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Phenol Content and Peroxidase Enzyme Activity in Soybean Infected with *Xanthomonas axonopodis* pv *glycines* with the Application of *Bacillus subtilis* JB12 and *Bacillus velezensis* ST32

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Abstract:

Xanthomonas axonopodis pv *glycines* (Xag) is a pathogen that causes pustule disease in soybeans. Many techniques for controlling this disease have been widely developed, one of which is the use of biological agents. *Bacillus* sp. from the soybean phyllosphere is a biological agent that has the potential to suppress the development of pustule disease. One of the biological control mechanisms is through biochemical induction of plant resistance which includes the accumulation of phenols, salicylic acid compounds, and peroxidase enzymes. *Bacillus subtilis* JB12 and *Bacillus velezensis* ST32 are two bacteria isolated from the soybean phyllosphere which have previously been known to suppress Xag through an antibiosis mechanism. This study aimed to determine the potential of *Bacillus subtilis* JB12 and *Bacillus velezensis* ST32 in inducing soybean resistance against Xag infection. This research was carried out in two stages, the induction of resistance to soybean germination and an experiment in a greenhouse. This study consisted of 4 treatments and 5 replications, including P0 (Xag inoculation), P1 (*Bacillus subtilis* JB12 inoculation), P2 (*Bacillus velezensis* ST32), and P3 (*Bacillus subtilis* JB12 + *Bacillus velezensis* ST32 inoculation). Observations were made on the content of phenolic compounds, peroxidase enzyme activity, and the development of soybean pustule disease. The results showed differences in phenol content and peroxidase activity at the two stages of the study. Seed treatment with both isolates of *Bacillus* sp was able to increase the phenol content of soybean sprouts up to 3 - 5 days after inoculation (dai). Phenol content then decreased and was followed by an increase in the peroxidase activity up to 7 dai. The application of Xag and two isolates of *Bacillus* sp. in soybean plants caused the phenol content to fluctuate and peroxidase activity to decrease. *Bacillus subtilis* JB12 in general played a better role in increasing phenol content and peroxidase enzyme activity in soybean than *Bacillus velezensis* ST32. The application of two isolates of *Bacillus* sp. was not able to prolong the incubation period and reduce the severity of the pustule disease 14 days after inoculation.

Keywords: *Bacillus subtilis*, *Bacillus velezensis*, Induce resistance, Soybean, *Xanthomonas axonopodis* pv *glycines*.

Introduction:

Xanthomonas axonopodis pv *glycines* (Xag) is a bacterium that causes pustule diseases which are becoming a problem in soybean cultivation. This disease is commonly found in various soybean-producing countries in the world. Xag-infected leaves show symptoms of pustule disease which is characterized by yellowish-green spots with a raised center. The spots then develop into brown pustules and in severe infections cause the leaves to fall[1,2]. The spread of this disease is quite fast and can cause yield losses of 15 – 50% at disease intensities above 75%[3]. The risk of decreased production caused by this disease causes the need for effective control techniques.

Biological control of pustules disease currently has the potential to be developed. This environmentally-friendly control technique aims to reduce the negative impact of using chemical pesticides on the environment and non-target organisms. Various types of biological control agents have been isolated and used to control soybean pustules. Habazar et al[4] in their research isolated St4E1.1 and St1E1.1 endophytic bacteria which were able to reduce the severity of leaf pustules up to 19.4% and 23%. These two isolates also played a role in increasing the growth and number of soybean pods. A similar result was reported by Algar et al[5], 4 bacteria isolates namely *P. fluorescens* N21.4, *S. maltophilia* N5.18, *C. balustinum* Aur9, and *Curtobacterium* sp. M84 which was able to induce soybean resistance against Xag.

The utilization of plant growth-promoting bacteria (PGPB) from the soybean phyllosphere has advantages for controlling soybean pustule disease. This is because PGPB isolated from the soybean phyllosphere has similar growing conditions to Xag. Thus, this facilitates the adaptation process of biological control agents when applied to plant's phyllosphere and has the potential to increase their effectiveness[6]. Nurcahyanti et al[7] previously isolated 11 isolates of phyllosphere bacteria that could suppress the growth of Xag in vitro. These eleven isolates belonged to the genus *Bacillus* and had a variety of characteristics, growth abilities, and the ability to inhibit Xag. *Bacillus* spp is a potential bacterium as a biocontrol agent for plant pathogens. *Bacillus* spp can suppress the development of plant pathogens by producing bacteriocins, surfactin, fengycin, chitinase, proteases, glucanases, and siderophores[8].

Biological control mechanisms include antibiosis, growin competition, parasitism, and induction of resistance[9]. The induction of resistance by biological agents can be biochemical or structural. Biochemical induction of plant resistance includes the mechanism of systemic acquired resistance (SAR) and induced systemic resistance (ISR). The activation of plant defense can be seen by the increase in salicylic acid compounds, jasmonic acid, peroxidase enzyme activity, and phenol content. Non-pathogenic bacteria are generally used as biotic elicitors to activate plant resistance based on induced systemic resistance (ISR). This resistance is triggered by bacteria through the production of various metabolites such as antibiotics, siderophores, volatile organic compounds, and others[9].

The results of research by Nurcahyanti et al[7] showed that *Bacillus subtilis* JB12 and *Bacillus velezensis* ST32 from the soybean phyllosphere were able to suppress the development of Xag in vitro through an antibiosis mechanism. *B. subtilis* JB12 can grow rapidly, providing an opportunity to compete with Xag in addition to being able to suppress it with an antibiosis mechanism. *B. velezensis* ST32 has a high antagonist ability against Xag by producing antimicrobial compounds. Tests in the greenhouse showed *B. subtilis* JB12 was able to reduce the severity of pustules to 21.47% at 70 days after inoculation, while *B. velezensis* ST32 was 23.41%. Both isolates were also able to increase soybean growth[10]. The ability of these two strains to induce plant resistance was not previously known. This study will examine the ability of the two *Bacillus* sp. isolates to induce resistance to Xag-infected soybeans by measuring phenol levels and peroxidase enzyme activity.

Materials and Methods:

Experimental design

This research consisted of testing soybean seed treatment in the laboratory and testing on soybean plants in the greenhouse. The study on greenhouse used a completely randomized design with 4 treatments and 5 replications, each consisting of 4 plants. The seed treatment used the same design but each replication consisted of 100 soybean seeds. The treatments used in the two stages of the study were P0: Control (Xag inoculation only), P1: Xag + *Bacillus subtilis* JB12, P2: Xag + *Bacillus velezensis* ST32, and P3: Xag + *Bacillus subtilis* JB12 + *Bacillus velezensis* ST32.

Bacterial Reculture

Bacteria *Bacillus subtilis* JB12, *Bacillus velezensis* ST32, and *Xanthomonas axonopodis* pv *glycines* (collection of Laboratory Plant Protection, Faculty of Agriculture, University of Jember) which had been stored in paraffin were taken one loop and put in 5 ml of sterile water. After the suspension was vortexed, one loop was taken and streaked on YPGA medium. The medium was incubated for 48 hours at room temperature. Bacteria *B. subtilis* JB12 (Fig.1A), *B. velezensis* ST32 (Fig. 1B), and Xag (Fig. 1C) were further purified and tested for gram, hypersensitive reaction on tobacco, and pathogenicity on soybean to confirm characteristics of each bacterium.

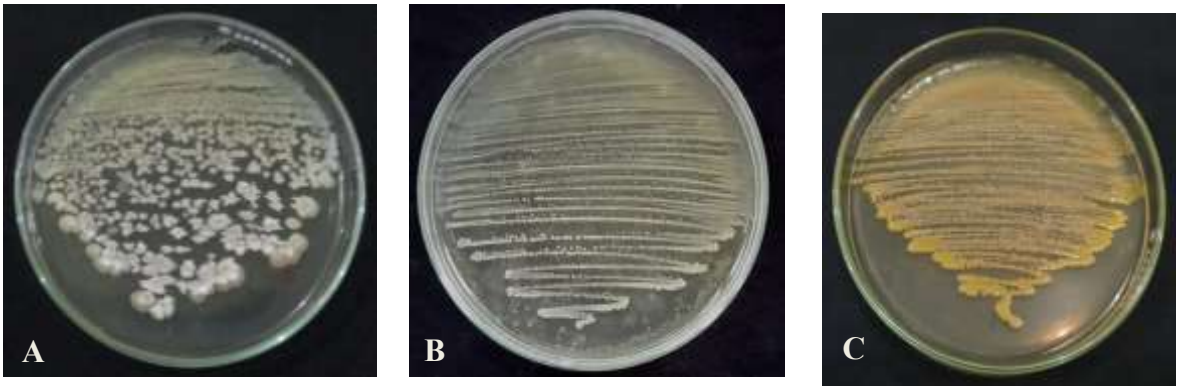

Figure 1. Bacterial colonies on YPGA medium : (A) *Bacillus subtilis* JB12, (B) *Bacillus velezensis* ST32, and (C) *Xanthomonas axonopodis* pv *glycines.*

Soybean seed treatment test with *Bacillus* sp.

Soybean seeds (Anjasmoro cultivar) as many as 100 seeds were soaked in *Bacillus* sp. suspension according to treatment with a bacterial density of 10^9 CFU/ml for 15 minutes. The soaked seeds are then dried for 15 minutes. Furthermore, the seeds were soaked in Xag suspension for another 15 minutes with a bacterial density of 10^8 CFU/ml. Seeds for control were only soaked in sterile water and Xag bacteria for 15 minutes. The seeds were then placed in a plastic container lined with sterile filter paper and incubated for 7 days with maintained humidity.

Application of *Bacillus* sp on soybean plants
Soybean planting

Soybean seeds (Anjasmoro cultivar) were planted in experimental pots containing 4 seeds/pot. The planting medium was a mixture of compost and soil with a ratio of 2:1. After the seeds grew, only 2 plants with good vigor were left. Fertilization was carried out 2 times, at the same time as planting and 15 days after planting according to the recommended dose for soybean.

***Bacillus* sp. application on soybean**

Applications of *B. subtilis* and *B. velezensis* were carried out according to the combination of treatments by spraying bacterial suspension when the plants were 20 days old. Each plant was sprayed with 10 ml of bacterial suspension with a density of 10^9 CFU/ml.

Inoculation of *Xanthomonas axonopodis* pv *glycines* on Soybean

Xag inoculation was carried out the day after *Bacillus* sp. application by spraying 10 ml of bacterial suspension with a density of 10^8 CFU/ml for each plant. Carborundum was added to the suspension to facilitate the inoculation process. The plants were then covered with plastic for 24 hours.

Measurement of phenol content and peroxidase enzyme activity in soybean sprouts and leaves

Measurements carried out on soybean sprouts and soybean leaves included phenol content, peroxidase enzyme activity, and disease development. Measurement of phenol content and peroxidase activity in soybean sprouts was carried out before seed treatment and on days 1, 3, 5, and 7 after treatment. Measurement of phenol content and peroxidase activity in soybean leaves was carried out before *Bacillus* sp. and Xag application, then on days 1, 3, 5, 7, 9, 12, and 15.

Extraction and measurement of phenol content

Extraction and fractionation of soybean leaves were carried out using the method by Otálvaro et al[11]. Soybean leaf samples of as much as 100 grams were washed and dried using an oven at 40ºC until the remaining leaf moisture content was 10%. The leaves were ground until smooth and filtered using a 180 µm filter. A total of 10 grams of filtered soybean leaf flour is then put into a bottle. Ethyl acetate as much as 100 ml was added to the bottle for the soaking process for 3 days. The soaking process with 100 ml of ethyl acetate was carried out 2 times. Then the solution was filtered and evaporated using a rotary evaporator until the remaining volume was 25%.

The phenol content of the ethyl acetate extract was measured using the Folin – Ciocalteu method[12] with modification. A total of 0.001 grams of ethyl acetate fraction was added with 1 ml of methanol. The resulting filtrate was taken 250 µl and added 2.75 ml of Folin – Calceur reagent (dilution 1:20) and 2 ml of N_2CO_3. The solution was incubated for 10 minutes at 40ºC. Then, the total phenol content was measured using a spectrophotometer at a wavelength of 765 nm. The standard curve calibration for gallic acid was calculated as equivalent per gram of fresh leaf weight. The absorbance value obtained was then substituted in

the standard curve regression equation for gallic acid, Y = 0.0156x – 0.0195; R_2 = 0,9992 (Y = Phenol content, X = absorbance value.

Measurement of peroxidase enzyme activity

The measurement of peroxidase enzyme activity was carried out based on the method by Saravanan[13]. A total of 1.5 ml of 0.05 M pyrogallol solution and 0.5 ml of 1% H_2O_2 were mixed with the enzyme extract from soybean leaves. The solution was then precipitated at room temperature for 10 minutes. The absorbance value of the peroxidase enzyme was measured using a spectrophotometer at a wavelength of 420 nm. Enzyme activity was calculated in units/minute. One unit is the activity of changing the optical density (OD) value of 420 nm on a spectrophotometer per minute.

Data analysis

Data on the content of phenol in soybeans were analyzed for variance and continued with Duncan Multiple Test Range (DMRT) at a 5% level using IBM SPSS Statistical software version 22.

Results:

Effect of seed treatment with *B. subtilis* JB12 and *B. velezensis* ST32 on phenol content of soybean sprouts.

Soybean seed treatment with *Bacillus* sp and Xag inoculation affected the phenol content of soybean sprouts (Fig. 2). Seed treatment with one *Bacillus* sp. isolate, combination or Xag inoculation (control) was able to increase phenol content 3 days after inoculation (dai). This increase occurred since 1 dai, except in the treatment of *Bacillus velezensis* JB12 which decreased in phenol content and only increased at 3 dai. The highest phenol content of 3 dai was shown by treatment of *B. velezensis* JB12, which was 63.18 mg/g (Table 1). The treatment of single *Bacillus* sp. isolates began to experience a decrease in phenol content at 5 dai, in contrast to control and combination treatment of *Bacillus* sp. which was still increasing.

Furthermore, the phenol content of all seed treatments with *Bacillus* sp. decreased at 7 dai, while the control continued to increase. At the end of observation, the control treatment showed the highest phenol content of 57.41 mg/g. The highest phenol content in *Bacillus* sp. treatment was recorded in the combination of JB12 and ST32, namely 35.42 mg/g. These results indicate that inoculation of Xag is the main trigger for the increase of phenol content. *Bacillus* sp. treatment, either alone or in combination only increased the phenol content up to 3 – 5 dai and then decreased until the end of observation.

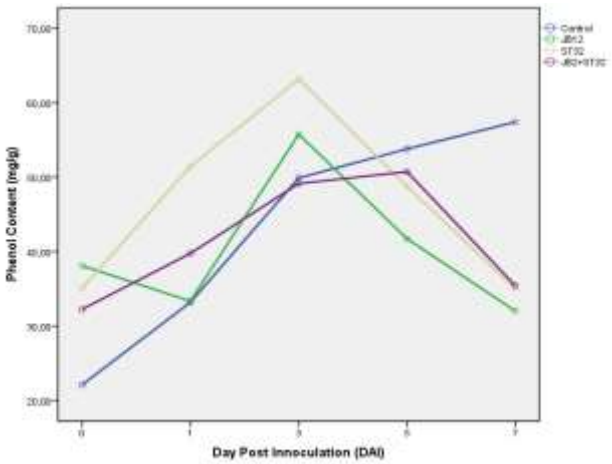

Figure 2. The content of phenol in soybean sprouts with seed treatment using Xag, *B. subtilis* JB12, and *B. velezensis* ST32.

Table 1. The content of phenol in soybean sprouts with seed treatment using Xag, *B. subtilis* JB12, and *B. velezensis* ST32.

Treatment	Phenol content in soybean sprouts (mg/g)				
	Before Seed Treatment	After Seed Treatment (DAI)			
		1	3	5	7
P0 : Control	22.10**d**	33.19**d**	49.89**c**	53.80**a**	57.41**a**
P1 : JB12	38.10**a**	33.35**c**	55.76**b**	41.72**d**	32.01**d**
P2 : ST32	35.09**b**	51.45**a**	63.18**a**	48.74**c**	34.98**c**
P3: JB12+ST32	32.24**c**	39.76**b**	49.14**d**	50.73**b**	35.42**b**

Effect of seed treatment with *B. subtilis* JB12 dan *B. velezensis* ST32 on peroxidase enzyme activity in soybean sprouts

Seed treatment with both strains of *Bacillus* sp. was able to affect the activity of peroxidase enzyme in soybean sprouts (Fig. 3). The activity of peroxidase tends to decrease 1 dai in all treatments, but on the next day, it increased. The increase in peroxidase activity with a single treatment of *B. subtilis* JB12 or *B. velezensis* ST32 occurred slowly until 5 dai. However, on the 7th day, the peroxidase activity increased significantly; 0.407 units/minute in JB12 and 0. 375 units/minute in ST32.

The treatment with the combination of JB12 and ST32 had a similar pattern to the control. The increase in enzyme activity in the combination

treatment was greater than in the single isolate *Bacillus* sp. treatment on day 5, which was 0.155 units/minute. The peroxidase activity on day 7 in the control and mixed *Bacillus* sp. treatment was lower than the single isolate *Bacillus* sp treatment with the same value of 0.23 units/minute. Xag inoculation triggers an increase in peroxidase enzyme activity in soybean sprouts. However, seed treatment with *B. subtilis* JB12 or *Bacillus velezensis* ST32 alone or in combination was able to trigger an increase in peroxidase activity higher than Xag as an elicitor.

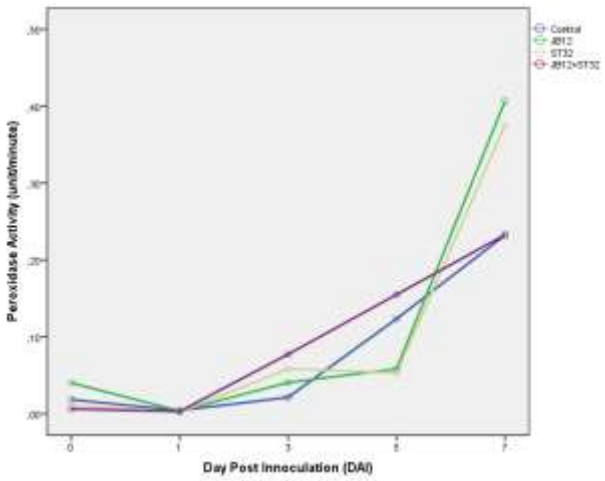

Figure 3. Peroxidase enzyme activity on soybean sprouts with seed treatment using Xag, *B. subtilis* JB12, and *B. velezensis* ST32.

Effect of application *B. subtilis* JB12 and *B. velezensis* ST32 on soybean leaves phenol content

The application of *Bacillus* sp JB12 and ST32 to soybeans affected the phenol content in the leaves. The phenol content on the leaves for 15 days was recorded to fluctuate in all treatments (Fig. 4). Phenol content in the control and application of *B. velezensis* ST32 increased on the third day after inoculation. The phenol content 3 days after inoculation in the control was recorded at 52.89 mg/g. while the single application *B. velezensis* ST32 48,81 mg/g (Table. 2). The combination of two *Bacillus* sp. isolates and a single application of *B. subtilis* JB12 caused a decrease in phenol content at 3 dai. Phenol content in all treatments except the single application of *B. velezensis* ST32 increased at 6 dai. The control condition still showed the highest phenol content at 6 dai which was 56.55 mg/g/

Fluctuations occurred on days 9 – 15, and the phenol content in the control decreased until then increased dramatically at 15 dai. The treatment of *B. subtilis* JB12 and the combination of JB12 and ST32 showed the same pattern, which was decreased on the 9 dai, increased on the 12 dai, and decreased again on the 15 dai. The fluctuations that occurred in JB12 were more significant than in the combination treatment. Different results occurred in the ST32 treatment, which only decreased on day 9, then continued to increase until day 15. Overall, the accumulated phenol on day 15 tended to be higher than before bacterial inoculation, except for the combination treatment of JB12 and ST32. The highest phenol content at 15 dai was shown in the control treatment, which was 80.77 mg/g, while the highest value in *Bacillus* sp treatment was 77.46 mg/g in a single inoculation of *B. velezensis* ST32.

Inoculation of Xag and two isolates *Bacillus* sp. acted as triggers that increase soybean phenol content in leaves. Phenol content in each treatment tended to fluctuate and the increase occurred at different time. The similar pattern of phenol content until 12 dai in JB12 and combination treatment was due to the role of *B. subtilis* JB12 as the main trigger. This can be seen from the high phenol content in a single treatment of JB12 at 12 dai 83.44 mg/g, whereas when combined with ST32 it was lower 57.93 mg/g. The similar pattern in control and single treatment of *B. velezensis* ST32 because Xag was more dominant as elicitor.

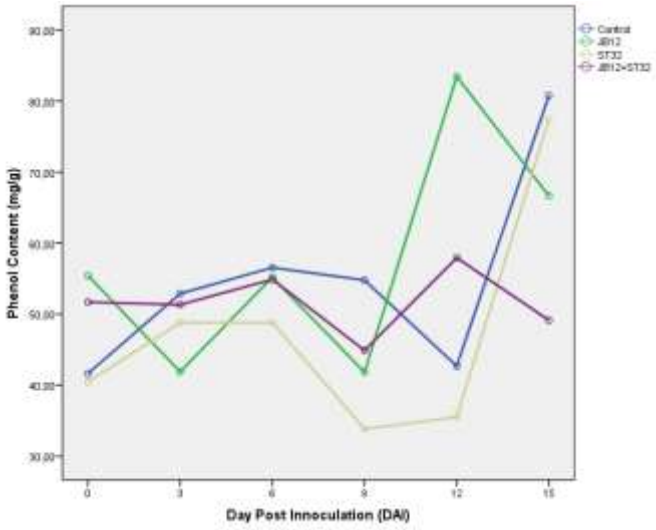

Figure 4. Changes in phenol content in soybean leaves inoculated with Xag, *B. subtilis* JB12, and *B. velezensis* ST32.

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Table 2. Phenol content in soybean leaves inoculated with Xag, *B. subtilis* JB12, and *B. velezensis* ST32.

Treatment	Phenol content in soybean leaves (mg/g)					
	Before application of *Bacillus* sp.	After application of *Bacillus* sp.				
		3	6	9	12	15
P0 : Control	41.64c	52.89a	56.55a	54.78a	42.69c	80.77a
P1 : JB12	55.43a	41.89d	55.18b	41.82c	83.44a	66.66c
P2 : ST32	40.45d	48.81c	48.81d	33.83d	35.51d	77.46b
P3: JB12+ST32	51.69b	51.33b	54.89c	44.90b	57.93b	49.14d

Effect of application *B. subtilis* JB12 and *B. velezensis* ST32 on soybean leaves peroxidase enzyme activity

Peroxidase activity in soybean leaves tended to decrease in all treatments. A similar pattern was noted in the control and application of *B. velezensis* ST32. Peroxidase activity in both treatments increased at 3 dai, but decreased drastically at 6 dai(Fig.5). The increase occurred at 9 dai, followed by a decrease again until the end of observation at 15 dai. Then the observation showed that the activity of peroxidase enzyme in *B. velezensis* ST32 treatment was 0.126 units/minute, higher than control (0.121 units/minute).

Different results were shown in the application of *B. subtilis* JB12. Enzyme activity continued to decrease up to 12 dai but was higher than control and *B. velezensis* ST32 treatment. The increase at 15 dai tends to be insignificant and much lower than before inoculation. The activity of peroxidase in *B. subtilis* JB12 treatment at 15 dai was 0.817 units/minute.

The highest enzyme activity was recorded in the combination treatment of JB12 and ST32. The activity of peroxidase enzyme in this combination treatment continued to increase until 6 dai, decreased until day 12, and increased at 15 dai. The highest activity occurred on day 6 (0.471 units/minute), while at the end of the observation 0.246 units/minute. Overall, the enzyme activity in this combination treatment was higher than in all treatments during the 15 days of observation. The treatment of *B. subtilis* JB12, either alone or in combination was able to cause higher peroxidase activity than Xag alone as the trigger. *B. velezensis* ST32 did not show a significant role in triggering the activity of the peroxidase enzyme, as seen from the decrease in activity and similarity of pattern with control.

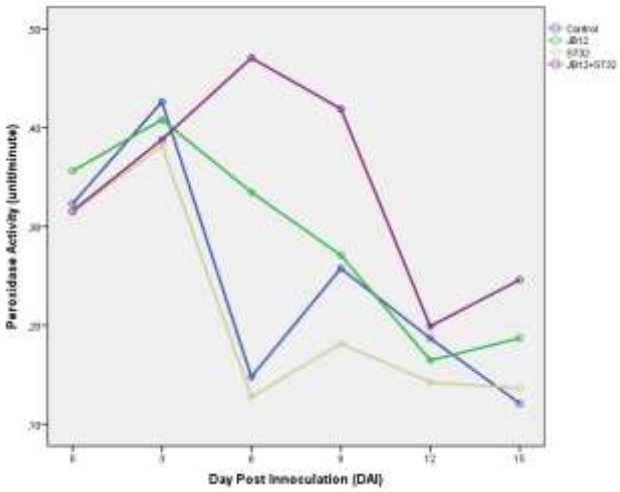

Figure 5. Peroxidase activity on soybean leaves inoculated with Xag, *B. subtilis* JB12, and *B. velezensis* ST32.

Effect of Application *B. subtilis* JB12 and *B. velezensis* ST32 on the development of soybean pustule disease

Early symptoms of pustule disease that appear on soybean leaves infected with Xag are yellowish-green spots. These spots will then develop into brown pustules with a raised center (Fig 6A and 6B). Symptoms of pustule disease appeared on soybean leaves in all treatments. The application of *B. subtilis* JB12 and *B. velezensis* ST32 did not prolong the incubation period and incidence of soybean pustules. This condition also affected the severity of pustule disease at 14 dai. There was no significant difference between the application of *Bacillus* sp. either alone or in combination compared to the control (Table. 3). The incubation period for all treatments ranged from 8 to 10 days after Xag inoculation. Disease incidence on 14 days of observation showed the same number 25%. Disease severity in combination with treatment of *B. subtilis* JB12 and *B. velezensis* ST32 was numerically lower than the control, as was the case with a single treatment of two *Bacillus* sp isolates. However, there was no significant difference in disease severity at 14 dai, which was around 2% in all treatments.

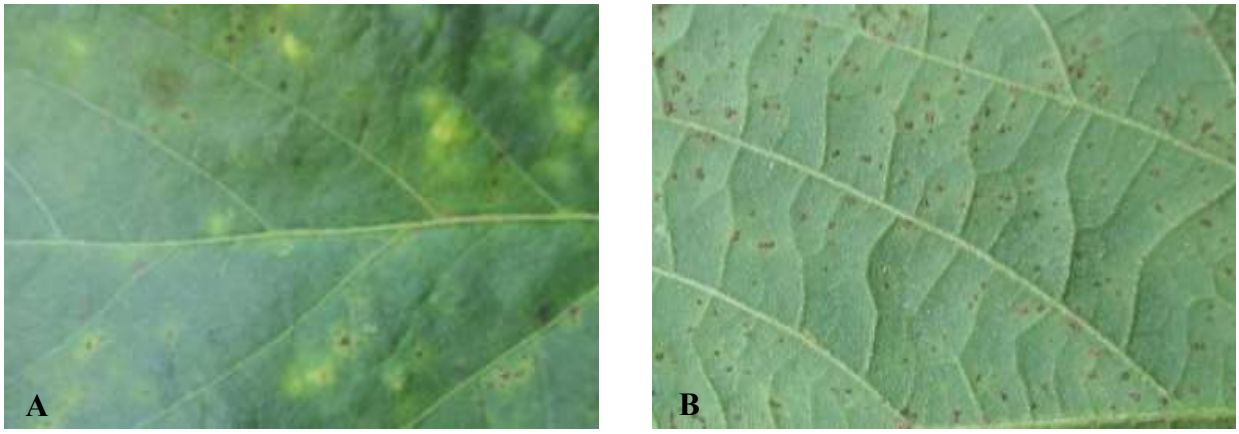

Figure 6. Symptoms of pustules disease on soybean leaves inoculated with *Xanthomonas axonopodis* pv *glycines*: (A) yellowish-green spot on the upper surface of the leaf, (B) The appearance of pustule symptoms from the underside of the leaf.

Table 3. Effect of application *B. subtilis* JB12 and *B.velezensis* ST32 on the development of soybean pustule diseases

Treatment	Development of soybean pustule diseases		
	The incubation period (dai)	Disease incidence (%) 14 (dai)	Disease severity (%) 14 (dai)
P0 : Control	8-10	25	2.49
P1 : JB12	8-10	25	2.36
P2 : ST32	8-10	25	2.47
P3: JB12+ST32	8-10	25	2.25

Note : dai = day after inoculation.

Discussion:

Phenol compounds are secondary metabolites that accumulate in plant tissues in response to pathogenic infections. Phenol is synthesized when receptors on plant cells recognize pathogenic microorganisms. This interaction then activates plant resistance based on pathogen-associated molecular patterns (PAMPS), so that the pathogen infection process will be inhibited[14,15]. The use of bacteria as resistance-inducing agents is generally able to increase phenol content that accumulates in plant tissues[16,17]. Rais *et al*[18] reported that *Bacillus* spp. can induce plant resistance from phyllosphere pathogens *Pyricularia oryzae* indicated by an increase in peroxidase content 3.5 – 4.1 times and polyphenol oxidase 3.0 – 3.8 times compared to control. Another study reported that the single application of 7 different *Bacillus subtilis* isolates and 8 other *Bacillus* sp isolates was able to increase total phenol, peroxidase levels, phenylalanine and polyphenol oxidase in potato plants[19].

Xag is thought to be the main trigger for the increase in phenol content of soybean sprouts. However, there is a role for *Bacillus* sp. in promoting higher phenol content at a certain period. Application of two *Bacillus* sp. isolates, either alone or in combination was able to increase the phenol content in soybean sprouts higher than the control up to 3 days after inoculation. This result indicated that *B. subtilis* JB12 and *B. velezensis* ST32 were able to trigger plant resistance by accumulating phenolic compounds. Similar results were reported by Ben *et al*[20], application of bacillus isolates B27 and B29 was able to increase total phenolic compounds in grape leaves higher than inoculation of pathogen *Uncila necator*. Jayapala *et al*[18] in their research explained that the application of *Bacillus* sp. BSp.3/aM was able to induce resistance of chili against *Colletrotichum capsica* infection which causes anthracnose disease. One of the signs of this resistance induction was the accumulation of phenolic compounds in the leaves higher than control treatment up to 120 hours after inoculation.

Peroxidase enzymes have quite diverse roles in the mechanism of plant resistance. Peroxidase acts as a catalytic enzyme that oxidizes phenolic compounds, breaking down H_2O_2 in the process of producing reactive oxygen species (ROS) and resistance-related compounds such as salicylic acid[21,22]. The decrease in phenol content of soybean sprouts was followed by an increase in peroxidase activity. Treatment with both isolates of *Bacillus* sp tended to increase the activity of peroxidase enzyme in soybean sprouts up to 7 dai compared to control. Increased peroxidase activity after bacterial inoculation as a resistance inducer was previously

reported by Khaeruni *et al*[23]. Soybeans infected with pustule disease were inoculated with a combination of rhizobacteria *Bacillus cereus* ST21e *Bacillus subtilis* ST21e, and *Serratia* sp SS29a. The results showed an increase in peroxidase activity by 1.07%.

The decrease in phenol content 5 days after inoculation was due to the application of two isolates of *Bacillus* sp. suppressing the development of Xag by increasing the activity of the peroxidase enzyme. This enzyme plays a role in the oxidation of phenol into quinines and hydrogen peroxide, which are antimicrobial compounds. This process also increases the rate of polymerization of phenolic compounds into lignin-like substances that are deposited on the cell walls[24]. This is thought to cause the accumulation of phenols to decrease. Xag infection tended to trigger plants to accumulate phenol, which continued to increase up to 7 dai. However, its peroxidase activity was lower than the *Bacillus* sp treatment.

In contrast to the seed treatment, the application of *Bacillus* sp. to soybeans showed fluctuating levels of phenol and peroxidase activity. In general, the phenol content of soybeans treated with *Bacillus* sp tended to be lower than control. This result is similar to the application of seed treatment which shows that Xag inoculation alone tends to trigger higher phenol accumulation. Likewise, the peroxidase enzyme activity was higher in soybean leaves with *Bacillus* sp treatment alone or in combination compared to controls. The main difference in the two stages of this study was that the phenol content and peroxidase activity in soybean leaves tend to fluctuate more than in soybean sprouts. Environmental conditions and different plant growth stages are thought to be factors that influence the pattern of phenol content and peroxidase activity in the two stages of this study.

Xag was suspected to be the main elicitor of increased phenol content in soybean sprouts and leaves, while the increase of peroxidase activity tended to be triggered by inoculation of two isolates of *Bacillus* sp. In general, the role of *Bacillus subtilis* JB12 was more significant in increasing phenol content and peroxidase activity than *Bacillus velezensis* ST32. The combination of these two *Bacillus* sp. isolates did not give a better effect than a single inoculation except on the activity of the peroxidase enzyme in soybean leaves. Previous studies also reported similar results[25]. Treatment with 2 strains of *Pseudomonas* GS4 and PhS1 was able to increase the activity of peroxidase in the leaves and roots of wheat. The increase also occurred in wheat that was only inoculated with the pathogen *Bipolaris sorokiniana*, but the activity was lower than in treatment with bacteria. Thus, it was concluded that both pathogen and bacteria as inducers were able to trigger an increase in peroxidase activity.

Increasing phenol content and peroxidase activity were not positively correlated with suppression of pustule disease development. The incubation period for *Bacillus* sp treatment and control was in the same range, 8 – 10 days. The severity and incidence of disease at 14 dai were not significantly different in all *Bacillus* sp treatments when compared with controls. This condition could be related to the accumulation and peroxidase at 12 – 15 dai. Phenol content in control tended to increase and was not much different from *Bacillus* sp treatment at 12 – 15 dai. Meanwhile, peroxidase activity during that time tended to decrease in all treatments. The activity of the peroxidase enzyme which was not positively correlated with disease progression was previously reported by Bashan *et al*[26]. *P. syringae* pv. tomato used to infect tomatoes in that study were able to trigger an increase in peroxidase activity. But the severity that occurs is quite diverse in each plant. So it can be concluded that an increase in peroxidase activity is not always able to contribute to the suppression of disease development.

Conclusion:

Seed treatment and application of *Bacillus* sp only triggered an increase in the phenol content of soybean sprouts and leaves in a limited time (3 – 5 dai) and then tended to decrease. In general, the application of *Bacillus sp* increased the activity of the peroxidase enzyme in soybean leaves and sprouts higher than the control until the end of observation. *Bacillus subtilis* JB12 isolate had a significant effect on increasing phenol content and peroxidase enzyme compared to *Bacillus velezensis* ST32. Application of *Bacillus* sp. did not prolong the incubation period nor reduce the severity of the disease in 14 dai.

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Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.

- Ethical Clearance: The project was approved by the local ethical committee in University of Jember, Indonesia.

Authors' contributions statement:

S. D. N., W. S. W., and R. M. designed the study, performed the laboratory and greenhouse experiment, analyzed phenol content and peroxidase enzyme activity. A. A. H. N. performed the laboratory and greenhouse experiment, analyzed the data using statistic software, interpret the data, wrote the paper with input from all authors, revision and proofreading.

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محتوى الفينول ونشاط إنزيم البيروكسيديز في فول الصويا المصاب بـ Xanthomonas axonopodis pv glycines مع تطبيق Bacillus subtilis JB12وBacillus velezensis ST32

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الخلاصة:

إن Xanthomonas axonopodis pv glycines (Xag) هو أحد مسببات الأمراض التي تسبب مرض البثور في فول الصويا. تم تطوير العديد من التقنيات للسيطرة على هذا المرض على نطاق واسع ، من بينها استخدام العوامل البيولوجية. Bacillus sp. من فولوسفير فول الصويا هو عامل بيولوجي لديه القدرة على قمع تطور مرض البثور. تتمثل إحدى آليات التحكم البيولوجي في الحث الكيميائي الحيوي لمقاومة النبات والتي تشمل تراكم الفينولات ومركبات حمض الساليسيليك وإنزيمات البيروكسيديز. Bacillus subtilis JB12 و Bacillus velezensis ST32 هما نوعان من البكتيريا المعزولة من طبقة فيلوسفير فول الصويا والتي كانت معروفة سابقًا بقمع Xag من خلال آلية مضادة لمضادات الحيوية. هدفت هذه الدراسة إلى تحديد إمكانات Bacillus subtilis JB12 و Bacillus velezensis ST32 في إحداث مقاومة فول الصويا ضد عدوى Xag. تم إجراء هذا البحث على مرحلتين، تحريض مقاومة إنبات فول الصويا وتجربة في صوبة زراعية. تكونت هذه الدراسة من 4 علاجات و 5 مكررات، بما في ذلك P0 (تلقيح Xag) ، P1 (Bacillus subtilis JB12)، P2 (Bacillus velezensis ST32)، P3 (Bacillus subtilis JB12 + Bacillus velezensis ST32). تم إجراء ملاحظات على محتوى المركبات الفينولية ، ونشاط إنزيم البيروكسيديز ، وتطور مرض بثور فول الصويا. أظهرت النتائج اختلافات في محتوى الفينول ونشاط البيروكسيداز في مرحلتي الدراسة. استطاعت معاملة البذور بكلا العزلتين من Bacillus sp زيادة محتوى الفينول في براعم فول الصويا حتى 3-5 أيام بعد التلقيح (داي). ثم انخفض محتوى الفينول وأعقب ذلك زيادة في نشاط البيروكسيداز حتى 7 أيام. تم تطبيق Xag واثنين من عزلات Bacillus sp. في نباتات فول الصويا تسبب في تذبذب محتوى الفينول وانخفض نشاط البيروكسيداز. لعبت Bacillus subtilis JB12 بشكل عام دورًا أفضل في زيادة محتوى الفينول ونشاط إنزيم البيروكسيديز في فول الصويا من Bacillus velezensis ST32. تم تطبيق عزلتين من Bacillus sp. لم يكن قادرًا على إطالة فترة الحضانة وتقليل شدة مرض البثور في 14 يومًا بعد التلقيح.

الكلمات المفتاحية: Bacillus subtilis، Bacillus velezensis، تحريض المقاومة، فول الصويا، Xanthomonas axonopodis pv glycines.