AGRIVITA VOLUME 35 No. 2 *JUNE - 2013* *ISSN : 0126-0537*

THE POTENCY OF *Bacillus* sp. AND *Pseudomonas* sp. AS BIOLOGICALCONTROL AGENTS AGAINST CORN LEAF BLIGHT DISEASE CAUSED BY *Pantoea* sp.

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Received: Februari 12, 2013/ Accepted: May 2, 2013

ABSTRACT

One of new biotic constraints in corn production in Indonesia is leaf blight disease caused by *Pantoea* sp*.* which needs to be controlled. The purpose of this research was to study the potential of *Bacillus* sp.and *Pseudomonas* sp. as biological control agents against corn leaf blightcaused by *Pantoea* sp.In this study, several assays were conducted, including antagonistics assays on agar plate, characterization of the type of antibiosis produced by antagonists and pot experiment in controlling leaf blight disease (*Pantoea* sp.) by using*Bacillus*sp. and *Pseudo-monas* sp. The results showed that all bacterial strains of *Bacillus* sp.and *Pseudomonas* sp.had potential in inhibiting the growth of *Pantoea* sp. by showing the clear zone on the agar plate. The antibiosis typeswere bactericide or bacteriostatic. On pot experiment all bacterial strains showed the reduction of the disease incidence at the same level compared to streptomycin suphate. All bacterial strains as well as bactericide reduced the disease incidence at 18-24% compared to control (aquades treatment only). The results suggest that all bacterial strains are potential as biological control agents against leaf blight disease on corn leaf caused by *Pantoea* sp.

Keywords: Biological control, *Bacillus* sp., *Pseudomonas* sp. and *Pantoea* sp.

INTRODUCTION

Corn (*Zea mays* L.) isa source of carbo-hydrate, and is widely used in food industries and livestock.Based on the data of Indonesia's Statistics Agency (BPS), East Java Province is the major of corn producer producing as much as6,295,301 ton in 2012. One of the new biotic constraints on corn production in East Java is leaf blight disease caused by *Pantoea* sp. Affecting corn production. In Brazil, for example, the leaf blight disease caused by *Pantoea* sp.resulted inthe loss of productivity up to 60% (Cota *et al.*, 2010).

Recently, Suryani *et al.*(2012) found leaf blight diseases on corn in Kediri regency. By physiological, biochemical and molecular method, it has been suggested that the disease was supposed to be caused by *Pantoea* sp.The bacterium had characterictic of oval cell shape with 0.4-2.2 µm size, and the colonies showed yellow flattened round with smooth edge. It has been known that this bacterium canspread through water, seed and insect vector. This bacterium lives on corn as a host (Krawczyk *et al.*, 2010). Several common host plants such assugarcane, sorghum, tomatoes, wheat, cotton, melon(Cota *et al.*, 2010) and rice (Mondal *et al.*, 2011) are attacked. Pataky (2004) stated that the management ofthis disease could be done in integrated manner, including the use of resistance cultivar, eradication and exclusion. Some research on biological control against this pathogenhas been conducted.

Bacillus sp. and *Pseudomonas* sp. are widely known to have potential as biological control agents in inhibiting several kinds of plant pathogens (Cook and Baker, 1996).*Bacillus* sp. and *Pseudomonas* sp. are known to have an ability in competing over nutritions, and producing secondary metabolites such as antibiotics, siderophores, bacteriocin and extracellular enzymes. The aim of this study was to elucidate the potential of *Bacillus* sp. and *Pseudomonas* sp. strains as biological control againstleaf blight disease caused by *Pantoea* sp.

Accredited SK No.: 81/DIKTI/Kep/2011

http://dx.doi.org/10.17503/Agrivita-2013-35-2-p103-109

MATERIALS AND METHODS

The experiment was conducted in Plant Pathology laboratory and glass house of Agricultural Faculty University of Brawijaya from September 2012 toFebruary 2013. Bacterial strains used in this study were *Pantoea* sp. strain KD1A, *Bacillus* sp.Strainsof UB-ABS1, UB-ABS4, UB-ABS5 and *Pseudomonas* sp. strains of UB-PF1, UB-PF3 and UB-PF4, the collections of Plant Disease laboratory, Faculty of Agriculture, University of Brawijaya. Other materials used in this study were sweet corn seed cultivar Jago (East West Seed, Indonesia), nutrient agar medium, King's B medium, bactericide (streptomycin sulphate 20%) and chloroform. The tools involved to support this experiment were petridish, analytic balances, hand sprayer, spectrophotometer vortex mixer, orbital shaker and micropipette.

Antagonistic Assays of *Bacillus* sp. and *Pseudomonas* Sp. Against *Pantoea* sp. in Agar Plate

The antagonisticassay was conducted by spray inoculation methods (Kawaguchi *et al.*, 2008). Cell suspensions of all strains of *Bacillus* sp. And *Pseudomonas* sp. were prepared from 48 hours cultures on nutrient agar medium, and adjustedat about 10^9Colony Forming Unit (CFU)/mL using spectrophotometry. A sterile paper disk (5 mm diameter) was dipped in the bacterial suspension for one minute and air dried for two hours. The paper disk was then, put on nutrient agar plate in Petridish and incubatedfor two days at 27^{o}C. Then the *Bacillus* sp. and *Pseudomonas* sp. were killed by putting 1 mL chloroform on the lid of petridish in the reversed position. The plates were then misted with a suspension of *Pantoea* sp. (approximately 10^9 CFU/mL) by using hand sprayer and werethen incubated for two days. The diameter of clear zone around the disk indicating the antibiosis activity was measured. The clear zone index was measured according to the method described by Sugiyono *et al*. (2008).

Characterization ofthe Type of Antibiosis Produced by Antagonist

A part of clear zone area was taken from the agar plate an then dipped in 10 mL of 0.05 % pepton solution in the test tube. The tube was then shaked for 24 hours. If the pepton solution was still clear, then the type of antibiosis was bactericidal, whereas if the solution became turbid then the type of antibiosis was bacterio-static (Djatmiko *et al*., 2007). Futher analysis was done by growing all strains of *Bacillus* sp. and *Pseudomonas* sp. in 10 mL Nutrient Broth medium at 27^{o}C in rotary shaker at 150 rpm for 24h,48h and 72h. The cells were harvested by centrifugation at 10,000 rpm for 10 minutes and the culture supernatant was sterilized by filtration with 0.45 µm milipore membranes (Dismic-25cs, Advantec, Tokyo). The super-natant was heated at 100 ^{o}C and then filled in the well (0.5 mm diameter) of nutrient agar plate containing *Pantoea* sp. at density of 10^9 CFU/mL. If the clear zone was developed, it suggests that antibiosis substance was the typical of antibiotic which is commonly resistant to heat treatment. If the clear zone was not developed, the type of antibiosis substance was enzyme, sidephore or bacteriocin which were composed from protein (Awais *et al*., 2010; Motta *et al.*, 2008; Bizani and Brandelli, 2004).

Potential of *Bacillus* sp.and *Pseudomonas* sp.in Controlling Leaf Blight Diseasecausedby *Pantoea* sp.

Fourteen days old corn plants (four-leaf stage) were inoculated with the suspensions of *Bacillus* sp. or*Pseudomonas* sp. at the density of 10^9 CFU/mL. Bacterial suspension was inoculated evenly by spraying ca. 5 mL/plant on leaves using hand sprayer. The next day, the leaves were inoculated with the suspension of bacterial pathogen *Pantoea* sp. After the inoculation, the plants were incubated in the plastic chamber for 12 h at 23^{o}C and 90%relative humidity (RH). After that the plants were moved to glass house with the average of temperature and humidity of 27^{o}C and 60% RH, respectively. The intensity of blight disease was evaluated on day 7 after inoculation of the pathogen by scoring method according to Lee and Hong (2010).

The population of *Bacillus* sp. or *Pseudomonas* sp. was also counted on day 7 after inoculation. Corn leaf discs were collected from three parts of the leaf i.e. the top, the center and the base of leaf using cork borer (diameter 0.8 mm) and put in the 15 mL test tube containing 10 mL of sterile potassium phosphate buffer.

The tubes were then vortex mixed thoroughly for 10 minutes. The supernatant was

then subjected to serial dilution and plated either on nutrient agar medium or King's B agar medium for viable plate counting method modified from Beattie and Marcell. (2002).

Statisticals Analysis

The data from the observations of antagonistics assay on agar plate, intensity of leaf blight disease on corn, and the estimationof bacterial population on leaves were analyzed with analysis of variance (ANOVA) using Microsoft Excel software and continued with Duncan multiple range test (DMRT) on 0.01 confidence level.

RESULTS AND DISCUSSION

The Antagonistic Potential of *Bacillus* sp.and *Pseudomonas* sp. against *Pantoea* sp. in Agar Plate Assay

Based on the result of the antagonistics assays on nutrient agar plate, all bacterial strains as well as streptomycin sulphate could inhibit the growth of *Pantoea* sp. There was no clear zone developed in the control (aquades only) *Bacillus* sp. and *Pseudomonas* sp. showing the antagonistic activity at the similar level or even higher than streptomycin sulphate (Figure 1).*Bacillus* sp.strains of UB-ABS4 and *Pseudomonas* sp. strains of UB-PF3 showed higher antagonistics activity compared to the other treatments.

In the antagonistic assay on agar plate, the developed clear zone was an indicator of antibiosis activity produced by bacterial antagonist against pathogens (Figure 2). It has been reported that *Bacillus* sp. and *Pseudomonas* sp. produced some metabolit compound in the form of antibiotics, siderophores, bacteriocins or enzymes which formed the clear zone when inoculated on the agar medium against other pathogenic bacteria (Sood *et al.*, 2007). Salerno and Sagardoy (2003) reported the similar results on antagonistic assay of *X. campestris* pv. *glycines* by antagonistic bacterium *Bacillus subtilis,* which could produce the clear zone from 6 to 7 mm in diameter.

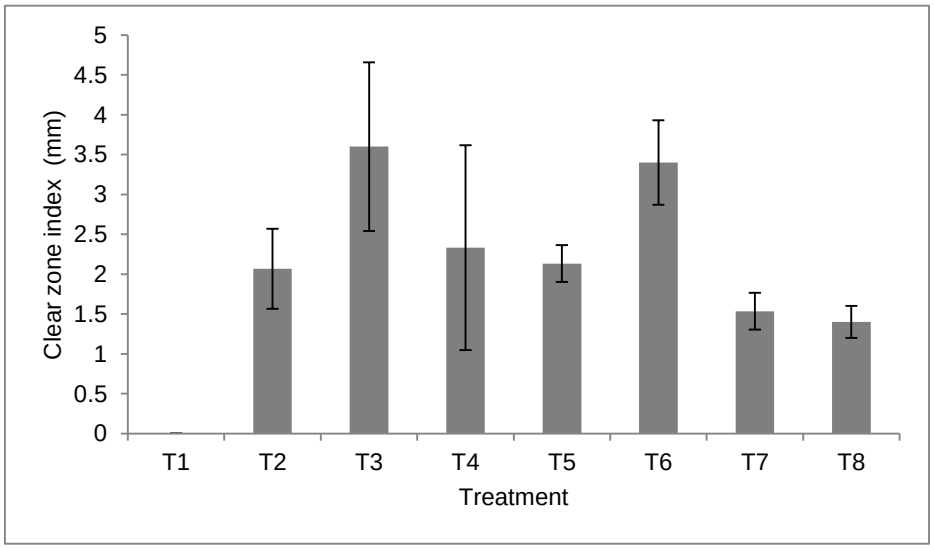

Figure 1. Clear zone index of antagonistic activity against *Pantoea* sp. T1 represents steril aquades, T2 represents streptomycin sulphate, T3 represents *Bacillus* sp*.* strain UB-ABS4, T4 represents *Bacillus* sp*.* strain UB-ABS5, T5 represents *Bacillus* sp*.* strain UB-ABS1, T6 represents *Pseudomonas* sp*.* strain UB-PF3, T7 represents *Pseudomonas* sp*.* strain UB-PF4, T8 represents *Pseudomonas* sp*.* strain UB-PF1. The error bars indicate the standard deviations.

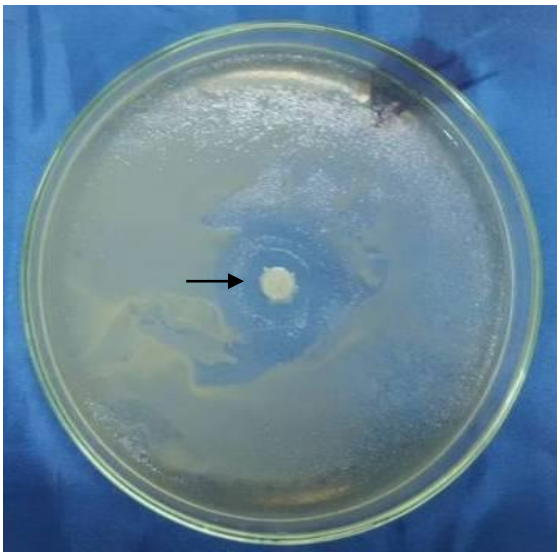

Figure 2. The representative result of antagonistic assay on agar plate *Bacillus* sp. strain UB-ABS4 against *Pantoea* sp. The arrow indicates the clear zone around the paper disk

Characterization of Antibiosis Type of Bacterial Antagonists

Two action modes of antibiosis produced by bacterial antagonist were shown in Table 1. Two strains of *Bacillus* sp. i.e. UB-ABS1 and UB-ABS4 showed bactericidal activity, whereas *Bacillus* sp. strain UB-ABS5 and all strains of *Pseudomonas* sp. showed bacteriostatic activity. The type of antibiosis i.e. bactericide or bacteriostatic could be influenced by the amount of antimicrobial substances released by antagonists. The type of antibiosis is bactericidal if the amount of antimicrobial compound released by antagonist is high which is able to kill and stop the vegetatif growth of thecell ofpathogens. However when the antimicrobial compound is in low amount, the mode of action will be bacteriostatic which just inhibits the vegetative growth of the pathogens (Pankey and Sabath, 2004).

Bacillus species is largely known to be capable of producing antibiotic as well as other antimicrobial compound i.e enzymes or bacteriocin in higher concentration which could result in the killing of other bacteria or microbes. Motta *et al*. (2008) reported that *Bacillus* species produced bacteriocins such as tochicin, lichenin and thuricin with different mode of action which had bactericidal effect, whereas subtilisin, an antibiotic produced by *Bacillus* sp. inhibited vegetative cell by bacteriostatic mechanism. Antagonistic *Pseudomonas* sp. such as *Pseudomonas fluorescens* is widely known to be able to produce siderophore as its mechanism in inhibiting other microbes. Siderophores are the protein substances that function in chelating metal ionic compound such as Fe^{2+} in the surrounding of bacterial cells, resulting in the unavaibility of metal ion required by other microbes growth. Thus, the mode of action of siderophore indirectly affects the growth of other microbes surrounding the bacterial antagonist, in which this is the typical of bacteriostatic (Diaz*et et al.*, 2002). Djatmiko *et al.*(2007) reported that *Pseudomonas fluores-cens* and *Bacillus* sp. could control plant pathogenic bacteria *Ralstonia solanacearum,* the causal agent of lincat disease on tobacco by bacteriostatic manner.

Tabel 1. The Antibiosis types of bacterial strains of*Bacillus* sp. and *Pseudomonas* sp.

Bacterial strain	Typesof antibiosis	Inhibition activity after heat treatmentof filtrate of antagonist cultured at different times		
		24h	48h	72h
Bacillus sp. strain UB-ABS4	Bactericide	+	+	+
*Bacillus*sp. strain UB-ABS5	Bacteriostatic	-	-	-
*Bacillus*sp. strain UB-ABS1	Bactericide	-	-	-
*Pseudomonas*sp. strain UB-PF3	Bacteriostatic	+	+	-
*Pseudomonas*sp. strain UB-PF4	Bacteriostatic	-	-	-
*Pseudomonas*sp. strain UB-PF1	Bacteriostatic	+	+	+

Based on the heat treatment assay of filtrate of bacterial culture showed in Table 1, itsuggests that antibiosis compounds produced by *Bacillus* sp. strain UB-ABS5 and UB-ABS1 and *Pseudomonas* sp. strain UB-PF4are composed from protein since its antibiosis activity was affected after heat treatment at 100^{o}C. Protein is widely known to be unstable after heat treatment due to the occurence of protein denaturation. Several protein based antibiosis compounds produced by antagonistic bacteria are enzyme, bacteriocin or toxin (Karimi *et al.*, 2012). Antibiosis compounds produced by *Bacillus* sp. strain UB-ABS4 and *Pseudomonas* sp. strain UB-PF3 and UB-PF1 are stable after heat treatment assay.Thus, it can be concluded that anti microbial compound produced by those strains are type of antibiotic known to be more stable when treated with heat. On *Pseudomonas* sp. strain UB-PF3, the clear zone was lost after 72 h culture whereas at 24 h and 48 h culture the clear zone still appeared. This result was probably caused the antimicrobial compound produced by *Pseudomonas* sp. strain UB-PF3 which stopped and decreased after 72 hour culture incubation. Many factors could damagethe secondary metabolism compounds released by microbes such as pH, temperature, media growth, carbon source and enzyme concentration (Bizani and Brandelli, 2004). It suggests that at 72 hour culture, the condition in the medium had changed such as pH thus causing the decrease of antimicrobial compound released by *Pseudomonas* sp. strain UB-PF3.

The Effect of Bacterial Antagonist Application on Blight Disease on Corn Leaves

The application of all strains of *Bacillus* sp. and *Pseudomonas* sp. could reduce the intensity of leaf blight disease caused by *Pantoea* sp. at 18%-24% compared to that of control (Figure 4).

The ability of bacterial antagonists to reduce the intensity of blight disease was comparable to that of bactericide Streptomycin sulphate. This result is in line with the work of Zeller (2006) which reported that the application of *Pseudomonas fluorescens* A506 and *Bacillus subtilis* BsBD 170 could reduce the fire blight disease intensity at 40-60% after artificial infection of *Erwinia amylovora*.

Antagonistic bacteria could form micro-colonies, a dense population of antagonist bacteria on the leaf surface. The existence of antagonistic microcolinies could inhibit the infection by pathogenic bacteria by releasing, antimicrobial compounds surrounding the micro-colonies. Hence, the intensity of infection by pathogenic bacteria could be reduced (Beattie and Lindow, 1999). Reduction of the disease intensity of leaf blight disease caused by *Pantoea* sp. on corn plant may have similar manner where the antagonistic bacteria were able to form micro colonies on corn leaf surface and then antimicrobial compounds released by microco-lonies can protect the leaves against the infection of *Pantoea* sp. Salerno and Sagardoy (2003) reported that *Bacillus subtilis* could control pustule disease caused by *X. campestris* pv. *glycines*in glass house experiment because *B. subtilis* could establish colonization on soy bean leaves and led to decreasing the intensity of pustule disease. Similar result was also reported by Saravanan *et al.* (2013) that *Pseudomonas fluorescens* and *Pseudomonas putida* were shown to be succesful in serving as biocontrol of several plant pathogens by producing siderophore.

Bacillus sp. and *Pseudomonas* sp. survived on day 7 after inoculation (Table 2). The population of *Bacillus* sp. and *Pseudomonas* sp. was high at the average of 10 million cells per cm^2 on day 7 after inoculation, suggesting that all bacterial antagonists have high epiphytic fitness when applied on corn leaves. Survival is the ability of organisms to cope with the varied environmental stress conditios, including fluctuating water ability, heat, osmotic stress and exposure to solar ultra-violet radiation. It was reported that *Pseudomonas* sp.was commonly found in thephyllosphere communities (Lindow and Brandl, 2003). *Bacillus* sp. was also reported that it could survive by producing spores that enhanced their epiphytic fitness (Jacobs and Sundin, 2001).

Table 2. The population of bacterial antagonists on corn leaves

Bacterial strain	LOG (CFU/cm^2)*
Bacillus sp. strain UB-ABS4	7.49
Bacillus sp. strain UB-ABS5	7.66
Bacillus sp. strain UB-ABS1	7.54
Pseudomonas sp. strain UB-PF3	7.43
Pseudomonas sp. strain UB-PF4	7.34
Pseudomonas sp. strain UB-PF1	7.28

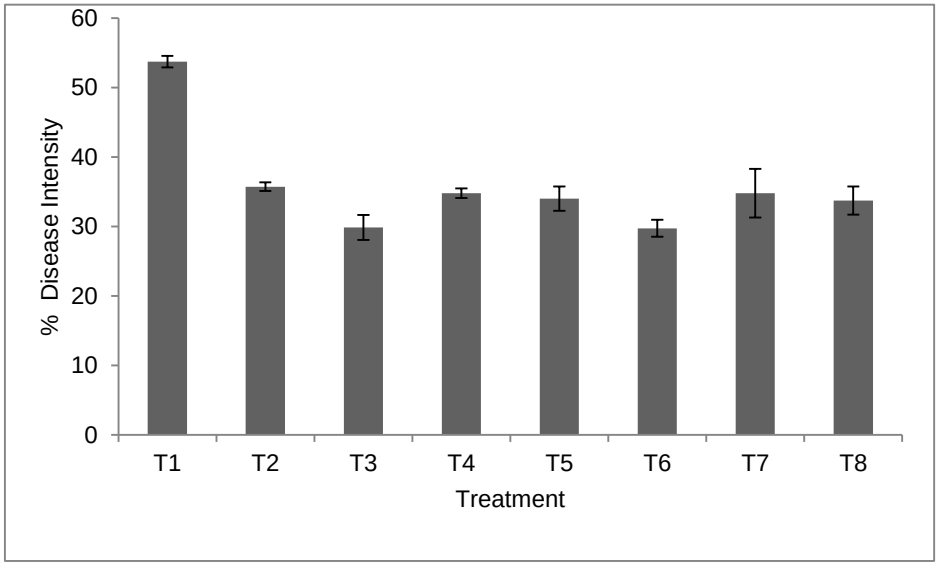

Figure 4. Intensity of blight disease on corn leaves caused by *Pantoea* sp. The error barsindicate the standard deviations

CONCLUSION

Based on antagonistic assay on agar plate and blight disease assay in planta, it is concluded that all strainsof *Bacillus* sp.and *Pseudomonas* sp. have potential in controlling corn leaf blight disease by *Pantoea* sp.,bactericide and bacterio-static manner. Strains of *Bacillus* sp.and *Pseudomonas* sp.were proven to be able to survive on corn leaves and contribute to reducing the intensity of corn leaf blight caused by *Pantoea* sp. at 18-24%.

ACKNOWLEDGEMENT

This research was funded by BPKLN DIKTI and Mayze Research Center Brawijaya University.

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