Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.5, No.13, 2015

Biocontrol of Wilt Disease on Pepper Using Endophytic Bacteria in Malang Indonesia

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Abstract
Wilt disease is one important disease on pepper that cause by *Ralstonia solanacearum,* be potential limiting factor of product and impact on economic country. Control of it using some technique have not been success. Biocontrol of it using endophytic bacteria is one alternative control methods and support sustainable agriculture. The object of these experiment are to select of endophytic bacteria that was pepper bacterial wilt disease. There are 10 isolates successfully isolated and two isolates are Ps_1 and Ps_8 can inhibit of wilt disease based on antagonistic test *in vitro* using seed coat method and forming inhibition zone size 4-7 mm so ability suppress of wilt disease *in vivo* test in green house. *In vivo* test in green house using pepper plant that 30 days age and aplicate of endophytic bacteria using root soak technique. The result show the two isolates are Ps_1 and Ps_8 have incubation period 15-16 days and significantly suppress disease incidence until 8.07-9.19%.
Keywords: *wilt disease, endophytic bacteria, biocontrol*

1. Introduction

Wilt disease is one important disease on pepper that cause by *Ralstonia solanacearum* be potential limiting factor of product and impact on economic country. Farmers have controled the disease using several methods including cultivation techniques, physical treatment, use of resistant varieties, bactericide and antibiotics application but all of them is not reducing the level of disease (CABI, 2013). Depend on it, biocontrol method using biocontrol agents was investigated. Several endophytic bacteria have been explored for biocontrol agents for the disease.

Endophytic bacteria is a group of microbes that live in plant tissue, without plant disease and related plants in mutualism simbiosis. Endophytic bacteria is in healthy tissues such as seeds, roots, stems and leaves. Plants benefit from the presence of endophytic bacteria because it produces compounds or secondary metabolites and antibiotics that stimulate growth hormone to affect plant growth and increases plant resistance to pathogens (Hundley, 2005; Bandara, *et al*., 2006). Endophytic bacteria can be isolated from the roots, stems, flowers and cotyledons, the space between cells in a tissue or vessel (Athman, 2006).

The object of these experiment are to select endophytic bacteria isolated from pepper stems, root and to investigate of them as biocontrol agents of pepper bacterial wilt disease.

2. Materials and Methods

Isolates of *R. solanacearum*. *R.solanacearum* used for research is a personal collection of Dr. Arika Purnawati then was isolation to obtain isolates was 48 hours.

Isolation of Endophytic Bacteria. The stem with 50 cm long from the base was washed with water to clean the surface then the parts were cut into 1 cm long. Specimen was sterilized using NaOCl 1.25% for 1 minute, dye absolute alcohol for 5 sec and do 3 times, dipp sterile water for 5 sec and do 3 times then the specimen is dried on sterile filter paper in laminar air flow (LAF) for 5 minutes (Widayanto, 2008). Spesimen was splited into two parts and inoculated on PDA medium (Difco): 39 g / l, pH 7.0 in a sterile Petri dish with position of inner surface touch medium or tummy position then is incubated in incubator at room temperature (28^0C) for 48 hours. After endophytic microbes grow, inoculated on sterile PDA medium for fungi, NA medium (Difco): 8 g / l, pH 7.0 for bacteria to obtained pure cultures. Isolation from root. All parts of the roots was washed with water to clean the surface then the parts were cut into 1 cm long. Specimen is sterilized using NaOCl 1.25% for 1 minute, dye absolute alcohol for 5 sec and do 3 times, dipp sterile water for 5 sec and do 3 times then the specimen was dried on sterile filter paper in laminar air flow (LAF) for 5 minutes (Widayanto, 2008). Spesimen was inoculated on PDA medium an upright position on the medium so the pieces touch the bottom of the medium then is incubated in incubator at room temperature (28^0C) for 48 hours. After endophytic microbes grow, inoculated on sterile PDA medium for fungi, the NA medium for bacteria to obtained pure cultures.

Antagonistic Test *in Vitro*. Antagonism test is done in laboratorium use seed coat method and the procedure are : pepper seeds that will be used are sterilized using klorok 1% for 30 sec then rinsed 3 times sterile water and dry on sterile tissue paper. Seed soak into suspension of endophytic bacteria with concentration 10^8 cfu / ml. Soaking of seed for 30, 60 then inoculated on NA medium and were incubated in incubator for 24 hours at room temperature (28^0C). After 24 hours, cup of Petridish was spilled 1 ml of chloroform about 2 hours then cup was closed and Petridish back to it's original position. Observed variable is inhibition zone around the seed and was

done at 24, 48, 72, 96 h. Antagonism test use completely randomized design with 2 factors are endophytic bacteria isolates (Ps_1, Ps_8) and seed soaking time (30, 60 min) with 3 replications. The data is analyzed statistically using LSD 5%.

Effectiveness test *in Vivo*. Effectiveness test *in vivo* was done in green house use root soak method and the procedure are : inoculation of *R. solanacearum* was done at 2 days before planting tomato plants using flush technique of suspension *R. solanacearum* to soil. Endophytic bacteria was inoculated a using root soak techniques in 100 ml suspension of endophytic bacteria for 30, 60 min then the tomato plants is planted in soil that have been inoculated *R. solanacearum*. Observed variable are incubation period and disease incidence during 60 days. Effectiveness test use completely randomized design with 2 factors are endophytic bacteria isolates (Ps_1, Ps_8) and root soaking time (30, 60 min) with 3 replications. The data is analyzed statistically using LSD 5%.

3. Results and Discussion

Antagonistic Test *in Vitro*

Results of antagonistic test of two isolates endophytic bacteria show that there's no different at LSD 5% analysis, but there's increase in size of inhibition zone at 48 hours of observation (Fig 1) and inhibiton mechanism is antibiosis as bacteriostatic.

The reason of the result because at the time production antibiotic by endophytic bacteria is enough and the molecule can adhere the surface of tomato seed coat that are semipermeable.

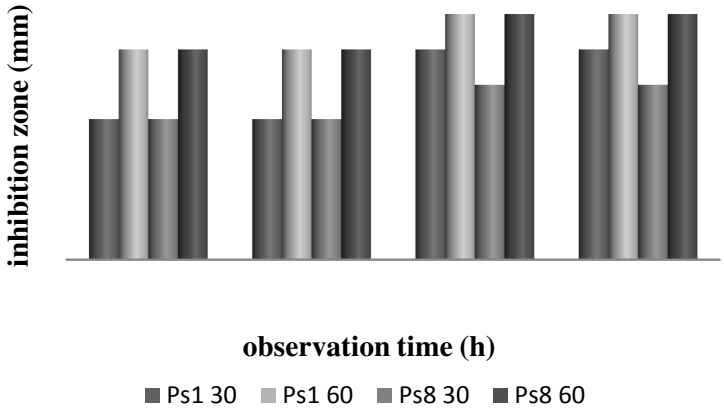

Fig.1. Grafic of Inhibition Zone of Endophytic Bacteria to *R. solanacearum*

Another reason maybe it's product siderophore that is a compound containing hidroksamat and catechol molecules, two molecules adhere the surface of tomato seed coat that are semipermeable. Both of them maybe have smaller molecular size than fat and protein so they are able to diffuse and adhere the surface of the tomato seeds coat. Oseni *et al.* (2011) and state that seed coat is composed of Ca, Na Fe, Mg, proteins, phenols and are semipermeable, Zhou *et al*. (2013) state that there are diversified chemical composition of the semipermeable layer seed coat of *R. nutans*, *A. inebrians*, *H. vulgare* var. *nudum* and *Triticale* is found to contain lipids in the semipermeable layer of seed coat. *F. sinensis* seed coat have pectin and *B. inermis* have cellulose in semipermeable layer of seed coat. Djatmiko *et al*. (2007) state that the inhibition zone in antagonistic test against *R. solanacearum* is antibiosis mechanism as bacteriostatic and is evidenced by take the zone then inoculate into peptone 0.5% solution and the solution is turbid.

Effectiveness test *in Vivo*

Results of effectiveness test of two isolates endophytic bacteria are incubation period 15-16 days and they significantly suppress disease incidence until 8.07-9.19% (Table 1) although there's no different at LSD 5% analysis for treatment.

Table 1. Disease Incidence of Wilt *Disease in Vivo*

Treatments	Disease Incidence (%) at …dat						
	14	21	28	35	42	49	56
Control	3,52 a*	13,50 a	13,50 a	18,13 a	18,13 a	18,13 a	18,13 a
Ps_1	0,17 b	3,75 b	3,75 b	7,50 b	7,50 b	7,50 b	9,50 b
Ps_8	0,17 b	5,25 b	5,25 b	8,75 b	8,75 b	8,75 b	10,25 b
BNT 5%	0,18	1,50	1,50	2,13	2,13	2,13	2,96

Note: * figures in the same column and follow by the same letter is not significantly different at LSD 5% ,
dat : days after treatment

The reason of the result because the disease development is influenced by environment are temperature is 34^0C, humidity is 80%, so maybe they production antibiotic and siderophore that can systemic resistance induction so that disease incidence is decrease. Fajinmi and Fajinmi (2010) state that temperature supports the development teh disease is 25-35^0C with humidity is 86% so Sastra (2009) states that the incubation period *R. solanacearum* on potato varieties is 6.5- 21.3 days.

Another reason because they production antibiotic and siderophore that induction resistance gen of tomato plants against *R. solanacearum* and effectiveness of biological control using biocontrol agents can be enhanced through the application techniques like soaking the roots. Mulya *et al*. (2006) states that P. PfG32 fluorescens that is isolated from rhizosphere of onion are actively suppress the incidence of bacterial wilt in tomatoes because produce siderophores and antibiotics.

The effectiveness of biological control using biocontrol agents can be enhanced through the application of different techniques like soaking the roots or seeds before transplanting and it's give the effect of more rapid induction of resistance (Hallmann, 2001).

4. Conclusion

From the research, two isolates of endophytic bacteria (Ps_1, Ps_8) can inhibit of wilt disease *in vitro* 4-7 mm and *in vivo* significantly suppress wilt disease until 8.07-9.19% with incubation period 15-16 days.

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