BIOLOGICAL SEED TREATMENT FOR CONTROLLING ANTHRACNOSE DISEASE OF HOT PEPPER

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Abstract

Three rhizobacteria (*Bacillus polymixa* BG25, *Pseudomonas fluorescens* PG01 and *Serratia liquefaciens* SG01) which inhibited the conidia germination of *Colletotrichum capsici* and produced the phytohormone indoleacetic acid (IAA) were selected and evaluated as biocontrol agents for controlling *Colletotrichum capsici*, the causal agent of hot pepper anthracnose and for improving the growth, yield and seed quality of hot pepper in glasshouse and field conditions. Two cultivars of hot pepper, cv. Lokal Brebes and cv. Tit Super were also used in this experiment. The result of the experiment showed that seed treatments with *P. fluorescens* PG01 either alone or in combination with *B. polymixa* BG25 increase yields and seed quality under glasshouse and field conditions. However, the most effective treatment was the combination of both agents. Moreover, biological seed treatment with *P. fluorescens* PG01 either alone or in combination with *B. polymixa* BG25 led to induction of resistance against *C. capsici*, as a result of increase in peroxidase activity and biosynthesis of phytoalexins that have been considered as resistance mechanisms against plant diseases.

Keywords: Hot pepper; Anthrachnose; Rhizobacteria; Yield; Seed quality

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INTRODUCTION

Hot pepper (*Capsicum annuum* L.) is an economically important spice crops and widely cultivated around the world for its pungent flavor and aroma (Obidiebub et al., 2012). In Indonesia, hot pepper is commonly cultivated by small farmers for increasing income and their living standards. In spite of the importance of hot pepper, unfortunately in the field, this plant also tends to suffer from infections by a host of pathogens throughout its life cycle. Anthracnose caused by seedborne pathogen *Colletotrichum capsici* is the most damaging postharvest disease on hot pepper fruits and cause serious yield losses under favorable environmental conditions (Than et al., 2008). Yield loss reaching 100% has been reported (Liestiani and Fikri, 2012). Conventional methods of disease control include cultural practices and fungicidal seed treatments (Jean et al., 2013). However, increased concern regarding the fate of synthetic pesticides in the environment, the development of fungicide-resistance in pathogenic strains and the public demand for fungicide-free produce, alternative methods of disease control are under investigation. Biological control has potential for the management of these diseases as an alternatif methods, which is also ecologically sound and environmentally safe.

Several antagonistic micro organism have been shown to reduced fungal decay of plant. Rhizobacterial strains of fluorescent pseudomonad, *Bacillus* spp. and *Serratia* spp. have been used to reduce disease caused by variety pathogens. *Pseudomonas fluorescens* effective in controlling grey mould disease on strawberry plants caused by *Botrytis cinere* (Haggag et al., 2012), bacterial wilt of Eggplant caused by *Ralstonia solanacearum* (Gargi et al., 2012; Chakravarty & Kalita, 2012), *P. fluorescens* combined with *B. licheniformis* dan *Chryseobacterium balustinum* effective on *Pythium aphanidermatum* the causal agent of hot pepper *dumping-off* (Domenech et al., 2006); *Bacillus subtilis* effective in controlling *Ralstonia solanacearum,* the causal agent of tomato wilt (Chen et al., 2013), and phytophthora blight caused by *Phytophthora capsici* (Lee et al., 2008); *Serratia marcescens* effective on *Colletotrichum gloeosporioides* the causal agent of anthracnose (Gutiérrez-Román et al., 2012); *Serratia plymuthica* strain C-1 effective in controlling phytophthora blight caused by *Phytophthora capsici* (Kim & Jung, 2008).

Little has been reported as to the control of anthracnose disease by biocontrol agents may because of latent phenomenom of the disease. However study of induced resistance using *Bacillus subtilis* EXB-123 significantly reduced anthracnosa of chilli plants caused by *Colletotrichum capsici* (Ramanujam et al., 2012). Another recent study showed that *Pseudomonas fluorescence*, *Trichoderma harzianum* and *Trichoderma viride* apparently suppressed the growth of bean anthracnose disease (Amin et al., 2014). The objective of this researches were to evaluated effectiveness of biological seed treatment for controlling anthracnose and for improving yield and seed quality of hot pepper.

MATERIALS AND METHODS

Isolation and preparation of C. capsici

Hot pepper fruit indicated anthracnose disease was extracted to get seeds, and then seed dried to obtain 8% of water content for the subsequent detection of the presence of *C. capsici*. Isolation was done by using Potato Dextrose Agar (PDA) medium. Before cultured, seed are disinfected with natrium hypochlorite of 2% for 5 minutes to remove other microorganisms' contaminants. After that, seed incubated for ± 7 days at room temperature with Near Ultra Violet (NUV) radiation for 12 hours of light and

12 hours of dark. Next, it's identified based on microscopic observation of the culture character (Watanabe, 2002). The obtained colonies of *C. capsici* were subcultured for several times until get the pure isolates for the next experiment.

Isolation and evaluation of rhizobacteria to inhibit conidia germination of C. capsici and to produce IAA

Bacteria exploration conducted on the rhizosfer of healthy hot pepper. Exploration target was to get non-pathogenic rhizobacteria from *Pseudomonas fluorescens* species, *Bacillus* spp, and *Serratia* spp. Isolation was done by serial dilution method using King's B medium (to isolate *P. fluorescens*) and Tryptic Soy Agr (TSA) 0.1 medium (to isolate *Bacillus* spp and *Serratia* spp). To get the isolate potentially as biocontrol agent, it was done some stages of testing the effectiveness of rhizobacteria to *C. capsici*. The potential isolate as biocontrol agent then identified by using standard test procedures (Schaad et al., 2001). One of the selection methods used to obtain a potential bacterial isolates was testing of bacterial filtrate to the germination of *C. capsici*. For each bacterial isolate used, as much as full sphere of ose needle inserted into the Erlenmeyer flask containing 10 ml of *Triptych Soy Broth* (TSB) medium, and placed on a shaker, then it swindle with a speed around 150 rpm at a temperature of 28^{o}C for 24 hours. The filtrate is separated from bacterial cell with speed centrifuge 10000 rpm for 15 minutes, then supernatant was filtrated with 0.2 um nitrocellulose membrane. At the 7 ages of *C. capsici* conidia on PDA medium were harvested by adding 20 ml of sterile aquades. Conidia suspension was given nutrient that contain 0.5% glucose and 0.5% asparagines and left for one hour at a temperature of 28^{o}C. The bacterial filtrate was then mixed with suspension of conidia *C. capsici* 1 : 1 (v/v). As a controlling, it was used suspension of conidia *C. capsici* without bacterial filtrate. The mixture of bacterial filtrate and conidia suspension were dropped into the glass of object and incubated for 24 hours at room temperature. The conidia have normal germination if the size of germination tube is a half of conidia length and it is not swelling. The observation of *C. capsici* conidia germination is done on five fields of view with the light microscope 100 times magnification. Several types of rhizobacteria were known have ability to produce the growth hormones. In this experiment, it was conducted a test of bacterial ability to produce the growth hormones *indoleacetic acid* (IAA) as a part of selection method to obtain a potential biocontrol agents. IAA test applied both Glickman & Dessaux method (1995). The content of IAA in the sample was calculated by regression made of pure IAA with concentration 0, 6.25, 12.5, 25, 50, 75, 100, 150 and 200 μgml^{-1}.

Inoculums preparation of biocontrol agents and seed treatment

Before used for seed treatment, bacterial isolates was cultured in medium King's B (for *P. fluorescens*) and TSA (for *Bacillus* spp. and *Serratia* spp.). The population density of bacterial used for seed treatment application is 10^9 cfu/ml (Bai et al., 2002) or equivalent reading to OD_{600} = 0.164 (for *Bacillus* spp.) OD_{600} = 0.072 (for *Serratia* spp), and OD_{600} = 0.192 (for *P. fluorescens*) based on the measurement of absorbance on the spectrophotometer. For the experiment in the greenhouse and in the field used two 2 varieties of hot pepper are susceptible to anthracnose namely Local Brebes and Tit Super. Seeds that will be used for this experiment are the hot pepper from the field which infected *C. capsici* with 90 – 95% range of infection (based on the blotter test result). Before giving of biocontrol agents treatment, seeds disinfected with natrium hypochlorite 2% for five minutes, then washed three times with sterile aquades and dried in laminar flow for an hour. A total of 50 ml bacterial suspension is mixed with 1 g of hot pepper seed in cultural bottles, stir thoroughly and then incubated for 24 hours at a temperature of 28°C. After treatment, seed was drained and dried. Then, it's ready to use.

Evaluation the effect of biocontrol agents on yield and seed quality of hot pepper in greenhouse

For this experiment, it was used three types of potential biocontrol agents that identified as *Bacillus polymixa* BG25, *Pseudomonas fluorescens* PG01 and *Serratia liquefaciens* SG01 based on the result of previous experiment. This experiment used split plot design in completely randomized design pattern, and continued with Duncan Multiple Range Test (DMRT) on level of $\alpha = 5\%$. As the main plot is hot pepper variety consisting of two levels: Local Brebes and Tit Super, while the sub plot is the seed treated by biocontrol agents that consists of ten levels: *B. polymixa* BG25 (BG25), *P. fluorescens* PG01 (PG01), *S. liquefaciens* SG01 (SG01), a mixture of *B. polymixa* BG25 + *P. fluorescens* PG01 (BG25 + PG01), a mixture of *B. polymixa* BG25 + *S. liquefaciens* SG01 (PG01 + SG01), a mixture of *B. polymixa* BG25 + *P. fluorescens* PG01 + *S. liquefaciens* SG01 (BG25 + PG01 + SG01), Dithane M-45 0.2% (Dhitane), the seed infected without biocontrol agents treatment (disease seed) and the seed uninfected without biocontrol agents (healthy seed). Each treatment was repeated for three times so there are 60 experimental units overall. Before planting, the hot pepper seed was sown in the nursery box (20 cm x 10 cm x 5 cm). Then, seed was moved to polybag (30 cm x 45 cm) after the age of four weeks. The media used was a mixture of soil and manure (1 : 4). Fertilizing plants conducted twice, the first fertilization was given together with a dose or Urea, SP36 and KCl which each of them are 7 g/plant, while the second fertilization was done after 6 weeks with a dose of Urea, SP36 and KCl respectively 10 g/plant. The observed variable such as the consumption weight of hot pepper per plant, percentage of disease incidence rate, vigor index of seed yield and contamination level of seed yield by *C. capsici*.

Evaluation the effect of biocontrol agents on yield and seed quality of hot pepper in the field

Field experiment was a continuation of the greenhouse experiment. Based on the agronomic characters and the percentage of disease incidence in greenhouse experiment, only *B. polymixa* BG25 and *P. fluorescens* PG01 then used in this experiment. This experiment applied split plot design in randomized block design pattern and continued with Duncan Multiple Range Test (DMRT) on level of α = 5%. As the main plot is hot pepper variety consisting of two levels: Local Brebes and Tit Super, while the sub plot is the seed treated by biocontrol agents that consists of six levels: BG25, PG01, a mixture of BG25 + PG01, Dithane, Infected seed and Healthy seed. Each treatment is repeated for three times so there are 36 experimental units and each of it consists of five plants. Preparation of planting, plant maintenance and observed variables in this experiment are same as in the greenhouse experiment.

Physiological response of plant due to biocontrol agents treatment

Physiological response of plant as a result of the biocontrol agents application was indicated by the increased of activity several enzymes and the biosynthesis accumulation of antimicrobial metabolic compounds related to plant resistance to the pathogen infection (Chen *at al.,* 2000). In this experiment, there were performed the measurement of peroxidase enzyme activity on hot pepper sprout, and the analysis of phytoalexin compound on hot pepper that have been given biocontrol agents treatment. The measurement of peroxidase activity is performed according to Hammerschmidt *et al.,* (1982) procedure which has been modified (Silva,

2004), whereas the content of phytoalexin on hot pepper analyzed by HPLC based on Lyion (1984) method in Linkens & Jackson (1987).

RESULTS AND DISCUSSION

Isolation and evaluation of rhizobacteria to inhibit conidia germination of C. capsici and to produce IAA

Exploration of rhizobacterial on the healthy hot pepper plant produced some isolates potentially as biocontrol agents. The effect of bacterial filtrate on conidia germination of *C. capsici* showed that bacterial filtrate can inhibit the conidia germination of C. capsici and caused abnormalities sprout (Figure 1)

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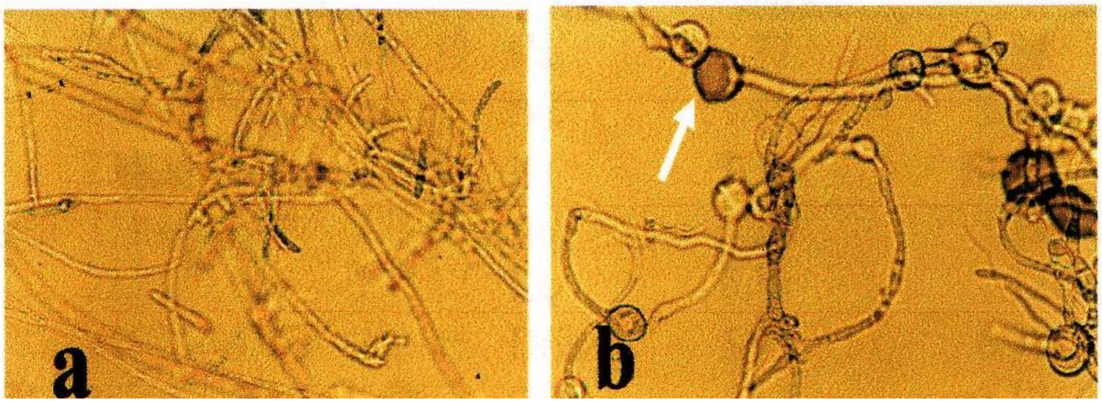

Figure 1. The inhibitory effect conidia germination of *C. capsici* by rhizobacterial filtrate, (a) Conidia germination without rhizobacterial filtrate, (b) with rhizobacterial filtrate (malformation hypha).

All the tested isolates able to produce IAA. From Pseudomonas group, *P. fluorescens* PG01 can produce the highest IAA than all the bacterial tested. Meanwhile, from the Bacillus and Serratia group, *B. polymixa* BG25 and *S. liquefaciens* SG01 tend to produce IAA more but not significantly different with the other isolates within the same group (Figure 2).

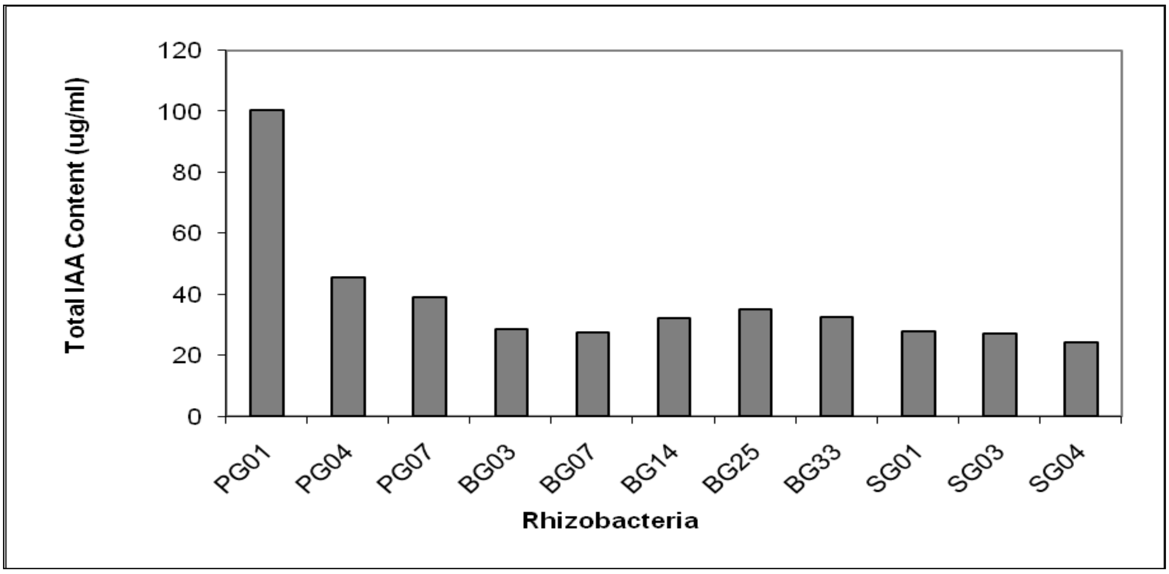

Figure 2. Total content of IAA produced by rhizobacteria

Evaluation the effect of biocontrol agents on yield and seed quality of hot pepper in greenhouse

The interaction between seed treatment and varieties that are used can increase the consumption weight of hot pepper. There is a very significant difference between the seeds that is given biocontrol agents treatment and the seed that is not given one to the consumption weight of hot pepper, however the best result obtained in the treatment of seed with a mixture of *B. polymixa* BG25 + *P. fluorescens* PG01 (Table 1).

Table 1 The effect of seed treatment with biocontrol agents to the consumption fruit weight and percentage of disease incidence of hot pepper. The numbers followed by the same capital letter in the same column, and the numbers followed by the same small letters on the same line showed no significantly different at the level of 5% DMRT test. BG25 (*Bacillus polymixa* BG25), PG01 (*Pseudomonas fluorescens* PG01), SG01 (*Serratia liquefaciens* SG01).

Seed Treatment	Consumption Fruit Weight (g/plant)		Disease Incidence Percentage (%)		
	Local Brebes	Tit Super	Local Brebes	Tit Super	Average
BG25	257.33 ± 4.43 Ca	254.30 ± 6.32 Ca	3.77 ± 1.39	3.94 ± 0.53	3.86 ± 0.94 E
PG01	296.72 ± 9.39 Bb	314.52 ± 3.05 Ba	3.48 ± 0.53	3.66 ± 0.55	3.57 ± 0.49 E
SG01	234.53 ± 2.76 CDa	223.86 ± 4.53 Eb	9.38 ± 2.08	10.25 ± 0.9	9.81 ± 1.51 C
BG25+PG01	331.48 ±11.71 Ab	358.28 ± 3.51 Aa	3.28 ± 0.61	3.39 ± 0.17	3.33 ± 0.41 E
BG25+SG01	225.31 ± 8.60 Da	222.57 ± 7.05 Ea	6.19 ± 1.63	7.12 ± 0.81	6.65 ± 1.26 D
PG01+SG01	245.69 ±13.83 CDa	233.08 ± 5.45 DEa	5.99 ± 1.05	6.97 ± 0.87	6.48 ± 1.01 D
BG25+PG01+SG01	257.68 ±21.47 Ca	242.48 ± 1.51 CDa	6.17 ± 1.48	7.17 ± 1.18	6.67 ± 1.32 D
Dithane	109.08 ±16.11 Eb	156.17 ± 4.33 Fa	15.65 ±1.81	19.88 ± 3.63	17.77 ± 3.45 B
Seed Infected	91.43 ±10.46 Ea	110.94 ± 17.55 Ga	26.07 ± 4.69	36.77 ± 8.21	31.42 ± 8.37 A
Healthy Seed	103.25 ±16.87 Eb	148.57 ± 7.37 Fa	18.82 ± 3.07	21.4 ± 0.87	20.11 ± 2.46 B
Average	215.25 ± 2.22	226.48 ± 72.59	9.88 ± 7.69 B	12.05±10.69 A	10.97 ± 9.3

There is a decline in the percentage of disease incidence significantly on seed that have application of biocontrol agents treatment both *B. polymixa* BG25 and *P. fluorescens* PG01 than the seed control (Table 1). The best result is obtained in a mixture of these two agents that able to reduce the percentage of disease incidence up to 87% on Local Brebes variety and 91% on Super Tit variety.

The observation result of seed physiological test showed that seed treatment with biocontrol agents can improve seed quality. The best result obtained in the treatment of seed with a mixture of *B. polymixa* BG25 and *P. fluorescens* PG01 (Table 2). As the increased in crop production due to treatment of biocontrol agents, improving the quality of seeds produced is also caused by plant growth improvement. The plant growth that more vigor will be better able to maximize their growth capability to produce maximum food reserve which a part of it will be accumulated in some seeds that produce more seeds vigor too.

Application of biocontrol agents treatment can also reduce contamination level of seed yield by anthracnose pathogen. The level of *C capsici* contamination in seed yield grown from seeds that were treated by biocontrol agents decreased significantly than the control one (Table 2). The low contamination level of this seed yield have correlation with the low disease incidence level of plant treated with biocontrol agents (Table 1).

Table 2 The effect of seed treatment with biocontrol agents to the vigor index and level of seed contamination. The numbers followed by the same capital letter in the same column, and the numbers followed by the same small letters on the same line showed no significantly different at the level of 5% DMRT test. BG25 (*Bacillus polymixa* BG25), PG01 (*Pseudomonas fluorescens* PG01), SG01 (*Serratia liquefaciens* SG01).

Seed Treatment	Vigor Index (%)						Seed Contamination Level (%)			
	Local Brebes		Tit Super		Average		Local Brebes		Tit Super	
BG25	32.67 ± 3.06		29.33 ± 3.06		31.00 ± 3.29	B	2.22 ± 1.54	Ea	2.67 ± 0.00	Da
PG01	32.00 ± 4.00		28.67 ± 1.15		30.33 ± 3.20	B	1.78 ± 0.77	Ea	2.22 ± 0.77	Da
SG01	28.00 ± 2.00		20.00 ± 2.00		24.00 ± 4.73	C	6.67 ± 1.33	Ca	7.11 ± 0.77	Ca
BG25+PG01	38.00 ± 3.46		38.00 ± 2.00		38.00 ± 2.53	A	1.78 ± 1.54	Ea	0.44 ± 0.77	Da
BG25+SG01	30.67 ± 1.15		26.67 ± 1.15		28.67 ± 2.42	B	6.22 ± 0.77	Cb	8.89 ± 0.77	Ca
PG01+SG01	31.33 ± 1.15		26.67 ± 3.06		29.00 ± 3.29	B	4.00 ± 0.00	DEa	5.78 ± 2.04	Ca
BG25+PG01+SG01	30.67 ± 3.06		27.33 ± 3.06		29.00 ± 3.29	B	5.33 ± 1.33	CDa	6.22 ± 0.77	Ca
Dithane	24.67 ± 2.31		24.67 ± 3.06		24.67 ± 2.42	C	14.67 ±1.33	Ba	17.33 ±2.67	Ba
Seed Infected	19.33 ± 3.06		17.33 ± 1.15		18.33 ± 2.34	D	25.33 ±1.33	Aa	32.89 ±3.36	Aa
Healthy Seed	24.67 ± 1.15		26.00 ± 2.00		25.33 ± 1.63	C	16.00 ±1.33	B	18.67 ±2.67	Ba
Average	29.20 ± 5.50	A	26.47 ± 5.67	B	27.83 ± 5.71		8.40 ± 7.58		10.22 ±9.76	

Evaluation the effect of biocontrol agents on yield and seed quality of hot pepper in the field

Based on evaluation result of some observation variables in the greenhouse, *B. polymixa* BG25 and *P. fluorescens* PG01 or a mixture of them show the best result, so that only the two agents are used in field experiment. As in the greenhouse experiment, application of biocontrol agents in field experiment can increase the consumption weight of hot pepper than the control one. The application of mixture *B. polymixa* BG25 and *P. fluorescens* PG01 give better result than single agent application (Table 3). The plant production increased is the accumulation of plant growth improvement is also very significant differences between the seed treated biocontrol agents than the control one (data not shown). The increased growth by these biocontrol agents occur because they have ability to produce growth hormone as see in Figure 2.

In the field condition are optimum for the development of anthracnose disease (rainy season), in fact biocontrol agents treatment capable in protecting plants from *C. capsici* infection. A mixture of *B. polymixa* BG25 and *P. fluorescens* PG01 can reduce percentage of disease incidence up to 89% in Local Brebes variety and 88% in Super Tit variety. This suggests that biocontrol agents capable in inducing plants systematically to improve defense against pathogen infection through increased enzyme activity or biosynthesis of antimicrobial compound. From the result of this research, it prove that there is an increase of peroxidase enzyme activity and biosynthesis of phytoalexin compound on the seed treated biocontrol agents than the control one (Figure 3a,b).

Table 3. The effect of seed treatment with biocontrol agents to the consumption weight of hot pepper and percentage of disease incidence. The numbers followed by the same capital letter in the same column, and the numbers followed by the same small letters on the same line showed no significantly different at the level of 5% DMRT test. BG25 (*Bacillus polymixa* BG25), PG01 (*Pseudomonas fluorescens* PG01).

Seed Treatment	Consumption Fruit Weight (g/tan)				Disease Incidence Percentage (%)			
	Local Brebes		Tit Super		Local Brebes		Tit Super	
BG25	267.67 ± 8.64	Ba	262.55 ± 11.75	Ba	10.05 ± 0.70	Cb	13.83 ± 0.60	Ca
PG01	286.37 ± 6.52	Ba	271.43 ± 6.44	Bb	9.84 ± 0.38	Cb	13.25 ± 0.89	Ca
BG25+PG01	327.37 ± 10.17	Aa	307.18 ± 12.76	Aa	8.48 ± 0.5	Ca	10.02 ± 1.06	Da
Dithane	151.00 ± 10.33	Ca	168.36 ± 14.01	Ca	58.18 ± 4.06	Ba	61.54 ± 2.44	Ba
Seed Infected	114.07 ± 23.3	Da	96.85 ± 11.54	Da	78.77 ± 1.21	Aa	82.27 ± 2.17	Aa
Healthy Seed	144.71 ± 9.71	Ca	160.82 ± 2.71	Ca	60.67 ± 0.68	Ba	62.71 ± 2.56	Ba

Table 4. The effect of seed treatment with biocontrol agents to the vigor index and seed contamination level. The numbers followed by the same capital letter in the same column, and the numbers followed by the same small letters on the same line showed no significantly different at the level of 5% DMRT test. BG25 (*Bacillus polymixa* BG25), PG01 (*Pseudomonas fluorescens* PG01).

Seed Treatment	Vigor Index (%)						Seed Contamination Level (%)			
	Local Brebes		Tit Super		Average		Local Brebes		Tit Super	
BG25	42.00 ± 2.00		23.33 ± 2.31		32.67 ± 10.41	B	4.00 ± 0.80	Cb	5.33 ± 1.22	Ca
PG01	42.00 ± 6.00		22.67 ± 3.06		32.33 ± 11.41	B	4.00 ± 1.39	Ca	5.07 ± 0.46	Ca
BG25+PG01	44.00 ± 6.00		29.33 ± 4.16		36.67 ± 9.27	A	2.40 ± 0.80	Ca	3.20 ± 0.80	Ca
Dithane	27.33 ± 3.06		16.67 ± 1.15		22.00 ± 6.20	C	15.47 ± 1.85	Bb	21.80 ± 0.72	Ba
Benih sakit	24.67 ± 3.06		10.67 ± 1.15		17.67 ± 7.94	D	27.20 ± 0.80	Ab	34.40 ± 3.20	Aa
Benih sehat	26.00 ± 4.00		15.33 ± 1.15		20.67 ± 6.41	CD	17.07 ± 0.46	Bb	23.20 ± 1.60	Ba
Average	34.33 ± 9.36	A	19.67 ± 6.62	B	27.00 ± 10.92		11.69 ± 9.33		15.5 ± 12.10	

In line with the result of greenhouse experiment, biocontrol agents treatment on field experiment can improve quality of seed yield. Either single or mixed, *B. polymyxa* BG25 and *P. fluorescens* PG01 produce the highest of vigor index than seed control (Table 4). The application of biocontrol agents treatment can also reduce contamination level of seed yield. Contamination level of *C. capsici* in seed of yield are planted from the seed that treated biocontrol agents decrease significantly than the seed control (Table 4). The low level of this seed yield contamination correlated to the low of disease incidence level of plants that treated biocontrol agents (Table 3). As we know that *C. capsici* is the contagious pathogens of seed, therefore if in the field production there is pathogen infection on the hot pepper, it's most likely that generated seed also infected by this pathogen.

Peroxidase activity

Plants have ability to increase the activity of some enzymes that associated with the resistance against pathogen infection after giving treatment of biocontrol agents. The result found in this experiment, seeds were given biocontrol agents treatment show its peroxidase activity is higher than seed control (Figure 3).

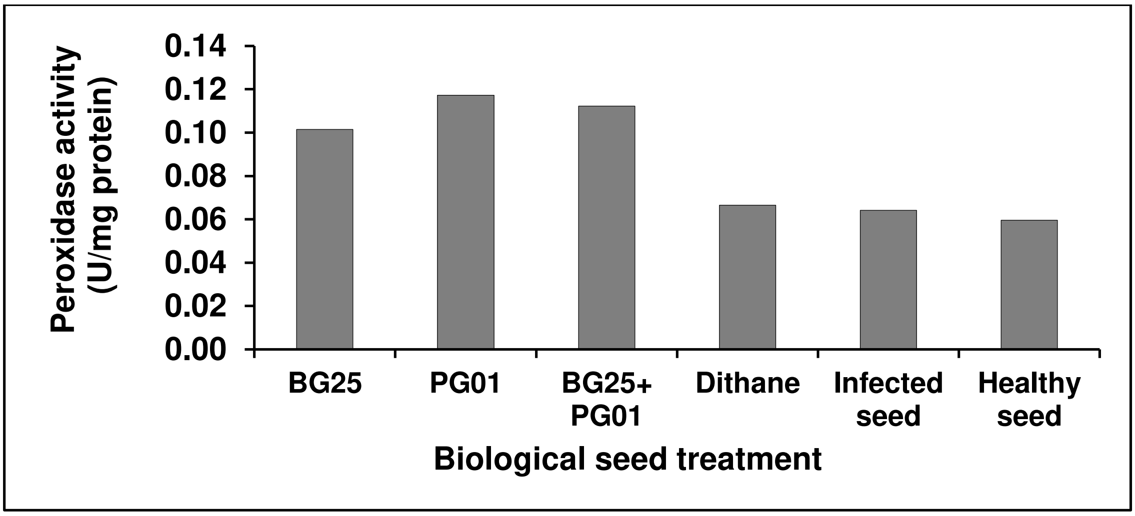

Figure 3. Peroxidase activity in hot pepper plants after treated by biocontrol agents. BG25 (*Bacillus polymixa* BG25), PG01 (*Pseudomonas fluorescens* PG01)

Phytoalexin accumulation

One indicator of resistance induction in plant is an increased of biosynthesis accumulation of antimicrobial metabolic compound. From the analysis result of three types phytoalexin (phytuberin, solavetivone and rishitin), it appear that an increased of compound at the hot pepper are from seeds that is given biocontrol agents treatment than the seed control (Figure 4). Phytoalexin are metabolic compound associated with plant defense against pathogen infection. It is negatively correlated to the percentage of disease incidence; that is percentage of disease incidence is lower in treatment with higher phytoalexin content, and vice versa.

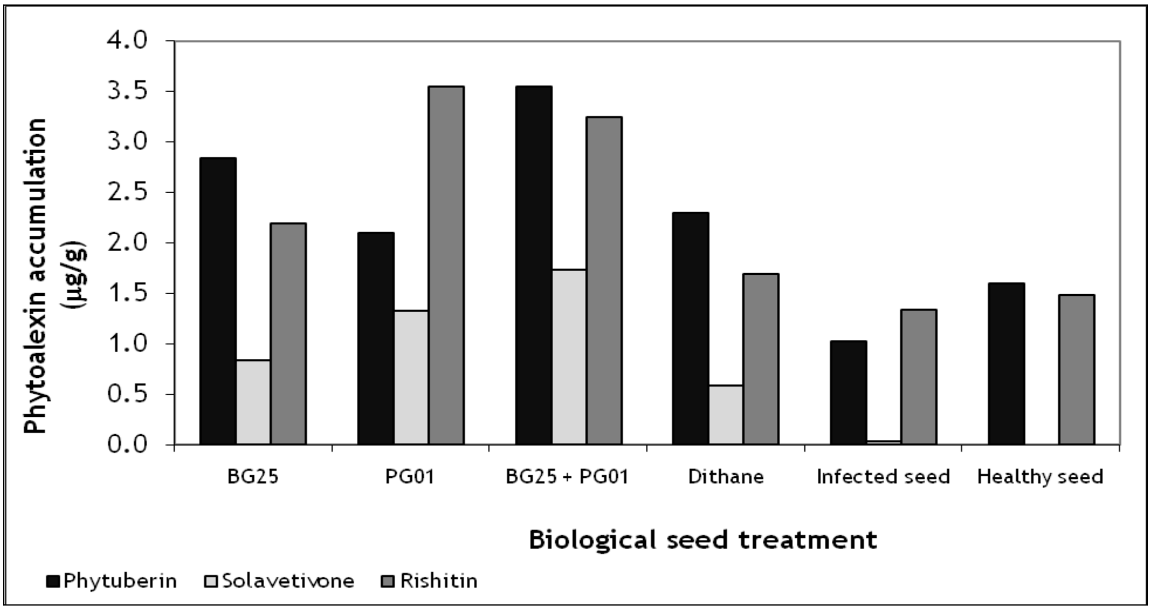

Figure 4. Phytoalexin accumulation of hot pepper fruit after treated by biocontrol agents

Rhizobacteria explorated from rhizosfer healthy hot pepper plant were potential as biocontrol agents. Evaluation the effect of rhizobacterial filtrate on conidia germination of *C. capsici* showed that rhizobacterial filtrate can inhibit the conidia germination of *C. capsici* and caused abnormalities hypha. Malformation hypha of *C. capsici* caused by antibiotic compounds that bacteria released (Ann, 2014).

All the tested isolates able to produce IAA. Previous study also found that *Bacillus* spp. (Acuna et al., 2012), *Pseudomonas* spp. (Pandey et al., 2013), dan *Serratia* spp. (Lwin et al., 2012) can produce IAA. From Pseudomonas group, *P. fluorescens* PG01 can produce the highest IAA than all the bacterial tested. Meanwhile, from the Bacillus and Serratia group, *B. polymixa* BG25 and *S. liquefaciens* SG01 tend to produce IAA more but not significantly different with the other isolates within the same group. The same study also showed that *Pseudomonas* spp. group can produce IAA more than another rhizobacteria (Ahmad et al., 2008).

The interaction between seed treatment and varieties that are used can increase the consumption fruit weight of hot pepper. There is a very significant difference between the seeds that is given biocontrol agents treatment and the seed that is not given one to the consumption weight of hot pepper, however the best result obtained in the treatment of seed with a mixture of *B. polymixa* BG25 + *P. fluorescens* PG01. The same result with this study also reported that the application of biocontrol agents increase production of pepper (Mirik et al., 2008) and chilli (Datta et al., 2011).

There is a decline in the percentage of disease incidence significantly on seed that have application of biocontrol agents treatment both *B. polymixa* BG25 and *P. fluorescens* PG01 than the seed control.

The best result is obtained in a mixture of these two agents that able to reduce the percentage of disease incidence up to 87% on Local Brebes variety and 91% on Super Tit variety. These results are consistent with the study by Zhang et al. (2010) that the mixture of two biocontrol agents which have ability to synergize each other will give better result in protecting plants from pathogen infection than the single agents.

Seed treatment with biocontrol agents can improve seed quality. The best result obtained in the treatment of seed with a mixture of *B. polymixa* BG25 and *P. fluorescens* PG01. As the increased in crop production due to treatment of biocontrol agents, improving the quality of seeds produced is also caused by plant growth improvement. The plant growth that more vigor will be better able to maximize their growth capability to produce maximum food reserve which a part of it will be accumulated in some seeds that produce more seeds vigor too.

Application of biocontrol agents treatment can also reduce contamination level of seed yield by anthracnose pathogen. The level of *C capsici* contamination in seed yield grown from seeds that were treated by biocontrol agents decreased significantly than the control one. The low contamination level of this seed yield have correlation with the low disease incidence level of plant treated with biocontrol agents. Based on evaluation result of some observation variables in the greenhouse, *B. polymixa* BG25 and *P. fluorescens* PG01 or a mixture of them show the best result, so that only the two agents are used in field experiment.

As in the greenhouse experiment, application of biocontrol agents in field experiment can increase the consumption weight of hot pepper than the control one. The application of mixture *B. polymixa* BG25 and *P. fluorescens* PG01 give better result than single agent application (Table 3). The plant production increased is the accumulation of plant growth improvement is also very significant differences between the seed treated biocontrol agents than the control one (data not shown). The increased growth by these biocontrol agents occur because they have ability to produce growth hormone as see in Figure 2. The similar result are also reported by Acuna et al. (2012), Pandey et al. (2013), and Lwin et al. (2012). Two that there is an increased in plant growth and yield after receiving biocontrol agents treatment which can produce the growth hormone.

In the field condition are optimum for the development of anthracnose disease (rainy season), in fact biocontrol agents treatment capable in protecting plants from *C. capsici* infection. A mixture of *B. polymixa* BG25 and *P. fluorescens* PG01 can reduce percentage of disease incidence up to 89% in Local Brebes variety and 88% in Super Tit variety. This suggests that biocontrol agents capable in inducing plants systematically to improve defense against pathogen infection through increased enzyme activity or biosynthesis of antimicrobial compound. From the result of this research, it prove that there is an increase of peroxidase enzyme activity and biosynthesis of phytoalexin compound on the seed treated biocontrol agents than the control one. Peroxidase is a key enzyme in lignin formation process that has function as a physical defense of plants against pathogen infection, while phytoalexin is antimicrobial compound as a form of plant chemical defense against pathogen infection (Ahuja et al., 2012; Jeandet et al., 2013).

The application of biocontrol agents' treatment can also reduce contamination level of seed yield. Contamination level of *C. capsici* in seed of yield are planted from the seed that treated biocontrol agents decrease significantly than the seed control. Plants have ability to increase the activity of some enzymes that associated with the resistance against pathogen infection after giving treatment of biocontrol agents. Chen et al. (2000) reported that the application of biocontrol agents can increase production of enzyme peroxidase, phenylalanine ammonia-liase (PAL) and polyphenol oxidase, as well as Silva et al. (2004) stated that enzyme peroxidase, PAL, and lipoksigenase increasing after it is given biocontrol agents treatment. The relevant result found in this experiment, seeds were given biocontrol agents treatment show its peroxidase activity is higher than seed control.

One indicator of resistance induction in plant is an increased of biosynthesis accumulation of antimicrobial metabolic compound. From the analysis result of three types phytoalexin (phytuberin, solavetivone and rishitin), it appear that an increased of compound at the hot pepper are from seeds that is given biocontrol agents treatment than the seed control. Phytoalexin are metabolic compound associated with plant defense against pathogen infection. It is negatively correlated to the percentage of disease incidence; that is percentage of disease incidence is lower in treatment with higher phytoalexin content, and vice versa. The same result is reported by Chen et al. (2000) that biocontrol agents can induce plants to enhance biosynthesis of lignin, phenolic compound, salicylic acid and phytoalexin.

We conclude that exploration rhizobacteria from the rhizosfer of healthy hot pepper plant have two types of biocontrol agents such as *Bacillus polymixa* BG25 and *Pseudomonas fluorescens* PG01 which are effective in controlling anthracnose disease and improving yield and seed quality of hot pepper. Inhibitory effect of biocontrol agents against *C. capsici* occurs through the mechanism of antibiosis and induction of hot pepper plant resistance.

Biocontrol agents application on hot pepper seed can increase peroxidase enzyme activity and phytoalexin biosynthesis which is an indicator of induction of hot pepper plant resistance to *C. capsici* infection. The metabolite compound are excreated by biocontrol agents related to the ability of these agents in inducing plant resistance to inoculation of *C. capsici* and enhancing the plant growth, yield and seed quality of hot pepper.

CONCLUSION

The *P. fluorescens* PG01 either alone or in combination with *B. polymixa* BG25 increase yields and seed quality under glasshouse and field conditions. Biological seed treatment with *P. fluorescens* PG01 either alone or in combination with *B. polymixa* BG25 led to induction of resistance against *C. capsici*, as a result of increase in peroxidase activity and biosynthesis of phytoalexins that have been considered as resistance mechanisms against plant diseases

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