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NON-PATHOGENIC PHYLLOSPHERE BACTERIA PRODUCING BIOACTIVE COMPOUNDS AS BIOLOGICAL CONTROL OF *Xanthomonas oryzae* pv oryzae

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ABSTRACT

Xanthomonas oryzae pv oryzae (Xoo) is the causal agent of leaf blight disease of rice. The use phyllosphere bacteria of rice plant as biocontrol agent is an alternative ecofriendly strategy to control Xoo. The aims of this study were to isolate and screen phyllosphere bacteria of rice plant which has an ability to produce bioactive compounds against Xoo. A total of 400 phyllosphere bacterial isolates were obtained from rice plant leaves, 171 isolates produced bioactive compounds that inhibited *Xoo*, and 79 isolates performed negative response of hypersensitivity on tobacco leaf and 57 isolates among them showed negative pathogenicity on rice leaf. Molecular identification based on 16S rRNA genes of nine non-pathogenic isolates revealed that 6 isolates were identified as genus *Bacillus*, whereas isolates BFF62, BFV63, and BFF75 were identified as *Myroides odoratimimus, Pontibacter niistensis*, and *Delftia tsuruhatensis*, respectively. Isolate BFF84 that showed the highest anti-*Xoo* activity was identified as *Bacillus aerophilus*.

KEYWORDS: Phyllosphere bacteria, bioactive compound, *Xanthomonas oryzae* pv oryzae (Xoo), anti-*Xoo*, 16S rRNA.

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INTRODUCTION

Bacterial leaf blight caused by *Xanthomonas oryzae* pv oryzae (Xoo) is one of the most destructive diseases of rice in Asia. The disease can reduce rice production by over 50%[1]. *Xoo* can infect every phases of plant growth, threrefore it is very hard to be controlled[2]. Disease resistance of plants and antibacterial pesticides have been introduced to control bacterial leaf blight disease, but recent years the focus to control disease has shifted to the usage of biocontrol agents, which were safer and more promising alternative than synthetic chemicals. However, eco-friendly strategies have been developed to suppress the diseases in increasing the yield. The rice plant represents a habitat for the diverse microorganisms. They colonize at the aerial parts (phyllosophere), the root surface (rhizoplane), and around the root zone (rhizosphere) [3]. The phyllosphere comprises the aerial parts of leaf. It is known that many potential interactions of phyllosphere bacteria with rice plants, such as plant growth promotion, plant hormone production[4], or biocontrol agent[5]. Bacterial community was located on the same leaf surface could compete for the same nutrient sources or habitats. This competition among microorganisms plays an important role in determining the population density of phyllosphere bacteria[6]. As a biocontrol agent, these bacteria can produce antimicrobial compound that very useful to protect plant from pathogen-attacked[7]. Data of the phyllosphere bacteria diversity from rice plant in Indonesia especially in particular those who produce anti-*Xoo* activity are still limited. In addition, the potency of these bacteria as biocontrol agent has not been studied completely. So, the objective of this study was to isolate and screen the potential non-pathogenic phyllosphere bacteria of rice plant that have a highly inhibitory effect against *Xanthomonas oryzae* pv oryzae.

MATERIALS AND METHODS

(i) Sampling and isolation of phyllosphere bacteria[8]

Rice plants were collected from rice fields in Situ Gede (Bogor, Indonesia). The leaf samples were taken from different phases of growth (vegetative, flowering, and ripening). Sampling was carried out in the morning,the samples were transported to the laboratory, and analyzed in less than 2 hours for isolation of phyllosphere bacteria. The phyllosphere bacteria of rice plant were isolated using Nutrient Agar (NA), TryptoneSoyAgar (TSA), Luria Bertani Agar (LA), and Kings B Agar (KBA) by serial dilution method. One gram of leaf sample was cut into small pieces and immersed in 25 ml of sterile 0.85% NaCl suspension. The suspension were homogenized by vortex for 15 min. Serial dilution technique was performed up to 10^{-7} dilution. The cultures were incubated and the colonies were observed after 3 days incubation at 28°C. Individual colonies were selected, picked, and recultured on fresh media for further experiments.

(ii) In vitro antagonism against Xoo

The Xoo pathogen STG21 was obtained from the previous sudy[9]. The antagonistic activity of phyllosphere bacteria against Xoo was tested by dual culture technique[5]. The isolates of phyllosphere bacteria were streaked on LA plates containing Xoo (±10^8cfu/ml) and incubated at 28°C for 2-3 days with appropriate control. Three replications were maintained for each isolate and inhibition zone was recorded.

(iii)Hypersensitive response (HR) and pathogenicity tests in planta[10]

Selected phyllosphere bacteria which had anti-Xoo activity, were grown in Luria Bertani broth (±10^8cfu/ml) and infiltrated into tobacco leaves by using needleless syringes and inoculated into one month-old rice plants (IR64) leaves by using leaf clipping method. Plant responses were scored at 48 h (for HR) and 14 days (for pathogenicity test) after inoculation. All plants were grown in growth chambers at 28-30ºC with a 12 h of photoperiod. Xoo wild type was used as positive control, while *Eschericia coli* DH5α and sterile water were used as negative control. Experiments were carried out for three replications.

(iv) Bacterial DNA genome extraction and 16S rRNA gene amplification[11]

Bacterial genome was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method. The bacterial 16S-rRNA genes were amplified by using PCR technique with universal primers 67F(5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3'). PCR was performed in 50 µl reaction mixture consisted of 25 µl Go Taq Green Master Mix (Promega®, USA), 4 µl (10 pmol/ µl) of each primer, 3 µl of DNA template, and 14 µl of nuclease free water. The procedure for PCR was pre-denaturation at 94ºC for 4 minutes, followed by 25 cycles: denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, and extention at 72ºC for 1 min. The final extention was at 72ºC for 7 min. The PCR products were purified, sequenced, and analyzed by comparing the sequences with the GenBank database (NCBI) by using the BlastN program to obtain the nearest phylogenetic neighbours. Phylogenetic trees were constructed using neighbor-joining algorithm, implemented in MEGA version 5.0 (Tempe, AZ, USA), with 1000 bootstrap replicates.

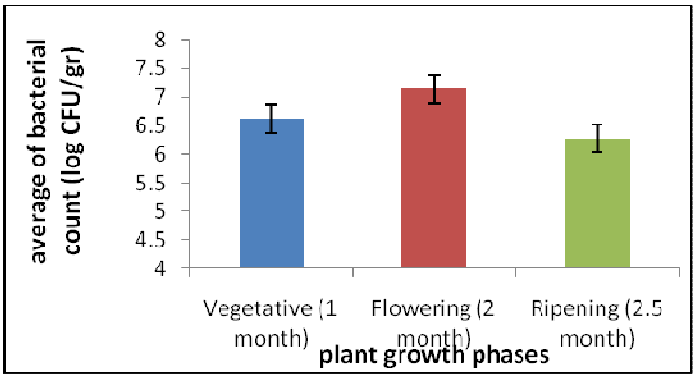
RESULTS

1. Isolation of Phyllosphere Bacteria

The colony forming units (CFU) numbers of bacteria from leaf samples during vegetative phases, flowering phases, and ripening phases were closely similar, at densities varied from 6,28 to 7,14 log CFU/g (fresh weight). (Graph 1). Using the selected medium described previously, a total of 400 isolates were purified from 3 different leaf samples. The flowering phases give the highest number of isolates (147 isolates).

Graph 1

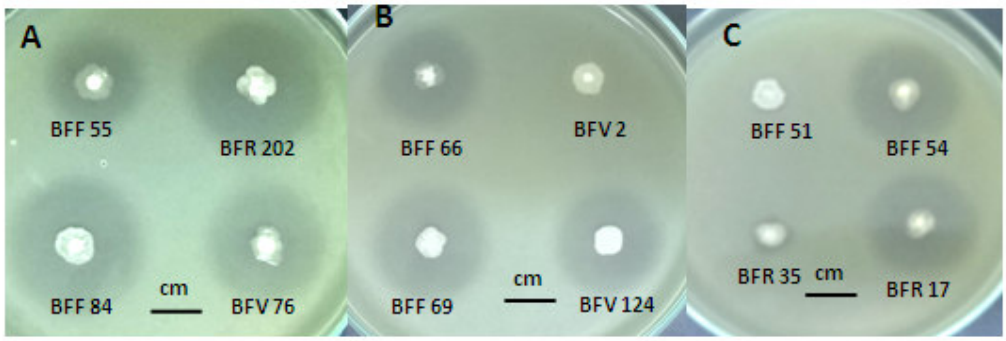
Numbers of phyllosphere bacteria isolated from leaf of rice plant at three different growth phase as determined by plate counts.



2. Screening of Antagonistic Bacteria Against Xoo

A total of 400 phyllosphere bacterial isolates, 171 isolates demonstrated antimicrobial activity against Xoo. These isolates had varying zones of inhibition ranging from large (20-30 mm) to small inhibition zones (0-10 mm) (Figure 1). Table 1 shows isolates which have anti-Xoo activity ranging from 10-34 mm.

Figure 1
Growth inhibition of Xoo by phyllosphere bacteria producing bioactive compound



Bioactive compound indicated by clear zone around colony of phyllosphere bacteria. (A)isolates of BFF 84, BFF 55, BFR 202, BFV 76, (B) isolates ofBFF 66, BFF 69, BFV 2, BFV 124, (C) isolates ofBFF 54, BFF 51, BFR 35, BFR 17.

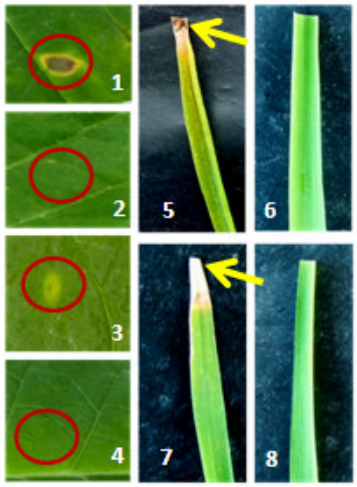
Table 1
Diameter of inhibition zone produced by phyllosphere bacteria against Xoo.

Vegetative phases (1 month)		Flowering phases (2 month)		Ripening phases (2.5 month)	
Isolate code	Diameter of inhibition zone (mm)	Isolate code	Diameter of inhibition zone (mm)	Isolate code	Diameter of inhibition zone (mm)
BFV 30	22	BFF 15	15	BFR 8	14
BFV 31	34	BFF 24	24	BFR 17	22
BFV 33	23	BFF 38	20	BFR 18	20
BFV 53	20	BFF 45	24	BFR 20	12
BFV 54	21	BFF 50	17	BFR 22	18
BFV 55	31	BFF 54	22	BFR 36	13
BFV 63	13	BFF 65	32	BFR 55	16
BFV 65	15	BFF 66	17	BFR 64	14
BFV 76	22	BFF 62	30	BFR 69	20
BFV 78	27	BFF 75	15	BFR 99	14
BFV 79	21	BFF 84	28	BFR 152	30
BFV 80	25	BFF 110	17	BFR 162	10
BFV 92	26	BFF 124	13	BFR 202	20
BFV 110	14	BFF 130	25	BFR 220	13
BFV 124	18	BFF 147	23	BFR 238	22

3 *HR and pathogenecity test*

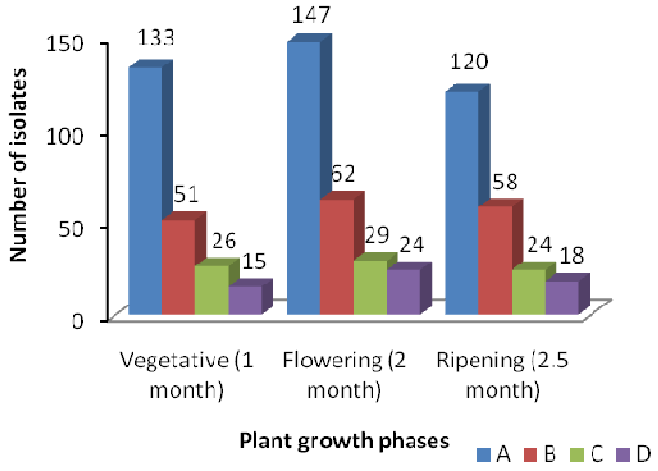
Selection of biocontrol agent was done through hypersensitivity test on tobacco and pathogenecity assay on rice plants (IR 64). Hypersensitivity reaction results showed 26 isolates from vegetative phases, 29 isolates from flowering phases, and 24 isolates from ripening phases had negative results indicated by no disease symptoms occured (Figure 2). *Xoo* wild type was used as positive control, while sterile water were used as negative control. One month-old rice plants was inoculated with isolates which chosen based on negative respond in hypersensitive test. *Xoo* wild type inoculation showed bacterial leaf blight disease symptoms which observed at 3-14 days after inoculation. A total of 57 non-phytopathogenic isolates had a potency as biocontrol agent against *Xoo* (Graph 2).

Figure 2
Hypersensitivity Reaction in tobacco and pathogenicity test on rice plant.



Hypersensitivity response in tobacco and pathogenicity test in 30-day-old of rice plant. Xoo as control positive in leaf (1,5), Sterile water as control negative (2,6), isolate BFF15 resulted in positive response indicated with any symptoms (3,7) and isolate BFF 84 resulted in negative response (4,8). Red circle and yellow arrow indicated the spot of injection and any disease symptoms.

Graph 2
Number of isolates that potential as biocontrol agent against Xoo.

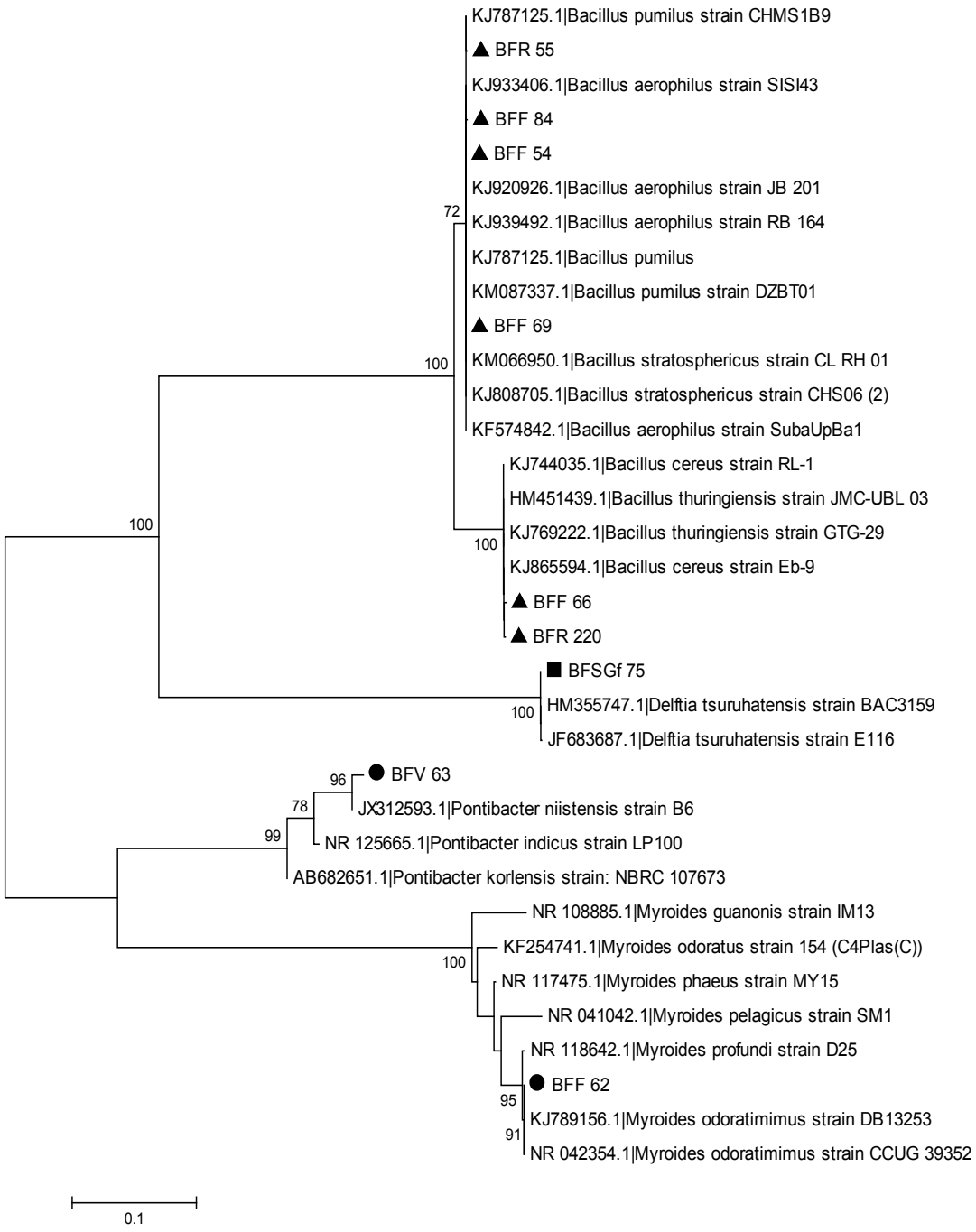


Total of phyllosphere bacteria isolated from rice plant (A), isolates that producing bioactive compound against Xoo (B), isolates resulted negative responses at hypersensitive test (C), and isolates resulted no symptoms at pathogenicity assay in one month-old rice plant (D).

4 Molecular taxonomy of isolates as biocontrol agent

The phylogenetic relationship determined by 16S rRNA gene sequences of the 9 selected isolates are presented in Figure 3. A comparison of the nearly 16S rRNA gene sequences of the isolate BFF84, BFF54, BFR55, BFF69, BFF66, and BFF220 here against sequences in the GenBank database revealed homologies of more than 99% to members of the *Bacillus* sp., while 2 isolate BFF62 and BFV63 revealed they had homologies to *Pontibacter niistensis* (96%) and 95% to *Myroides odoratimimus*, respectively. These bacteria belong to the group of bacteroidetes. One isolate, BFF75, had momology revealed homologies to *Delftia tsuruhatensis* (100%).

Figure 3
Phylogenetic tree of non-pathogenic phyllosphere bacteria based on 16S rRNA sequence



Phylogenetic tree based on 16S rRNA gene of 9 potential isolates producing bioactive compound as biocontrol agent against Xoo. Scale shows distance evolution on the branch length, while the numbers on the branches indicate bootstrap values.

DISCUSSION

The desired achievement of this study was to control the spread of bacterial leaf blight disease caused by *Xoo* by looking for antagonistic bacteria that are able to produce bioactive compound which inhibit the growth of pathogen in their natural habitat, phyllosphere of rice. Like other biological surfaces, the phyllosphere harbors a large numbers and diversity of microorganisms including bacteria, yeast, fungi, and algae[6]. Bacteria are the most numerous and diverse colonists of leaves, with culturable counts in

the range of 10^2 and 10^{12} cells per gram of leaf[12]. Changes in microbial phyllosphere communities are driven by changing environmental conditions such as heavy rainfall, temperature, wind, and radiation[13]. In the present study, we found that growth phases in rice plant effected the diversity of the bacterial phyllosphere, in which flowering phases gave the highest number of total bacteria in rice phyllosphere. The plant growth phases were linked to age of the leaves. The aging of leaves contributed to seasonal changes in microbial phyllosphere communities. Leaf age affects leaf properties, both with respect to the leaf morphology[14] and physiological properties, such as photosynthetic activity[15]. These changes affect the size of the organic compound pool, sites of exudation and the quality of organic compounds exudated over the cuticle and, in turn, affect the microbial community composition[16]. Not all of the isolated phylloshere bacteria had anti-*Xoo* activity. Only 42,75% of isolates were able in inhibiting the growth of pathogen by producing antimicrobial compound. Antimicrobial compound is produced by microorganisms to remain competitive in their environment by diminishing growth of other bacteria. In the case the other bacteria are pathogen, the plant benefits as well[17]. Table 1 shows diameter of inhibition zone produced by non-pathogenic phyllosphere bacteria in the range of 10 mm to 35 mm, which some isolates were looked like very effective to inhibit the growth of *Xoo* by producing antimicrobial compound, otherwise some isolates grew widely even the zone inhibition were very small. The antagonist mechanisms are competition of space and nutrients. An antagonist which is succesfull in the colonization of an ecological niche, competes mostly for both space and nutrients[6]. The number of strains to be considered as putative biocontrol agents sometimes are indispensable to narrow down in planta assay. It is well known that in vitro antibiosis does not predict in planta antagonism[18]. We conducted some pre screening test in the plant to know the pathogenecity potency of bacterial phyllosphere that had ability to control the growth of *Xoo*. Plant respons to the presence of bacteria by the recognition called Microbe-Associated Molecular Patterns (MAMPs). Lipopolysacharide is an example of bacterial MAMPs that can trigger a host response[19]. In an incompatible interaction of plant would answer to a pathogen attack with a programmed cell death known as hypersensitive response (HR). In a compatible interaction (plants are not resistant), pathogenic bacteria have evolved strategies to overcome the plants defense response that evolves type III secretion system which is encoded by hrp gene cluster and considered as the key virulence determinant[20]. In this study, 57 isolates showed negative response in HR and pathogenecity test, these potential isolates could be recommended as biocontrol agents. We selected 9 isolates based on their variety of inhibition zone. Four isolates (BFF 62, BFF 84, BFF 54, BFF 69) were have large inhibition zone (20 mm-30 mm), three isolates (BFF 66, BFR 55, BFF 75) were have moderate inhibition zone (15 mm-20 mm), and two bacterial isolates (BFR 220, BFV 63) were have small inhibition zone (13 mm). The selected isolates were further identified using 16S rRNA gene sequence approach. Identification and phylogenic tree analysis revealed the dominance of genera Firmicutes, followed by Proteobacteria and Bacteroidetes. Isolate BFF 84 and BFF 54 were identified as *Bacillus aerophilus* with high homology more than 99%, while isolate BFF 69 and BFR 55 were closely similar to *Bacillus stratosphericus* and *Bacillus pumilus*, respectively. Two isolates BFF66 and BFF 220 were identified as *Bacillus cereus*. The member of gram negative bacteria (BFF 75, BFV 63, and BFF 62) was identified as *Delftia tsuruhatensis*, *Ponibacter niistensis*, and *Myroides odoratimimus*, respectively. *Delftia tsuruhatensis* is commonly found in rhizoplane or rhizosphere of plant. It has been reported that these diazotrophic bacteria could suppress the growth of various plant pathogen effectively, especially the three main rice pathogens (*Xanthomonas oryzae* pv oryzae, *Rhizoctonia solani*, and *Pyricularia oryzae*)[21]. The presence of two members of Bacteroidetes can be believed that it is related to insects activity in environmental of rice fields. Simultaneously or sequentially attack by herbivores is common in plants. Plant defence signalling trade-offs can have important ecological consequences in nature that may be reflected in a positive correlation

between herbivory and phyllosphere bacterial abundance and diversity[22]. *Myroides odoratimimus* is a gram negative bacteria, rod, and live aerobically. This yellowish orange colour bacteria has been isolated from gut of diptera has been characterized resistance to some antibiotic compound and is able to control the growth of clinical pathogen[23]. A pink pigmented bacteria, *Ponibacter niistensis* isolated from phyllosphere of rice plant was one of the many pigmented bacteria found in phyllosphere habitat. Phyllosphere is a habitat that is quickly changed by the environmental factors. One of the ways for phyllosphere bacteria withstand high UV radiation is by producing pigment pink or orange or by secreting exopolysaccharide (EPS)[24]. By dual culture assay, six isolates BFF 84, BFF 54, BFR 55, BFF 66, BFF 69, BFR 220 showed superior anti-*Xoo* activity. *Bacillus* strains which are known have the potential use to protect the plant from pathogens or pests, and stimulating plant growth are attributed to two groups i.e. the *B. cereus* group and *B. subtilis* group. The *B. cereus* group includes *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanesis*; while the *B. subtilis* group includes *B. subtilis*, *B. pumilus*, *B. atrophaeus*, *B. licheniformis* and *B. amyloliquefaciens*[25]. For several diseases caused by genera of *Xanthomonas*, colonization processes and biofilm formation are the important part for the development of the pathogen in phyllosphere. Applying antagonistic Bacilli to rice leaves may be associated with their ability to interfere those process. *B. subtilis* and *B. amyloliquefaciens* have been proven as biocontrol agents to *Xanthomonas axonopodis* pv citri, the causal agent of citrus cancer disease[26]. By producing some antimicrobial compounds such as bacteriocins and lipopeptides, *Bacillus* strains can act as potential biological suppressers of phytopathogens[27].

CONCLUSION

Antagonistic phyllospere bacteria of rice plant against pathogenic microbes are important as biocontrol agents of bacterial leaf blight disease caused by Xoo.171 isolates of phyllospere bacteria performed antagonistic activities against Xoo. 57 isolates among them are important to be developed as biological control of Xoo. Nine selected bacterial isolates revealed *Bacillus* spp. were more dominately antagonistic bacteria encountered based on phylogenetic tree analysis. It was exciting to discover member of *Delftia tsuruhatensis*, a pink bacteria *Pontibacter niistensis*, and a flavobacterium *Myroides odoratimimus* were also active to inhibit the growth of Xoo. These non-pathogenic phylosphere bacteria can be recommended as biocontrol agents of Xoo.

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