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Diversity of *Bacillus* spp. from soybean phyllosphere as potential antagonist agents for *Xanthomonas axonopodis* pv. *glycines* causal of pustule disease

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Abstract. *Nurcahyanti SD, Wahyuni WS, Masnilah R, Nurdika AAH. 2021. Diversity of* Bacillus *spp. from soybean phyllosphere as potential antagonist agents for* Xanthomonas axonopodis *pv.* glycines *causal of pustule disease. Biodiversitas 22: 5003-5011.* Pustule disease caused by *Xanthomonas axonopodis* pv. *glycines* (Xag) is an important disease in soybean. Bacteria from soybean phyllosphere is one of the potential biological agents against this disease. This is because the microorganisms have similarity. This study aimed to determine the diversity and species of bacteria from the soybean phyllosphere that have potential as biological agents. The research was carried out by morphological observation, physiological - biochemical testing, and molecularly with BOX AIR primer. Molecular identification was carried out by amplifying the 16S-rRNA gene with 27F and 1492R primers. The results showed that 11 isolates could inhibit Xag *in vitro* which showed morphological, biochemical, and molecular diversity. These bacteria were identified as *Bacillus* spp. which consisted of 4 groups, namely *Bacillus siamensis, B. subtilis. B. amyloliquifaction* and *B. velezensis*. The diversity of phyllosphere bacteria allows them to be used as biological agents because they do not inhibit each other and have diverse living abilities in various environmental conditions.

Keywords: Biological control, microbial diversity, phyllosphere bacteria, soybean, *Xanthomonas axonopodis* pv. *glycines*

INTRODUCTION

Xanthomonas axonopodis pv. g*lycines* (Xag) is the causal agent of pustule disease on soybean crops in Indonesia. This disease was widespread globally and a major disease in soybean producing countries (Wrather et al. 2001). The early symptoms of pustule disease are leaves have yellowish-green spots and develop into brown pustules surrounded with yellow halo (Prathuangwong and Choethana 1998; Salerno and Sagardoy 2003). The characteristic of Xag colony on nutrient agar media was yellow, smooth, convex, and with the entire margin glistening. This bacteria can hydrolyze starch with clear zones of 4-7 mm on SX media, 0.9 x 1.6 µm in size, negative gram, single polar flagellum, forming capsules, and acid-fast negative (Sain and Gour 2013).

Biological control of plant diseases using antagonistic bacteria is widely used. Xag is an airborne pathogen and inhabits the leaves, the biological agents were also be sought in the plant phyllosphere. Generally, plant leaf surfaces are colonized by bacteria with a density of 10^6 - 10^7 cfu/cm^2 (Vorholt 2012). On the leaves, there were various bacteria with the number of colonization 10^2-10^{12} cfu/g leaf (Thompson et al. 1993; Inácio et al. 2002 in Whipps et al. 2008). Salerno and Sagardoy (2003) succeeded in isolating 175 bacterial isolates from the soybean phyllosphere, bacterial strains with code 210 showed great antagonism and reduced the severity of soybean pustule disease.

The microbial communities are influenced by many factors such as plant species, seasons, geography, and differences in environmental conditions (Luo et al. 2019). The location of the planting field and the phase of plant growth have a more significant influence than cultivars or genetic modification (Leveau 2018). Many factors affect the life of phyllosphere bacteria such as relative humidity, UV light intensity, fluctuations of temperature and humidity, nutrient limitations, microbial interactions, plant phenotype, and genotype (Lindow and Brandl 2003; Chaudhary et al. 2017).

Microbes in the phyllosphere can protect plants from pathogens (Arnold et al. 2003; Rastogi et al. 2013) because they can produce antimicrobial compounds and compete with pathogens (Braun et al. 2010). Several bacteria capable of producing antifungal and antibacterial compounds have been isolated from paddy leaves (Wiraswati et al. 2018). Kumar et al. (2018) isolated 46 bacterial isolates from the phyllosphere of several plants that produce antifungal compounds. This bacteria isolate showed the ability to increase the growth and yield of potato production. Batool et al. (2016) isolated *Bacillus, Microbacterium, Acinetobacter, Proteus, Psycrobacter, Pseudomonas, Streptomyces*, and *Kineococcus* from wheat to increase plant growth. The use of phyllosphere bacteria and their antimicrobial compounds as biological agents in

the future can be developed for biotechnology applications (Zhang et al. 2010). This study aims to determine the diversity of bacteria in the soybean phyllosphere that can be used as a biological control agent for soybean bacterial pustules.

MATERIALS AND METHODS

Xanthomonas axonopodis pv. *glycine* reculture

The experiment was done at Plant disease laboratory, Departement of Plant Protection, Agriculture Faculty, Jember University, Indonesia. The isolate of Xag was a collection from Plant Disease Laboratory. The Culture of Xag was grown on Yeast Peptone Glucose Agar (YPGA) media with the streak inoculation. After 48 hours of incubation, a colony with yellow color was taken and purified as pure culture (Sain and Gour 2013). Confirmation of bacterial strain was carried out by gram test using 3% KOH, starch hydrolysis test, hypersensitive reaction test, and pathogenicity test (Schaad et al. 2001).

Isolation of phyllosphere bacteria

The healthy soybean plants were taken from Jember, Situbondo, and Lumajang, East Java, Indonesia. The sample was consisted of a mixture of the top, middle, and bottom leaves. The cutting leaves were grounded in 20 mL sterile water and then put into 250 mL erlenmeyer. The extract was centrifuged at the speed of 50 rpm for 24h. The supernatant was serially diluted into 10^{-5} and 10^{-6}. HundredµL of each dilution was grown on Yeast Peptone Glucose Agar (YPGA) and Kings'B medium then incubated at room temperature for 48 hours. A single colony was selected based on the morphology criteria and the purified bacteria was kept as a preserved culture in sterile liquid paraffin (Akter et al. 2014). Hypersensitive reaction and pathogenicity tests are carried out to ensure that the bacteria are not pathogenic to the plant. This was done by injecting 10^8 cfu/mL bacterial suspension into the mesophyll of tobacco leaves for HR test and soybean leaves for pathogenicity test. Furthermore, it was incubated at a humid temperature and observed for necrotic symptoms in tobacco and pustules in soybeans leaves.

Antimicrobial activity of the phyllosphere bacteria against Xag

An antimicrobial activity test was carried out by the dual plating method. Phyllosphere bacteria were dripped on YPGA and Kings'B media (fluorescent bacteria) and incubated for 48 hours. The Petri dish was put upside down and 1 mL chloroform was poured into the lid. After 2 hours, the petri dish was reversed again. Then the 48 hours aged Xag suspension was mixed with 4 mL 0.6% water agar and poured on the top of YPGA and KingsB media. The media was then incubated for 24 hours. The diameter of clear zones from the edge of the phyllosphere bacteria colony was measured. Detection mechanism of inhibition of phyllosphere bacteria against Xag was done by putting agar from the clear zone into a test tube containing 0.5% peptone water and incubating up to 5 days to see the turbidity of peptone water which was determined by the Xag growth (Rahman et al. 2012). The compatibility test was carried out on potential isolates using the dual plating method.

Characteristic of the potential phyllosphere bacteria isolates

Morphological observations were carried out visually to determine the characteristics of the bacterial colony including color, shape, edge, and elevation (Salle 1979; Cappuccino and Welsh 2017). Physiology and biochemistry test are based on the results of molecular identification related to the bacterial genus. This test includes the gram test, catalase, oxidase, starch hydrolysis, utilization of carbon sources, and growth at different levels of NaCl and pH (Schaad et al. 2001). Compatibility tests of the phyllosphere bacteria isolates were carried out using the dual plating method as described in the antimicrobial activity test

Data analysis

The result of antimicrobial activity was analyzed for variance and continued with the Duncan Multiple Range Test at a 5% confidence level using IBM SPSS Statistics software version 22.

Genetic diversity

DNA extraction

Bacteria DNA was extracted using the CTAB method (Ausubel et al. 2010). Bacterial isolates on 5 mL Nutrient Broth media were incubated for 24 hours. After incubation, the bacterial liquid culture was shaken at 125 rpm for 16-24 hours at room temperature. The bacterial culture was then put in a 1.5 mL tube and centrifuged at 12,000 rpm. The supernatant was discarded. The pellets were added to 540 µL of TE buffer and 30 µL 10% SDS, then incubated for 1 hour at 37°C in an oven. The suspension was added with 100 µL 5 M NaCl and 80 µL CTAB/NaCl, shaken, and then incubated at 65°C for 10 minutes in a water bath. A total of 750 µL CIAA was added and shaken to the suspension, then centrifuged at 12,000 rpm for 5 minutes. The supernatant was transferred into a new tube with 600 µL of PCIAA, and then centrifuged at 12,000 rpm for 5 minutes. The supernatant was transferred again in a new tube with 96% ethanol (1 M) as much as 0.6 x the volume of the supernatant obtained. Then it was centrifuged at 12,000 rpm for 5 minutes and the pellet was washed with 300 µL of 70% cold ethanol, then centrifuged at 12,000 rpm for 5 minutes. The pellets obtained were dried and added with 20 µL of TE buffer and stored at -20°C.

Analysis of phyllosphere bacteria diversity with Rep PCR

Phyllosphere bacterial isolates were analyzed for genetic diversity using the repetitive based-sequenced polymerase chain reaction (rep-PCR) method. The primer used was BOX A1R (5'-CTACGGCAAGGCGACGCTG ACG-3'). DNA amplification was carried out using a total volume of 25 µL, consisting of 12,5 µL GoTaq green, 3 µL primer BOX-A1R, 2,5 µL DNA template, and 7 µL ddH_2O. The DNA amplification reaction was carried out under pre-

denatured PCR conditions at 95°C for 15 minutes, followed by 35 cycles consisting of denaturation at 94ºC for 1 minute, annealing at 53°C for 1 minute, the initial DNA band doubling reaction at 72°C for 2,5 minutes and doubling of the final DNA band at 72°C for 10 minutes. Then the final temperature was set at 4°C.

The PCR product was electrophoresed on agarose gel with a concentration of 1.5% in 1x TBE buffer. A total of 8 µL of PCR product and 5 µL of DNA marker were injected into each well on the gel and the electrophoresis machine was run at a voltage of 90 mV for 80 minutes. Next, the gel was immersed in ethidium bromide solution for 10 minutes. The DNA bands were visualized on a UV transilluminator.

16S-rRNA. gene amplification

Amplification of the 16S-rRNA gene was conducted using universal primer pairs, 27F (5` AGAGTTTGAT (AC) TGGCTCAG 3`) and 1492R (5`CGG (CT) TACC TTGTTACGACTT 3`). PCR mixture contained 25 µL GoTaq Green Master Mix, 1µL of each primer, 1 µL DNA template, and 22 µL nuclease-free water. Amplification of 16SrRNA gene was conducted using the following reaction: predenaturation (94°C, 4 min), 30 cycles of denaturation (94°C, 1 min), annealing (52°C,1 min), elongation (72°C, 1 min), post elongation (72°C, 10 min). The PCR product was then visualized on 1% agarose under UV light.

DNA sequence analysis

Sequencing was carried out by the services of PT Genetika Science Indonesia. The sequence analysis was carried out using the NCBI BLAST-N program to see its relationship with the bacterial sequences in Genbank. Phylogenetic tree was constructed using MEGA 11 software.

RESULTS AND DISCUSSION

Characteristic of *Xanthomonas axonopodis* pv. *glycines*

The characteristic bacterial pathogen was yellow on YPGA media (Figure 1.A), gram-negative, positive to hydrolyze starch, positive hypersensitive reaction test, and positive pathogenicity test. The bacteria show a positive reaction in the starch hydrolysis test in the presence of a clear zone around the colony (Figure 1.B). The tobacco leaves infiltrated with bacteria pathogen in hypersensitive reaction test were pale green color 24 hours after inoculation then turned to yellow and brown spots (Figure 1.C). The first symptoms on pathogenicity tests were shown by yellow to brown spots (Figure 1.D), then from those spots arose pustules (Figure 1.E) after 8 days after inoculation **(**dai**)**. Based on that characteristic (Figure 1), it suggested that this bacteria was *X. axonopodis* pv. *glycines.* This characteristic also fits with the description according to Prathuangwong and Choethana (1998).

Isolation of phyllosphere bacteria

A total of 69 isolates of bacteria were obtained from soybean leaves. Eight isolates grew in Kings'B media and 51 isolates in YPGA media. Based on the hypersensitive reaction test and pathogenicity tests, negative results indicated that the phyllosphere bacteria were not plant pathogens.

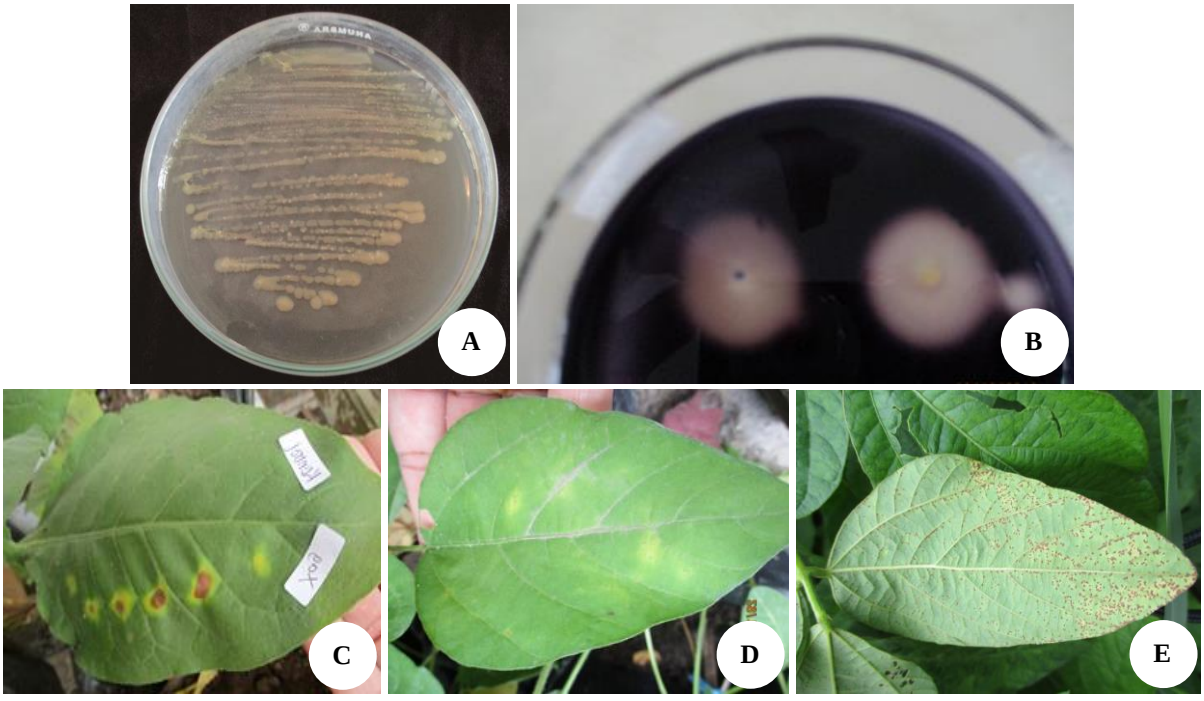


Figure 1. Xag bacteria. A. Bacteria colonies, B. Starch hydrolysis test, C. Hypersensitive reaction test, D. Pathogenicity test, E. Pustules on the underside of soybean leaves

Growth of colony and antimicrobial activity

All antagonist bacteria grown on YPGA medium inhibition zone against Xag (Tables 1 and 2, Figure 3). For example, the ST32 isolate has the largest inhibition zone of 23.2 mm. All bacteria grown on Kings'B medium were not able to inhibit Xag. Based on these results, 11 from 69 isolates were selected because they have a large inhibition zone (more than 10 mm) with a bacteriostatic inhibition mechanism. The bacteriostatic mechanism was confirmed by the change of peptone water turbidity after 24 hours of incubation, as a sign that Xag was able to grow and not killed by antimicrobial compound. These isolates were JB4, JB5, JB6, JB7, JB12, JB13, ST4, ST31, ST32, LB2, and LB3. The eleven isolates were the result of isolation growing on YPGA media.

These 11 selected isolates have different growth abilities indicated by the size of the colony's diameter and diverse colony types (Table 2, Figure 3). The JB12 isolate has the largest colony diameter of 58.40 mm and the smallest was the ST32 isolate with a diameter of 8.07 mm. The colony diameter was not correlated positively with the inhibition ability to Xag.

Compatibility of 11 isolates of phyllosphere bacteria

The test results showed that the 11 isolates on YPGA media did not inhibit each other except for isolates JB4 and JB 5 inhibited JB12 but the opposite did not happen (Table 3).

Morphological characteristics of 11 phyllosphere bacteria isolates

Eleven potential phyllosphere bacteria isolates had similar morphological characteristics including rough, flat surfaces and color differences. Some tended to be white and dull, except for the ST32 isolate, whose colonies were round, convex, and mucoid (Table 4; Figure 2).

Physiological and biochemical characterization of 11 phyllosphere bacteria isolates

Based on the results of molecular identification which showed that all isolates belonged to the genus *Bacillus*, the physiological and biochemical tests carried out were based on the characteristics of the *Bacillus* genus (Schaad et al. 2001). All isolates showed gram-positive reactions and had varying abilities in other tests (Table 5). All isolates hydrolyzed starch with different abilities. JB4, JB6, and ST32 had better starch hydrolyzing ability than the others. At room temperature and 45ºC, all isolates were also able to grow, indicated with the turbidity of the media, and the bacteria were evenly suspended. But, isolates JB5, JB7, ST31, and LB2 showed that the bacteria grew to form aggregates.

Growth at 65ºC showed that isolates JB4, JB5, JB6, JB7, JB13, LB2, and LB3 could grow in suspended form, while the other isolates were unable to grow. All isolates showed a positive reaction in the catalase test, which was able to break down H_2O_2 into $H_2O + O_2$, indicated by the formation of air bubbles. All isolates grew at 7% NaCl and were evenly suspended on media, except isolate ST31 with an aggregate growth pattern. All isolates were also able to grow at pH 5.7 by forming aggregates. All 11 isolates have the same ability to use mannitol as a carbon source but have a different ability to use glucose and dextrose. The ST32 isolate was different from the others because it could not use glucose and dextrose as a carbon source. Isolates JB4, JB6, JB7, JB13, LB2, and LB3 can better utilize glucose than the others.

Genetic characteristics of 11 phyllosphere bacteria isolates

The amplification results of 11 phyllosphere bacteria isolates with 16S rRNA primers showed that all were amplified at 1500 bp (Figure 4.A). The results of genetic diversity analysis using BOX AIR primers showed that there were 4 groups of bacteria, the first group consisted of isolates JB4, JB5, JB6, JB7, JB13, ST4, ST31, the second group isolated JB12, the third group was isolates ST32 and the fourth group consisted of isolates LB2 and LB3 (Figure 4.B).

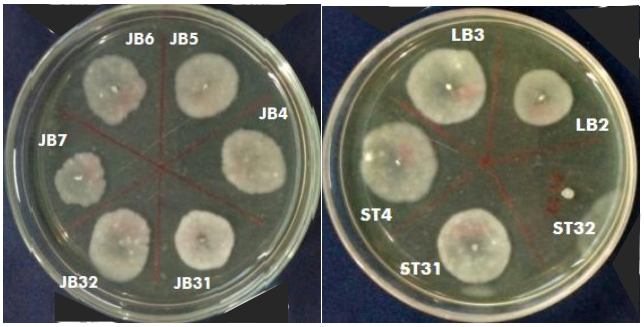


Figure 2. Morphological characteristic of 11 selected phyllosphere bacteria isolates.

Table 1. Antimicrobial activity of phyllosphere Bacteria against Xag.

Inhibition zone diameter (mm)	Criteria of inhibition	Amount
0	No inhibition	41
1 – 5,9	Low inhibition	12
6 – 10	Medium inhibition	5
>10	High inhibition	11

Table 2. Growth and antimicrobial activity of 11 isolate phyllosphere bacteria

Isolate code	Colony diameter on 48 hours growth (mm)	Radius of inhibition zone (mm)	Inhibition mechanism
JB4	29.90b	12.27f	Bacteriostatic
JB5	10.43f	12.23f	Bacteriostatic
JB6	20.27d	16.40b	Bacteriostatic
JB7	31.27b	12.07g	Bacteriostatic
JB12	58.40a	10.47h	Bacteriostatic
JB13	30.07b	13.27e	Bacteriostatic
ST4	16.30e	15.50c	Bacteriostatic
ST31	12.17f	13.03e	Bacteriostatic
ST32	8.07g	23.20a	Bacteriostatic
LB2	25.43c	14.50d	Bacteriostatic
LB3	19.10d	16.33b	Bacteriostatic

Note: The same letters in one column showed no significant difference at a 5% confidence level

Based on these results, four representative isolates were selected for sequencing and the results were compared with the reference sequences from the Gene bank (Table 6). The first group represented by ST4 was *Bacillus siamensis*, JB 12 was *Bacillus subtilis*, ST32 was *Bacillus velezensis* and LB2 was *Bacillus amyloliquefaciens*. Phylogenetic tree was constructed based on this data (Figure 5).

Table 4. Morphological characteristics of phyllosphere bacteria

Isolates code	Characteristic of morphology
JB4	Dull white colonies, thin, flat, stringy surfaces, irregular edges
JB5	Dull white colonies, thin, flat, rough surfaces, flat edges
JB6	White colonies, thin, flat, stringy surfaces, irregular edges
JB7	Dull white colonies, thin, flat, stringy surfaces, irregular edges
JB12	Dull white colonies, thin, flat, stringy surfaces, irregular edges
JB13	White, thin, flat, surface colonies, irregular edges
ST4	White colonies, thin, flat, irregular rough edges
ST31	White colonies, thin, flat, rough surfaces, irregular edges
ST32	White colonies, convex, mucoid surfaces, regular edges
LB2	Dull colonies, thin, flat, irregular shapes, irregular edges
LB3	White colonies, thin, flat, stringy surfaces, irregular edges

Table 6. Sequence reference of phyllosphere bacteria based on BLAST

Accession number	Query cover (%)	Ident. (%)	Isolate name
Isolate ST4			
MK291446.1	100	98.92	*Bacillus sp*. (in: Bacteria) strain BO53
MH236612.1	100	98.92	*Bacillus siamensis* strain ML093-4
MH236611.1	100	98.92	*Bacillus siamensis* strain ML091-2
MH236604.1	100	98.92	*Bacillus siamensis* strain ML065-2
CP024897.1	100	98.92	*Bacillus velezensis* strain CN026
Isolate JB12			
KU510073.1	99	98.08	*Bacillus subtilis* strain BA14
MN094375.1	99	97.98	*Bacillus velezensis* strain isolate BS-9
MK392030.1	99	97.98	*Bacillus sp.* (in: Bacteria) strain SN3
MF114390.1	99	97.98	*Bacillus subtilis* strain BsA1I
MF114382.1	99	97.98	*Bacillus subtilis* strain BsP1IS
Isolate ST32			
MT560280.1	100	99.80	*Bacillus amyloliquefaciens* strain SDF-005S
MT539153.1	100	99.80	*Bacillus velezensis* strain HFBPR51
MT538583.1	100	99.80	*Bacillus velezensis* strain 3726
MT538532.1	100	99.80	*Bacillus velezensis* strain 3668
MT538490.1	100	99.80	*Bacillus velezensis* strain 3618
Isolate LB2			
MT107183.1	100	99.19	Bacterium strain MP4S2
MT560280.1	100	99.06	*Bacillus amyloliquefaciens* strain SDF-005S
MT559810.1	100	99.06	*Bacillus amyloliquefaciens* strain 21/IV
MT559808.1	100	99.06	*Bacillus subtilis* strain 20.10
MT559807.1	100	99.06	*Bacillus amyloliquefaciens* strain 28.3

Table 3. Compatibility 11 isolates of phyllosphere bacteria

Isolate code	Compatibility 11 isolates of phyllosphere bacteria										
	JB4	JB5	JB6	JB7	JB12	JB13	ST4	ST31	ST32	LB2	LB3
JB4	+	+	+	+	+	+	+	+	+	+	+
JB5	+	+	+	+	-	+	+	+	+	+	+
JB6	+	+	+	+	-	+	+	+	+	+	+
JB7	+	+	+	+	+	+	+	+	+	+	+
JB12	+	+	+	+	+	+	+	+	+	+	+
JB13	+	+	+	+	+	+	+	+	+	+	+
ST4	+	+	+	+	+	+	+	+	+	+	+
ST31	+	+	+	+	+	+	+	+	+	+	+
ST32	+	+	+	+	+	+	+	+	+	+	+
LB2	+	+	+	+	+	+	+	+	+	+	+
LB3	+	+	+	+	+	+	+	+	+	+	+

Note: (+): compatible; (-): not compatible

Table 5. Physiological and biochemical characterization of 11 phyllosphere bacteria isolates

Isolate code	Physiological and biochemical test result										
	Gram	Growth at room temp.	Catalase	Growth at 45ºC (5 days)	Growth at 65ºC (5 days)	pH 5,7	NaCl 7% (5 days)	Glucose broth (14 days)	Mannitol	Dextrose (14 days)	Starch
JB4	Positive (+)	+ (g)	+	+ (g)	+ (s)	++ (g)	+ (s)	++++++	+	+	+ ***
JB5	Positive (+)	+ (g)	+	+ (s)	+ (s)	++ (g)	+++ (s)	+++++	+	+	+ **
JB6	Positive (+)	+ (g)	+	+ (g)	+ (s)	++ (g)	+ (s)	+++++++	+	+	+ ***
JB7	Positive (+)	+ (g)	+	+ (s)	+ (s)	++(g)	+ (s)	+++++++	+	+++	+ **
JB12	Positive (+)	+ (g)	+	+ (g)	-	+ (g)	+++ (s)	++++	+	+++	+ **
JB13	Positive (+)	+ (g)	+	+ (g)	+(s)	++(g)	+++ (s)	+++++++	+	+++	+ **
ST4	Positive (+)	+ (g)	+	+ (g)	-	+ (g)	++ (s)	++	+	+++	+**
ST31	Positive (+)	+ (g)	+	+ (s)	-	++ (g)	++ (g)	++++++	+	+++	+ *
ST32	Positive (+)	+ (s)	+	+ (g)	-	+ (g)	+ (s)	-	+	-	+ ***
LB2	Positive (+)	+ (g)	+	+ (s)	+ (s)	++ (g)	+++ (s)	+++++++	+	++	+ **
LB3	Positive (+)	+ (g)	+	+(g)	+ (s)	++ (g)	++ (s)	+++++++	+	+++	+ **

Note: General: (+): Positive test result; (-): negative test result; Starch test: (*) Clear zone 1-3 mm, (**) clear zone 4-7 mm and (***) clear zone more than 8 mm; Temperature test: (s):suspended, (g): aggregate

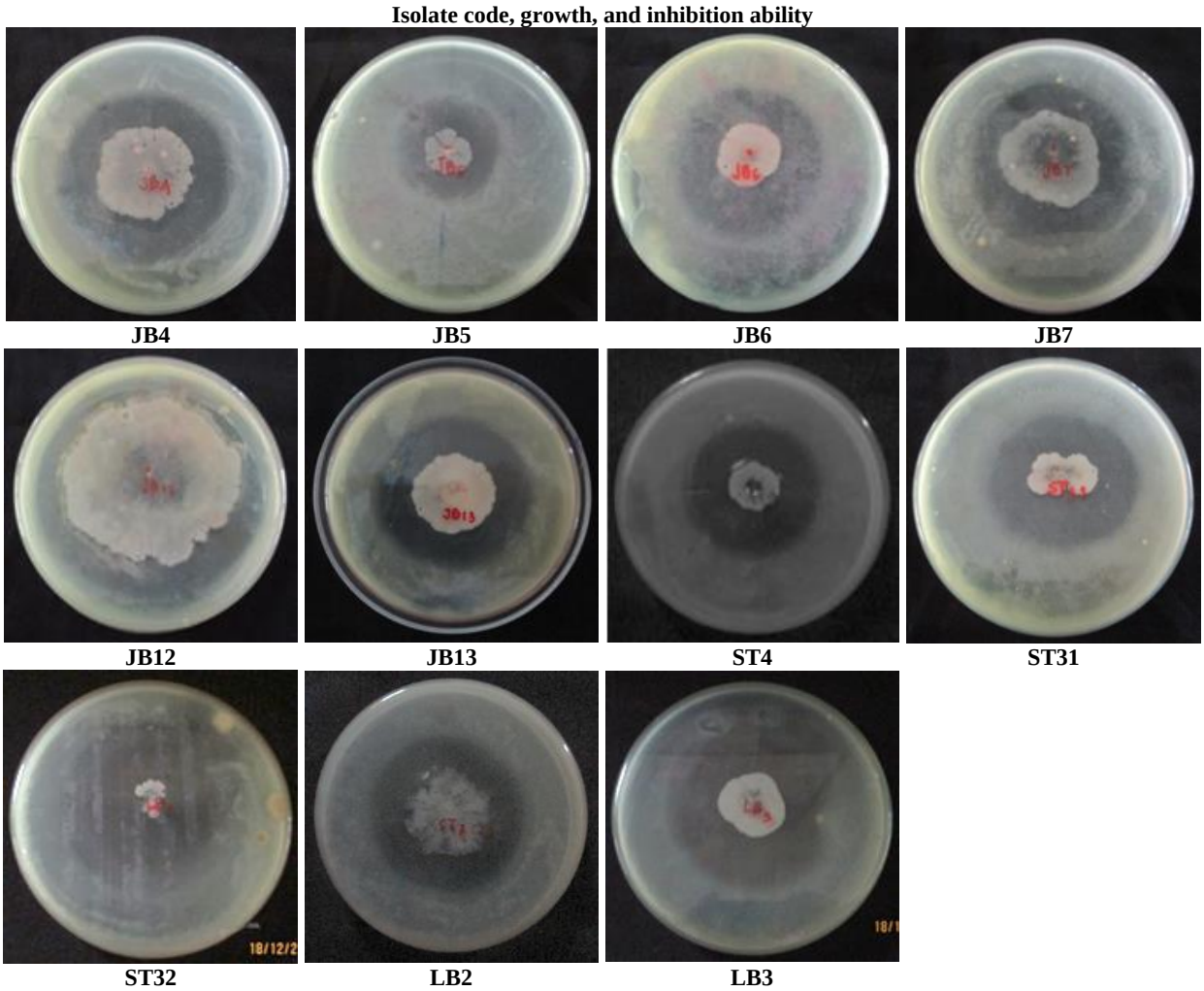
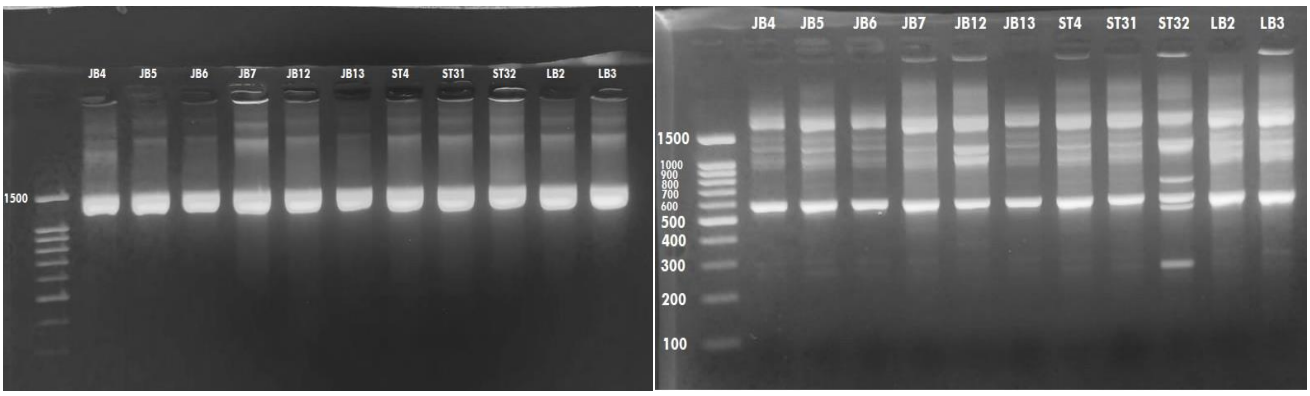


Figure 3. Growth and antimicrobial activity of phyllosphere bacteria against Xag



A **B**

Figure 4. The genetic diversity of phyllosphere bacteria, 1500 bp band in red boxes: A. 16S rRNA primer, B. BOX AIR primer

Discussion

Exploration and development of bacteria as antagonist agents for biological control of plant diseases is important to reduce chemical pesticides. Soybean pustule disease caused by Xag is an airborne disease. Biological agents derived from the phyllosphere provide advantages because they are similar and highly adaptable to phyllosphere conditions full of environmental stresses. High survival rates in the field are a determining factor for success in biological control. Phyllosphere bacteria can stimulate plant growth, colonize the phyllosphere and suppress plant pathogens (Wiraswati et al. 2018). *Bacillus* in the phyllosphere plays a role in plant growth and health (Mazinani et al. 2017) because it can control the disease by producing antimicrobial compounds.

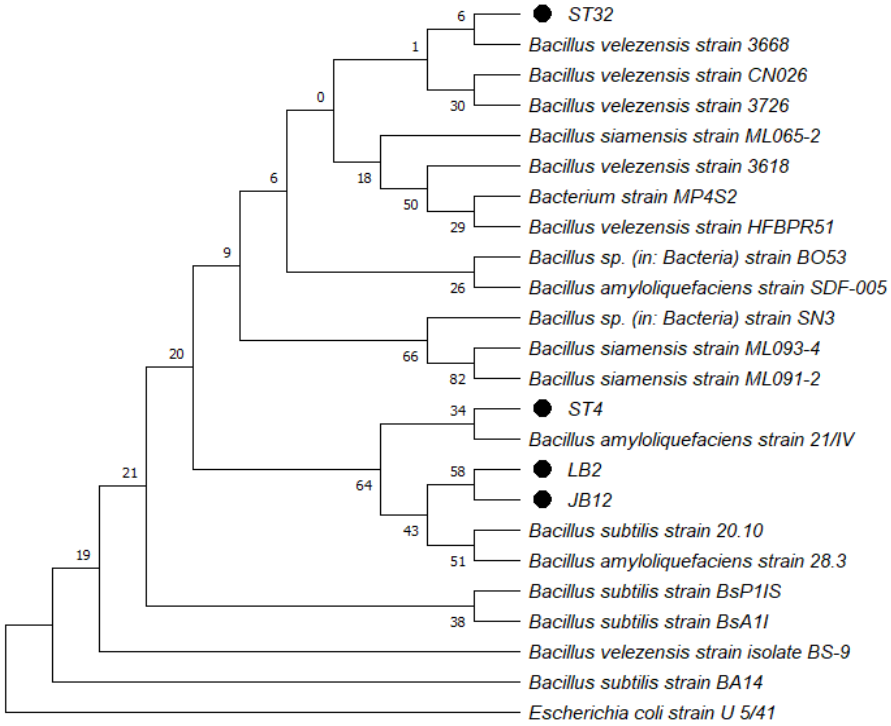


Figure 5. Phylogenetic tree from 4 bacteria isolates (ST4, ST32, JB12, and LB2) and the closest relatives of other bacteria species.

Several previous studies reported that many bacteria were isolated from the phyllosphere. Valverde et al. (2017) succeeded in isolating chestnut phyllosphere bacteria, 58% were gram-positive bacteria, and 10% were from Firmicutes consisting of *Paenybacillus*, *Bacillus*, *Psycrobacillus*, and *Staphyllococcus*. *Bacillus* and *Anthrobacter* were isolated from the paddy phyllosphere and showed inhibition against *Xanthomonas oryzae* pv. oryzae (Prabawati et al. 2019). Endophytic bacteria from long bean plants consisting of *Bacillus*, *Delfitia*, *Methylobacterium*, *Microbacterium*, *Paenybacillus*, *Stapylococcus*, and *Stenotrophomonas* with a population density of 4.5 x 10^2 – 2.8 x 10^3 cfu/g leaf (Costa et al. 2012). Several groups of bacteria consisting of several species were isolated from various vegetable plants, namely *Proteobacteria*, *Cyanobacteria*, *Bacteroidetes*, and *Firmicutes* (Zhang et al. 2010).

A total of 69 isolates were isolated from the soybean phyllosphere and 11 isolates were potential as antagonistic agents against Xag by producing antimicrobial compounds. The eleven isolates were molecularly identified as genus *Bacillus* and have morphological diversity, growth rate, inhibition of Xag, physiology-biochemistry, and genetics. The microbial phyllosphere community is very high in the diversity of type, characteristic, and potential antagonist (Chaudhary et al. 2017). From the 11 isolates based on the analysis of the 16S rRNA gene sequence, there were 4 groups of *Bacillus*, namely *Bacillus siamensis*, *B. subtilis*, *B. velezensis*, and *B. amyloliquefaciens*. According to Sivakumar et al. (2020), geography condition affects the diversity of phyllosphere bacteria more than the growth phase of plants.

In biological control, the mechanism of antagonistic agents is antibiosis, space competition, and induce resistance (Cook and Baker 1998). The antibiosis mechanism depends on the ability of bacteria to produce antimicrobial compounds that can inhibit the development of pathogens. The competition mechanism depends on the bacteria's growth so that they can dominate space and nutrients. Induce resistance in addition to being influenced by the ability of antagonistic bacteria to trigger plant resistance mechanism also depends on the response of plants in responding to signals given by antagonistic agents. The rapid growth of phyllosphere bacteria *in vitro* correlated with its ability to colonize existing space if the phyllosphere condition supports its growth, both nutrition and environment. Therefore, it was expected these bacteria were able to compete in using space of leaf surfaces and nutrient availability. This colonization will provide plant protection against pathogenic infections. *B. subtilis* strain JB12 showed the fastest growth with a colony diameter of 58.40 mm.

The eleven *Bacillus* have high inhibition, which is above 10 mm. *Bacillus velezensis* ST32 showed the largest inhibition zone of 23.20 mm, followed by JB6 and LB3 with inhibition zones of 16.40 mm and 16.33 mm, respectively. All *Bacillus* showed bacteriostatic inhibitory mechanism. This large inhibition zone of Xag indicated a good ability to suppress Xag growth *in vitro* with the mechanism of antibiosis. It was suggested that the ability of different phyllosphere bacteria to inhibit Xag related to the type and amount of antimicrobial compounds or secondary

metabolites produced. This was because the phyllosphere bacteria have a high sensitivity to these compounds. For example, Psip22d epiphytic bacteria produce *syringomycin, syringopeptin, and 3-methylarginine* (MeArg) compounds which able to inhibit *Pseudomonas syringae* pv. *glycines* (Psg) (Braun et al. 2010). *Bacillus amyloliquefaciens* BZ6-1 endophytes in peanut produce *surfactin* and *Fengysin* A which can inhibit *Ralstonia solanacearum* (Wang and Liang 2014). Research from Milijasevic-Marcic et al.(2018) showed that as many as 10 isolates of *B. subtilis* inhibited *Xanthomonas vesicatoria*, but *Bacillus pumilus* isolates did not affect *X. vesicatoria* growth. *B. subtilis* strains B-319, B-325 and B-358 showed the largest inhibition zone and B-319 showed the greatest inhibition. Strain B-358 is a candidate biological agent which in vitro inhibits *X. vesicatoria, Clavibacter michiganensis* subsp. *michiganensis* and *Verticillium.*

Based on the compatibility test, most *Bacillus* did not inhibit each other's growth. This is because the *Bacillus* strain is not sensitive to antimicrobial compounds produced by other strains. According to Simons et al. (2020) that bacteriocins as ribosomally synthesized peptides directed against bacteria closely related to producer strain. The genus Bacillus produces different bacteriocins, especially the types of polypeptides referred to as *subtilin* and *coagulin*. *B. licheniformis* ZJU12 produces peptides similar to *bacteriocins* and have broad-spectrum antagonists (Cesa-Luna et al. 2020).

The eleven *Bacillus* showed diversity in morphology, physiology, biochemistry, and genetics. Although there were differences, ten *Bacillus* spp isolates showed flat and rough surface morphology, while *B. velezensis* ST32 looked convex, mucoid with small colonies. *Bacillus* had different survival abilities under certain environmental conditions such as acidity, salinity, and temperature. All *Bacillus* isolate grew at an acidic pH of 5.7 and a high NaCl content of 7%. It is advantageous to be used as biological agents for Xag whose application is in the phyllosphere because it is resistant to high salt levels in rainwater. *Bacillus* JB4, JB5, JB6, JB7, JB13, LB2, and LB3 can grow at a temperature of 65ºC. This shows its ability to be able to live better in the phyllosphere with high-temperature stress. The ability of *Bacillus* to use carbon sources will affect its ability to survive by utilizing carbon sources in the phyllosphere, especially from plant secretions. In some tests, bacteria showed growth by forming suspensions and aggregates. According to Leveau (2018), bacteria on the leaf surface can sometimes form aggregates or live solitary.

Genetic diversity of *Bacillus* spp. isolates are thought to affect its ability to decompose compounds, use nutritional source, and adaptation to environmental conditions. This was indicated by its diversity in biochemical and physiology test. The different abilities of these diverse phyllosphere bacteria could be a factor that caused many types of bacteria to survive together in the phyllosphere community to make a balanced phyllosphere ecosystem. This fact provides an opportunity for the phyllosphere bacteria to be applied together as antagonist agents. According to Mazinani et al. (2017), *Bacillus* produces antimicrobial compounds and forms spores to survive in most habitats. Further research is needed regarding the ablity of the *Bacillus* spp. isolates to control soybean pustule disease in the glasshouse and in the field.

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