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Research Article
Suppression of *Colletotrichum gloeosporioides* by Indigenous Phyllobacterium and its Compatibility with Rhizobacteria

[1]Siti Nur Aisyah, [1]Sulastri Sulastri, [1]Retmi Retmi, [1]Rahmi Henda Yani, [1]Elly Syafriani, [1]Lily Syukriani, [2]Fatchiyah Fatchiyah, [3]Amri Bakhtiar and [1]Jamsari Jamsari

[1]Laboratory of Biotechnology, Faculty of Agriculture, Andalas University, 2nd Floor, Campus Limau Manis, 25163 Padang, West Sumatera, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, East Java, Indonesia
[3]Faculty of Pharmacy, Andalas University, Padang, West Sumatera, Indonesia

Abstract
Background and Objective: Plant-associated bacteria, such as Phyllobacterium play a significant role in protecting plant from pathogenic fungal infection. These Phyllobacterium are known to be existed in such a microbial community with various microbes thus leading to elevated disease suppression. The aim of this study was to assess the fungal suppression activity of indigenous Phyllobacterium isolates and its strain/species compatibility with rhizobacteria. **Materials and Methods:** Two indigenous Phyllobacterium isolates were identified using 16S rRNA gene sequence and its antifungal activities were tested against several phytopathogenic fungus. Further antagonistic assay was performed to compare the efficacy of cell culture and cell-free supernatants. Its compatibility was assayed by performing the antifungal assay using the combination of these Phyllobacterium isolates with rhizobacteria ones. Data were statistically analyzed using one-way analysis of variance and the significance was further processed using Duncan's new multiple range test with a $p<0.05$. **Results:** Both isolates (UBCF_01 and UBCF_13), identified as *Serratia plymuthica*, exhibited higher suppression activity against *Colletotrichum gloeosporioides* compared to *Fusarium oxysporum* and *Sclerotium rolfsii*. Both isolates revealed opposite trend in their activities resulted from cultured cells and cell-free supernatants. Furthermore, the better suppression efficacy of the culture supernatants was resulted from single cultured cells, instead of co-culture. However, both isolates displayed quite poor compatibility with rhizobacteria isolates. **Conclusion:** These indigenous Phyllobacterium showed promising ability to be used as biocontrol agents for anthracnose. The application of its culture supernatants offered the less hazardous option of biological control implementation. However, their poor compatibility, even with the same species (rhizospheric UBCR_12) might be occurred due to habitat differences.

Key words: Phyllobacterium, *Colletotrichum gloeosporioides*, anthracnose, indigenous, *Serratia plymuthica*, culture supernatant, compatibility, rhizobacteria

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Corresponding Author: Jamsari Jamsari, Laboratory of Biotechnology, Faculty of Agriculture, Andalas University, 2nd Floor, Campus Limau Manis, 25163 Padang, West Sumatera Indonesia Tel: +62 751-72776 Fax: +62 751-72702

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Phyllosphere located in aerial parts of plants is known to be one of habitat predominantly occupied by bacteria (with a density up to 10^8 cm^{-2} leaf)[1,2]. This habitat is considered as one of vital and ubiquitous environment, not only for bacteria but also for diverse microbial community[1,3]. However, unlike other plant parts, phyllosphere is a very harsh environment since it is exposed to rapid and fluctuating environmental stresses[4]. This hostility contributes to great microbial heterogeneity in the phyllosphere due to various mechanisms used by the colonizers to avoid or tolerate with this kind of habitat[4,5]. It also provides a broad array of plant-microbe interactions comprising of mutualism, commensalism, parasitism as well as pathogenicity[2,3].

The presence of Phyllobacterium displayed symbiotic effects to their host by influencing plant growth and functions, such as production of growth promoting compounds and protection against pathogen infections[6-10]. As a biocontrol agent, these Phyllobacterium utilize its outstanding colonization ability to create preemptive exclusion of the pathogen[11]. Numerous species of Phyllobacterium, such as *Paenibacillus* sp., *Pseudomonas* sp., *Serratia* sp., *Pantoea* sp. and *Myroides* spp., have been reported for their capability in suppressing several phytopathogens[11-15].

Colletotrichum is genus of pathogenic fungus which has been reported to infect numerous crops[16]. One of its species, *C. gloeosporioides* has become one of major pathogens worldwide by causing fruit rots (anthracnose) to at least 1000 plant species[17]. This species is also known as the most frequent species causing anthracnose in chili pepper, especially in tropical regions[18-22]. Due to this disease infection, significant yield loss and unreliable product quality become unavoidable[23]. Various biocontrol agents have been reported for its ability in controlling this fungal attack, i.e., *Bacillus subtilis*, *Pseudomonas fluorescens*, *Streptomyces hygroscopicus* and *Oudemansiella mucida*[24-27].

The utilization of Phyllobacterium to overcome anthracnose infection is still far less documented. Regarding to its high tolerance against harsh environment, the competitive trait of Phyllobacterium could be utilized to managing this disease. Furthermore, this disease is difficult to be early managed due to its long-term incubation period known as quiescence phase until the symptom appeared visually during fruit ripening stage[28]. Outstanding colonization ability of this Phyllobacterium might block the symptom development through nutrient and space competition. Additionally, this study also examined the efficacy of cell-free supernatant application instead of living cell to develop more eco-friendly plant disease management. This study was aimed to investigate *in vitro* antifungal activity of two West Sumatera indigenous Phyllobacterium isolates against the anthracnose causing fungus *C. gloeosporioides* and its compatibility with rhizobacteria isolates.

MATERIALS AND METHODS

Isolation of Phyllobacterium isolates: In total of 120 isolates of indigenous Phyllobacterium were collected from phyllosphere of *Brassica juncea* L. in 2012 from District of Solok, West Sumatera, Indonesia. About 5 g of leaves were surface sterilized 3.5% sodium hypochlorite for 5 min and rinsed thoroughly three times using sterile distilled water. Leaves were subsequently grounded and cultured on a NaCl solution for 24 h under shaking condition. The bacteria were then isolated through serial dilutions and grown on Nutrient Agar (NA) medium for 2 days. Further selection was performed according to the morphological characteristics before subjected to antagonist assay. Bacterial isolates showing the highest antifungal activity would be used for further analysis.

Culture of fungi and bacterial isolates: Fungi used in this study consisted of three species of pathogenic fungi, i.e., *Colletotrichum gloeosporioides*, *Sclerotium rolfsii* and *Fusarium oxysporum*. *Colletotrichum gloeosporioides* was obtained from internal collection of Biotechnology Laboratory (Andalas University), while *S. rolfsii* and *F. oxysporum* were obtained from Phytopathology Laboratory (Andalas University) collection. Fungal mycelial disk (5×5 mm) was cultured onto PDA (Potato dextrose agar) medium (pH 7.0) and incubated at room temperature for 7 days in darkness.

In addition, two rhizobacteria isolates used in this study were *Pseudomonas lurida* strain UBCR_36 and *Serratia plymuthica* strain UBCR_12 collected from Biotechnology Laboratory (Andalas University). Both phyllo and rhizobacteria were grown on NA medium (pH 7.0) for 18 h at room temperature in darkness as well.

Species identification using 16S rRNA gene sequences: Two Phyllobacterium isolates showing the best suppression were molecularly identified using 16S rRNA gene sequences. This gene from each isolate was amplified using 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') primers. This analysis was conducted using the same protocol as described by Syafriani *et al.*[29].

Evaluation of bacterial inhibition range against pathogenic fungi: Inhibition range of these two isolates against three pathogenic fungi was tested through dual culture assay. The fungal mycelial disk was grown onto PDA (pH 7.0) for 48 h. Bacterial isolates were cultured in Luria Broth (LB) (pH 7.0) at 160 rpm room temperature for 12 h ($OD_{600\ nm}$ = 1.0). Paper disks (diameter 5 mm) were impregnated with 20 µL of cell culture and applied at 4 different positions located 3 cm away from the center of fungal growth. The plate was subsequently incubated at room temperature for 7 days. Inhibition was measured daily using this following formula[30]:

$$\text{Inhibition (\%)} = \frac{DC - DT}{DC} \times 100 \quad (1)$$

where, DC is the diameter of untreated fungus spot and DT is the diameter of fungus spot treated with bacteria[30]. Each treatment was performed in five replicates.

Co-cultivation of bacterial isolates and chili pepper isolated *C. gloeosporioides*: The protocol used for this co-cultivation was adapted from Zhang *et al.*[31] with several modifications. The culture of *C. gloeosporioides* was performed using a disk (5×5 mm) in 100 mL potato dextrose broth (pH 7.0) medium for 72 h under shaking condition at 100 rpm and room temperature. Bacteria cells were then cultured in LB medium (pH 7.0) until it reached $OD_{600\ nm}$ = 1.0. Bacterial pellet was collected through centrifugation (20,000×g at 4°C for 15 min) and washed three times with dH_2O. About 5 mL of cells suspension (3×10^8 CFU mL^{-1}) was then inoculated into the fungal culture and grown for 24 h at room temperature under agitated condition (100 rpm). Fungal mycelia were discarded and the bacteria was pelleted by centrifugation (20,000×g at 4°C for 15 min). The supernatants were then used for inhibition assay.

Antifungal assay of bacterial culture supernatants against *C. gloeosporioides*: The CSN of UBCF_01 and UBCF_13 were subsequently tested for its antifungal activity against chili pepper isolated *C. gloeosporioides*. The CSN used for this assay were divided into two categories, single culture and co-culture supernatants. This assay was performed using agar well plate method and each treatment was performed in five replicates. CSN, either from single culture or co-culture were harvested by centrifugation (20000×g at 4°C for 15 min) and subsequently filtered using a membrane with a pore size of 0.22 µm. An aliquot (50 µL) of the cell-free filtrate was then applied on 3 cm away from 2 days-aged *C. gloeosporioides* at four different positions.

Evaluation of CSN compatibility between phyllo and rhizobacteria isolates: The bacterial CSN compatibility for consortium type application was evaluated by combining the culture supernatants of phyllo and rhizobacteria isolates obtained from co-culture. Cell-free culture supernatants from each isolate were applied in the form of dual, triple and quadruple strain consortium with the mixing ratio 1:1 (v/v). Mixed culture supernatants (50 µL) was then subjected to dual culture medium containing 2 days-aged *C. gloeosporioides* and grown for 7 days at room temperature. The resulting inhibition was routinely measured using the formula as described previously.

Statistical analysis: Data were statistically analyzed using SPSS version 23.0 and presented with mean and standard deviation. Data from inhibition range assay and strains compatibility was processed by one-way analysis of variance (ANOVA). Significant differences from each treatment group were further processed using Duncan's New Multiple Range Test (DNMRT) with a $p<0.05$[32].

RESULTS

Species identity of Phyllobacterium isolates: Of 120 bacterial isolates collected from *B. juncea* leaves, 23 isolates showed antifungal activity against *C. gloeosporioides* and the highest suppression was resulted by two isolates, named UBCF_01 and UBCF_13. Based on the sequence of 16S rRNA genes, these two isolates were identified as *Serratia plymuthica*. Gene sequence from both isolates which covered 1534 bp has been deposited in the NCBI database with accession number KX394778 (UBCF_01) and KX394779 (UBCF_13). From a BLAST homology search, high homology (99%) of the gene sequence was shown between *S. plymuthica* UBCF_01 (KX394778) with other *S. plymuthica* species, such as *S. plymuthica* UBCF_13 (KX394779), *S. plymuthica* UBCR_12 (KU299959) and *S. plymuthica* AS9 (CP002773). Based on the phylogenetic tree, *S. plymuthica* UBCF_01 displayed closer genetic relationship with *S. plymuthica* UBCR_12 (KU299959) than with *S. plymuthica* UBCF_13 (Fig. 1).

Antifungal activity range of Phyllobacterium isolates: Both Phyllobacterium isolates, UBCF_01 and UBCF_13 displayed antifungal activity against *C. gloeosporioides* but no

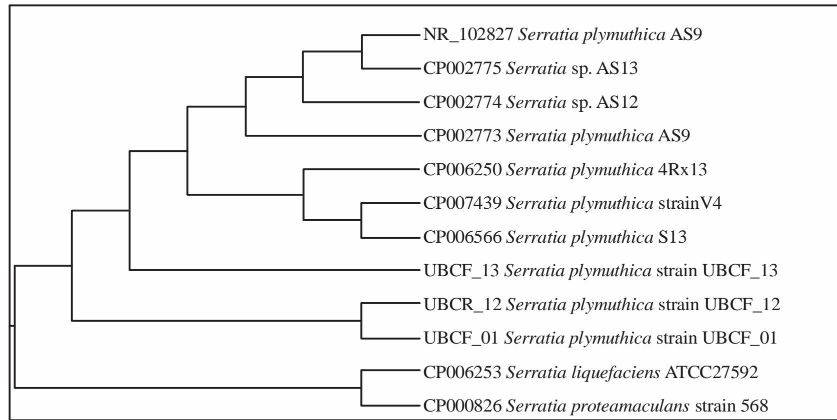

Fig. 1: Genetic relationship of both Phyllobacterium isolates with other *Serratia* species based on the sequence of 16S rRNA gene

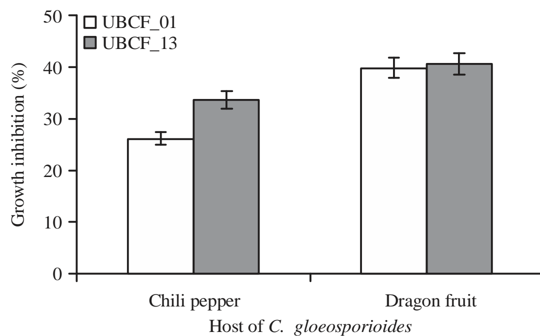

Fig. 2: Inhibition spectrum of Phyllobacterium isolates against *C. gloeosporioides* from different host
Values are Means±SD (n = 5)

inhibition of *F. oxysporum* and *S. rolfsii* was observed (Fig. 2). Of the two *C. gloeosporioides* phytovars used, the suppression by both Phyllobacterium was stronger against the one isolated from dragon fruit compared to the chili pepper one. However, each isolate showed similar inhibitory effect against dragon fruit isolate of *C. gloeosporioides*. Unlike the dragon fruit phytovar, UBCF_13 showed significantly higher suppression ($p<0.05$) than UBCF_01 at 7 days post application (DPA), respectively 34 and 26% (Fig. 2). However, the best suppression was still obtained from UBCF_13 about 41% against dragon fruit phytovar of *C. gloeosporioides* (Fig. 2). This result suggested that the antifungal activities of UBCF_01 and UBCF_13 are very specific for isolates of *C. gloeosporioides*, while they have no measurable inhibitory effect on *F. oxysporum* and *S. rolfsii*.

Effect of culture supernatants (CSN) on fungal suppression: Application of CSN from cultured Phyllobacterium showed similar inhibitory effects as the complete cell culture (Fig. 2) on *C. gloeosporioides*. Figure 3 shows that the single-cultured CSN from each isolate exhibited similar suppression trend. Of these two isolates, CSN from UBCF_01 exhibited higher inhibition at 7 DPA (30%) compared to the one from UBCF_13 CSN (26%) (Fig. 3). Despite having similar overall suppressions, the effect of UBCF_01 CSN was observed early (2 DPA) (Fig. 3), while inhibition by UBCF_13 was only detected at 4 DPA. The results imply that the two isolates suppress fungal growth by different substances or mechanisms. The UBCF_01 had a rapid effect but this was not very sustainable and it appeared that the fungus adapted. In contrast, UBCF_13 showed a delayed effect but this was better maintained.

In contrast, CSN effect from single-cultured UBCF_13 started to retard the fungal growth and displayed a measurable suppression at 4 DPA (Fig. 3). According to the suppression pattern, it showed that there might be a sudden effect at 4 DPA that was successfully maintained until 7 DPA, so that the suppression seemed to increase continuously. However, since no inhibition recorded at previous days, it is assumed that the distance between the CSN application site and the fungal growth might be one of the barriers. Thus, the fungal enabled to grow freely but then started to be suppressed when its growth was more closer to the CSN application site.

Unlike the single-cultured CSN, fungal growth was less affected by the CSN from bacteria co-cultured with fungus (Fig. 3). These results showed that the antifungal activity of both bacterial strains is not stimulated by the presence of the pathogen. Even though the suppression was clearly occurred during the co-culture and marked by the remarkable mycelial mass difference but it still could not be correlated with the CSN efficacy used in the antagonistic assay. Due to various unspecified substances contained in the CSN, the measurement to determine the concentration is seemingly unable to be performed.

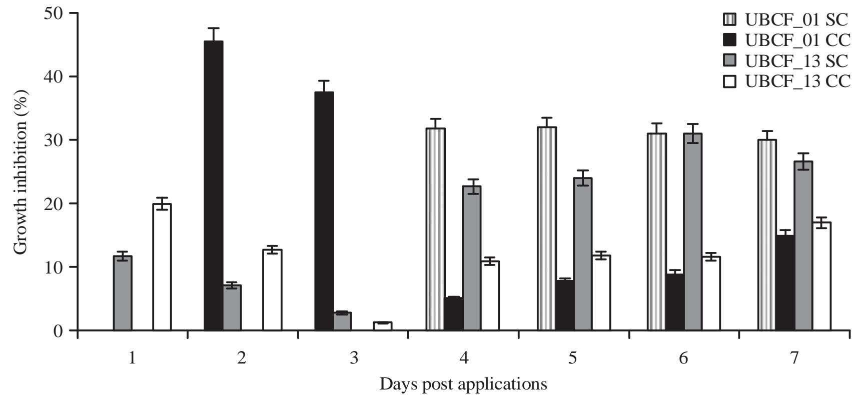

Fig. 3: Difference in fungal suppression efficacy of each Phyllobacterium culture supernatant resulted from different types of culture

SC: Single culture and CC: Co-culture, values are Mean±SD (n = 5)

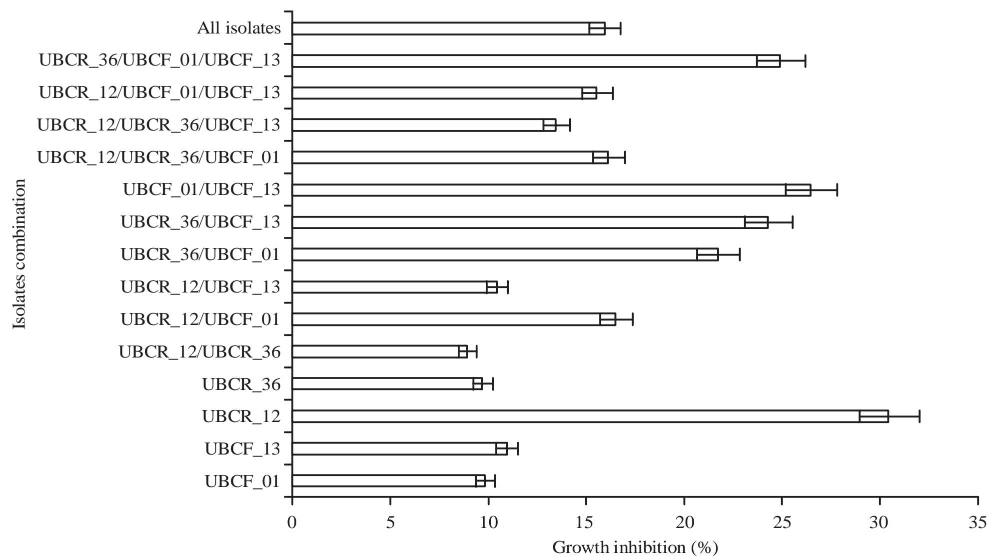

Fig. 4: Effect of strain/species compatibility towards the resulting suppression against *C. gloeosporioides* at 7 days post application

Values are Mean±SD (n = 5)

CSN compatibility between phyllo and rhizobacteria isolates: To improve the inhibition efficacy of the Phyllobacterium isolate, the CSN were applied in a mixture with rhizobacteria isolates. Figure 4 shows that both single and mixed applications resulted in different effects regarding the fungal suppression activity against chili pepper isolated *C. gloeosporioides*. This may indicate various compatibilities or mutual inhibition of antifungal effects. It showed clearly that combination of Phyllobacterium with rhizospheric UBCR_36 resulted higher inhibitory effect rather than the single isolate one. In contrast, mixed CSN application involving rhizospheric UBCR_12 showed relatively lower suppression than other combinations.

Compared to the single application, both Phyllobacterium isolates exhibited higher inhibition with consortium application. The combination of both Phyllobacterium strains resulted in the highest suppression (about 26%) (Fig. 4). Both isolates were also well compatible with *P. lurida* UBCR_36 although the suppression was not significantly enhanced. Double consortium of UBCR_36 with each Phyllobacterium isolate displayed quite similar suppression activity as the consortium consisted of these three isolates (Fig. 4).

Unlike *P. lurida* UBCR_36, rhizobacteria isolate *S. plymuthica* UBCR_12 exhibited weak synergistic effect with other isolates, including the same *S. plymuthica* species. Furthermore, CSN mixtures of both rhizobacteria isolates

repressed its inhibitory activity. Compared to UBCF_13 and UBCR_36, UBCR_12 showed better compatibility with UBCF_01. From the consortium of UBCR_12-UBCF_01, the highest suppression was observed compared to other UBCR_12 combinations. The presence of UBCF_01 in the consortium of triple isolates might improve the weak compatibility between UBCR_12 and UBCR_36. Nonetheless, the resulting inhibitions under consortium did not surpass its suppression when applied singly (30.54%) (Fig. 4). Combining all four isolates showed a weak effect on fungal inhibition indicating that there was no additive or synergistic mechanism. In the contrary, it rather appeared that the antifungal effects negatively interfered with each other.

DISCUSSION

Result of this study had proven that phyllospheric *S. plymuthica* could be developed as one of choice for anthracnose biocontrol agents (Fig. 2). This species is rarely reported for managing *C. gloeosporioides*. Recent studies reported that the rhizospheric *S. plymuthica* UBCR_12 displayed high suppression against *C. gloeosporioides*[29,33]. Compared to the rhizosperic *S. plymuthica* from previous work[33], these phyllospheric species exhibited different characteristic in suppressing *C. gloeosporioides*. It might be associated with lifestyle difference as the consequence of habitat difference, even the genetic relationship between both strains were closely related (Fig. 1). Antifungal activity of both Phyllobacterium isolates seemed to be more independent and specific only against *C. gloesporioides* (Fig. 2). Such characteristic had been previously reported by Zachow *et al.*[34] where the phyllospheric *Pseudomonas* sp. only showed remarkable suppression against *R. solani* of four pathogens tested.

Phyllobacterium is also known as highly competitive colonizer. This trait affects their ability to protect the host plant by suppressing the pathogen growth. Their prominent plant-protective effect is occurred due to their high abundance. Although this abundance related to the total cell number is not the key factor determining their protection but this high density population may be associated to the resources competition[9,11]. This kind of mechanism is termed as pre-emptive colonization which is mainly stimulated by niche occupation[35]. Several studies reported that nutritional similarity could bottle up the pathogen's secondary colonization and lead to better antagonistic effect[9,36,37].

This study was also proved that cell-free culture supernatants (CSN) of both Phyllobacterium isolates were able to suppress the growth of *C. gloeosporioides* (Fig. 3). In addition, both isolates showed that higher antifungal effect was resulted only from single culture. This result suggested that these two isolates did not require to be induced by fungal cells to obtain higher suppression efficacy. Regarding to this characteristic, these Phyllobacterium isolates displayed better option for the ease of mass production compared to rhizospheric UBCR_12 from previous study[33]. According to this result, the utilization of biocontrol agents for large-scale field application could be applied in more eco-friendly way through this CSN, instead of using living cell. Since it is cell free, it could reduce the risk of uncontrolled bacterial spreading and effects on ecosystem balance when applied in high volume. The CSN is mostly composed of secreted secondary metabolites that play a crucial role in defense mechanisms against biotic and abiotic stresses. Its production is usually occurred during the late growth phase of bacteria and is greatly stimulated by several factors, such as nutrient compositions[38].

To develop an efficient CSN application, CSN effect should be measured by a proper method that could accurately explain the CSN efficacy on pathogenic fungus. Figure 3 shows that the inhibitory effect of CSN from both isolates was appeared quite slowly. This slow occurrence could be one of limitations that affect the measurement, so that, the exact efficacy of this CSN could be precisely determined. It was also predicted that this slow effect could be associated to diffussion problem. The selection of agar diffusion method to be used in this study was considered due to its simplicity, low cost, low amount of sample required and easy to interpret[39]. However, this method showed some limitations, such medium incompatibility and substance instability problems occurred during diffusion process[40].

Application of microbial consortium had been widely studied to observe the possibility of obtaining higher suppression efficacy through the combination of diverse mechanisms from each microbial component[11,41,42]. However, application of microbial consortium using bacterial CSN as conducted in this study, was still less documented. Figure 4 shows that CSN combination composed of both Phyllobacterium isolates resulted in elevated suppression compared to single isolate application. Moreover, both isolates exhibited remarkable compatibility with rhizospheric *P. lurida* UBCR_36. However, this synergistic effect was quite unseen from the CSN combination of both phyllo and rhizospheric *S. plymuthica*. Successful consortium demands the high compatibility among the involving strain or species to cooperate synergistically or at least additive on the disease suppression. Incompatible combinations might result in the decrease or even loss of inhibitory effects[42-44].

CONCLUSION AND FUTURE RECOMMENDATIONS

It is concluded that the Phyllobacterium isolates UBCF_01 and UBCF_13 are promising alternative biocontrol agents against *C. gloeosporioides*. Supernatants from cultures of these bacteria were also effective. Field application of bacterial CSN instead of living cells would provide a less hazardous approach to plant disease management. However, these supernatants were only tested under controlled conditions in the absence of plants. The supernatants should be further tested directly with infected plants both in the greenhouse and then in the field.

This present study presented new option of biocontrol agent against *C. gloeosporioides* in chili pepper by utilizing the indigenous Phyllobacterium. It also offered safer way in biocontrol application through bacterial cell-free CSN to minimize the risk of uncontrolled bacterial spreading when applied in large-scale. Elevated suppression resulted from strain/species combinations suggested that presence of other antagonist bacteria could maximize the resulting inhibitory effect.

SIGNIFICANCE STATEMENTS

This study discovers an easily accessible yet eco-friendly choice for biological agents to manage the anthracnose infection in chili pepper. It will help the researchers to develop better plant diseases management system for field scale with lower risk of biological contaminations due to direct application of biocontrol agents that was not fully explored, yet. Thus, a new idea on these applications of biocontrol agents and possibly other improvement, may be arrived at.

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