Potential of *Bacillus subtilis* potato isolate as biocontrol agent of *Xanthomonas oryzae* pv. *oryzae* and candidate for nanosuspension formula

Abstract. *Bacillus subtilis* potato isolate was able to be an antagonist of plant pathogens. The aims of this study were (1) to examine the potential of potato isolate *B. subtilis* as a control for *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) as a pathogen rice bacterial leaf blight, (2) determine the inhibition mechanism of their isolates, (3) Evaluating the best *B. subtilis* to make nanosuspension formula. The method was carried out in a completely randomized design with 5 treatments of *B. subtilis* potato isolates B46, B209, B211, B298 and B315, repeated 4 times. *Xoo* and five *B. subtilis* were grown in nutrient broth medium in a shaker. *Xoo* and *B. subtilis* were grown in yeast extract peptone glucose agar medium by the diffusion method. *Xoo* was inoculated on medium, suspension of *B. subtilis* was immersed in 5 mm diameter filter paper and placed on the medium. The best *B.subtilis* as a controller was used to formulate into nanosuspension. The variables are zone of inhibition, inhibition mechanism and antibiosis index. The results showed that only three *B. subtilis* were able to inhibit the growth of *Xoo*, namely isolates of B211, B298 and B315 with the largest inhibition zone being 10 mm by *B. subtilis* B315 isolates. The mechanism of inhibition of the three isolates of *B. subtilis* against *Xoo* was bacteriostatic. The antibiosis index of *B. subtilis* B315 against *Xoo* was 1 (strong). The selected *B. subtilis* B315 was developed as a nanosuspension formula with inhibition zone of 2.1 mm and antibiosis index was 0.42.

Key words: Antagonist, antibiosis, nanosuspension, *Xanthomonas oryzae*

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INTRODUCTION

Bacillus subtilis is known as a useful bacterium that can be used as an antagonist against plant pathogens. *B. subtilis* can be explored from soil, water, air, plant surfaces and endophytes from plant tissues such as roots, stems, sheath, and leaves. *B. subtilis* is an antagonistic bacterium that is Gram positive, rod-shaped, 7-8 x 2-3 µm, flagellated, aerobic, not encapsulated, have endospores that are ovoid to cylindrical, measuring 0.6-0.9 x 1.0-1.5 µm, thin walled. Spores are formed generally within 24 hours (Gordon et al. 1973). One of the strong characteristics bacteria are classified into Bacillus is their ability to form endospores under extreme conditions. Based on the endospore morphology, Bacillus can be divided into 3, namely: (I) oval or cylindrical spores located in the middle, subterminal or terminal, swollen rods, or not swollen, (II), oval, slightly cylindrical spores, subterminal or terminal, stems do not swell, (III) spores are spherical, subterminal or terminal, rods are swollen (Chun and Vidaver 2001).

B. subtilis isolated from potato rhizosphere, has been selected to obtain five superior isolates, there are *B. subtilis* B46, B209, B211, B298 and B315. Molecularly *Bacillus* isolates B46, B209, B211, B298 are homologous to *B. subtilis* subsp. *spizizeni* RRLKE2, while B315 is homologous to *B. subtilis* strain WIFD5 (Prihatiningsih et al. 2020a). *B. subtilis* potato isolate has been tested for control of bacterial wilt disease by *Ralstonia solanacearum* of potatoes, tomatoes, tobacco, and chilies. Control effectiveness varies both in the greenhouse and in different fields. This shows that its effectiveness depends on weather conditions, plant age and population density of *B. subtilis*.

Control of potato *R. solanacearum* with *B. subtilis* B315 was 64.9% effective, while tomato, chili and eggplant in the lowlands reached 74.6% with a population density of 10^8 cfu.mL^{-1} (Prihatiningsih, 2013; Prihatiningsih and Djatmiko, 2016). *B. subtilis* is also reported to be able to produce IAA (*Indole Acetic Acid*) in the range of 57.56-79.33 ppm, that stimulate plant growth (Prihatiningsih et al. 2020b). *Bacillus* spp. consortium indigenous can control *Colletotrichum capsici* and increase chili growth (Yanti et al. 2020). *B. amyloliquefaciens* can reduce the incidence of tomato bacterial wilt, suppress the population of *R. solanacearum* and improve the growth of tomato plants (Singh et al. 2016).

The mechanism of controlling pathogens by *Bacillus* is antibiosis, which is to inhibit pathogens by producing compounds such as antibiotics, enzymes, and other volatile compounds. In vitro testing of mechanism character antibiosis is characterized by the formation of a clear zone around the antagonist colony. The liquid formula of *B. subtilis* B315 as a controller of *R. solanacearum* in vitro showed the effectiveness with an inhibition zone of 18 mm with an antibiosis inhibition mechanism indicated by the enzymes amylase, chitinase and proteases produced (Prihatiningsih and Djatmiko 2016; Lestari et al. 2017; Prihatiningsih et al. 2020b; Prihatiningsih et al. 2021).

Based on the ability of these *B. subtilis* potato isolate, in this study it was cross tested to another plants, against *Xanthomonas oryzae* pv. *oryzae* which causes bacterial leaf blight of rice. Evaluation of pathogen inhibition by antagonistic bacteria can be measured by the size of the inhibition zone, such as measuring the diameter of the clear zone to calculating the area of the inhibition potential. Thus according to these data, an antibiotic index can be calculated. The mechanism of antagonistic bacteria inhibiting pathogenic bacteria is bacteriostatic and bactericidal (Goto 1992).

Rice bacterial leaf blight caused by *X. oryzae* pv. *oryzae* (*Xoo*) is an important disease and has long been recognized as a bacterial disease of rice in Asia (Naqvi et al. 2014) thoroughly in several locations of rice cultivation. Yield losses varied up to 20% under moderate conditions and more than 50% in conducive conditions to disease development such as varieties, plant growth stages and weather conditions. This bacterial rice blight disease was once an epidemic during 1998 in the Palakkad area of Kerala and since then several proportions have been observed each year (Swamy et al. 2006; Kala et al. 2015). Several disease management strategies used to solve these problems such as using resistant plants based on a single gene (monogenic), hence break its resistance gradually.

Symptoms of bacterial leaf blight caused elongated spots starting from the tips of the leaves are white to gray with irregular wavy edges, dry, occurring during the vegetative period of the plant called crackle symptoms, and during the generative period of the plant the symptoms are called blight (Shanti et al. 2010; Lia 2018). If the pathogen attacks the plant approaching mature, the leaves turn pale yellow. So far, there is no control yet to solve this *Xoo* attack problem. Based on the description above, it is necessary to carry out a biological control with *B. subtilis.*

Nanoparticles are one of the pesticide formulations with nano technology, also known as nanobiopesticides. Nanobiopesticides can be in the form of nanosuspensions, nanoemulsions and nanoparticles (Lade et al. 2019) which have been developed since the 19th century, but have only been noticed since 1959. Nanotechnology in the field of plant disease management can be used for disease diagnosis and detection of pathogens, evaluation of pathogens suppression, and pesticides reformulating. Application and effectiveness of plant disease control using nanobiopesticides is application to soil, seeds or leaves to protect plants from pathogen invasion (Alghuthaymi et al. 2015). Nanotechnology has advantages as pesticides by reducing the toxicity, improve shelf-life and solubility of pesticides that are less soluble in water, and have a positive effect on plants and the environment (Worrall et al. 2018).

The aims of this study were (1) to test of the potential *B. subtilis* potato isolate as a control for *X. oryzae* pv. *oryzae* pathogenic rice bacterial leaf blight, (2) determine the suppression mechanism of *B. subtilis* potato isolate against *Xoo*, (3) Evaluating the best *B. subtilis* potato isolate to make a nanosuspension formula.

MATERIALS AND METHODS

Preparation of bacteria

The research materials used were *B. subtilis* B46, B209, B211, B298 and B315 potato isolates (Prihatiningsih collection) were growth in yeast peptone glucose agar (YPGA), *Xoo* in Sucrose Peptone Agar (SPA), and they were growth in nutrient broth (NB) for dual culture inhibition test. *Xoo* bacteria isolate from rice field at Karangwangkal Purwokerto. The research was performed from February to June 2021. All the experiments at the Plant Protection Laboratory, Faculty of Agriculture Universitas Jenderal Soedirman (UNSOED) Purwokerto.

Bacterial antagonist test of *B. subtilis* against Xoo

Five isolates of *B. subtilis* from potato rhizosphere, namely *B. subtilis* B46, B209, B211, B298 and B315 were tested for their inhibition against *Xoo* by the agar diffusion method using filter paper with a diameter of 5 mm according to the method of Balouiri et al. (2016). *Xoo* as the tested pathogenic bacteria was grown on NB medium shaken at 150 rpm at room temperature for 24 hours. Bacterial density *Xoo* 10^8cfu/mL was inoculated by pour plate on YPGA medium. The 5 mm diameter filter paper was dripped with 10 µL suspension, then placed on the YPGA medium in 3 parts. The clear zone formed around the filter paper indicated the inhibition of *Xoo* growth.

Evaluation of inhibition mechanism

To evaluate the ability of antagonistic bacteria to inhibit *Xoo* the percentage of inhibition was detemined by the inhibition zone formed. Determination of inhibition using the method of Balouiri et al. (2016) by measuring the diameter of the clear zone, and the diameter of the colony (Figure 1). Inhibition measurement (P) was performed after 24 hours.

P = {diameter of clear zone (A) - diameter of bacterial colony (B)} (Balouiri et al. 2016)

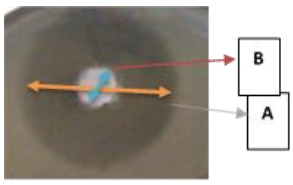

Figure 1. Antibiotic index measurement.

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(A). Diameter of the clear zone, (B). diameter of the colony or filter paper

Antibiosis index

The antibiosis index was determined according to the enzyme index using the formula for measuring the chitinolytic index refer to (Halimahtussadiyah et al. 2017) or phosphate solvent produced by antagonistic bacteria, by measuring the diameter of the clear zone, and the diameter of the colony using the following formula:

$$AI = \frac{\text{(diameter of clear zone - diameter of bacterial colony)}}{\text{diameter of bacterial colony}}$$

or

AI = (A-B) : B (Halimahtussadiyah et al. 2017)

(A). Diameter of the clear zone, (B). diameter of the colony or filter paper

According to Azizah (2017), determination of the inhibition category grouped based on the index of inhibition or index of antibiosis, namely:

a. Very Strong (>2.0) with symbol (+++)
b. Strong (1.0 – 1.9) with symbol (++)
c. Weak (0.1 – 0.9) with the symbol (+)
d. Has no antagonistic ability (0.0) with the symbol (-)

Preparation of nanosuspension formula from supernatant of selected *Bacillus subtilis*

B. subtilis B315 was grown in NB medium, shaken 2x24 hours at 150 rpm at room temperature, then centrifuged at 3000 rpm for 15 minutes at room temperature, the supernatant obtained was used as the active ingredient. Chitosan was dissolved in 1% acetic acid. 0.5 mL of *B. subtilis* B315 supernatant was put into a microcentrifuge tube containing 490 µL of chitosan solution in 1% acetic acid pH 4.0-5.0, shaken with a vortex for 20 seconds. The homogeneous mixture was added with 10 µL of 0.1% tripolyphosphate (TPP) solution and continuing homogenized by vortex for 20 seconds. The nanoparticle preparation was performed characterized, the morphology, the particle size and the distribution of particle size.

Evaluation of inhibition effect of *B. subtilis* supernatant nanosuspension formula to suppress *Xoo*

The inhibition of *B. subtilis* supernatant in nanosuspension formula against *Xoo* was determined by agar diffusion method using filter paper according to the method of Balouiri et al. (2016). *Xoo* as the tested pathogenic bacteria was grown on NB medium shaken at 150 rpm at room temperature for 24 hours. Bacterial density *Xoo* $10^8 cfum L^{-1}$ was inoculated on YPGA medium. The 5 mm diameter filter paper was dripped with nanosuspension formula, then placed in the YPGA medium in 3 parts. The clear zone around the filter paper showed the effect inhibition of *B. subtilis* supernatant nanosuspension formula to suppress *Xoo* (Balouiri et al. 2016).

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Data analyses

Data were analysis by descriptive methods and comparative to references. The results were expressed as the mean ± sd of mean. Raw data were imported to Microsoft Excel program for graphical representation.

RESULTS AND DISCUSSION

Inhibition assay of *Bacillus subtilis*. to *Xoo*

The results of this study included the selection of 5 isolates of *B. subtilis* (B46, B209, B211, B298 and B315) to be able to determine which isolates would be formulated into nanoparticles for the next research stage. Selection of *B. subtilis* against pathogenic bacteria *Xoo* is shown in Table 1 and Figure 2. *B. subtilis* B315 isolate was highest with 10±0.12 mm inhibition zone than another isolates.

Table 1. Inhibition of five isolates of *B. subtilis* against *Xoo*

B. subtilis isolate	Inhibition Zone (mm)	Inhibition mechanism
B46	-	-
B209	-	-
B211	9±0.14	bacteriostatic
B298	8±0.12	bacteriostatic
B315	10±0.12	bacteriostatic

Figure 2. Inhibition of *B. subtilis* against *Xoo*

The mechanism of inhibition of *B. subtilis* against *Xoo* is bacteriostatic antibiosis, it's mean *B. subtilis* only able to inhibit the growth of *Xoo* but it is not lethal effect. It was demonstrated by the cloudy peptone water which indicated that *Xoo* was still growing, while the bactericidal mechanism occurred when the peptone water remained clear or it's as same as the control (Figure 3), which was a lethal. The bacteriostatic mechanism of these five *B. subtilis* isolates was also shown against the pathogenic bacterium *Ralstonia solanacearum* that causes potato bacterial wilt disease (Prihatiningsih 2013).

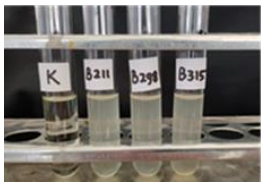

Figure 3. Bacteriostatic mechanism of three isolates *B. subtilis* to *Xoo*

Antibiosis index

The highest antibiosis index was shown by isolate *B. subtilis* B315 of 1 and according to the category of inhibition index it was strong (++) (Figure 4). This result indicates that *B. subtilis* B315 from potato rhizosphere has good prospects to be developed as a biological agent for biopesticides to control pathogenic bacteria not only in the habitat of *R. solanacearum* which causes potato bacterial wilt, but also against *Xoo* which causes rice bacterial leaf blight. *B. subtilis* isolates B46 and B209 in the inhibition test against potato *R. solanacearum* were able to inhibit, but after cross-testing against rice pathogenic bacteria the two isolates were unable to inhibit (-).

Antibiosis index is useful as an indicator that bacteria are capable of being antagonists against pathogens, both bacteria and fungi. Bacteria are able to control pathogenic fungi by producing the enzyme chitinase as an inhibitory mechanism since this enzyme degrades the cell walls of pathogenic fungi which consists mostly of chitin (Halimahtussadiyah et al. 2017). Chitinase activity of *B. subtilis* B298 was detected at 15 hours of incubation, optimum temperature of 40ºC and optimum pH of 5.0, respectively, at 6.936 UmL^{-1}, 5.764 $U.mL^{-1}$ and 6.813 $U.mL^{-1}$ (Lestari et al. 2017). *B. subtilis* also produce proteases and siderophores hence able to inhibit pathogenic bacteria such as rice *Xoo* and potato *R. solanacearum* (Prihatiningsih et al. 2017; Prihatiningsih et al. 2021).

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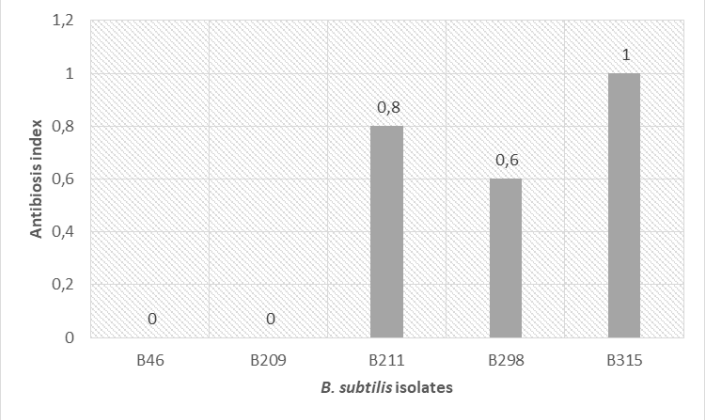

Figure 4. Antibiosis index of the five isolates of *B. subtilis* against Xoo

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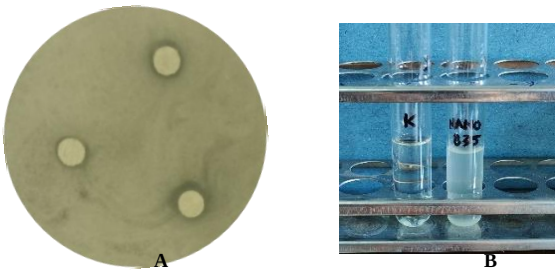
Nanosuspensi formula of *B. subtilis* B315 supernatant to control Xoo

The inhibition of *Xoo* growth by nanosuspension formula of *B. subtilis* B315 supernatant was 2.1 mm (Figure 5) in diameter and in accordance with Marcic et al. (2018) that *B. subtilis* can suppress *X. vesicatoria* in tomatoes with an inhibition zone of 5-9 mm but without in nanosuspension formula. The antibiosis index of this nanosuspension formula was 0.41 (+) which was classified as inhibiting but weak. The mechanism of inhibition is strong bacteriostatic because the zone formed is clearer than *B subtilis* B315 without nanosuspension (Figure 2 and 5).



A B

Figure 5. Inhibition of nanosuspension formula of *B. subtilis* B315 against Xoo (A) and its bacteriostatic mechanism (B).

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In conclusion *B. subtilis* B315 is an isolate of *Bacillu*s from potato rhizosphere that could suppresses *Xoo* in vitro with an antibiosis index was 1 and is categorized strong. The mechanism of inhibition is bacteriostatic antibiosis, which only inhibits the growth of *Xoo* bacteria and it is not a lethal effect. *B. subtilis* B315 supernatant nanosuspension formula was able to inhibit *Xoo* with an antibiosis index of 0.42 (+). The nanosuspension formula was prospective to developed as biocontrol against *Xoo* and bacterial leaf blight on fields.

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REFERENCES

Alghuthaymi MA, Almoammanarb H, Raic M, Said-Galievd E, Abd-Elsalame KA. 2015. Myconanoparticles: Synthesis and their role in phytopathogens management Biotechnol Biotechnol Equipt. Taylor & Francis, 29(2): 221–236. doi: 10.1080/13102818.2015.1008194.

Azizah, N. 2017. Uji antagonis *Corynebacterium* sp, *Bacillus amyloliquefaciens*, *Trichoderma* sp, dan *Gliocladium* sp, terhadap bakteri patogen *Burkholderia glumae* pada tanaman padi secara In-Vitro. (Thesis). Fakultas Pertanian Universitas Hasanudin, Makassar (Indonesian).

Balouiri, M., Sadiki, M. and Ibnsouda, S. K. 2016. Methods for in vitro evaluating antimicrobial activity : A review, JPharm Anal. 6(2): 71–79. doi: 10.1016/j.jpha.2015.11.005.

Chun, W. and Vidaver, A. K. 2001. Gram-positive bacteria: *Bacillus* In: Schaad NW, Jones JB, Chun W (eds) Plant Pathogenic Bacteria APS Press, St. Paul Minnesota.

Goto M. 1992 *Fundamental of Bacterial Plant Pathology*. Academic Press New York.

Halimahtussadiyah R, Natsir M, Kurniawati D, Puspita S, Utamy. 2017. Isolation and identification of chitinolytic bacteria of pohara river of south east sulawesi and the optimization production of chitinase enzyme, AIP Conf. Proc. 1823, 020062-1–020062-7; pp. 1–7. doi: 10.1063/1.4978135.

Kala A, Soosairaj S, Mathiyazhagan S, Raja P. 2015. Isolation and Identification of *Xanthomonas oryzae* pv . *oryzae* the causal agent of rice bacterial leaf blight and its activities against of six medicinal plants, Asian J. Plant Sci. Res 5(6):80-83.

Lade BD, Gogle DP, Lade DB, Moon GM, Nandeshwari SB, Kumbhare SD. 2019 Nanobiopesticide formulations: Application strategies today and future perspectives' In: Koul O (eds) Nano-Biopesticides Today and Future Perspectives. Publisher, City. doi: 10.1016/B978-0-12-815829-6.00007-3.

Lestari P, Prihatiningsih N, Djatmiko HA. 2017 Partial biochemical characterization of crude extract extracellular chitinase enzyme from *Bacillus subtilis* B 298, IOP Conf. Series: materials Sci Engin, 012041. doi: 10.1088/1757-899X/172/1/1/012041.

Lia R. 2018. Pathotype profile of *Xanthomonas oryzae* pv . *oryzae* isolates from North Sumatera. IOP Conf. Series: Earth Environ Sci 122 (2018) 012142 doi :10.1088/1755-1315/122/1/012142

Marcic SM, Todorovic V, Stanojevic O, Beric T, Stankovic S, Todorovic B, Potocnic I. 2018 Antagonistic potential of *Bacillus* spp. isolates against bacterial pathogens of tomato and fungal pathogen of pepper, *Pesticide i fitomedicina*, 33(1): 9–18. doi: 10.2298/pif1801009m.

Naqvi SAH, Perveen R, Umer UD, Malik O, Rehman A, Wazeer S, Majid T. 2014 Determination of antibacterial activity of various broad spectrum antibiotics against *Xanthomonas oryzae* pv . *oryzae* , a cause of bacterial leaf blight of rice', *InterJMicrobiol Mycol* 2(3): 12–19.

Prihatiningsih N. 2013. Aktivitas antibiosis *Bacillus* sp. B315 sebagai agens pengendali hayati *Ralstonia solanacearum* pada kentang. (Dissertasion). Universitas Gadjah Mada Yogyakarta (Indonesian).

Prihatiningsih N. and Djatmiko HA. 2016. Enzim amilase sebagai komponen antagonis *Bacillus subtilis* B315 terhadap *Ralstonia solanacearum kentang*. J HPT Tropika 16(1): 10–16.

Prihatiningsih N, Djatmiko HA,Lestari P. 2017. Aktivitas siderofor *Bacillus subtilis* sebagai pemacu pertumbuhan dan pengendali patogen tanaman terung. J HPT Tropika 17(2): 170–178.

Prihatiningsih N, Djatmiko HA, Lestari P. 2020b. Screening of competent rice root endophytic bacteria to promote rice growth and bacterial leaf blight disease control.. J HPT Tropika 20(1):78–84.

Prihatiningsih N, Arwiyanto T, Hadisutrisno B, Widada J. 2020a. Characterization of *Bacillus* spp.. from the rhizosphere of potato Granola varietiy as an antibacterial against *Ralstonia solanacearum*. Biodiversitas 21(9): 4199–4204. doi: 10.13057/biodiv/d210934.

Prihatiningsih N, Asnani A, Djatmiko HA. 2021 Extracellular protease from *Bacillus subtilis* B315 with antagonistic activity against bacterial wilt pathogen (*Ralstonia solanacearum*) of chili. Biodiversitas 22(3): 1291–1295. doi: 10.13057/biodiv/d220327.

Shanti ML, Shenoy VV, Devi GL, Kumar VM, Premalatha P, Kumar GN, Shaahidhar HE, Zehr UB, Barwale FWH. 2010. Marker-assisted breeding for resistance to bacterial leaf blight in popular cultivar and parental lines of hybrid rice, *J Plant Pathol* 92 (2):495-501.

Singh D, Yaday DK, Chaudhary G, Rana VS, Sharm RK. 2016. Potential of *Bacillus amyloliquefaciens* for Biocontrol of Bacterial Wilt of Tomato Incited by *Ralstonia solanacearum*, J Plant Pathol Microbiol 7(1): 1–6. doi: 10.4172/2157-7471.1000327.

Swamy P, Panchbha AN, Dodiya P, Naik V, Panchbhai SD, Zehr UB, Azhakanandam K, Char BR. 2006. Evaluation of bacterial blight resistance in rice lines carrying multiple resistance genes and Xa21 transgenic lines, Curr Sci 90 (6): 818-824

Worrall EA, Hamid A, Mody KT, Mitter N, Pappu HR. 2018. Nanotechnology for plant disease management. *Agronomy*, 8(12): 1–24. doi: 10.3390/agronomy8120285.

Yanti Y, Hamid H, Habazar T. 2020. The ability of indigenous *Bacillus* spp . consortia to control the anthracnose disease (*Colletrotricum capsici*) and increase the growth of chili plants. Biodiversitas 21(1): 179–186. doi:

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INTRODUCTION

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Control of potato *R. solanacearum* with *B. subtilis* B315 was 64.9% effective, while tomato, chili and eggplant in the lowlands reached 74.6% with a population density of 10^8 cfu.mL^{-1} (Prihatiningsih, 2013; Prihatiningsih and Djatmiko, 2016). *B. subtilis* is also reported to be able to produce IAA (*Indole Acetic Acid*) in the range of 57.56-79.33 ppm, that stimulate plant growth (Prihatiningsih et al. 2020b). *Bacillus* spp. consortium indigenous can control *Colletotrichum capsici* and increase chili growth (Yanti et al. 2020). *B. amyloliquefaciens* can reduce the incidence of tomato bacterial wilt, suppress the population of *R. solanacearum* and improve the growth of tomato plants (Singh et al. 2016).

The mechanism of controlling pathogens by *Bacillus* is antibiosis, which is to inhibit pathogens by producing compounds such as antibiotics, enzymes, and other volatile compounds. In vitro testing of mechanism character antibiosis is characterized by the formation of a clear zone around the antagonist colony. The liquid formula of *B. subtilis* B315 as a controller of *R. solanacearum* *in vitro* showed the effectiveness with an inhibition zone of 18 mm with an antibiosis

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inhibition mechanism indicated by the enzymes amylase, chitinase and proteases produced (Prihatiningsih and Djatmiko 2016; Lestari et al. 2017; Prihatiningsih et al. 2020b; Prihatiningsih et al. 2021).

Based on the ability of these *B. subtilis* potato isolate, in this study it was cross tested to another plants, against *Xanthomonas oryzae* pv. *oryzae* which causes bacterial leaf blight of rice. Evaluation of pathogen inhibition by antagonistic bacteria can be measured by the size of the inhibition zone, such as measuring the diameter of the clear zone to calculating the area of the inhibition potential. Thus according to these data, an antibiotic index can be calculated. The mechanism of antagonistic bacteria inhibiting pathogenic bacteria is bacteriostatic and bactericidal (Goto 1992).

Rice bacterial leaf blight caused by *X. oryzae* pv. *oryzae* (*Xoo*) is an important disease and has long been recognized as a bacterial disease of rice in Asia (Naqvi et al. 2014) thoroughly in several locations of rice cultivation. Yield losses varied up to 20% under moderate conditions and more than 50% in conducive conditions to disease development such as varieties, plant growth stages and weather conditions. This bacterial rice blight disease was once an epidemic during 1998 in the Palakkad area of Kerala and since then several proportions have been observed each year (Swamy et al. 2006; Kala et al. 2015). Several disease management strategies used to solve these problems such as using resistant plants based on a single gene (monogenic), hence break its resistance gradually.

Symptoms of bacterial leaf blight caused elongated spots starting from the tips of the leaves are white to gray with irregular wavy edges, dry, occurring during the vegetative period of the plant called crackle symptoms, and during the generative period of the plant the symptoms are called blight (Shanti et al. 2010; Lia 2018). If the pathogen attacks the plant approaching mature, the leaves turn pale yellow. So far, there is no control yet to solve this ~~-~~*Xoo* attack problem. Based on the description above, it is necessary to carry out a biological control with *B. subtilis*.

Nanoparticles are one of the pesticide formulations with nano~~-~~technology, also known as nanobiopesticides. Nanobiopesticides can be in the form of nanosuspensions, nanoemulsions and nanoparticles (Lade et al. 2019) which have been developed since the 19th century, but have only been noticed since 1959. Nanotechnology in the field of plant disease management can be used for disease diagnosis and detection of pathogens, evaluation of pathogens suppression, and pesticides reformulating. Application and effectiveness of plant disease control using nanobiopesticides is application to soil, seeds or leaves to protect plants from pathogen invasion (Alghuthaymi et al. 2015). Nanotechnology has advantages as pesticides by reducing the toxicity, improve shelf-life and solubility of pesticides that are less soluble in water, and have a positive effect on plants and the environment (Worrall et al. 2018).

The aims of this study were (1) to test of the potential *B. subtilis* potato isolate as a control for *X. oryzae* pv. *oryzae* pathogenic rice bacterial leaf blight, (2) determine the suppression mechanism of *B. subtilis* potato isolate against *Xoo*, (3) ~~Evaluating~~ evaluating the best *B. subtilis* potato isolate to make a nanosuspension formula.

MATERIALS AND METHODS

Preparation of bacteria

The research materials used were *B. subtilis* B46, B209, B211, B298 and B315 potato isolates (Prihatiningsih collection) that were ~~growth~~ grown in yeast peptone glucose agar (YPGA), *Xoo* in ~~Sucrose~~ sucrose ~~Peptone~~ peptone ~~Agar~~ agar (SPA), and further they were ~~growth~~ grown in nutrient broth (NB) for dual culture inhibition test. *Xoo* bacteria isolate was collected from rice field at Karangwangkal Purwokerto. The research was performed from February to June 2021. All the experiments at the Plant Protection Laboratory, Faculty of Agriculture Universitas Jenderal Soedirman (UNSOED) Purwokerto.

Bacterial antagonist test of *B. subtilis* against *Xoo*

Five isolates of *B. subtilis* from potato rhizosphere, namely *B. subtilis* B46, B209, B211, B298 and B315 were tested for their inhibition against *Xoo* by ~~the~~ agar diffusion method using filter paper with a diameter of 5 mm according to the method of Balouiri et al. (2016). *Xoo* as the tested pathogenic bacteria was grown on NB medium shaken at 150 rpm at room temperature for 24 hours. Bacterial density *Xoo* 10^8cfu/mL was inoculated by pour plate on YPGA medium. The 5 mm diameter filter paper was dripped with 10 µL suspension, then placed on the YPGA medium in 3 parts. The clear zone formed around the filter paper indicated the inhibition of *Xoo* growth.

Evaluation of inhibition mechanism

To evaluate the ability of antagonistic bacteria to inhibit *Xoo* the percentage of inhibition was ~~determined~~ determined by the inhibition zone formed. ~~Determination of inhibition~~ Inhibition was determined using the method of Balouiri et al. (2016) by measuring the diameter of the clear zone, and the diameter of the colony (Figure 1). Inhibition measurement (P) was performed after 24 hours.

P = {diameter of clear zone (A) - diameter of bacterial colony (B)} (Balouiri et al. 2016)

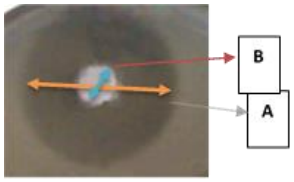

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Figure 1. Antibiotic index measurement.
(A). ~~Diameter~~ diameter of the clear zone, (B). diameter of the colony or filter paper

Antibiosis index

The antibiosis index was determined according to the enzyme index using the formula for measuring the chitinolytic index refer to (Halimahtussadiyah et al. 2017) or phosphate solvent produced by antagonistic bacteria, by measuring the diameter of the clear zone, and the diameter of the colony using the following formula:

$$AI = \frac{\text{(diameter of clear zone - diameter of bacterial colony)}}{\text{diameter of bacterial colony}}$$

or

AI = (A-B) : B (Halimahtussadiyah et al. 2017)

(A). ~~Diameter~~ diameter of the clear zone, (B). diameter of the colony or filter paper

According to Azizah (2017), determination of the inhibition category grouped based on the index of inhibition or index of antibiosis, namely:

a. Very Strong (>2.0) with symbol (+++)
b. Strong (1.0 – 1.9) with symbol (++)
c. Weak (0.1 – 0.9) with the symbol (+)
d. Has no antagonistic ability (0.0) with the symbol (-)

Preparation of nanosuspension formula from supernatant of selected *Bacillus subtilis*

B. subtilis B315 was grown in NB medium, shaken 2~~×~~24 hours at 150 rpm at room temperature, then centrifuged at 3000 rpm for 15 minutes at room temperature, the supernatant obtained was used as the active ingredient. Chitosan was dissolved in 1% acetic acid. 0.5 mL of *B. subtilis* B315 supernatant was put into a micro centrifuge tube containing 490 µL of chitosan solution in 1% acetic acid pH 4.0-5.0, shaken with a vortex for 20 seconds. The homogeneous mixture was added with 10 µL of 0.1% tripolyphosphate (TPP) solution and continuing homogenized by vortex for 20 seconds. The nanoparticle preparation was performed characterized, the morphology, the particle size and the distribution of particle size.

Evaluation of the ~~inhibition~~ inhibitory effect of *B. subtilis* supernatant nanosuspension formula to suppress *Xoo*

The inhibition of *B. subtilis* supernatant in nanosuspension formula against *Xoo* was determined by agar diffusion method using filter paper according to the method of Balouiri et al. (2016). *Xoo* as the tested pathogenic bacteria was grown on NB medium shaken at 150 rpm at room temperature for 24 hours. Bacterial density of *Xoo* (10^8cfumL^{-1}) was inoculated on YPGA medium. The 5 mm diameter filter paper was dripped with nanosuspension formula, then placed in the YPGA medium in 3 parts. The clear zone around the filter paper showed the effect inhibition of *B. subtilis* supernatant nanosuspension formula to suppress *Xoo* (Balouiri et al. 2016).

Data ~~analyses~~ analysis

Data were analysis by descriptive methods and comparative to references. The results were expressed as the mean ± sd of mean. Raw data were imported to Microsoft Excel program for graphical representation.

RESULTS AND DISCUSSION

Inhibition assay of *Bacillus subtilis*~~.~~ to *Xoo*

The results of this study included the selection of 5 isolates of *B. subtilis* (B46, B209, B211, B298 and B315) to be able to determine which isolates would be formulated into nanoparticles for the next research stage. ~~Selection of *B. subtilis* against pathogenic bacteria *Xoo* is shown in Table 1 and Figure 2.~~ Result revealed that *B. subtilis* B315 isolate ~~was~~ showed highest (10+0.12 mm) ~~with 10+0.12 mm~~ inhibition zone than another isolates (Table 1 and Figure 2).

Table 1. Inhibition of the five isolates of *B. subtilis* against *Xoo*

B. subtilis isolates	Inhibition ~~Zone~~ zone (mm)	Inhibition mechanism
B46	-	-
B209	-	-

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B211	9±0.14	bacteriostatic
B298	8±0.12	bacteriostatic
B315	10±0.12	bacteriostatic

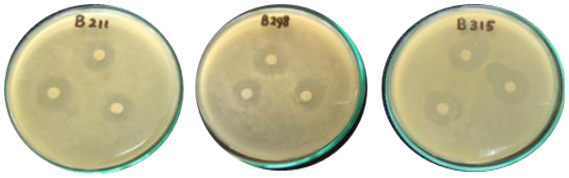

Figure 2. Inhibition of *B. subtilis* against *Xoo*

The mechanism of inhibition of *B. subtilis* against *Xoo* ~~is~~ was bacteriostatic antibiosis, it's mean that *B. subtilis* only able to inhibit the growth of *Xoo* but it ~~is~~ was not lethal effect. It was demonstrated by the cloudy peptone water which indicated that *Xoo* was still growing, while the bactericidal mechanism occurred when the peptone water remained clear or it's as same as the control (Figure 3), which was a lethal. The bacteriostatic mechanism of these five *B. subtilis* isolates was also shown against the pathogenic bacterium *Ralstonia solanacearum* that causes potato bacterial wilt disease (Prihatiningsih 2013).

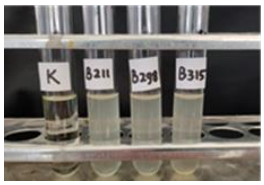

Figure 3. Bacteriostatic mechanism of three isolates of *B. subtilis* ~~to~~ against *Xoo*

Antibiosis index

The highest antibiosis index was shown by isolate *B. s ubtilis* B315 of 1 and according to the category of inhibition index it was strong (++) (Figure 4). This result indicates that *B. subtilis* B315 from potato rhizosphere has good prospects to be developed as a biological agent for biopesticides to control pathogenic bacteria not only in the habitat of *R. solanacearum* which causes potato bacterial wilt, but also against *Xoo* which causes rice bacterial leaf blight. *B. subtilis* isolates B46 and B209 in the inhibition test against potato *R. solanacearum* were able to inhibit, but after cross-testing against rice pathogenic bacteria the two isolates were unable to inhibit (-).

Antibiosis index is useful as an indicator that bacteria are capable of being antagonists against pathogens, both bacteria and fungi. Bacteria are able to control pathogenic fungi by producing the enzyme chitinase as an inhibitory mechanism since this enzyme degrades the cell walls of pathogenic fungi which consists mostly of chitin (Halimahtussadiyah et al. 2017). Chitinase activity of *B. subtilis* B298 was 6.936 UmL^{-1}, 5.764 $U.mL^{-1}$ and 6.813 $U.mL^{-1}$, which were detected at 15 hours of incubation, optimum temperature of 40 ~~°~~°C and optimum pH of 5.0, respectively~~, at 6.936 UmL^{-1}, 5.764 $U.mL^{-1}$ and 6.813 $U.mL^{-1}$~~ (Lestari et al. 2017). *B. subtilis* also produce proteases and siderophores hence able to inhibit pathogenic bacteria such as rice *Xoo* and potato *R. solanacearum* (Prihatiningsih et al. 2017; Prihatiningsih et al. 2021).

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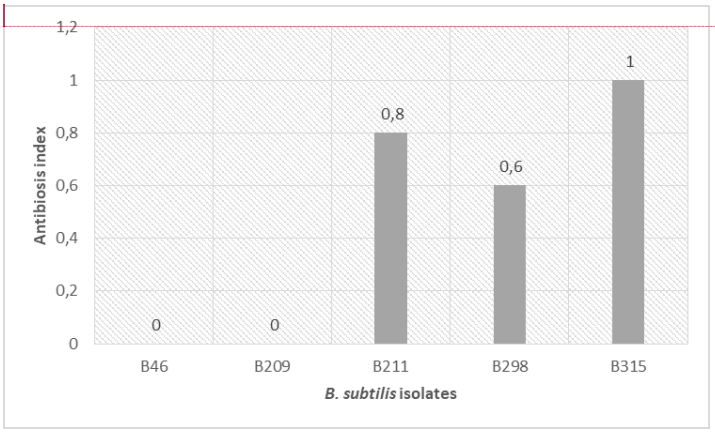

Figure 4. Antibiosis index of the five isolates of *B. subtilis* against *Xoo*

Nanosuspension formula of *B. subtilis* B315 ~~supernatant~~ to control *Xoo*

The inhibition of *Xoo* growth by nanosuspension formula of *B. subtilis* B315 supernatant was 2.1 mm (Figure 5) in diameter. ~~and in accordance~~ According to ~~with~~ Marcic et al. (2018), ~~that~~ *B. subtilis* can suppress *X. vesicatoria* in tomatoes with an inhibition zone of 5-9 mm but without ~~in~~ nanosuspension formula. The antibiosis index of this nanosuspension formula was 0.41 (+) which was classified as weak inhibitor ~~inhibiting but weak~~. The mechanism of inhibition ~~is~~ was strong bacteriostatic because the zone formed ~~is~~ was clearer than *B subtilis* B315 without nanosuspension (Figures 2 and 5).

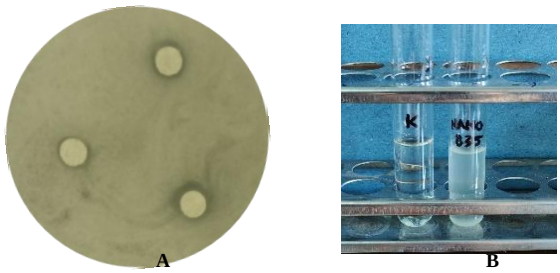

Figure 5. (A). Inhibition of the nanosuspension formula of *B. subtilis* B315 against *Xoo* ~~(A)~~ (B) ~~and~~ its bacteriostatic mechanism ~~(B)~~.

In conclusion *B. subtilis* B315 ~~is~~ was an isolate of *Bacillus* isolated from potato rhizosphere that could suppresses *Xoo in vitro*, ~~with~~ had an antibiosis index ~~was~~ of 1 and ~~is~~ was categorized as strong. The mechanism of inhibition ~~is~~ was bacteriostatic antibiosis, which only inhibits the growth of *Xoo* bacteria ~~and~~ but it ~~is~~ was not ~~a~~ lethal ~~effect~~. *B. subtilis* B315 supernatant nanosuspension formula was able to inhibit *Xoo* with an antibiosis index of 0.42 (+). The nanosuspension formula ~~was~~ has the prospective to be developed as biocontrol against *Xoo* and bacterial leaf blight on

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REFERENCES

Alghuthaymi MA, Almoammanarb H, Raic M, Said-Galievd E, Abd-Elsalame KA. 2015. Myconanoparticles: Synthesis and their role in phytopathogens management. Biotechnol Biotechnol Equipt. ~~Taylor & Francis,~~ 29(2): 221–236. doi: 10.1080/13102818.2015.1008194.

Azizah, N. 2017. Uji antagonis *Corynebacterium* sp, *Bacillus amyloliquefaciens*, *Trichoderma* sp, dan *Gliocladium* sp, terhadap bakteri patogen *Burkholderia glumae* pada tanaman padi secara *In-~~Vitro~~vitro*. (Thesis). Fakultas Pertanian Universitas Hasanudin, Makassar (Indonesian).

Balouiri, M., Sadiki, M. and Ibnsouda, S. K. 2016. Methods for *in vitro* evaluating antimicrobial activity : A review. JPharm Anal. 6(2): 71–79. doi: 10.1016/j.jpha.2015.11.005.

Chun, W. and Vidaver, A. K. 2001. Gram-positive bacteria: *Bacillus* In: Schaad NW, Jones JB, Chun W (eds) Plant Pathogenic Bacteria APS Press, St. Paul Minnesota.

Goto M. 1992 *Fundamental of Bacterial Plant Pathology*. Academic Press New York.

Halimahtussadiyah R, Natsir M, Kurniawati D, Puspita S, Utamy. 2017. Isolation and identification of chitinolytic bacteria of pohara river of south east sulawesi and the optimization production of chitinase enzyme. AIP Conf. Proc. 1823, 020062-1–020062-7; pp. 1–7. doi: 10.1063/1.4978135.

Kala A, Soosairaj S, Mathiyazhagan S, Raja P. 2015. Isolation and Identification of *Xanthomonas oryzae* pv . *oryzae* the causal agent of rice bacterial leaf blight and its activities against of six medicinal plants. Asian J. Plant Sci. Res 5(6):80-83.

Lade BD, Gogle DP, Lade DB, Moon GM, Nandeshwari SB, Kumbhare SD. 2019 Nanobiopesticide formulations: Application strategies today and future perspectives' In: Koul O (eds) Nano-Biopesticides Today and Future Perspectives. Publisher, City. doi: 10.1016/B978-0-12-815829-6.00007-3.

Lestari P, Prihatiningsih N, Djatmiko HA. 2017 Partial biochemical characterization of crude extract extracellular chitinase enzyme from *Bacillus subtilis* B 298, IOP Conf. Series: materials Sci Engin. 012041. doi: 10.1088/1757-899X/172/1/1/012041.

Lia R. 2018. Pathotype profile of *Xanthomonas oryzae* pv . *oryzae* isolates from North Sumatera. IOP Conf. Series: Earth Environ Sci 122 (2018) 012142 doi :10.1088/1755-1315/122/1/012142

Marcic SM, Todorovic V, Stanojevic O, Beric T, Stankovic S, Todorovic B, Potocnic I. 2018 Antagonistic potential of *Bacillus* spp. isolates against bacterial pathogens of tomato and fungal pathogen of pepper. *Pesticide i fitomedicina*. 33(1): 9–18. doi: 10.2298/pif1801009m.

Naqvi SAH, Perveen R, Umer UD, Malik O, Rehman A, Wazeer S, Majid T. 2014 Determination of antibacterial activity of various broad spectrum antibiotics against *Xanthomonas oryzae* pv . *oryzae* , a cause of bacterial leaf blight of rice'. InterJMicrobiol Mycol 2(3): 12–19.

Prihatiningsih N. 2013. Aktivitas antibiosis *Bacillus* sp. B315 sebagai agens pengendali hayati *Ralstonia solanacearum* pada kentang. (Dissertasion). Universitas Gadjah Mada Yogyakarta (Indonesian).

Prihatiningsih N. and Djatmiko HA. 2016. Enzim amilase sebagai komponen antagonis *Bacillus subtilis* B315 terhadap *Ralstonia solanacearum kentang*. J HPT Tropika 16(1): 10–16.

Prihatiningsih N, Djatmiko HA,Lestari P. 2017. Aktivitas siderofor *Bacillus subtilis* sebagai pemacu pertumbuhan dan pengendali patogen tanaman terung. J-HPT Tropika 17(2): 170–178.

Prihatiningsih N, Djatmiko HA, Lestari P. 2020b. Screening of competent rice root endophytic bacteria to promote rice growth and bacterial leaf blight disease control. J HPT Tropika 20(1):78–84.

Prihatiningsih N, Arwiyanto T, Hadisutrisno B, Widada J. 2020a. Characterization of *Bacillus* spp. from the rhizosphere of potato Granola varietiy as an antibacterial against *Ralstonia solanacearum*. Biodiversitas 21(9): 4199–4204. doi: 10.13057/biodiv/d210934.

Prihatiningsih N, Asnani A, Djatmiko HA. 2021 Extracellular protease from *Bacillus subtilis* B315 with antagonistic activity against bacterial wilt pathogen (*Ralstonia solanacearum*) of chili. Biodiversitas 22(3): 1291–1295. doi: 10.13057/biodiv/d220327.

Shanti ML, Shenoy VV, Devi GL, Kumar VM, Premalatha P, Kumar GN, Shaahidhar HE, Zehr UB, Barwale FWH. 2010. Marker-assisted breeding for resistance to bacterial leaf blight in popular cultivar and parental lines of hybrid rice. J Plant Pathol 92 (2):495-501.

Singh D, Yaday DK, Chaudhary G, Rana VS, Sharm RK. 2016. Potential of *Bacillus amyloliquefaciens* for Biocontrol of Bacterial Wilt of Tomato Incited by *Ralstonia solanacearum*. J Plant Pathol Microbiol 7(1): 1–6. doi: 10.4172/2157-7471.1000327.

Swamy P, Panchbha AN, Dodiya P, Naik V, Panchbhai SD, Zehr UB, Azhakanandam K, Char BR. 2006. Evaluation of bacterial blight resistance in rice lines carrying multiple resistance genes and Xa21 transgenic lines. Curr Sci 90 (6):

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Worrall EA, Hamid A, Mody KT, Mitter N, Pappu HR. 2018. Nanotechnology for plant disease management. Agron~~omy,~~ 8(12): 1–24. doi: 10.3390/agronomy8120285.
Yanti Y, Hamid H, Habazar T. 2020. The ability of indigenous *Bacillus* spp . consortia to control the anthracnose disease (*Colletotricum capsici*) and increase the growth of chili plants. Biodiversitas 21(1): 179–186. doi: 10.13057/biodiv/d210123.

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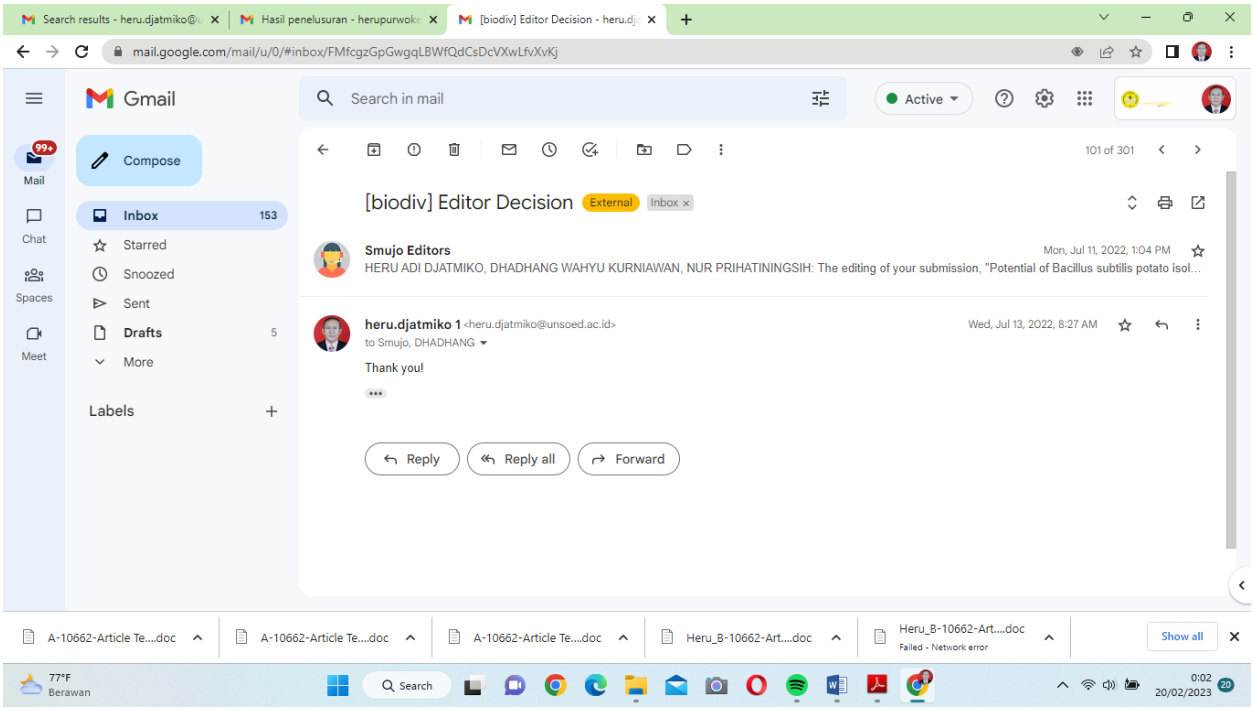

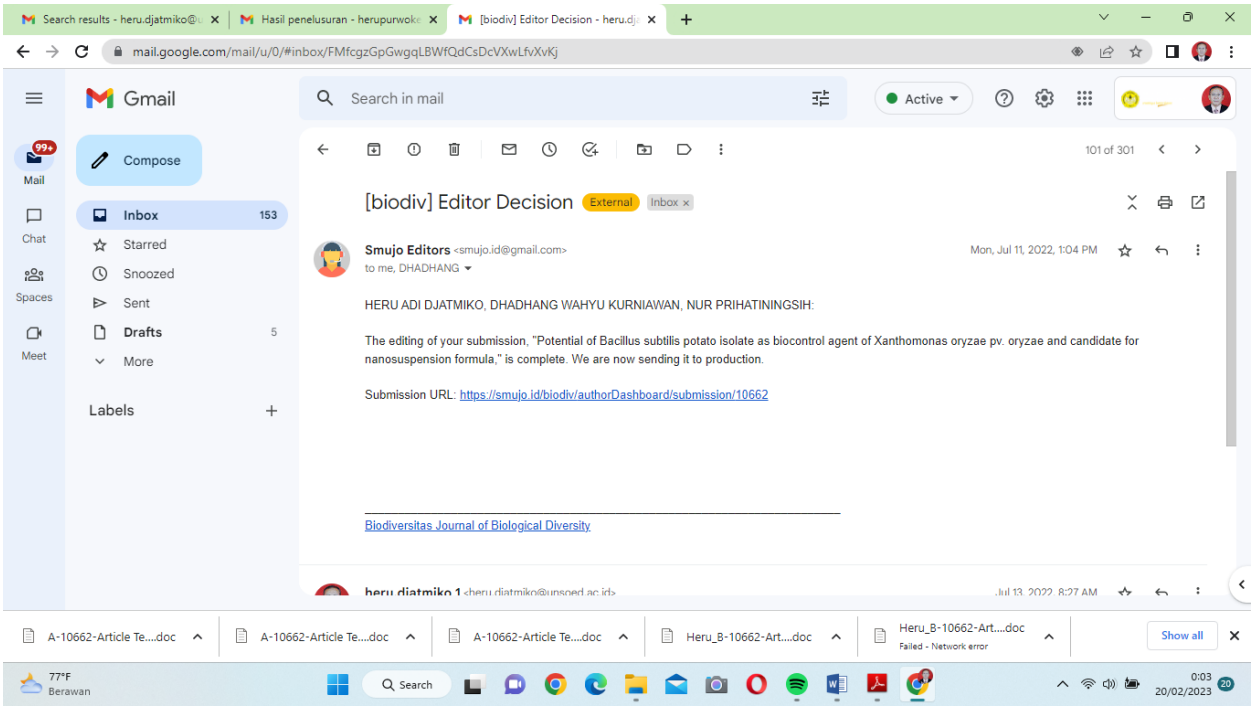

Potential of *Bacillus subtilis* potato isolate as biocontrol agent of *Xanthomonas oryzae* pv. *oryzae* and candidate for nanosuspension formula

Abstract. *Bacillus subtilis* potato isolate was able to be an antagonist of plant pathogens. The aims of this study were (1) to examine the potential of potato isolate *B. subtilis* as a control for *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) as a pathogen rice bacterial leaf blight, (2) determine the inhibition mechanism of their isolates, (3) evaluating the best *B. subtilis* to make nanosuspension formula. The method was carried out in a completely randomized design with 5 treatments of *B. subtilis* potato isolates B46, B209, B211, B298 and B315, repeated 4 times. *Xoo* and five *B. subtilis* were grown in nutrient broth medium in a shaker. *Xoo* and *B. subtilis* were grown in yeast peptone glucose agar medium by the diffusion method. *Xoo* was inoculated on medium, and then a 5 mm diameter filter paper immersed with *B. subtilis* suspension was placed on the medium. The best *B.subtilis* as a controller was used to formulate into nanosuspension. The variables were zone of inhibition, inhibition mechanism and antibiosis index. The results showed that only three *B. subtilis* were able to inhibit the growth of *Xoo*, namely B211, B298 and B315 isolates. The highest (10 mm) inhibition zone was recorded by *B. subtilis* B315 isolate. The mechanism of inhibition of the three isolates of *B. subtilis* against *Xoo* was bacteriostatic. The antibiosis index of *B. subtilis* B315 against *Xoo* was 1 (strong). The selected *B. subtilis* B315 was developed as a nanosuspension formula with inhibition zone of 2.1 mm and antibiosis index was 0.42.

Key words: Antagonist, antibiosis, nanosuspension, *Xanthomonas oryzae*

INTRODUCTION

Bacillus subtilis is known as a useful bacterium that can be used as an antagonist against plant pathogens. *B. subtilis* can be explored from soil, water, air, plant surfaces and endophytes from plant tissues such as roots, stems, sheath, and leaves. *B. subtilis* is an antagonistic bacterium that is Gram positive, rod-shaped, 7-8 × 2-3 µm, flagellated, aerobic, not encapsulated, have endospores that are ovoid to cylindrical, measuring 0.6-0.9 × 1.0-1.5 µm, thin walled. Spores are formed generally within 24 hours (Gordon et al. 1973). One of the strong characteristics bacteria are classified into Bacillus is their ability to form endospores under extreme conditions. Based on the endospore morphology, Bacillus can be divided into 3, namely: (I) oval or cylindrical spores located in the middle, subterminal or terminal, swollen rods, or not swollen, (II), oval, slightly cylindrical spores, subterminal or terminal, stems do not swell, (III) spores are spherical, subterminal or terminal, rods are swollen (Chun and Vidaver 2001).

B. subtilis isolated from potato rhizosphere, has been selected to obtain five superior isolates, there are *B. subtilis* B46, B209, B211, B298 and B315. Molecularly *Bacillus* isolates B46, B209, B211, B298 are homologous to *B. subtilis* subsp. *spizizeni* RRLKE2, while B315 is homologous to *B. subtilis* strain WIFD5 (Prihatiningsih et al. 2020a). *B. subtilis* potato isolate has been tested for control of bacterial wilt disease by *Ralstonia solanacearum* of potatoes, tomatoes, tobacco, and chilies. Control effectiveness varies both in the greenhouse and in different fields. This shows that its effectiveness depends on weather conditions, plant age and population density of *B. subtilis*.

Control of potato *R. solanacearum* with *B. subtilis* B315 was 64.9% effective, while tomato, chili and eggplant in the lowlands reached 74.6% with a population density of 10^8 cfu.mL^{-1} (Prihatiningsih, 2013; Prihatiningsih and Djatmiko, 2016). *B. subtilis* is also reported to be able to produce IAA (*Indole Acetic Acid*) in the range of 57.56-79.33 ppm, that stimulate plant growth (Prihatiningsih et al. 2020b). *Bacillus* spp. consortium indigenous can control *Colletotrichum capsici* and increase chili growth (Yanti et al. 2020). *B. amyloliquefaciens* can reduce the incidence of tomato bacterial wilt, suppress the population of *R. solanacearum* and improve the growth of tomato plants (Singh et al. 2016).

The mechanism of controlling pathogens by *Bacillus* is antibiosis, which is to inhibit pathogens by producing compounds such as antibiotics, enzymes, and other volatile compounds. In vitro testing of mechanism character antibiosis is characterized by the formation of a clear zone around the antagonist colony. The liquid formula of *B. subtilis* B315 as a controller of *R. solanacearum in vitro* showed the effectiveness with an inhibition zone of 18 mm with an antibiosis inhibition mechanism indicated by the enzymes amylase, chitinase and proteases produced (Prihatiningsih and Djatmiko 2016; Lestari et al. 2017; Prihatiningsih et al. 2020b; Prihatiningsih et al. 2021).

Based on the ability of these *B. subtilis* potato isolate, in this study it was cross tested to another plants, against *Xanthomonas oryzae* pv. *oryzae* which causes bacterial leaf blight of rice. Evaluation of pathogen inhibition by antagonistic bacteria can be measured by the size of the inhibition zone, such as measuring the diameter of the clear zone to calculating the area of the inhibition potential. Thus according to these data, an antibiotic index can be calculated. The mechanism of antagonistic bacteria inhibiting pathogenic bacteria is bacteriostatic and bactericidal (Goto 1992).

Rice bacterial leaf blight caused by *X. oryzae* pv. *oryzae* (*Xoo*) is an important disease and has long been recognized as a bacterial disease of rice in Asia (Naqvi et al. 2014) thoroughly in several locations of rice cultivation. Yield losses varied up to 20% under moderate conditions and more than 50% in conducive conditions to disease development such as varieties, plant growth stages and weather conditions. This bacterial rice blight disease was once an epidemic during 1998 in the Palakkad area of Kerala and since then several proportions have been observed each year (Swamy et al. 2006; Kala et al. 2015). Several disease management strategies used to solve these problems such as using resistant plants based on a single gene (monogenic), hence break its resistance gradually.

Symptoms of bacterial leaf blight caused elongated spots starting from the tips of the leaves are white to gray with irregular wavy edges, dry, occurring during the vegetative period of the plant called crackle symptoms, and during the generative period of the plant the symptoms are called blight (Shanti et al. 2010; Lia 2018). If the pathogen attacks the plant approaching mature, the leaves turn pale yellow. So far, there is no control yet to solve this *Xoo* attack problem. Based on the description above, it is necessary to carry out a biological control with *B. subtilis.*

Nanoparticles are one of the pesticide formulations with nanotechnology, also known as nanobiopesticides. Nanobiopesticides can be in the form of nanosuspensions, nanoemulsions and nanoparticles (Lade et al. 2019) which have been developed since the 19th century, but have only been noticed since 1959. Nanotechnology in the field of plant disease management can be used for disease diagnosis and detection of pathogens, evaluation of pathogens suppression, and pesticides reformulating. Application and effectiveness of plant disease control using nanobiopesticides is application to soil, seeds or leaves to protect plants from pathogen invasion (Alghuthaymi et al. 2015). Nanotechnology has advantages as pesticides by reducing the toxicity, improve shelf-life and solubility of pesticides that are less soluble in water, and have a positive effect on plants and the environment (Worrall et al. 2018).

The aims of this study were (1) to test of the potential *B. subtilis* potato isolate as a control for *X. oryzae* pv. *oryzae* pathogenic rice bacterial leaf blight, (2) determine the suppression mechanism of *B. subtilis* potato isolate against *Xoo*, (3) evaluating the best *B. subtilis* potato isolate to make a nanosuspension formula.

MATERIALS AND METHODS

Preparation of bacteria

The research materials used were *B. subtilis* B46, B209, B211, B298 and B315 potato isolates (Prihatiningsih collection) that were grown in yeast peptone glucose agar (YPGA), *Xoo* in sucrose peptone agar (SPA), and further they were grown in nutrient broth (NB) for dual culture inhibition test. *Xoo* bacteria isolate was collected from rice field at Karangwangkal Purwokerto. The research was performed from February to June 2021. All the experiments at the Plant Protection Laboratory, Faculty of Agriculture Universitas Jenderal Soedirman (UNSOED) Purwokerto.

Bacterial antagonist test of *B. subtilis* against *Xoo*

Five isolates of *B. subtilis* from potato rhizosphere, namely *B. subtilis* B46, B209, B211, B298 and B315 were tested for their inhibition against *Xoo* by agar diffusion method using filter paper with a diameter of 5 mm according to the method of Balouiri et al. (2016). *Xoo* as the tested pathogenic bacteria was grown on NB medium shaken at 150 rpm at room temperature for 24 hours. Bacterial density *Xoo* 10^8cfu/mL was inoculated by pour plate on YPGA medium. The 5 mm diameter filter paper was dripped with 10 µL suspension, then placed on the YPGA medium in 3 parts. The clear zone formed around the filter paper indicated the inhibition of *Xoo* growth.

Evaluation of inhibition mechanism

To evaluate the ability of antagonistic bacteria to inhibit *Xoo* the percentage of inhibition was determined by the inhibition zone formed. Inhibition was determined using the method of Balouiri et al. (2016) by measuring the diameter of the clear zone, and the diameter of the colony (Figure 1). Inhibition measurement (P) was performed after 24 hours.

P = {diameter of clear zone (A) - diameter of bacterial colony (B)} (Balouiri et al. 2016)

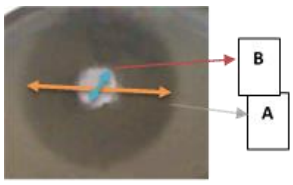

Figure 1. Antibiotic index measurement.

(A). diameter of the clear zone, (B). diameter of the colony or filter paper

Antibiosis index

The antibiosis index was determined according to the enzyme index using the formula for measuring the chitinolytic index refer to (Halimahtussadiyah et al. 2017) or phosphate solvent produced by antagonistic bacteria, by measuring the diameter of the clear zone, and the diameter of the colony using the following formula:

$$AI = \frac{\text{(diameter of clear zone - diameter of bacterial colony)}}{\text{diameter of bacterial colony}}$$

or

AI = (A-B) : B (Halimahtussadiyah et al. 2017)

(A). diameter of the clear zone, (B). diameter of the colony or filter paper

According to Azizah (2017), determination of the inhibition category grouped based on the index of inhibition or index of antibiosis, namely:

a. Very Strong (>2.0) with symbol (+++)
b. Strong (1.0 – 1.9) with symbol (++)
c. Weak (0.1 – 0.9) with the symbol (+)
d. Has no antagonistic ability (0.0) with the symbol (-)

Preparation of nanosuspension formula from supernatant of selected *Bacillus subtilis*

B. subtilis B315 was grown in NB medium, shaken 2×24 hours at 150 rpm at room temperature, then centrifuged at 3000 rpm for 15 minutes at room temperature, the supernatant obtained was used as the active ingredient. Chitosan was dissolved in 1% acetic acid. 0.5 mL of *B. subtilis* B315 supernatant was put into a micro centrifuge tube containing 490 µL of chitosan solution in 1% acetic acid pH 4.0-5.0, shaken with a vortex for 20 seconds. The homogeneous mixture was added with 10 µL of 0.1% tripolyphosphate (TPP) solution and continuing homogenized by vortex for 20 seconds. The nanoparticle preparation was performed characterized, the morphology, the particle size and the distribution of particle size.

Evaluation of the inhibitory effect of *B. subtilis* supernatant nanosuspension formula to suppress *Xoo*

The inhibition of *B. subtilis* supernatant in nanosuspension formula against *Xoo* was determined by agar diffusion method using filter paper according to the method of Balouiri et al. (2016). *Xoo* as the tested pathogenic bacteria was grown on NB medium shaken at 150 rpm at room temperature for 24 hours. Bacterial density of *Xoo* (10^8cfumL^{-1}) was inoculated on YPGA medium. The 5 mm diameter filter paper was dripped with nanosuspension formula, then placed in the YPGA medium in 3 parts. The clear zone around the filter paper showed the effect inhibition of *B. subtilis* supernatant nanosuspension formula to suppress *Xoo* (Balouiri et al. 2016).

Data analysis

Data were analysis by descriptive methods and comparative to references. The results were expressed as the mean ± sd of mean. Raw data were imported to Microsoft Excel program for graphical representation.

RESULTS AND DISCUSSION

Inhibition assay of *Bacillus subtilis* to *Xoo*

The results of this study included the selection of 5 isolates of *B. subtilis* (B46, B209, B211, B298 and B315) to be able to determine which isolates would be formulated into nanoparticles for the next research stage. Result revealed that *B. subtilis* B315 isolate showed highest (10±0.12 mm) inhibition zone than another isolates (Table 1 and Figure 2).

Table 1. Inhibition of the five isolates of *B. subtilis* against *Xoo*

B. subtilis isolates	Inhibition zone (mm)	Inhibition mechanism
B46	-	-
B209	-	-
B211	9±0.14	bacteriostatic
B298	8±0.12	bacteriostatic
B315	10±0.12	bacteriostatic

Figure 2. Inhibition of *B. subtilis* against *Xoo*

The mechanism of inhibition of *B. subtilis* against *Xoo* was bacteriostatic antibiosis, it's mean that *B. subtilis* only able to inhibit the growth of *Xoo* but it was not lethal effect. It was demonstrated by the cloudy peptone water which indicated that *Xoo* was still growing, while the bactericidal mechanism occurred when the peptone water remained clear or it's as same as the control (Figure 3), which was a lethal. The bacteriostatic mechanism of these five *B. subtilis* isolates was also shown against the pathogenic bacterium *Ralstonia solanacearum* that causes potato bacterial wilt disease (Prihatiningsih 2013).

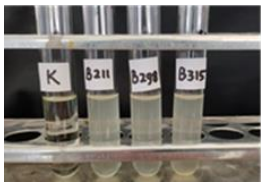

Figure 3. Bacteriostatic mechanism of three isolates of *B. subtilis* against *Xoo*

Antibiosis index

The highest antibiosis index was shown by isolate *B. s_ubtilis* B315 of 1 and according to the category of inhibition index it was strong (++) (Figure 4). This result indicates that *B. subtilis* B315 from potato rhizosphere has good prospects to be developed as a biological agent for biopesticides to control pathogenic bacteria not only in the habitat of *R. solanacearum* which causes potato bacterial wilt, but also against *Xoo* which causes rice bacterial leaf blight. *B. subtilis* isolates B46 and B209 in the inhibition test against potato *R. solanacearum* were able to inhibit, but after cross-testing against rice pathogenic bacteria the two isolates were unable to inhibit (-).

Antibiosis index is useful as an indicator that bacteria are capable of being antagonists against pathogens, both bacteria and fungi. Bacteria are able to control pathogenic fungi by producing the enzyme chitinase as an inhibitory mechanism since this enzyme degrades the cell walls of pathogenic fungi which consists mostly of chitin (Halimahtussadiyah et al. 2017). Chitinase activity of *B. subtilis* B298 was 6.936 U.mL^{-1}, 5.764 U.mL^{-1} and 6.813 U.mL^{-1}, which were detected at 15 hours of incubation, optimum temperature of 40˚C and optimum pH of 5.0, respectively (Lestari et al. 2017). *B. subtilis* also produce proteases and siderophores hence able to inhibit pathogenic bacteria such as rice *Xoo* and potato *R. solanacearum* (Prihatiningsih et al. 2017; Prihatiningsih et al. 2021).

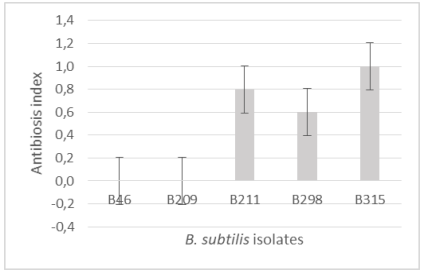

Figure 4. Antibiosis index of the five isolates of *B. subtilis* against *Xoo*

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Nanosuspension formula of *B. subtilis* B315 to control *Xoo*

The inhibition of *Xoo* growth by nanosuspension formula of *B. subtilis* B315 supernatant was 2.1 mm (Figure 5) in diameter. According to Marcic et al. (2018), *B. subtilis* can suppress *X. vesicatoria* in tomatoes with an inhibition zone of 5-9 mm but without nanosuspension formula. The antibiosis index of this nanosuspension formula was 0.41 (+) which was classified as weak inhibitor-. The mechanism of inhibition was strong bacteriostatic because the zone formed was clearer than *B subtilis* B315 without nanosuspension (Figures 2 and 5).

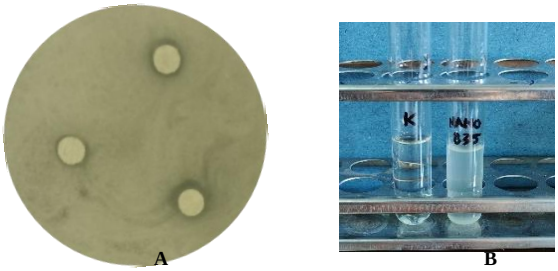

Figure 5. (A). Inhibition of the nanosuspension formula of *B. subtilis* B315 against *Xoo* (B) its bacteriostatic mechanism.

In conclusion *B. subtilis* B315 was an isolate of *Bacillus* isolated from potato rhizosphere that could suppresses *Xoo in vitro*, had an antibiosis index of 1 and was categorized as strong. The mechanism of inhibition was bacteriostatic antibiosis, which only inhibits the growth of *Xoo* bacteria but it was not lethal. *B. subtilis* B315 supernatant nanosuspension formula was able to inhibit *Xoo* with an antibiosis index of 0.42 (+). The nanosuspension formula has the prospective to be developed as biocontrol against *Xoo* and bacterial leaf blight on fields.

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REFERENCES

Alghuthaymi MA, Almoammanarb H, Raic M, Said-Galievd E, Abd-Elsalame KA. 2015. Myconanoparticles: Synthesis and their role in phytopathogens management. Biotechnol Biotechnol Equipt 29(2): 221–236. doi: 10.1080/13102818.2015.1008194.

Azizah, N. 2017. Uji antagonis *Corynebacterium* sp, *Bacillus amyloliquefaciens*, *Trichoderma* sp, dan *Gliocladium* sp, terhadap bakteri patogen *Burkholderia glumae* pada tanaman padi secara *In-vitro*. (Thesis) Fakultas Pertanian Universitas Hasanudin, Makassar (Indonesian).

Balouiri, M., Sadiki, M. and Ibnsouda, S. K. 2016. Methods for *in vitro* evaluating antimicrobial activity : A review. JPharm Anal. 6(2): 71–79. doi: 10.1016/j.jpha.2015.11.005.

Chun, W. and Vidaver, A. K. 2001. Gram-positive bacteria: *Bacillus* In: Schaad NW, Jones JB, Chun W (eds) Plant Pathogenic Bacteria APS Press, St. Paul Minnesota.Gordon RE, Haynes WC, Pang CH. 1973. *The Genus Bacillus*. United States Departement of Agriculture, Washington DC.

Goto M. 1992 *Fundamental of Bacterial Plant Pathology*. Academic Press New York.

Halimahtussadiyah R, Natsir M, Kurniawati D, Puspita S, Utamy. 2017. Isolation and identification of chitinolytic bacteria of pohara river of south east sulawesi and the optimization production of chitinase enzyme. AIP Conf Proc 1823, 020062-1–020062-7; pp. 1–7. doi: 10.1063/1.4978135.

Kala A, Soosairaj S, Mathiyazhagan S, Raja P. 2015. Isolation and Identification of *Xanthomonas oryzae* pv . *oryzae* the causal agent of rice bacterial leaf blight and its activities against of six medicinal plants. Asian J Plant Sci Res 5(6):80-83.

Lade BD, Gogle DP, Lade DB, Moon GM, Nandeshwari SB, Kumbhare SD. 2019 Nanobiopesticide formulations: Application strategies today and future perspectives' In: Koul O (eds) Nano-Biopesticides Today and Future Perspectives. Publisher, City. doi: 10.1016/B978-0-12-815829-6.00007-3.

Lestari P, Prihatiningsih N, Djatmiko HA. 2017 Partial biochemical characterization of crude extract extracellular

chitinase enzyme from *Bacillus subtilis* B 298, IOP Conf. Series: materials Sci Engin. 012041. doi: 10.1088/1757-899X/172/1/1/012041.

Lia R. 2018. Pathotype profile of *Xanthomonas oryzae* pv . *oryzae* isolates from North Sumatera. IOP Conf. Series: Earth Environ Sci 122 (2018) 012142 doi :10.1088/1755-1315/122/1/012142

Marcic SM, Todorovic V, Stanojevic O, Beric T, Stankovic S, Todorovic B, Potocnic I. 2018 Antagonistic potential of *Bacillus* spp. isolates against bacterial pathogens of tomato and fungal pathogen of pepper. *Pesticide i fitomedicina*., 33(1): 9–18. doi: 10.2298/pif1801009m.

Naqvi SAH, Perveen R, Umer UD, Malik O, Rehman A, Wazeer S, Majid T. 2014 Determination of antibacterial activity of various broad spectrum antibiotics against *Xanthomonas oryzae* pv . *oryzae* , a cause of bacterial leaf blight of rice'. InterJMicrobiol Mycol 2(3): 12–19.

Prihatiningsih N. 2013. Aktivitas antibiosis *Bacillus* sp. B315 sebagai agens pengendali hayati *Ralstonia solanacearum* pada kentang. (Dissertasion). Universitas Gadjah Mada Yogyakarta (Indonesian).

Prihatiningsih N. and Djatmiko HA. 2016. Enzim amilase sebagai komponen antagonis *Bacillus subtilis* B315 terhadap *Ralstonia solanacearum kentang*. J HPT Tropika 16(1): 10–16.

Prihatiningsih N, Djatmiko HA,Lestari P. 2017. Aktivitas siderofor *Bacillus subtilis* sebagai pemacu pertumbuhan dan pengendali patogen tanaman terung. J HPT Tropika 17(2): 170–178.

Prihatiningsih N, Djatmiko HA, Lestari P. 2020b. Screening of competent rice root endophytic bacteria to promote rice growth and bacterial leaf blight disease control. J HPT Tropika 20(1):78–84.

Prihatiningsih N, Arwiyanto T, Hadisutrisno B, Widada J. 2020a. Characterization of *Bacillus* spp. from the rhizosphere of potato Granola varietiy as an antibacterial against *Ralstonia solanacearum*. Biodiversitas 21(9): 4199–4204. doi: 10.13057/biodiv/d210934.

Prihatiningsih N, Asnani A, Djatmiko HA. 2021 Extracellular protease from *Bacillus subtilis* B315 with antagonistic activity against bacterial wilt pathogen (*Ralstonia solanacearum*) of chili. Biodiversitas 22(3): 1291–1295. doi: 10.13057/biodiv/d220327.

Shanti ML, Shenoy VV, Devi GL, Kumar VM, Premalatha P, Kumar GN, Shaahidhar HE, Zehr UB, Barwale FWH. 2010. Marker-assisted breeding for resistance to bacterial leaf blight in popular cultivar and parental lines of hybrid rice. J Plant Pathol 92 (2):495-501.

Singh D, Yaday DK, Chaudhary G, Rana VS, Sharm RK. 2016. Potential of *Bacillus amyloliquefaciens* for Biocontrol of Bacterial Wilt of Tomato Incited by *Ralstonia solanacearum*. J Plant Pathol Microbiol 7(1): 1–6. doi: 10.4172/2157-7471.1000327.

Swamy P, Panchbha AN, Dodiya P, Naik V, Panchbhai SD, Zehr UB, Azhakanandam K, Char BR. 2006. Evaluation of bacterial blight resistance in rice lines carrying multiple resistance genes and Xa21 transgenic lines. Curr Sci 90 (6): 818-824

Worrall EA, Hamid A, Mody KT, Mitter N, Pappu HR. 2018. Nanotechnology for plant disease management. Agron 8(12): 1–24. doi: 10.3390/agronomy8120285.

Yanti Y, Hamid H, Habazar T. 2020. The ability of indigenous *Bacillus* spp . consortia to control the anthracnose disease (*Colletrotricum capsici*) and increase the growth of chili plants. Biodiversitas 21(1): 179–186. doi: 10.13057/biodiv/d210123.

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