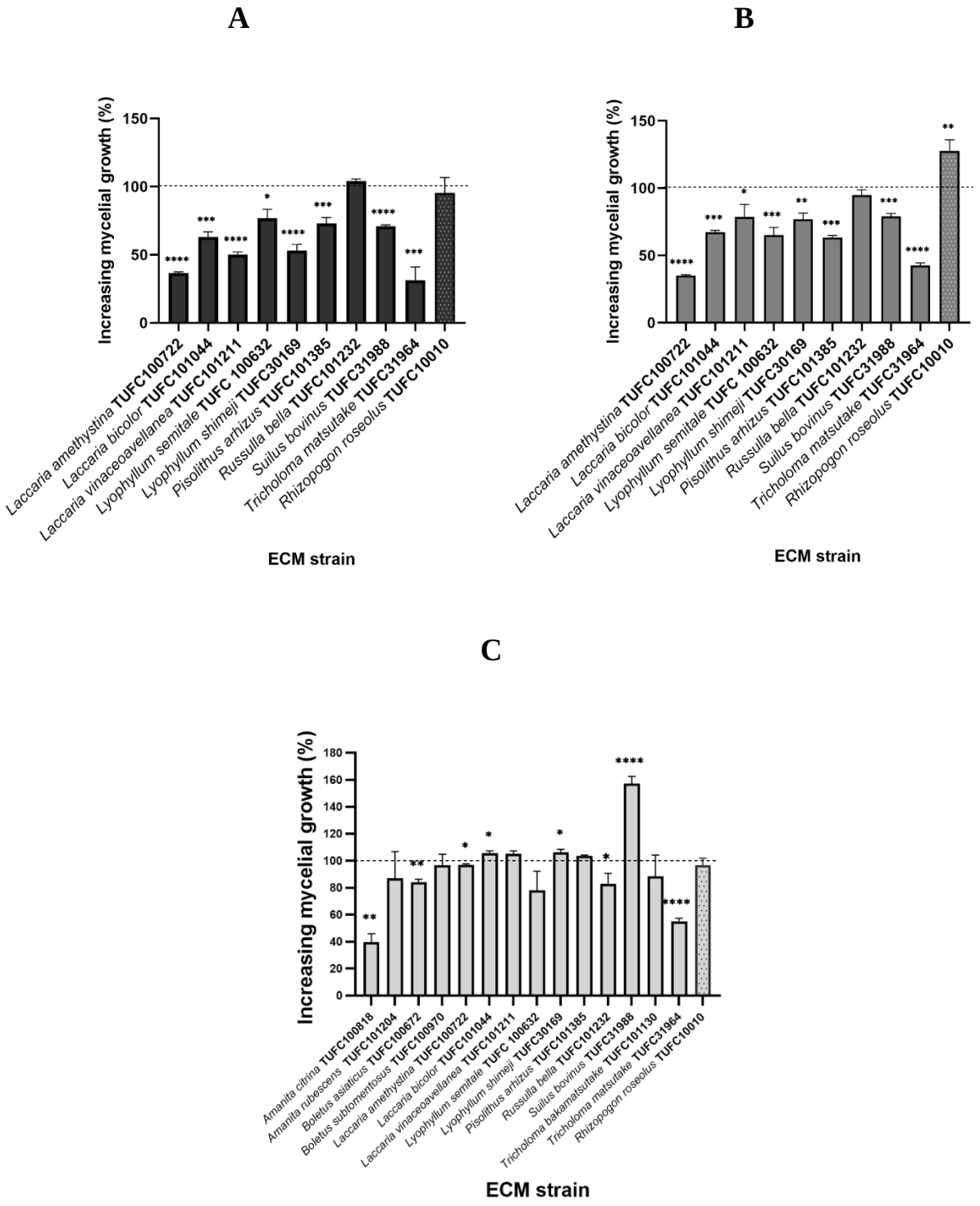



Fig. 4.1 The mycelium growth percentage of various genera of ECM fungal strain as the confrontation with *Paraburkholderia fungorum* GIB024 in diluted 1:5 MMN agar plate with addition of glucose 2 g/L (A), 1 g/L (B), and 0 g/L glucose (C) post 15-day of bacterial

inoculation. *Rhizopogon roseolus* as the sporocarp source isolation of the bacterium was emphasized with the pattern bar.

Dotted line (---) marked 100%, indicating the percentage of control, below the mark means inhibition, above means promotion. The asterisk sign was indicated the summary of the unpaired t test of the ECM monoculture versus treatment with the bacterium, * ($P < 0.05$), **(P: 0.05 – 0.01), ***(P: 0.01 – 0.001), ****($P > 0.001$). Without asterisk sign means not significantly different ($P > 0.05$).

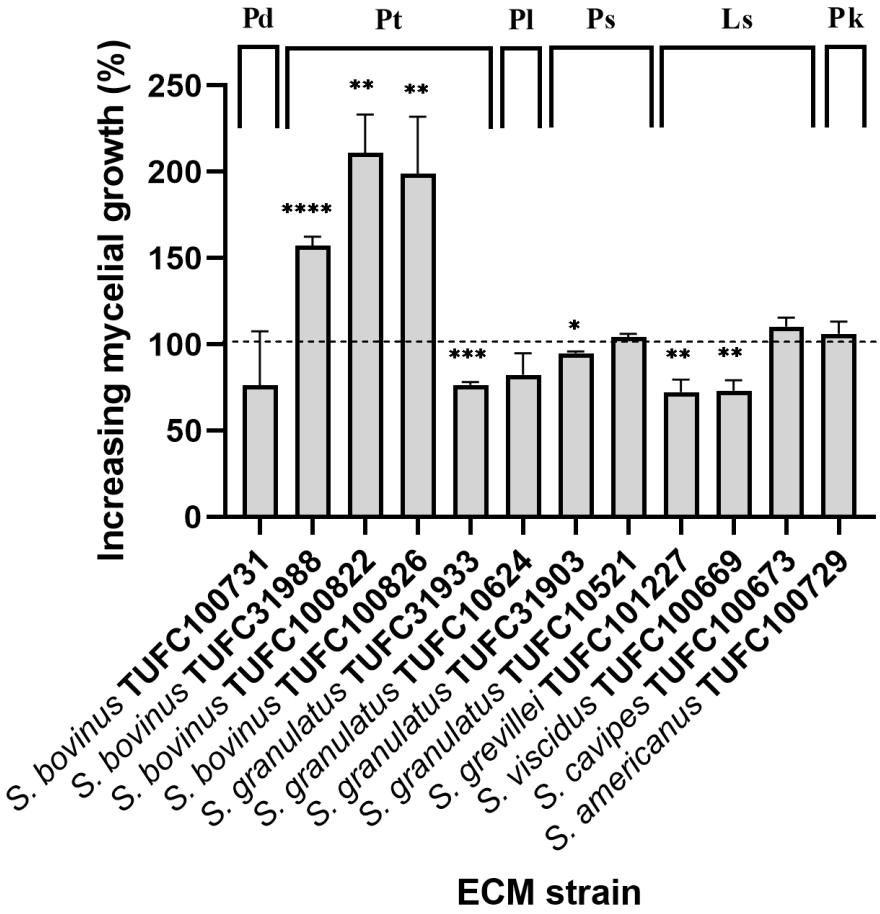
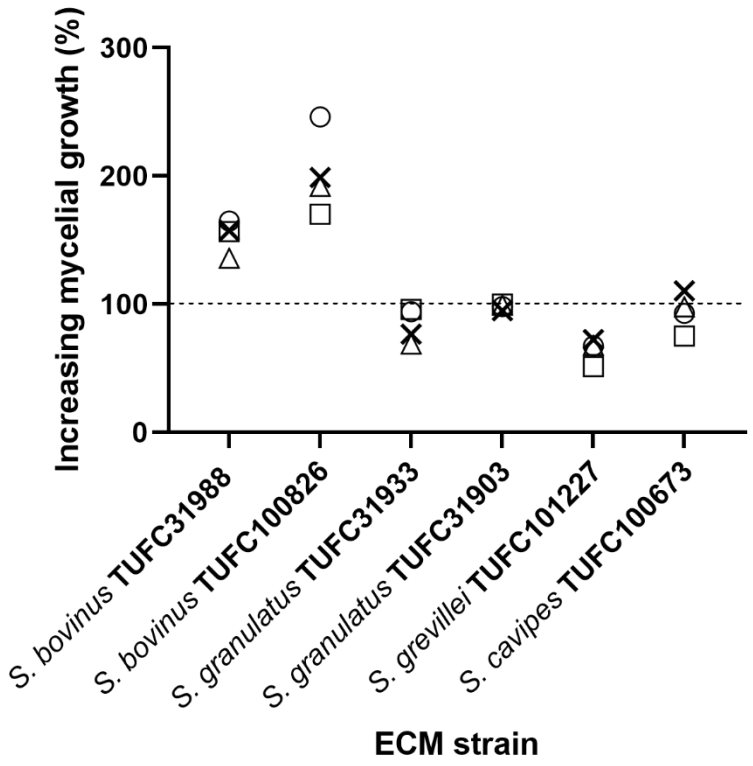



Fig. 4.2 The mycelium growth of *Suillus* spp. associated with various *Pinaceae* plant hosts (Pd = *Pinus densiflora*, Pt = *Pinus thunbergii*, Pl = *Pinus luchuensis*, Ps = other *Pinus* spp., Ls = *Larix* spp., and Pk = *Pinus koraiensis*) in the confrontation with *Paraburkholderia fungorum* GIB024 in diluted 1:5 MMN agar plate without glucose.

Dotted line (---) marked 100%, indicating the percentage of control, below the mark means inhibition, above means promotion. The asterisk sign was indicated the summary of the unpaired t test of the ECM monoculture versus treatment with the bacterium, * ($P < 0.05$), **(P: 0.05 – 0.01), ***(P: 0.01 – 0.001), ****($P > 0.001$). Without asterisk sign means not significantly different ($P > 0.05$).




| | %CV |
|---|---|
| TUFC31988 | 8.05 |
| TUFC100826 | 15.90 |
| TUFC31933 | 15.80 |
| TUFC31903 | 2.33 |
| TUFC101227 | 14.22 |
| TUFC100673 | 15.51 |

Fig. 4.3 The scattering plot of mycelium growth of *Suillus* spp. associated with various *Pinaceae* plant hosts in the confrontation with *Paraburkholderia fungorum* GIB024 as well as other Burkholderiales bacteria (*Cabaleronia sordidicola* GIB028, *Janthinobacterium agaricidamnosum* GIB029, and *Paraburkholderia caledonica* KN1) isolated from sporocarp of *Rhizopogon roseolus* in *Pinus thunbergii* forest. The direct confrontation assay was performed in in diluted 1:5 MMN agar plate without glucose.

Dotted line (---) marked 100%, indicating the percentage of control, below the mark means inhibition, above means promotion. The coefficient of variation percentage (%CV) of increasing mycelial growth as the response of confrontation with the all bacteria was calculated for each ECM fungal strain.

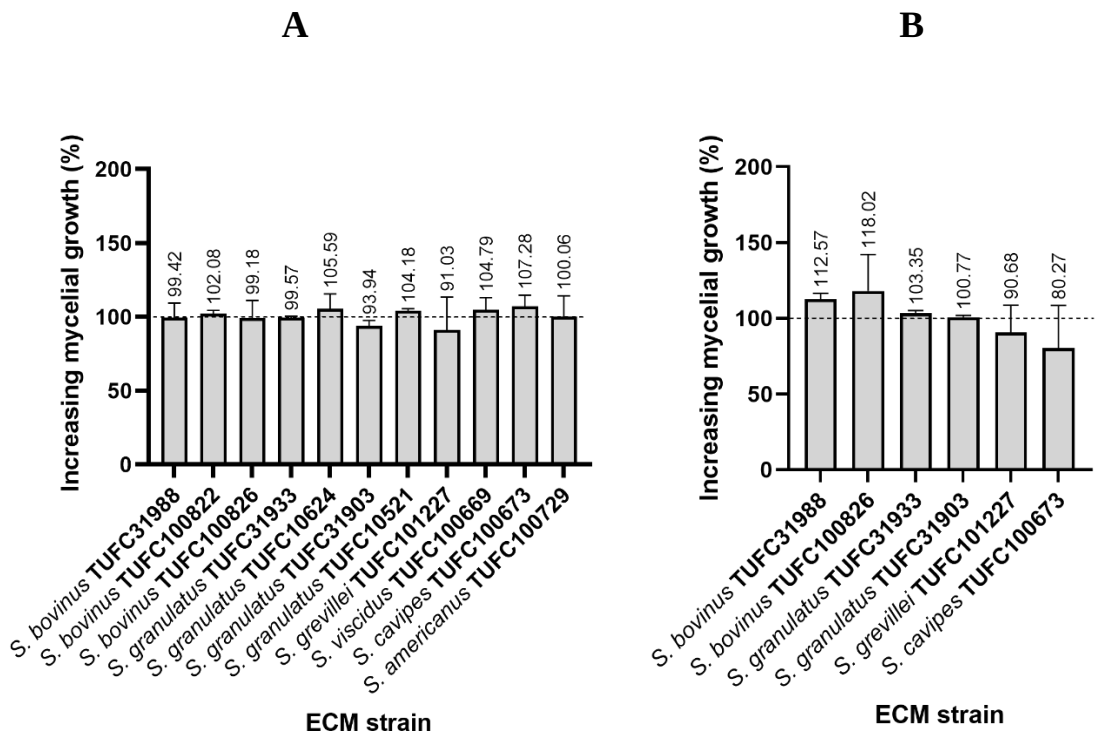



Fig. 4.4 The increasing of mycelium growth of *Suillus* spp. associated with various *Pinaceae* plant hosts post 15 days of indirect confrontation with *Paraburkholderia fungorum* GIB024 (A) and application of 40 µL of sterilized bacterial suspension (B).

The assay was performed in diluted 1:5 MMN agar plate without glucose. Dotted line (---) marked 100%, indicating the percentage of control, below the mark means inhibition, above means promotion. The increasing mycelial growth percentage is annotated above of each bar.

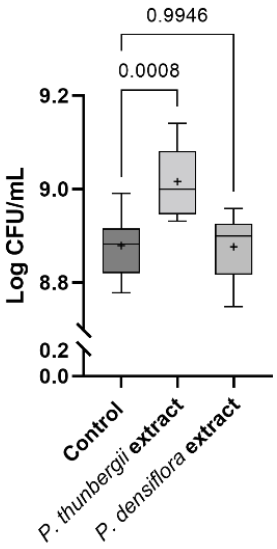



Fig. 4.5 The box and whiskers plot for growth profile of *P. fungorum* GIB024 in diluted 1:5 MMN without glucose with supplementation of *P. thunbergii* and *P. densiflora* extract (obtained from heat extraction of 0.5 % w/v fresh weight of pine in diluted 1:5 MMN without glucose).

Mean is shown as plus sign. The growth is express as log CFU/mL of liquid medium used. The medium without supplementation of pine extract was used as control. The Dunnett's multiple comparisons test of control vs plant extract was performed, and the P value numbers were shown in the plot.

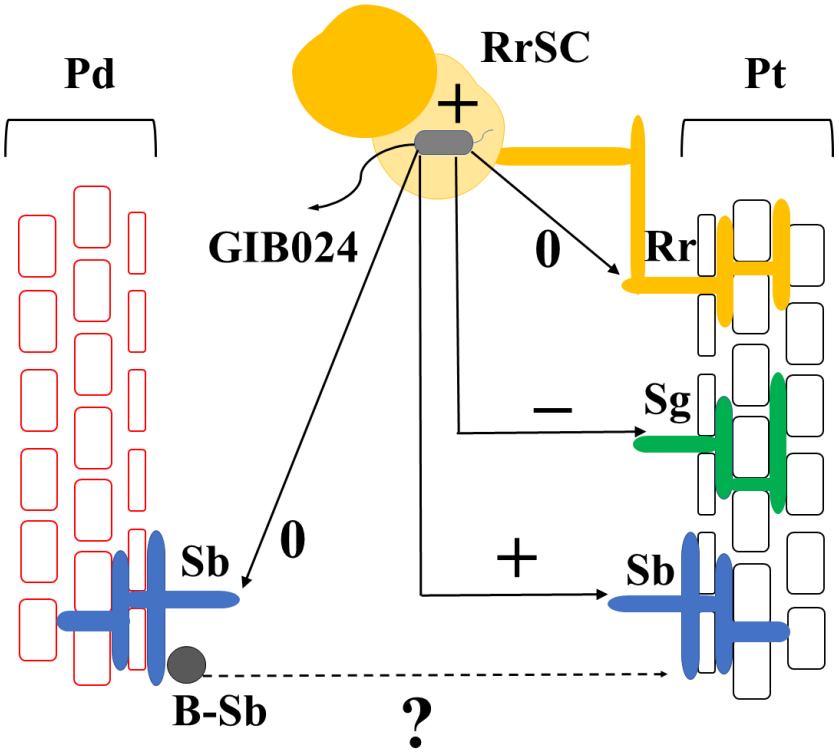



Fig. 4.6 A schematic figure showing the species- and plant-host- dependent pattern of mycelial growth-specificity selection, in the terms of inhibition (-), neutral (0), and promotion (+), of *P. fungorum* GIB024, a bacterium isolated from *R. roseolus* sporocarp (RrSC) in *P. thunbergii* (Pt) forest, toward various ECM fungi associated with Pt as well as *P. densiflora* (Pd). In ectomycorhizosphere scenario, GIB024 specifically promoted the vegetative growth of *S. bovinus* (Sb), but inhibited *S. granulatus* (Sg), and showed neutrality toward its ECM fungal host, *R. roseolus* (Rr). However, not all Sb was promoted, since plant host has an important role on determining the specificity of the bacterium as shown that GIB024 promoted Sb associated with Pt, but not Pd. GIB024 was likely promoted RrSC, the isolation source, as shown by the bacterium positive effect on Rr in high sugar medium. As future research, a possible crossed-specificity (?) of bacterium associated with Sb of Pd (B-Sb) toward Sb of Pt is projected to enhance our understanding toward BFI in ectomycorrhiza.

# Chapter 5

## General discussion and conclusion

Bacteria are ubiquitously presence in the nature and frequently occurred in interkingdom interaction, including with fungi. Generally, the bacterial-fungal interaction (BFI) takes form in mutualism, parasitism, or commensalism. In this study, the research was focused on the mutualistic interaction between an edible hypogeous ectomycorrhizal mushroom, *Rhizopogon roseolus* ("shouro" in Japanese), with a bacterium, *Paraburkholderia fungorum* strain GIB024, isolated from sporocarp (fruiting body) of *R. roseolus* in a *Pinus thunbergii* (Japanese black pine) dominance forest. Compared to mycorrhizosphere, fruiting body provides high specificity bacteria due to its unique chemistry and tight selection mechanism of bacteria from bulk soil as bacterial pool (Liu et al., 2018). Moreover, because of the absence of plant root influence, bacteria associated with fruiting body work exclusively in influencing their surrounding fungal mycosphere. Therefore, bacteria associated with fruiting body are expected to have high probability to form mutualistic interaction with their fungal counterpart, particularly in the mycelial growth-promoting ability. However, fruiting body bacteria are relatively unexplored compared with bacteria in mycorrhizosphere soil. Due to their uniqueness and specificity, ectomycorrhizal fruiting body bacteria might be having roles in mycorrhization or in sporocarp functions. One of the most distinct characterization outcomes of mutualistic interaction in BFI is increasing growth of both parties. However, in this study, the focus was mainly on the growth of fungal part due to impact of confrontation with the bacterium, *P. fungorum*. The growth was measured as elongation of mycelial colony. In the term of mycorrhiza symbiosis, the capacity of bacterium to enhanced the growth of fungal mycelia is an early indication of the roles of the

bacterium as mycorrhizal helper bacterium (MHB), since increasing mycelia growth resulting higher chance for promotion of mycorrhiza establishment.

The interaction in BFI can be occurred as physical as well as chemical way. In the physical interaction, the role of fungal hyphae as highway for bacterial dispersion and growth are essential particularly in the soil environment. However, prior to this cell-to-cell dependent contact, the chemical signal between these two kingdom organisms plays role on the recognition and suitability, thus allowing deeper interaction. The signals produced by bacteria or fungi can be in the form of soluble or volatile compounds. In the interaction of *R. roseolus* – *P. fungorum*, the soluble metabolites produced by the bacterium, instead of volatile metabolites, have mycelial growth-promoting ability toward the fungal mycelia. The signaling mechanism via soluble metabolites imply the urge to proceed in physical contact for further interaction. The growth-promoting signal released by the bacterium can be counted as inviting sensing, thus helping the bacterial to easily migrate and attached to the fungal hyphae. Therefore, the observation of the bacterial attachment toward fungal hyphae is projected for further investigation. There is a possibility of the biofilm formation by the bacterium post-hyphal attachment as well, as shown by Nazir et al., (2014). In this study, *Burkholderia terrae* was able to migrate through soil by attaching and forming biofilm in the hyphae of an ectomycorrhizal, *Lyophyllum* sp. Moreover, the bacterium and its biofilm provide protection for the fungus toward antifungal compound.

Metabolite exchange is possibly occurred during mutual interaction between *R. roseolus* and *P. fungorum*. Fungal mycelia exudates high energy carbonaceous compounds as subject to microbial utilization. Among those carbon sources, organic acids, mainly oxalic acid, are released abundantly by *R. roseolus* (Casarin et al., 2003). Additionally, in field condition, *Pinus thunbergii* root also exudated organic acids, with oxalic acid is the most abundance, followed by malic and citric acid (Brunner and Sperisen, 2013). This study showed that these

organic acids are used as preference carbon source by *P. fungorum*. As an exchange, the bacterium is appeared to synthesize a mycelial growth-promoting metabolite(s) by using organic acids exclusively. Furthermore, member of *Burkholderiaceae* bacteria were commonly inhabited and dominated soil niche with high amounts of organic acids such as in rhizosphere (Weisskopf et al., 2011) and mycorrhizosphere (Uroz et al., 2007). Although *P. fungorum* was able to utilize other carbon sources that presumably exudated by fungal mycelia or pine roots such as mannitol, trehalose, or glucose, the mycelial growth-promoting metabolite(s) were only produced when *P. fungorum* utilized organic acids. The selection of distinct fungal carbon source by bacterium in order to promote mycelial growth might be conserved for bacteria. On contrary to the finding of this study, *Pseudomonas fluorescens* utilized trehalose, not organic acids, to produced mycelial growth-promoting metabolite toward *Laccaria bicolor* (Deveau et al., 2010). Therefore, this specificity carbonaceous substrate is subjected to be explored further and might be play important role on the specificity interaction in BFI.

Due to the ability of *P. fungorum* to utilize oxalic acid or oxalate as sole carbon source, the bacterium is likely involved in biogeochemical cycle, in this case carbon cycle, through oxalate-carbonate pathway (OCP). Oxalotrophy, the ability of bacteria to use oxalic acid or oxalate as carbon source in OCP, is a widespread trait of *Burkholderiaceae* bacteria (Kost et al., 2014). In a study conducted by Weisskopf et al., (2011), *Burkholderia* species is the dominant member of bacterial community associated with white lupin root producing organic acid, and 98% of these *Burkholderia* bacteria were oxalotrophic, compared with only 2% of non-*Burkholderia*. Moreover, the study also showed that oxalotrophic characteristic is specific to plant beneficial *Burkholderia,* and not to the pathogenic strains such as *B. cepacia*. However, it is necessary to confirm whether *P. fungorum* strain GIB024 is an oxalotrophic bacterium by detecting the presence of oxalotrophic marker gene (Khammar et al., 2009). In

OCP, the oxalatrophic bacterium oxidized oxalate into carbonate, thus increasing soil pH. A study showed that soil saprophytic fungi supported the population and activity of oxalotrophic bacteria through cell-to-cell contact and exudating stimulatory compounds (Martin et al., 2012). The similar outcome might be happened in the interaction between *P. fungorum* – *R. rosoelus*, as shown by the supporting effect of ectomycorrhiza fungi toward *Streptomyces* sp. NJ10, an oxalotrophic bacterium isolated from an ectomycorrhizosphere of *P. massoniana* (Sun et al., 2019). As a pay-off mechanism, *P. fungorum* enhanced the growth of *R. roseolus* by producing mycelial-growth promoting metabolites by using oxalate as carbon source.

Regarding the mutual interaction and mycelial growth-promoting ability with other ectomycorrhizal fungi, *P. fungorum* GIB024 showed nutrient-, species-, and plant host-dependent. Such dependency defined the complexity of bacterial-fungal interaction in the field condition. *P. fungorum* GIB024 as a sporocarp bacterium stimulated the growth of *R. roseolus* in rich nutrient, presumably fitting the chemistry condition of sporocarp itself with abundance of carbonaceous compounds. Moreover, the bacterium showed closely associated with suilloid ectomycorrhizal fungi, known to be specific for pine as plant host, such as *Rhizopogon* sp. and *Suillus* sp by promoting their mycelial growth, but had neutral or even inhibition effect toward other group of ectomycorrhizal fungi. Several studies showed the co-occurrence and even dominancy of *Burkholderiaceae* toward suilloid ectomycorrhizal fungi. *Burkholderia* bacteria dominated mycorrhizosphere of *S. bovinus* – *P. sylvestris* (Timonen and Hurek, 2006) and showed mycelial growth-promoting as well as mycorrhization enhancer ability in *R. luteolus* – *P. radiata* association (Garbaye and Bowen, 1989). On the other hand, *Burkholderia* sp. showed inhibition toward the growth and mushroom formation of saprophytic *Lyophyllum* sp. (Nazir et al., 2014). Although *P. fungorum* GIB024 has strong preference toward suilloid ectomycorrhizal fungi, the mycelium growth-promoting ability is

also limited by the ectomycorrhizal plant host. *P. fungorum* GIB024 gave positive effect on *R. roseolus* and *S. bovinus* associated with *P. thunbergii*, but not *S. bovinus* associated with other pine species. Moreover, *S. granulatus* associated with *P. thunbergii* were not promoted, thus the specificity was narrowed to *R. roseolus* and *S. bovinus* associated with *P. thunbergii*, but not other *Suillus* spp. Therefore, *R. roseolus* and *S. bovinus* associated with *P. thunbergii* were promoted by the indigenous bacterium, *P. fungorum* GIB024.

The mutual interaction between bacterium and ectomycorrhizal fungi is strongly shaped by the plant host. Several previous investigations showed the prominent role of fungal species in determining the positive interaction between bacteria and their ectomycorrhizal fungal counterparts (Warmink et al., 2009; Boersma et al., 2010). Additionally, in these studies, carbonaceous compounds related to the fungal mycelial were shown as driving factors for the mutual BFI. However, in this current study, plant host, along with fungal species, were found to be involved in the suitability selection of bacteria toward their fungal host. Based on the results, *P. fungorum* GIB024 can be categorized as specific fungiphile, instead of universal, toward ectomycorrhizal mushrooms, as its mycelial growth-promoting ability was narrowed to *R. roseolus* and *S. bovinus* associated with *P. thunbergii* only. Nevertheless, the understanding related to the mechanism of plant host as determining factor for bacterial selection toward ectomycorrhizal fungi is still in infancy. Since *P. fungorum* GIB024 is an indigenous bacterium of *P. thunbergii,* the pine is likely supporting the growth of bacterium, thus enhancing its interaction with the indigenous ectomycorrhizal association with the *P. thunbergii*. However, more deeper investigations are needed to reveal this plant host roles on the selection of its association bacteria with the ectomycorrhizal association as well.

The findings of this study lead to further investigations related to BFI particularly between *P. fungorum* GIB024 and *R. roseolus* (Fig. 5.1). Since this study focused on chemical interaction, the investigation of physical interaction including possibility of

bacterial attachment and biofilm formation toward fungal mycelia is needed to complement our understanding. The bacterium has also a possibility to act as an endo-hyphal bacterium as shown by various *Burkholderiaceae* bacteria (Okrasińska et al., 2021). Since GC-content of several strains of *P. fungorum* is above 60% and the presence of secretion systems particularly type-3 and type-6 secretion system (Loong et al., 2019; Tan et al., 2020), *P. fungorum* GIB024 is likely acted as a facultative endosymbiont. However, environmental factors inciting the endosymbiotic trait as long as its ecological roles toward the bacterium and fungus need to be identified. As comparison, a work by Venkatesh et al., (2022) showed that bacteria reside inside the hyphae of some filamentous fungi have better survivability toward cold stress than planktonic bacteria. In the biogeochemical cycle, the involvement of *P. fungorum* GIB024 in OCP need to be clarified. Designed microcosms in in planta model systems are needed to confirm the roles of organic acids in tripartite symbiotic plant–ectomycorrhizal fungus–bacterium systems, with a focus on the mycorrhizosphere microenvironment. Moreover, the crossed-specificity study of bacteria and elucidation the chemical structure of possible bacterial metabolite(s) that responsible for mycelial growth promoting effect are recommended for further investigations as well.

In conclusion, the attempt to evaluate the ecological roles of of *P. fungorum* GIB024, a sporocarp bacterium isolated from the fruiting body of *R. roseolus* in *P. thunbergii* forest, during interaction with *R. roseolus* have been done through three consecutive experiments. Through chemical interaction, *P. fungorum* GIB024 excreted soluble metabolite(s), instead volatile metabolite(s), that are able to promote the mycelial growth of *R. roseolus*. There is a possible metabolite exchange mechanism between *P. fungorum – R. roseolus*. Fungal exudates, in the form of organic acids, were utilized by the bacterium to promote its growth, as an exchange, the bacterium synthesized mycelial growth-promoting metabolite(s). The ability of *P. fungorum* GIB024 to utilize organic acids, particularly oxalic acid, is an

indication the involvement of this bacterium in biogeochemical cycle, in this case carbon cycle, through oxalate-carbonate pathway (OCP). Regarding the mutual interaction and mycelial growth-promoting ability with other ectomycorrhizal fungi, *P. fungorum* GIB024 showed nutrient-, species-, and plant host- dependent. *P. fungorum* GIB024, isolated from *R. roseolus* sporocarp in *P. thunbergii* forest, is a specific fungiphile bacterium, as its mycelial growth-promoting ability was narrowed to ectomycorrhizal fungi associated with *P. thunbergii* only, namely *R. roseolus*, in rich nutrient, and *S. bovinus*, in poor nutrient.

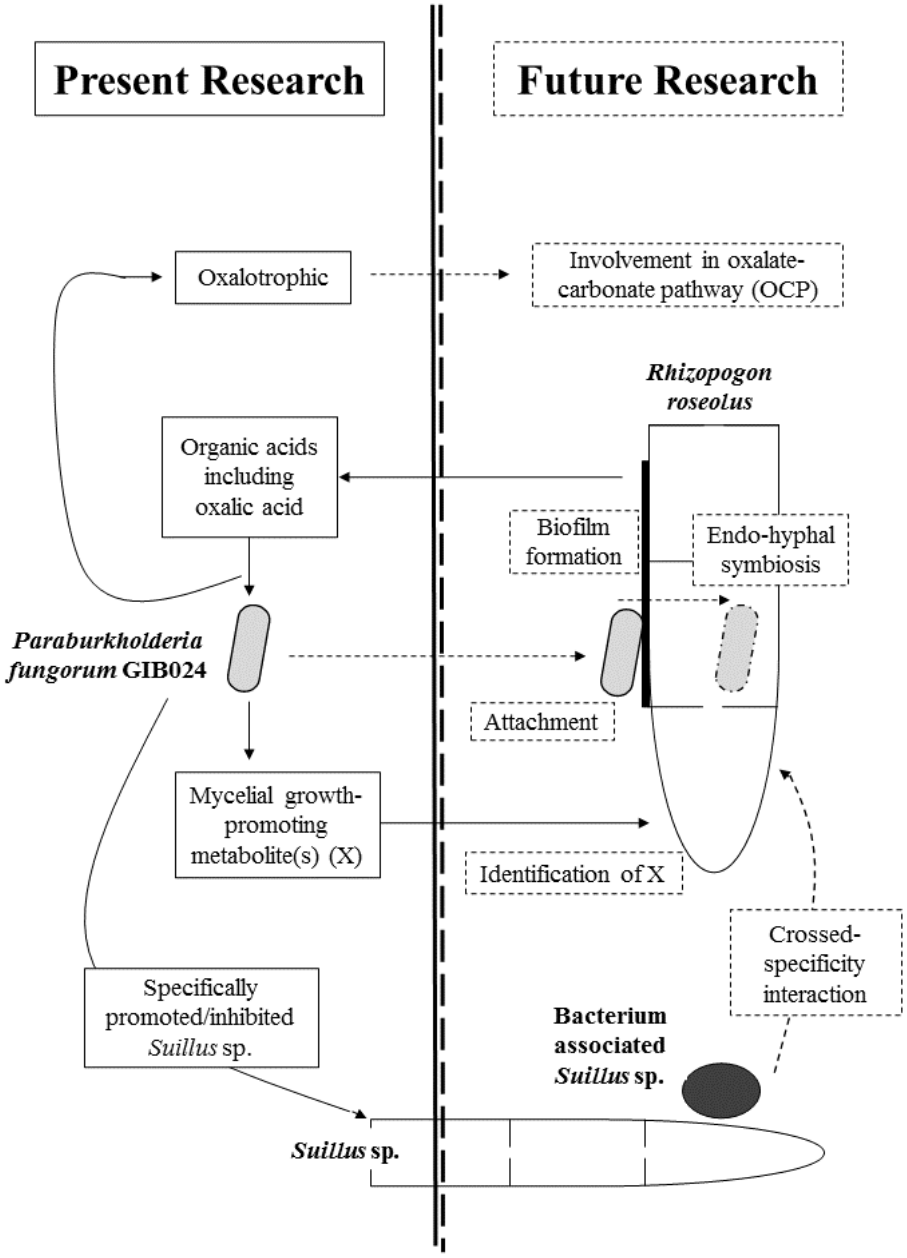



Fig. 5.1 The outline of the findings of present research (solid lines and boxes) and prospectus future research (dotted lines and boxes) regarding the ecological roles of *P. fungorum* GIB024 and its interaction with ectomycorrhizal fungi.

# Summary

*Rhizopogon roseolus* (“shouro” in Japanese) is an edible ectomycorrhizal (ECM) mushroom with high economic value. Previous study found that a sporocarp (fruiting body) of *R. roseolus* in a *Pinus thunbergii* (Japanese black pine) dominance forest harbored unique community of bacteria that might be committed in mutual bacterial-fungal interaction (BFI) toward its fungal host. One of the bacteria, *Paraburkholderia fungorum* strain GIB024, showed a mutual BFI by its ability to promote mycelial growth of *R. roseolus* trough in-vitro direct confrontation screening. However, the mycelial promoting mechanism of this bacterium was not revealed yet. Moreover, the literature studies showed that this bacterium was wide pervasive and co-occurance with various fungal host. In mutual BFI, the bacterium relies on fungal carbonaceous compounds as source of nutrients while extracellularly release mycelial growth-promotor, in the form of volatile or soluble compound. Therefore, the aim of this current study was to investigate the potential role of the extracellular mycelial growth-promoting metabolite, identified the possible fungal carbonaceous compounds during mutual BFI of *P. fungorum* – *R. roseolus* and specificity interaction of the bacterium with other ECM mushrooms.

First, I investigate the effect of potential metabolite(s) produced by *P. fungorum* GIB024. As comparison, I also selected other *R. roseolus* sporocarp bacterium from the same genera with GIB024, *P. caledonica* KN1. Direct confrontation assay at three different distances, a pour plate method that sampled bacterial spent broth either with and without agitation at 25 °C, and an indirect confrontation assay was carried out in order to assess the *R. roseolus* growth-promoting ability of *Paraburkholderia* spp. These assessments were carried out in a 1:5 diluted Melin-Norkran-modified medium with glucose (hs-dMMN) and without glucose (ls-dMMN). GIB024 promoted the growth of R. roseolus in ls-dMMN in short

distance, whereas KN1 inhibited the growth of the fungus in that condition. In hs-dMMN, both bacteria have neutral or slightly promotion effect toward *R. roseolus*. I determined from the spent broth analysis that *Paraburkholderia* spp. that grew axenically under static conditions had a more pronounced mycelial growth-promoting effect on *R. roseolus* than under agitation conditions. I also found that high concentration of spent broth resulted in a decrease in mycelial growth-promoting ability. Volatile metabolite(s) produced by both bacteria did not promote the mycelial growth of *R. roseolus.* In conclusion, *Paraburkholderia* spp. exhibited a species- and nutrient (sugar)-dependent ability to promote the mycelial growth of *R. roseolus*, and the bacterial soluble metabolite(s) play a crucial role in their growth-promoting ability.

Then, I investigate whether specific carbon sources are responsible for bacterial stimulation of mycelial growth of *R. roseolus*, by *P. fungorum* GIB024. I designed a two-compartment media in a non-separated Petri dish for a direct confrontation bioassay. The first compartment for growing the fungus was filled with 1:5 diluted MMN without glucose. The second compartment acted as the bacterial medium and was filled with various sole carbon sources, which were grouped into free sugars (glucose, fructose, trehalose, mannose, xylose, and arabinose), sugar alcohols (mannitol, glycerol, sorbitol), organic acids (malonic acid, maleic acid, citric acid, and oxalic acid), malt and yeast extract, soluble starch, chitin, and no addition of a carbon source. *R. roseolus* growth was stimulated when *P. fungorum* was grown in the organic acids rather than in other carbon source groups. Moreover, the bacterium grew better in organic acids than in free sugars or sugar alcohols. In the organic acid supplemented-medium, the bacterium increased the environmental pH and its sterilized suspension stimulated growth of *R. roseolus*. These results suggested a potential role of organic acids as a mediator during the mutual interaction between *P. fungorum* and *R. roseolus*.

Finally, I investigated the specificity among various ECM mushrooms regarding growth promotion by *P. fungorum* GIB024. In vitro agar-based plate approaches of confrontation assays as well as application of a sterilized bacterial suspension to ECM strains were employed. All assays were performed in 1:5 diluted Modified Melin-Norkrans (MMN) medium, with and without glucose. The bacterium significantly promoted the growth of *Suillus bovinus*, but not *R. roseolus*, in the medium without glucose. However, the opposite result was obtained in the medium supplemented with 1 g/L of glucose. I then extended the assay to focus on *Suillus* spp. in association with various plant hosts in medium without glucose. GIB024 significantly promoted *S. bovinus* growth in association with *P. thunbergii*, but not with *P. densiflora*. The bacterium had a neutral or inhibitory effect on the growth of other *Suillus* spp. associated with *P. thunbergii* as well as other *Pinaceae*. Furthermore, application of a sterilized bacterial suspension produced effects resembling those of the confrontation assay. These results suggest that combination of nutrient condition, ECM species, and plant host determines the mycelial growth-promoting specificity of ECM-associated bacteria.

The study indicated that there is a possible metabolite exchange between *P. fungorum* and *R. roseolus* modulating by fungal organic acids, and as an exchange, the bacterium produced soluble non-volatile mycelial growth-promoting metabolite(s) and suggested that *P. fungorum* GIB024 was a specific fungiphile bacterium, whose mycelial growth-promoting ability was specified against ECM fungal strains associated with *P. thunbergii*. The information will contribute to deepen understanding BFI in mushroom science and to promote application study in which GIB024 was effectively used in production of ectomycorrhizal pine trees and cultivation of shouro mushroom.

# 和文摘要

ショウロ *Rhizopogon roseolus* は食用きのこであり，クロマツ *Pinus thunbergii* と共生する外生菌根菌である．これまでの研究で本きのこ子実体からショウロ菌糸体生育を促進する細菌 *Paraburkhplderia fungorum* GIB024 および *P. caledonica* KN1 が分離されている．しかしながら，これらの細菌の菌糸生育促進に関与する物質やその作用メカニズムに関する知見は少ないのが現状である．そこで，本研究では，菌糸生育促進に関与する物質の探求と多様な外生菌根菌の菌糸生育に及ぼす特異的作用に関する調査をした．

先ずは，子実体から分離した *P. fungorum* GIB024 および *P. caledonica* KN1 細菌が示す菌糸生育促進効果を効率よくかつ安定的に検定するために，検定条件を再度検討した．GIB024 細菌とショウロ菌糸体を同一培地内で培養する二員培養検定における培地成分の影響について調査した．その結果，グルコースを除去した貧栄養条件下では，GIB024 細菌は著しい菌糸生育促進効果を示したが，KN1 は阻害効果を示した．一方，グルコース添加培地では，両細菌が若干の生育促進効果を示した．細菌培養液におけるショウロ菌糸体生育促進効果を検出するための細菌培養条件と菌糸生育促進効果との関連性を調査した．その結果，GIB024 細菌を攪拌培養し殺菌したろ液よりも静置培養し殺菌したろ液の方が，促進効果が高かった．さらには，殺菌細菌ろ液は，低濃度が有効であり，菌糸体培養培地へ添加する細

菌ろ液を増大させると，菌糸生育が阻害される傾向が認められた．以上のことから，菌糸生育促進効果は用いる細菌の種類や培地の栄養条件に依存していること，また，菌糸生育促進効果は低濃度の水溶性の物質が関与していることが示唆された．

次いで，GIB024 細菌がショウロ菌糸体生育を促進する作用に及ぼす炭素源の影響について調査した．本調査をするにあたり，2 区画寒天培地検定法を開発した．第 1 区画はグルコースを除く 1/5 濃度 MMN 寒天培地とし，ここでショウロ菌糸体を培養した．第 2 区画は様々な炭素源を含む寒天培地でありここで GIB024 細菌を培養した．これらの寒天培地を同一容器内で接合させ，ショウロ菌糸体生育に及ぼす GIB024 細菌の効果について調査した．その結果，ショウロの菌糸体生育は GIB024 細菌を有機酸含有培地で培養すると顕著に促進された．GIB024 細菌は有機酸含有培地で旺盛に増殖し，細菌培養培地の pH は上昇した．さらには，殺菌した細菌懸濁液でもショウロ菌糸生育を促進した．以上のことから，GIB024 細菌とショウロ菌糸体間の相互作用に有機酸が重要な役割を演じていると思われた．

最後に，菌糸生育促進効果の特異性について多様な外生菌根菌を用いて調査した．調査方法としては，きのこ菌糸と GIB024 細菌を同一シャーレ内で離して培養する二員培養検定法と細菌培養ろ液での検定法の両検定法を用いた．その結果，クロマツ林から分離したアミタケ *Suilus bovinus* の菌糸生育を著しく促進したが，アカマツ *P. densiflora* 林から分離したアミタケの菌糸生育は促進しなかった．このことは，同一のきのこ種であっても，共生する樹木種が

異なる菌株系統を用いると細菌への反応性が異なることを示している．さらに，GIB024 細菌はクロマツ林や他のマツ林から採取したチチアワタケ *S. granulatu* や他の *Suilus* 属のきのこ菌糸生育に対して促進効果を示さないか，あるいは反対に阻害的に作用した．また，本現象は，細菌培養ろ液でも同様の現象が認められた．以上のことから，GIB024 細菌はショウロを含め宿主クロマツと共生するきのこ菌株系統に対して特異的に生育促進すると考えられた．また，本きのこ菌株系統特異性に関与する物質は低濃度の水溶性物質であると考えられた．

以上の研究は，GIB024 細菌が生産する水溶性物質が外生菌根菌ショウロの菌糸生育促進に関連する物質であること，また，おそらくショウロ菌が分泌する有機酸を GIB024 細菌が利用して菌糸生育促進物質を生産している可能性があること，さらには，細菌が示す菌糸生育促進効果にはきのこ菌株系統特異性があり，その特異性には低濃度の水溶性物質が関与することを明らかにした．これらの一連の結果は，細菌と外生菌根菌との相互作用に関する理解を深めるための基礎的知見を提供し，さらには，GIB024 細菌を用いたショウロ感染苗木の育成やきのこ栽培等の応用研究の発展にも貢献する成果である．

# Acknowledgements

I would like to express my gratitude for my supervisor, Prof. Norihiro Shimomura for giving me the opportunity to obtain Ph.D. study at Tottori University, Japan. I would like to thank him for introducing me to bacterial-fungal interaction (BFI) and mushroom biotechnology topic. Moreover, I also want to appreciate his invaluable guidance for improving my experimental techniques, writing skills, and oral presentation capabilities.

I would like to extend my gratitude to Prof. Tadanori Aimi of Tottori University and Prof. Azakami of Yamaguchi University for their input throughout my study period. I also want to thank all fellow students of Prof. Shimomura laboratory for their valuable assistances and contributions during this research study. Furthermore, I am also grateful for supports from my superior, Prof. I Made Sudiana, and my colleague at Research Organization for Environmental and Life Sciences, National Research and Innovation Agency (BRIN), Indonesia.

The uttermost gratitude for the Japanese Government (Monbukagakusho:MEXT) Scholarships for the financial support under special program in bioresource utilization science of fungus and mushroom, Ministry of Education, Culture, Sport, Science and Technology, Governments of Japan. Finally, I also want to thanks my entire family for their supports during my Ph.D. study in Japan.

# References

Abeysinghe, G, Kuchira, M, Kudo, G, Masuo, S, Ninomiya, A, Takahashi, K, Utada, A S, Hagiwara, D, Nomura, N, Takaya, N and Obana, N: Fungal mycelia and bacterial thiamine establish a mutualistic growth mechanism, Life Sci. Alliance, **3**, e202000878 (2020).

Ahonen-Jonnarth, U L, Van Hees, P A, Lundström, U S and Finlay, R D: Organic acids

produced by mycorrhizal *Pinus sylvestris* exposed to elevated aluminium and heavy metal concentrations, New Phytol, **146**, 557-567 (2020)

Albarracín Orio, A G, Petras, D, Tobares, R A, Aksenov, A A, Wang, M, Juncosa, F, Sayago, P, Moyano, A J, Dorrestein, P C and Smania, A M: Fungal–bacterial interaction selects for quorum sensing mutants with increased production of natural antifungal compounds, Commun Biol, **3**, 1-9 (2020)

do Amaral, F P, Tuleski, T R, Pankievicz, V C, Melnyk, R A, Arkin, A P, Griffitts, J, Tadra-Sfeir, M Z, de Souza, E M, Deutschbauer, A, Monteiro, R A and Stacey, G: Diverse bacterial genes modulate plant root association by beneficial bacteria, MBio, **11**, e03078-20 (2020)

Andreolli, M, Lampis, S, Zenaro, E, Salkinoja-Salonen, M and Vallini, G: *Burkholderia fungorum* DBT1: a promising bacterial strain for bioremediation of PAHs-contaminated soils, FEMS Microbiol Lett, **319**, 11-18 (2011)

Andrino, A, Guggenberger, G, Kernchen, S, Mikutta, R, Sauheitl, L and Boy, J: Production of organic acids by arbuscular mycorrhizal fungi and their contribution in the mobilization of phosphorus bound to iron oxides, Front Plant Sci, **12**, 661842 (2021)

Antony-Babu, S, Deveau, A, Van Nostrand, J D, Zhou, J, Le Tacon, F, Robin, C, Frey-Klett, P and Uroz, S: Black truffle-associated bacterial communities during the development and maturation of *Tuber melanosporum* ascocarps and putative functional roles, Environ Microbiol, **16**, 2831-2847 (2014)

Aspray, T J, Frey-Klett, P, Jones, J E, Whipps, J M, Garbaye, J and Bending, G D: Mycorrhization helper bacteria: a case of specificity for altering ectomycorrhiza architecture but not ectomycorrhiza formation, Mycorrhiza, **16**, 533-541 (2006)

Aspray, T J, Jones, E E, Davies, M W, Shipman, M and Bending, G D: Increased hyphal branching and growth of ectomycorrhizal fungus *Lactarius rufus* by the helper bacterium P*aenibacillus* sp., Mycorrhiza, **23**, 403-410 (2013)

Axelrood, P E, Clarke, A M, Radley, R and Zemcov, S J: Douglas-fir root-associated microorganisms with inhibitory activity towards fungal plant pathogens and human bacterial pathogens, Can J Microbiol, **42**, 690-700 (1996)

Bais, H P, Weir, T L, Perry, L G, Gilroy, S and Vivanco, J M: The role of root exudates in rhizosphere interactions with plants and other organisms, Annu Rev Plant Biol, **57**, 233-266 (2006)

Barea, J M, Azcón, R and Azcón-Aguilar, C: Interactions between mycorrhizal fungi and bacteria to improve plant nutrient cycling and soil structure, In Varma, A and Buscot, F (eds), Microorganisms in soils: roles in genesis and functions, Springer, Berlin Heidelberg, Germany, pp. 195-212 (2005)

Bending, G D: What are the mechanisms and specificity of mycorrhization helper bacteria?, New Phytol, **174**, 707-710 (2007)

Boer, W D, Folman, L B, Summerbell, R C and Boddy, L: Living in a fungal world: impact of fungi on soil bacterial niche development, FEMS Microbiol Rev, **29**, 795-811 (2005)

Boersma, F G, Otten, R, Warmink, J A, Nazir, R and Van Elsas, J D: Selection of *Variovorax paradoxus*-like bacteria in the mycosphere and the role of fungal-released compounds, Soil Biol Biochem, **42**, 2137-2145 (2010)

Bonfante, P and Anca, I A: Plants, mycorrhizal fungi, and bacteria: a network of interactions, Annu Rev Microbiol, **63**, 363-383 (2009)

Brunner, I and Sperisen, C: Aluminum exclusion and aluminum tolerance in woody plants,

Front Plant Sci, **4**, 172 (2013)

Carlson, E U: Effect of strain of *Staphylococcus aureus* on synergism with *Candida albicans* resulting in mouse mortality and morbidity, Infect Immun, **42**, 285-292 (1983)

Casarin, V, Plassard, C, Souche, G and Arvieu, J C: Quantification of oxalate ions and protons released by ectomycorrhizal fungi in rhizosphere soil, Agronomie, **23**, 461-469 (2003)

Castanheira, N, Dourado, A C, Kruz, S, Alves, P I, Delgado-Rodríguez, A I, Pais, I, Semedo, J, Scotti-Campos, P, Sánchez, C, Borges, N and Carvalho, G: Plant growth-promoting *Burkholderia* species isolated from annual ryegrass in Portuguese soils, J Appl Microbiol, **120**, 724-739 (2016)

Coenye, T, Laevens, S, Willems, A, Ohlén, M, Hannant, W, Govan, J R, Gillis, M, Falsen, E and Vandamme, P: *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples, Int J Syst Evol Microbiol, **51**, 1099-1107 (2001)

Deveau, A, Brulé, C, Palin, B, Champmartin, D, Rubini, P, Garbaye, J, Sarniguet, A and Frey-Klett, P: Role of fungal trehalose and bacterial thiamine in the improved survival and growth of the ectomycorrhizal fungus *Laccaria bicolor* S238N and the helper bacterium *Pseudomonas fluorescens* BBc6R8, Environ Microbiol Rep, **2**, 560-568 (2010)

Deveau, A, Bonito, G, Uehling, J, Paoletti, M, Becker, M, Bindschedler, S, Hacquard, S, Herve, V, Labbe, J, Lastovetsky, O A and Mieszkin, S: Bacterial–fungal interactions: ecology, mechanisms and challenges, FEMS Microbiol Rev, **42**, 335-352 (2018)

Drever, J I and Vance, G F: Role of soil organic acids in mineral weathering processes, In

Pittman, E D and Lewan, M D (eds), Organic acids in geological processes, Springer, Berlin Heidelberg, Germany, pp 138-161 (1994)

Duponnois, R, Garbaye, J, Bouchard, D and Churin, J L: The fungus-specificity of mycorrhization helper bacteria (MHBs) used as an alternative to soil fumigation for ectomycorrhizal inoculation of bare-root Douglas-fir planting stocks with *Laccaria laccata*, Plant Soil, **157**, 257-262 (1993)

Duponnois, R, Galiana, A and Prin, Y: The mycorrhizosphere effect, a multitrophic interaction complex improves mycorrhizal symbiosis and plant growth, In Siddiqui, Z A, Akhtar, M S and Futai K (eds), Mycorrhizae: sustainable agriculture and forestry, Springer, Dordrecht, Germany, pp 227-240 (2008)

Duponnois, R and Kisa, M: The possible role of trehalose in the mycorrhiza helper bacterium effect, Botany, **84**, 1005-1008 (2006)

Dutton, M V and Evans, C S, Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment, Can J Microbiol, **42**, 881-895 (1996)

Emmett, B D, Lévesque-Tremblay, V and Harrison, M J: Conserved and reproducible bacterial communities associate with extraradical hyphae of arbuscular mycorrhizal fungi, The ISME Journal, **15**, 2276-2288 (2021)

De Felice, A, Di Lorenzo, F, Scherlach, K, Ross, C, Silipo, A, Hertweck, C and Molinaro, A: Structural investigation of the lipopolysaccharide O-chain isolated from *Burkholderia fungorum* strain DSM 17061, Carbohydr Res, **433**, 31-35 (2016)

Fillinger, S, Chaveroche, M K, Van Dijck, P, de Vries, R, Ruijter, G, Thevelein, J and d'Enfert, C: Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*, Microbiology, **147**, 1851-1862 (2001)

Frey-Klett, P, Burlinson, P, Deveau, A, Barret, M, Tarkka, M and Sarniguet, A: Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists, Microbiol Mol Biol Rev, **75**, 583-609 (2011)

Frey-Klett, P, Garbaye, J and Tarkka, M, The mycorrhiza helper bacteria revisited, New Phytol, **176**: 22-36 (2007)

Frey, P, Frey-Klett, P, Garbaye, J, Berge, O and Heulin, T: Metabolic and genotypic fingerprinting of fluorescent pseudomonads associated with the Douglas Fir-*Laccaria bicolor* mycorrhizosphere, Appl Environ Microbiol, **63**, 1852-1860 (1997)

Garbaye, J: Tansley review no. 76 helper bacteria: a new dimension to the mycorrhizal symbiosis, New Phytol, **128**, 197-210 (1994)

Garbaye, J and Bowen, G D: Stimulation of ectomycorrhizal infection of *Pinus radiata* by some microorganisms associated with the mantle of ectomycorrhizas, New Phytol, **112**, 383-388 (1989)

Garbaye, J and Duponnois, R: Specificity and function of mycorrhization helper bacteria (MHB) associated with the *Pseudotsuga menziesii-Laccaria laccata* symbiosis, Symbiosis, **14**, 335-344 (1992)

García-Rodríguez, J L, Pérez-Moreno, J, Ríos-Leal, D, Saez-Delgado, P, Atala-Bianchi, C, Sánchez-Olate, M and Pereira-Cancino, G: In vitro growth of ectomycorrhizal fungi associated with *Pinus radiata* plantations in Chile, Rev Fitotec Mex, **40**, 415-423 (2017)

Godhe, A and Rynearson, T: The role of intraspecific variation in the ecological and evolutionary success of diatoms in changing environments, Philos Trans R Soc B, **372**, 20160399 (2017)

Gotoh, T, Koyama, T and Ogura, K: Farnesy1 Diphosphate Synthase and Solanesyl Diphosphate Synthase reactions of diphosphate-modified allylic analogs: the significance of the Diphosphate linkage involved in the allylic substrates for Prenyltransferase, J Biochem, **112**, 20-27 (1992)

Hammarlund, S P and Harcombe, W R: Refining the stress gradient hypothesis in a microbial community, Proc Natl Acad Sci U S A, **116**, 15760-15762 (2019)

Van Der Heijden, M G, Bruin, S D, Luckerhoff, L, Van Logtestijn, R S and Schlaeppi K: A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity, plant nutrition and seedling recruitment, ISME J, **10**, 389-399 (2016)

Hoffman, M T, Gunatilaka, M K, Wijeratne, K, Gunatilaka, L and Arnold, A E: Endohyphal bacterium enhances production of indole-3-acetic acid by a foliar fungal endophyte, PLoS One, **8**, e73132 (2013)

Islam, F and Ohga, S: Effects of media formulation on the growth and morphology of ectomycorrhizae and their association with host plant, Int Sch Res Notices, **2013**, 317903 (2013)

Itoh, H, Jang, S, Takeshita, K, Ohbayashi, T, Ohnishi, N, Meng, X Y, Mitani, Y and Kikuchi, Y: Host–symbiont specificity determined by microbe–microbe competition in an insect gut. Proc Natl Acad Sci U S A, **116**, 22673-22682 (2019)

Izumi, H and Finlay, R D: Ectomycorrhizal roots select distinctive bacterial and ascomycete communities in Swedish subarctic forests, Environ Microbiol, **13**, 819-830 (2011)

Jabra-Rizk, M A, Falkler Jr, W A, Merz, W G, Kelley, J I, Baqui, A A and Meiller, T F: Coaggregation of *Candida dubliniensis* with *Fusobacterium nucleatum*, J Clin Microbiol, **37**, 1464-1468 (1999)

Jäderlund, L, Arthurson, V, Granhall, U and Jansson, J K: Specific interactions between arbuscular mycorrhizal fungi and plant growth-promoting bacteria: as revealed by different combinations, FEMS Microbiol Lett, **287**, 174-180 (2008)

Kai, M, Effmert, U, Berg, G and Piechulla, B: Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. Arch Microbiol, **187**, 351-360 (2007)

Kalač, P: A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms, J Sci Food Agric, **93**, 209-218 (2013)

Kang, Y M and Cho, K M: Identification of auxin from *Pseudomonas* sp. P7014 for the rapid growth of *Pleurotus eryngii* mycelium, Korean J Microbiol, **50**, 15-21 (2014)

Khammar, N, Martin, G, Ferro, K, Job, D, Aragno, M and Verrecchia, E: Use of the frc gene as a molecular marker to characterize oxalate-oxidizing bacterial abundance and diversity structure in soil, J Microbiol Methods, **76**, 120-127 (2009)

Kost, T, Stopnisek, N, Agnoli, K, Eberl, L and Weisskopf, L: Oxalotrophy, a widespread trait of plant-associated *Burkholderia* species, is involved in successful root colonization of lupin and maize by *Burkholderia phytofirmans*, Front Microbiol, **4**, 421 (2014)

Kumari, D, Reddy, M S and Upadhyay, R C: Diversity of cultivable bacteria associated with fruiting bodies of wild Himalayan *Cantharellus* spp, Ann Microbiol, **63**, 845-853 (2013)

Lehr, N A, Schrey, S D, Bauer, R, Hampp, R and Tarkka, M T, Suppression of plant defence response by a mycorrhiza helper bacterium, New Phytol, **174**, 892-903 (2007)

Leveau, J H and Preston, G M: Bacterial mycophagy: definition and diagnosis of a unique bacterial–fungal interaction, New Phytol, **177**, 859-876 (2008)

Li, G X, Wu, X Q, Ye, J R and Yang, H C: Characteristics of organic acid secretion associated with the interaction between *Burkholderia multivorans* WS-FJ9 and poplar root system, Biomed Res Int, **2018**, 9619724 (2018)

Li, Q, Zhao, J, Xiong, C, Li X, Chen, Z, Li, P and Huang, W: *Tuber indicum* shapes the microbial communities of ectomycorhizosphere soil and ectomycorrhizae of an indigenous tree (*Pinus armandii*), PloS one, **12**, e0175720 (2017)

Liu, X X, Hu, X, Cao, Y, Pang, W J, Huang, J Y, Guo, P and Huang, L: Biodegradation of phenanthrene and heavy metal removal by acid-tolerant *Burkholderia fungorum* FM-2. Front Microbiol, **10**, 408 (2019)

Liu, Y, Sun, Q, Li, J and Lian, B: Bacterial diversity among the fruit bodies of ectomycorrhizal and saprophytic fungi and their corresponding hyphosphere soils, Sci Rep, **8**, 11672 (2018)

Lohberger, A, Spangenberg, J E, Ventura, Y, Bindschedler, S, Verrecchia, E P, Bshary, R and Junier, P: Effect of organic carbon and nitrogen on the interactions of *Morchella* spp. and bacteria dispersing on their mycelium, Front Microbiol, **10**, 124 (2019)

Van Loon, L C: Plant responses to plant growth-promoting rhizobacteria, Eur J Plant Pathol, **119**, 243-54 (2007)

Loong, S K, Tan, K K, Zulkifle, N I and AbuBakar. S: Draft genome of *Paraburkholderia fungorum* sequence type 868 recovered from human synovial tissues, Data Brief, **25**, 104159 (2019)

Macias-Benitez, S, Garcia-Martinez, A M, Jimenez, P C, Gonzalez, J M, Moral M T and Rubio J P. Rhizospheric organic acids as biostimulants: monitoring feedbacks on soil microorganisms and biochemical properties, Front Plant Sci, **11**, 633 (2020)

Martín, J F: Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story, J Bacteriol, **186**, 5197-5201 (2004)

Martin, G, Guggiari, M, Bravo, D, Zopfi, J, Cailleau, G, Aragno, M, Job, D, Verrecchia, E and Junier, P: Fungi, bacteria and soil pH: the oxalate–carbonate pathway as a model for metabolic interaction, Environ Microbiol, **14**, 2960-2970 (2012)

Money, N P: Insights on the mechanics of hyphal growth, Fungal Biol Rev, **22**, 71-76 (2008)

Nally, E, Groah, S L, Pérez-Losada, M, Caldovic, L, Ljungberg, I, Chandel, N J, Sprague, B, Hsieh, M H and Pohl, H G: Identification of *Burkholderia fungorum* in the urine of an individual with spinal cord injury and augmentation cystoplasty using 16S sequencing: copathogen or innocent bystander?, Spinal Cord Ser Cases, **4**, 1-6 (2018)

Napitupulu, T P, Ramadhani, I, Kanti, A and Sudiana, I M: Diversity, phosphate solubilizing, and IAA production of culturable fungi associated with healthy and wilt banana, Arch Phytopathol Plant Prot, **54**, 2306-2332 (2021)

Navarro-Ródenas, A, Berná, L M, Lozano-Carrillo, C, Andrino, A and Morte, A: Beneficial native bacteria improve survival and mycorrhization of desert truffle mycorrhizal plants in nursery conditions, Mycorrhiza, **26**, 769-779 (2016)

Nazir, R, Warmink, J A, Boersma, H and Van Elsas. J D: Mechanisms that promote bacterial fitness in fungal-affected soil microhabitats, FEMS Microbiol Ecol, **71**, 169-185 (2009)

Nazir, R, Tazetdinova, D I and van Elsas, J D: *Burkholderia terrae* BS001 migrates proficiently with diverse fungal hosts through soil and provides protection from antifungal agents, Front Microbiol, **5**, 598 (2014)

Noble, R, Dobrovin-Pennington, A, Hobbs, P J, Pederby, J and Rodger, A: Volatile C8

compounds and pseudomonads influence primordium formation of *Agaricus bisporus*, Mycologia, **101**, 583-591 (2009)

Obase, K: Bacterial community on ectomycorrhizal roots of *Laccaria laccata* in a chestnut plantation, Mycoscience, **60**, 40-44 (2019)

Obase, K: Effects of bacterial strains isolated from the ectomycorrhizal roots of *Laccaria parva* on sporocarp production by the fungus in vitro, Mycoscience, **61**, 9-15 (2020)

Okrasińska, A, Bokus, A, Duk, K, Gęsiorska, A, Sokołowska, B, Miłobędzka, A, Wrzosek, M and Pawłowska, J: New endohyphal relationships between mucoromycota and *Burkholderiaceae* representatives, Appl Environ Microbiol, **87**, e02707-20 (2021)

de Oliveira-Longatti, S M, Martins de Sousa, P, Marciano Marra, L, Avelar Ferreira, P A and de Souza Moreira, F M: *Burkholderia fungorum* promotes common bean growth in a dystrophic oxisol, Ann Microbiol, **65**, 1825-1832 (2015)

Oliveira, J Q, Jesus, E D, Lisboa, F J, Berbara, R L, Faria, S M: Nitrogen-fixing bacteria and arbuscular mycorrhizal fungi in *Piptadenia gonoacantha* (Mart.) Macbr, Braz J Microbiol, **48**, 95-100 (2017)

de Oliveira Longatti, S M and de Souza Moreira, F M: Evaluation of plant growth-promoting traits of *Burkholderia* and *Rhizobium* strains isolated from Amazon soils for their co-inoculation in common bean, Afr J Microbiol Res, **7**, 948-959 (2013)

Olsson, P A and Wallander, H: Interactions between ectomycorrhizal fungi and the bacterial community in soils amended with various primary minerals, FEMS Microbiol Ecol, **27**, 195-205 (1998)

Ordoñez, Y M, Fernandez, B R, Lara, L S, Rodriguez, A, Uribe-Velez, D and Sanders, I R: Bacteria with phosphate solubilizing capacity alter mycorrhizal fungal growth both

inside and outside the root and in the presence of native microbial communities, PloS one, **11**, e0154438 (2016)

Panneerselvam, P, Saritha, B, Mohandas, S, Upreti, K K, Poovarasan, S, Sulladmath, V V and Venugopalan, R: Effect of mycorrhiza-associated bacteria on enhancing colonization and sporulation of *Glomus mosseae* and growth promotion in sapota (*Manilkara achras* (Mill) Forsberg) seedlings, Biol Agric Hortic, **29**, 118-131 (2013)

Pent, M, Põldmaa, K and Bahram, M: Bacterial communities in boreal forest mushrooms are shaped both by soil parameters and host identity, Front Microbiol, **8**, 836 (2017)

Pierce, E C, Morin, M, Little, J C, Liu, R B, Tannous, J, Keller, N P, Pogliano, K, Wolfe, B E, Sanchez, L M and Dutton, R J: Bacterial–fungal interactions revealed by genome-wide analysis of bacterial mutant fitness, Nat Microbiol, **6**, 87-102 (2021)

Pion, M, Spangenberg, J E, Simon, A, Bindschedler, S, Flury, C, Chatelain, A, Bshary, R, Job, D and Junier, P: Bacterial farming by the fungus *Morchella crassipes*, Proc R Soc B: Biol Sci, **280**, 20132242 (2013)

Pivato, B, Offre, P, Marchelli, S, Barbonaglia, B, Mougel, C, Lemanceau, P and Berta, G: Bacterial effects on arbuscular mycorrhizal fungi and mycorrhiza development as influenced by the bacteria, fungi, and host plant, Mycorrhiza, **19**, 81-90 (2009)

Pramoj Na Ayudhya, S, Riffiani, R, Ozaki, Y, Onda, Y, Nakano, S, Aimi, T and Shimomura, N: Isolation of bacteria from fruiting bodies of *Rhizopogon roseolus* and their effect on mycelial growth of host mushroom, Mushroom Science and Biotechnology, **27**, 134-139. (2019)

Pratama, A A, Jiménez, D J, Chen, Q, Bunk, B, Spröer, C, Overmann, J and van Elsas, J D: Delineation of a subgroup of the genus *Paraburkholderia*, including *P. terrae* DSM

17804T, *P. hospita* DSM 17164T, and four soil-isolated fungiphiles, reveals remarkable genomic and ecological features—proposal for the definition of a *P. hospita* species cluster, Genome Biol Evol, **12**, 325-344 (2020)

Pritsch, K and Garbaye, J: Enzyme secretion by ECM fungi and exploitation of mineral nutrients from soil organic matter, Ann For Sci, **68**, 25-32 (2011)

Rahman, M, Sabir, A A, Mukta, J A, Khan, M, Alam, M, Mohi-Ud-Din, M, Miah, M, Rahman, M and Islam, M T: Plant probiotic bacteria *Bacillus* and *Paraburkholderia* improve growth, yield and content of antioxidants in strawberry fruit, Sci Rep, **8**, 2504 (2018)

Rainey, P B: Effect of *Pseudomonas putida* on hyphal growth of *Agaricus bisporus*, Mycol Res, **95**, 699-704 (1991)

Ratzke, C and Gore, J: Modifying and reacting to the environmental pH can drive bacterial interactions, PLoS Biol, **16**, e2004248 (2018)

Ray, P and Adholeya, A: Correlation between organic acid exudation and metal uptake by ectomycorrhizal fungi grown on pond ash in vitro, Biometals, **22**, 275-281 (2009)

Riedlinger, J, Schrey, S D, Tarkka, M T, Hampp, R, Kapur, M and Fiedler, H P: Auxofuran, a novel metabolite that stimulates the growth of fly agaric, is produced by the mycorrhiza helper bacterium *Streptomyces* strain AcH 505, Appl Environ Microbiol, **72**, 3550-3557 (2006)

Rigamonte, T A, Pylro, V S and Duarte, G F: The role of mycorrhization helper bacteria in the establishment and action of ectomycorrhizae associations, Braz J Microbiol, **41**, 832-840 (2010)

Rousk, J, Brookes, P C and Baath, E: Contrasting soil pH effects on fungal and bacterial

growth suggest functional redundancy in carbon mineralization, Appl Environ Microbiol, **75**, 1589-1596 (2009)

Rúa, M A, Wilson, E C, Steele, S, Munters, A R, Hoeksema, J D and Frank, A C: Associations between ectomycorrhizal fungi and bacterial needle endophytes in *Pinus radiata*: implications for biotic selection of microbial communities, Front Microbiol, **7**, 399 (2016)

Rudnick, M B, Van Veen, J A and De Boer, W: Oxalic acid: a signal molecule for fungus-feeding bacteria of the genus C ollimonas?, Environ Microbiol Rep, **7**, 709-714 (2015)

Ruiz, B, Chávez, A, Forero, A, García-Huante, Y, Romero, A, Sánchez, M, Rocha, D, Sánchez, B, Rodríguez-Sanoja, R, Sánchez, S and Langley, E: Production of microbial secondary metabolites: regulation by the carbon source, Crit Rev Microbiol, **36**, 146-167 (2010)

Sabat, J and Gupta, N: Nutritional factors affecting the antifungal activity of *Penicillium steckii* of mangrove origin, Afr J Microbiol Res, **4**, 126-135 (2010)

Sánchez-Clemente, R, Guijo, M I, Nogales, J and Blasco, R: Carbon source influence on extracellular pH changes along bacterial Cell-Growth, Genes, **11**, 1292 (2020)

Schmidt, R, Etalo, DW, De Jager, V, Gerards, S, Zweers, H, De Boer, W and Garbeva, P: Microbial small talk: volatiles in fungal–bacterial interactions, Front Microbiol, **6**, 1495 (2016)

Schmidt, R, Jager, V D, Zühlke, D, Wolff, C, Bernhardt, J, Cankar, K, Beekwilder, J, Ijcken, W V, Sleutels, F, Boer, W D and Riedel, K: Fungal volatile compounds induce production of the secondary metabolite Sodorifen in *Serratia plymuthica* PRI-2C, Sci

Rep, **7**, 862 (2017)

Schrey, S D, Erkenbrack, E, Früh, E, Fengler, S, Hommel, K, Horlacher, N, Schulz, D, Ecke, M, Kulik, A, Fiedler, H P and Hampp, R: Production of fungal and bacterial growth modulating secondary metabolites is widespread among mycorrhiza-associated *Streptomycetes*, BMC Microbiol, **12**, 164 (2012)

Sharma, M P, Grover, M, Chourasiya, D, Bharti, A, Agnihotri, R, Maheshwari, H S, Pareek, A, Buyer, J S, Sharma, S K, Schütz, L and Mathimaran, N: Deciphering the role of trehalose in tripartite symbiosis among rhizobia, arbuscular mycorrhizal fungi, and legumes for enhancing abiotic stress tolerance in crop plants, Front Microbiol, **11**, 509919 (2020)

Shirakawa, M, Uehara, I and Tanaka, M: Mycorrhizosphere bacterial communities and their sensitivity to antibacterial activity of ectomycorrhizal fungi, Microbes Environ, **34**, 191-198 (2019)

Somerville, G A and Proctor, R A: Cultivation conditions and the diffusion of oxygen into culture media: the rationale for the flask-to-medium ratio in microbiology, BMC microbiol, **13**, 9 (2013)

De Sordi, L and Mühlschlegel, F A: Quorum sensing and fungal–bacterial interactions in *Candida albicans*: a communicative network regulating microbial coexistence and virulence, FEMS Yeast Res, **9**, 990-999 (2009)

Steffan, B N, Venkatesh, N and Keller, N P: Let's get physical: bacterial-fungal interactions and their consequences in agriculture and health: J. Fungi, **6**, 243 (2020)

Stopnisek, N, Zühlke, D, Carlier, A, Barberán, A, Fierer, N, Becher, D, Riedel, K, Eberl, L and Weisskopf, L: Molecular mechanisms underlying the close association between soil

*Burkholderia* and fungi, ISME J, **10**, 253-264 (2016)

Stuart E K and Plett, K L: Digging deeper: in search of the mechanisms of carbon and nitrogen exchange in ectomycorrhizal symbioses, Front Plant Sci, **10**, 1658 (2020)

Sun, Q, Li, J, Finlay, R D and Lian, B: Oxalotrophic bacterial assemblages in the ectomycorrhizosphere of forest trees and their effects on oxalate degradation and carbon fixation potential, Chem Geol, **514**, 54-64 (2019)

Sun, Y P, Unestam, T, Lucas, S D, Johanson, K J, Kenne, L and Finlay, R: Exudation-reabsorption in a mycorrhizal fungus, the dynamic interface for interaction with soil and soil microorganisms, Mycorrhiza. **9**, 137-144 (1999)

Tan, K Y, Dutta, A, Tan, T K, Hari, R, Othman, R Y and Choo, S W: Comprehensive genome analysis of a pangolin-associated *Paraburkholderia fungorum* provides new insights into its secretion systems and virulence, PeerJ, **8**, e9733 (2020)

Timonen, S and Hurek, T: Characterization of culturable bacterial populations associating with *Pinus sylvestris–Suillus bovinus* mycorrhizospheres, Can J Microbiol, **52**, 769-778 (2006)

Toljander, J F, Lindahl, B D, Paul, L R, Elfstrand, M and Finlay, R D: Influence of arbuscular mycorrhizal mycelial exudates on soil bacterial growth and community structure, FEMS Microbiol Ecol, **61**, 295-304 (2007)

Uehling, J K, Entler, M R, Meredith, H R, Millet, L J, Timm, C M, Aufrecht, J A, Bonito, G M, Engle, N L, Labbé, J L, Doktycz, M J and Retterer, S T: Microfluidics and metabolomics reveal symbiotic bacterial–fungal interactions between *Mortierella elongata* and *Burkholderia* include metabolite exchange, Front Microbiol, **10**, 2163 (2019)

Uroz, S, Calvaruso, C, Turpault, M P, Pierrat, J C, Mustin, C and Frey-Klett, P: Effect of the mycorrhizosphere on the genotypic and metabolic diversity of the bacterial communities involved in mineral weathering in a forest soil, Appl Environ Microbiol, **73**, 3019-3027 (2007)

Varesel, G C, Portinar, S, Trott, A, Scannerinil, S I, Luppi-Mosca, A N and Martinotti, M G: Bacteria associated with *Suillus grevillei* sporocarps and ectomycorrhizae and their effects on in vitro growth of the mycobiont, Symbiosis, **21**, 129-147 (1996)

Velez, P, Espinosa-Asuar, L, Figueroa, M, Gasca-Pineda, J, Aguirre-von-Wobeser, E, Eguiarte, LE, Hernandez-Monroy, A and Souza, V: Nutrient dependent cross-kingdom interactions: fungi and bacteria from an oligotrophic desert oasis, Front Microbiol, **9**, 1755 (2018)

Venkatesh, N, Greco, C, Drott, M T, Koss, MJ, Ludwikoski, I, Keller, N M and Keller, N P: Bacterial hitchhikers derive benefits from fungal housing, Curr Biol, **32**, 1523-1533 (2022)

Warmink, J A, Nazir, R and Van Elsas, J D: Universal and species-specific bacterial 'fungiphiles' in the mycospheres of different basidiomycetous fungi, Environ Microbiol, **11**, 300-312 (2009)

Weisskopf, L, Heller, S and Eberl, L: *Burkholderia* species are major inhabitants of white lupin cluster roots, Appl Environ Microbiol, **77**, 7715-7720 (2011)

Weisskopf, L, Schulz, S and Garbeva, P: Microbial volatile organic compounds in intra-kingdom and inter-kingdom interactions, Nat Rev Microbiol, **19**, 391-404 (2021)

Winkelmann, G: Ecology of siderophores with special reference to the fungi, Biometals, **20**, 379-392 (2007)

Yamanaka, T: The effect of pH on the growth of saprotrophic and ectomycorrhizal ammonia fungi in vitro, Mycologia, **95**, 584-589 (2003)

Yang, M, Zou, J, Liu, C, Xiao, Y, Zhang, X, Yan, L, Ye, L, Tang, P and Li, X: Chinese white truffles shape the ectomycorrhizal microbial communities of *Corylus avellana*, Annals of Microbiology, **69**, 553-565 (2019)

Yu, F, Liang, J F, Song, J, Wang, S K and Lu, J K: Bacterial community selection of *Russula griseocarnosa* mycosphere soil, Front Microbiol, **11**, 347 (2020)

Zagriadskaia, Y A, Lysak, L V, Sidorova, I I, Aleksandrova, A V and Voronina, E Y: Bacterial complexes of the fruiting bodies and hyphosphere of certain basidiomycetes, Biol Bull, **40**, 358-364 (2013)

Zahid, A, Ramzan, M, Bashir, M A, Khatana, M A, Akram, M T, Nadeem, S, Qureshi, M S, Iqbal, W, Umar, M, Walli, S and Tariq, R M: Effect of humic acid enriched cotton waste on growth, nutritional and chemical composition of oyster mushrooms (*Pluerotus ostreatus* and *Lentinus sajor-caju*), J King Saud Univ Sci, **32**, 3249-3257 (2020)

Zarenejad, F, Yakhchali, B and Rasooli, I: Evaluation of indigenous potent mushroom growth promoting bacteria (MGPB) on *Agaricus bisporus* production, World J Microbiol Biotechnol, **28**, 99-104 (2012)

Zhang, L, Ming-Xia, W A, Hua, L I, Ling, Y U, Huang, J G and Penfold C: Mobilization of inorganic phosphorus from soils by ectomycorrhizal fungi, Pedosphere, **24**, 683-689 (2014)

Zhang, R, Ran, Y, Dai, Y, Yang, H, Zhang, H, Lu, Y and Wang, L: Infectious granuloma caused by *Burkholderia fungorum* confirmed by laser-capture microdissection and polymerase chain reaction, Br J Dermatol, **171**, 1261-1263 (2014)

# List of Related Publications

Napitupulu, T P, Pramoj Na Ayudhya, S, Aimi, T and Shimomura, N: Mycelial Growth-promoting Potential of Extracellular Metabolites of *Paraburkholderia* spp. Isolated

from *Rhizopogon roseolus* Sporocarp, J Pure Appl Microbiol, **16**, 1154-1166 (2022) (The corresponding content is in Chapter 2)

Napitupulu, T P, Aimi, T and Shimomura, N: Potential role of organic acids in the mycelial growth promotion of *Rhizopogon roseolus* during interaction with the sporocarp bacterium, *Paraburkholderia fungorum* GIB024, Mushroom Science and Biotechnology, **30**, In press (The corresponding content is in Chapter 3)