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Induction of soybean resistance to bacterial pustule disease (*Xanthomonas axonopodis pv. glycines*) by rhizobacteria and organic material treatment

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Abstract. This study aimed to evaluate the role of different formulations and types of organic matter in improving yield and resistance of soybean plants to bacterial pustule disease. The study was prepared based on a randomized block design with a factorial pattern. The first factor was the application of rhizobacterial formulation (biofresh), ie F0 = without the application of rhizobacteria, F1 = application of biofresh in solid formulation, and F2 = application of biofresh in liquid formulation. The second factor was the application of organic materials, namely B1 = compost of soybean litter + cow dung, B2 = compost of rice straw + cow dung, B3 = compost of soybean litter + rice straw + cow dung. Observation of disease severity and soybean yield was conducted on five sample plants in each treatment. The results showed that the treatment of biological agent biofresh in solid formulation combined with compos of soybean litter, was the best treatment in increasing plant resistance to bacterial pustule disease and seed weight. Plant resistance induction occurred systemically characterized by salicylic acid increase of 0.3 mg and peroxidase increase of 0.07 unit / mL in the sample plants.

1. Introduction

Pustule bacterial disease *(Xanthomonas axonopodis pv. glycines*) is an important disease in soybean crop, and has been spread in soybean crop centers in Indonesia such as: West Java, Central Java, Yogyakarta, Lampung, South Sulawesi, Southeast Sulawesi and West Sumatera [1, 2]. The pathogen spreads through seeds [3], water and wind [4]. In humid environment conditions, the disease may decrease yields by about 21-40% [5]. Chemical disease control has been reported as one of the causes of environmental damage or resistance of pathogenic bacteria [6], while the supply of bacterial pustule resistant varieties is still very limited.

To minimize the use of chemicals in the control of plant diseases, the use of biological agents such as rhizobacteria is one of the alternative pathogen-friendly controls. Currently, biological fertilizers have been developed with three types of rhizobacteria contents, namely Bacillus cereus ST21b, B. subtilis ST21e, *Serratia* sp SS29a. This third mixture of rhizobacteria isolates produced a biological

agent formulation called "Biological agents biofresh". Biofresh has the ability as a biological antagonist and plant growth promoter to reduce the use of synthetic pesticides and chemical fertilizer [7].

The survival of biological agents is very important because it is associated with its ability to suppress pathogens and stimulate plant growth. Application of organic materials can improve microbial life and activity, since organic matter is a source of energy and microbial food in the soil [8]. In addition to organic contents in the soil, the materials and formulations of biological agents also affect the survival of a biological agent in the field [9]. The objective of this research was to evaluate the role of biofresh biodegradable formulation form and different organic materials in increasing plant resistance to bacterial pustule disease and soybean crop yield.

2. Material and methods

2.1 Materials

Soybean seeds of Gema variety were obtained from Indonesian Legumes and Tuber Crops Research Institute, Indonesian Agency for Agricultural Research and Development, Malang, Indonesia. *Bacillus cereus* ST21b, *B. subtilis* ST21e, *Serratia sp* SS29a, and *X. axonopodis* pv. *glycines* were obtained from Phytopathology Laboratory, Plant Protection Department of Halu Oleo University, Kendari.

2.2 Experimental design

This study used factorial randomized complete block design consisting of two factors. The first factor was a biological agent biofresh formulation which consisted of three levels i.e.: F0 = without biofresh (control), F1 = solid formulations, F2 = liquid formulations.The second factor was organic matter compost consisting of four levels i.e.: B1 = compost of soybean litter waste, B2= compost of rice straw, B3 = compost of soybean litter waste + rice straw. Overall, there were 9treatment combinations, each treatment was repeated 3 times so that there were 27treatment units and each treatment consisted of 5 units of the plant samples. Data were analyzed using the SAS 9.1.3 version statistical software. The difference of treatment means were separated by Duncan multiple range test at 5% level.

2.3 Preparation of growing media and cultivation of soybean seeds

The growing media used in this research was ultisols that has been sterilized with vapor sterilization. Soil thoroughly mixed with straw compost, soybeans compost and cow dung according to treatments, with a ratio of 1:7 [10]. The mixture were then placed in a polybag sized of 30 × 40 cm and placed on open land at Experimental Farm, of Halu Oleo University, Kendari according to the research design. Three soybean seeds were cultivated at depth of 5 cm from the surface of growing media. Two weeks after cultivation, two plants were selected and were retained as sample plants, while the rest was removed and discarded.

2.4 Formulation and application of biofresh

The rhizobacteria (*Bacillus cereus* ST21b, *B. subtilis* ST21e and *Serratia* sp SS29a) were used as raw materials of biofresh formulation. Before mixing with the formulation, the rhizobacteria were propagated on solid TSA medium separately and were incubated for 48 h. Bacteria colonies growth was suspended in sterile distilled water until it reached population density of 10^{10}cfu/mL and was used in two formulations of biofresh.

Solid formulation was prepared by mixing the third rhizobacteria suspension with carrier materials such as peat organic matter and animal manure with certain ratio. Furthermore, the mixture of biological agent and material formulation was dried for 48 h, was packed in plastic bags to 5 kg and was ready for application. For the liquid formulation, it was prepared by mixing the third rhizobacteria suspension with a carrier material in the form of a mixture of water, coconut water and Tryptic Soy Broth with certain ratio, in a 1 L plastic container, the mixture was then incubated for 48 h and was ready for application [10].

Applications of biological agent biofresh was conducted during planting process. Four week after planting (WAP), biofresh was sprinkled with liquid formulation around the planting hole as much as 10 mL and solid formulations for covering planting hole during planting and was spreaded around 10 grams per planting hole at the age of 4 WAP.

2.5 Preparation and inoculation of Xanthomonas axonopodis pv. *glycines*

X. axonopodis pv *glycines* purified on yeast extract dextrose agar (YDA) media for 4 days were suspended in aquadest solution until cell concentration reached 10^{10}-10^{11} cells/mL. Inoculation was performed on 3-week-old plants after planting (WAP) by spraying the pathogen suspension on the entire leaf surface using a 1 L sprayer. After inoculation, the plant was enclosed for 24 hours with transparent plates that had been hollowed both sides.

2.6 Salicylic acid and peroxidase *enzymes content analysis*

Analysis of salicylic acid and peroxidase enzymes was performed on the control treatment (F0B1) and best treatment (F1B1) through leaf composite, one day prior to pathogen inoculation and one week after pathogen inoculation. Analysis of salicylic acid levels was performed according to Warrier*et al*. [11], while the peroxidase enzyme activity analysis used Patra and Mishra method [12].

2.7 Observation variables

Observations were carried out on five plant samples of each treatment unit. The observed variables were:

(1) Disease severity.Disease severity was calculated using scoring method through assessment score of the plant leaves based on pustule symptoms formed in plant samples [10]. Scoring result was then used to calculate the severity of the disease by using the formula:

$$DS = \sum_{i=0}^{n} \left(\frac{n.V}{Z.N}\right) \text{x } 100\ \% \quad (1)$$

DS: disease severity (%); n: the number of leaves attacked in each category; N: the number of observed leaves; V: value scale of each category of that were attacked and Z: the highest scale value of attacked category. In this method, we measure disease severity(yi) collected at various times(ti). Besides disease severity values, the value of area under disease progress curve (AUDPC) was also calculated to observe the disease progress. Values was calculated based on the formula AUDPC of Van der Plank [13]

$$\text{AUDPC} = \sum_{i=1}^{n=1} \left(\frac{y_i + y_{i+1}}{2}\right) \text{x } (t_{i+1} - t_i) \quad (2)$$

(2) Measurement of salicylic acid content and peroxidase enzyme activity

Salicylic acid content was determined by measurement of absorbance value using UV-Vis spectrophotometer at 525 nm wavelength, while the determination of peroxidase enzyme activity was done by measuring absorbance at 420 nm wavelength in the same tool.

(3) Crop yield: Crop yield was observed when the plants were in harvested stage which was characterized by yellowing of the leaves as much as 80%. Observed crop yield variables included pods and crop seed weight.

3. Results and Discussion

3.1 Disease Severity of Pustule Bacterial Disease

The treatments significantly affected the severity of bacterial pustule disease at age 2, 3, 4, 5 and 6 weeks after inoculation. The rate of development of disease severity in each treatment combination is presented in Table 1.

Table 1. Combination effects of biofresh formulation and organic matter on disease severity of pustule bacterial disease on soybean

Treatments	Disease Severity (%) on *n*					AUDPC
	2 WAI	3 WAI	4 WAI	5 WAI	6 WAI	(unit/week)
F0B1	12 a	25 a	33 a	58 a	58 a	10.54
F0B2	11a	23 a	34 a	60 a	60 a	10.73
F0B3	12a	26 a	31 a	58 a	59 a	10.49
F1B1	6b	12 c	24 b	46 b	46 b	7.59
F1B2	9ab	19ab	33 a	51 a	52 b	9.30
F1B3	9ab	17ab	30 a	52 a	53 b	9.11
F2B1	7b	19ab	33 a	56 a	56 b	9.79
F2B2	7b	18ab	28 a	52 a	53 b	8.99
F3B3	8ab	18ab	28 a	55 a	55 b	9.26

Note : WAI = week after inoculation. Numbers followed by same letters at same column showed no significant different at α 5%.

The best treatment combination in reducing bacterial pustule infections was the treatment of solid formulation with the addition of soybeancompos (F1B1), with the lowest disease severity and AUDPC, ie 46% and 7.59 units/week, respectively. This indicated that biofresh biological agents applications in the form of solid formulations can reduce the development of bacterial pustule disease in soybean crops.

3.2 Salicylic Acid Content and Peroxidase Enzyme Activity

The results showed that there was a higher salicylic acid content and peroxidase enzyme activity in the biodegradation biofresh formulation (F1B1) treatment than non the biofresh treatments (F0B1), as presented in Table 2.

Table 2. Comparison of salicylic acid increases and peroxidase activity between biofresh treatment and compos soy litter with control

Treatments	Content of Salicylate acid (mg)		
	Before inoculation	After inoculation	Increase
F0B1	0.75	0.98	0.24
F1B1	1.10	1.40	0.30
	Peroxidase Activity (unit/mL)		
	Before inoculation	After inoculation	Increase
F0B1	1.30	1.54	0.23
F1B1	2.22	3.29	1.07

Increased accumulation of salicylic acid was a rapid reaction of plants to fight pathogenic bacterial infections. Salicylic acid is an important compound for plant defense against pathogens. This was reported by Zhang *et al*. [14], that the content of endogenous salicylic acid in tobacco plant sprouts treated with three strains of rhizobacteria increased significantly in the first week after treatment.

In other observations, peroxidase increased in the test plant of 1.07 units / mL. The peroxidase function is to strengthen the cell wall against the degradation of enzymes produced by pathogens through the formation of structural proteins in cell walls [15]. The use of PGPR as a biological induction agent has been widely reported. Application of B. megaterium in cucumber can inhibit the incidence of damping-off disease caused by Pythium aphanidermatum of 75.78% [16]. Increased levels of salicylic acid and peroxidase enzyme activation in plants treated with biodegradable biofresh

agents indicated that the resistance of the soybean plant to bacterial pustule disease was related to the mechanism of systemic resistance induction.

3.3 Crop yield

The results of observation on soybean crops given biofresh biofertilizer and different organic materials significantly affect the weight of dry pod and seed weight. The highest dry pod weight and seed was found in biodegradation treatment of biofresh in solid formulation and soy litter organic matter (F1B1), 64.17 g / plant and 50.03 g / plant, respectively (Table 3). This indicated that the application of biofresh in a solid formulation with the addition of organic compos soy litter can provide high yields.

Table 3. Combination effects of Biofresh formulation and organic matter on crop yield

Treatment	Pod Weight (g/plant)	Seed Weight (g/plant)
F0B1	55.83 b	39.10 b
F0B2	51.90 ab	39.07 b
F0B3	50.03 ab	38.17 b
F1B1	64.17 c	50.03 c
F1B2	49.97 ab	36.13 ab
F1B3	49.10 ab	34.67 a
F2B1	64.07 c	46.40 c
F2B2	38.77 a	37.43 ab
F3B3	52.27 ab	37.13 ab

Note : WAI = week after inoculation. Numbers followed by same letters at same column showed no significant different at α 5%.

4. Conclusion

Application of biological agent biofresh in a solid formulation combined with compos of soybean litter was the best treatment in increasing plant resistance to bacterial pustule disease and soybean crop yield. Induction of plant resistance occurred systemically, characterized by salicylic acid increase of 0.3 mg and peroxidase increase of 0.07 unit/mL in sample plants.

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