

SEED-DIPPING APPLICATION OF LOCAL ENDOPHYTIC BACTERIAL CONSORTIUM AGAINST BACTERIAL LEAF BLIGHT OF RICE

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ABSTRACT

SEED-DIPPING APPLICATION OF LOCAL ENDOPHYTIC BACTERIAL CONSORTIUM AGAINST BACTERIAL LEAF BLIGHT OF RICE. Bacterial leaf blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most important diseases of rice, and to date no effective control measure yet. Alternative controls by several antagonistic microbes have been used to suppress bacterial infection. The aim of this research was to investigate antimicrobial effect of bacterial consortium as the primary step to control bacterial blight of rice. A total of five endophytic bacterial consortiums as biocontrol agents were developed and further evaluated for their capability in reducing bacterial blight of rice under screen house condition. The bacterial consortium was applied by seed dipping method using bacterial suspension prior to transplanting. The result showed that bacterial consortia were able to inhibit bacterial blight disease development both in vitro and in vivo test. Consortium labeled as C 1 (containing isolates *E 64, *E 65, *E 66, *C 1A, *C 29D) and C 2 (containing isolates *E 64, *E 65, *E 76, *C 1A, *CPKKP 3.5) showed higher bacterial blight percent reduction by two seed-dipping treatments of 24 h and 48 h incubation time, whilst C 4 and C 5 only effective by seed-dipping of 72 h incubation; however none of these consortium affected plant height as well as yield. It was indicated that C 1 and C 2 consortium have relatively high antagonistic activity than that of control treatment and could be applied further to reduce bacterial blight disease development on rice.

Key words: *Xanthomonas oryzae* pv. *oryzae*, endophytic, rice, bacterial consortium, seed-dipping application

INTRODUCTION

Rice represents an important food crop in Indonesia and its productivity tends to leveling off due to several factors. Bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) was reported as one of the important major diseases of rice causing heavy losses, particularly in tropical rice countries, including Indonesia (Mew, 2004).

In the period of 2007-2009, bacterial blight disease incidence and severity was reported in several region and districts throughout the main islands in Indonesia causing average damage of 77,646 Ha annually (Anonymous, 2009). *Xanthomonas oryzae* pv. *oryzae* has several alternate host including weeds, hence the pathogen could survive under unsuitable condition in the field. The pathogen can infect rice plants by entering through stomata, hydrotodes or wounding (Ou, 1985). The bacteria multiplied in intracellular space and colonized xylem vessels (Suryadi and Machmud, 1990; Kaku, 2005).

Many efforts to control bacterial blight disease have been evaluated and used in the field by integrating cultural practices, planting resistant varieties and chemicals control. Although these measures

were effective, no single measure was effective to reduce bacterial blight disease development in the field. The use of biocontrol agents as environmentally safe microbial antagonists has been interested to reduce bacterial blight infection. Considerable attention has been given in evaluating the effectiveness of several endophytic bacteria in reducing bacterial blight infection on rice leaves. Many alternative studies on application of bacterial antagonist as biocontrol agent to reduce several plant pathogens have been reported and reviewed (Cook and Baker, 1996; Sakhtivel *et al.*, 2001; Gnanamanickam, 2009).

In the glass house experiment, it was reported that four time foliar sprays application using single biofungicide formulation of *Bacillus subtilis* (10^6 - 10^7 CFU/ml) on tea leaves infected by *Colletotrichum theae-sinensis* could effectively controlled the pathogen (Kim *et al.*, 2009). Use of *B. subtilis* was also reported effective against citrus disease (Singh and Deverall, 1984). Application of combination biocontrol agents may improve its effectiveness, increase level of protection and suppress multiple plant diseases (Singh and Deverall, 1984; Duffy and Weller 1995; Jetiyanon and Kloepper, 2002; Guetsky *et al.*, 2002).

Plant-microbe interactions have been studied at ecological, biological and biochemical levels, but

more complex interactions comprised of more than two biological components have received less attention (Guetsky *et al.*, 2002). To date, although research of biocontrol application has been established, limited studies on bacterial consortium using local isolates have been reported on suppressing of *Xoo* in Indonesia; hence application of their potential in reducing pathogen should therefore be investigated.

The aim of the research was to screen endophytic bacteria from indigenous isolates for their capability to inhibit major rice diseases, particularly bacterial blight of rice, so that it can provides more data information in terms of bacterial endophytic effectiveness and variability. This paper reported potential use of several local endophytic bacteria and development of bacterial consortium against bacterial blight of rice under controlled environment test (screen house condition).

MATERIALS AND METHODS

Bacterial Isolation and Cultivation

For detailed of the study, isolates were obtained from Indonesian Center of Biotechnology and Genetic Resources R&D (ICABIOGRAD) Culture Collection. General media for bacterial cultivation used were Nutrient Broth (NB), Nutrient Agar (NA), and Wakimoto Agar (WA). Samples isolates of bacteria were collected from various sources and locations (Table 1). These isolates had been characterized for various purposes such as biofertilizer as well as bio-control agents; for instance the isolates C 1A, C 29D and CPKKP 3.5, E76 showed chitinolytic activity by producing chitinolytic index of 2.19; 5.88, 2.43, and 0.98, respectively (Susilowati *unpublished*).

Bacterial colonies were obtained through a serial dilution by streaking on NA plates supplemented with 0.5% antibiotic Streptomycin. Each of isolates was inoculated into NB media on rotary shaker at 125 rpm (Stuart Scientific SI 50) which was incubated for 24-h at 37°C. The single pure bacterial colony each was cultured on NA slant and incubated for 48 h at room temperature.

In Vitro Assay

Culture broth containing endophytic bacteria (10 ml) was centrifuged at 10,000 rpm for 15 minutes (Eppendorf centrifuge 5410). The optical density was adjusted to bacterial concentration of 10^7 CFU/ml using spectrophotometer (Hitachi 150-20). The isolates for the consortium development were selected by specific criterion based on the previous test, such as ability to produce clearance zone and its efficacy to reduce pathogen (Suryadi *et al. unpublished*). A total of five bacterial consortiums was developed using combination of five to six isolates (Table 2).

Table 1. Source of endophytic bacterial isolates used in the study

No	Bacterial species	Code isolates	Source of origins	Years of collection
1	<i>Bacillus sp</i>	E 64	Rice, Cikembar, Sukabumi - W. Java	2004
2	<i>B. firmus</i>	E 65	Rice, Cikembar, Sukabumi - W. Java	2004
3	<i>Bacillus sp</i>	E 66	Rice, Cikembar, Sukabumi - W. Java	2004
4	<i>Burkholderia sp</i>	E 76	Rice, Cikembar, Sukabumi - W. Java	2004
5	<i>Citrobacter sp</i>	C 1A	Sugarcane, Kb Agung, Malang - E. Java	2007
6	<i>Klebsiella sp</i>	C 29D	Soil, Kenjeran, Surabaya - E. Java	2007
7	<i>Pseudomonas aeruginosa</i>	CPKKP 3.5	Soil, Pantai kukup, - Yogyakarta	2007

Notes: All bacterial isolates used were obtained from ICABIOGRAD microbial germplasm culture collection These isolates have been characterized and identified based on 16S rRNA sequencing. (Susilowati *unpublished*).

Table 2. Treatment of isolates selected in forming the bacterial consortium to control bacterial blight of rice

No	Treatments code	Isolates combination/consortium
1	C 1	*E 64,*E 65,*E 66, *C 1A, *C 29D
2	C 2	*E 64,*E 65,*E 76, *C 1A, *CPKKP 3.5
3	C 3	*E 64,*E 65,*E 66, *C 1A, *CPKKP 3.5
4	C 4	*E 64,*E 65,*E 76, *C 1A, *C 29D, *CPKKP 3.5
5	C 5	*E 65,*E 76, *C 1A, *C 29D, *CPKKP 3.5
6	Chemical control (streptomycin sulphate)	-
7	Untreated control (sterile water)	-

A loopful of the selected isolates was individually inoculated into NB medium for 24 h to form bacterial consortium. A 20% (v/v) aliquots of the culture was mixed and transferred into 250 ml Erlenmeyer flask containing 100 ml NB medium and allowed mixture to grow under agitated condition (125 rpm) using a rotary shaker (Stuart Scientific SI 50).

Antagonistic activity was tested using diffusion agar method (Madigan *et al.* 2000). A 100 µl *Xoo* bacterial consortium (24-h old grown on WA) was spread on NA plates, then 3 MM Whatman filter paper disc (diameter 5 mm) dipped in bacterial consortium (10^7 CFU/ml) was placed on top of surface media plates containing *Xoo*. The plates were incubated for 24-48 h at room temperature. Observations were done by measuring clearance zone.

Effect of Consortium in Reducing Bacterial Blight in Screen House Test

Selected bacterial isolates were developed to established consortium and used to inhibit bacterial blight disease development under screen house test. All of bacterial consortiums were mixed following treatments with ratio 1:1 (v/v), and they were grown on NB media agitated at constant speed (125 rpm) using rotary shaker (Stuart Scientific SI 50). Cells were harvested and centrifuged briefly at 6400 g for 5 min at room temperature to release bacterial filtrates. Bacterial concentration was measured using McFarland standard (10^{7-8} CFU/ml).

Rice seeds cultivar (cv.) IR 64 were dipp-inoculated within bacterial consortium suspension for 24 h, 48 h and 72 h at room temperature, respectively; then planted in plastic pots (diameter 30cm) containing rice soil from Muara Experiment Station, Bogor, West Java, Indonesia. Plants were inoculated with *Xoo* race VIII (10^7 CFU/ml) at maximum tillering stage using clipping method. Each treatment was carried out in four replications. Sterilized water was used for the negative control.

Data analysis and observation

Disease progress curve of bacterial blight of rice was measured based on lesion length at different period of observation (1 week interval) until

harvesting time. Area under disease progress curve (AUDPC) was calculated as described by Shaner and Finney (1977) using formula $AUDPC = \sum_i (X_i + X_{i+1})/2 (t_{i+1} - t_i)$; where X_i = bacterial blight disease intensity (measured as lesion length), t_i = observation time i , and n = total number of observations. The percent of control reduction was calculated using formula $I = (a-b)/a \times 100\%$, where I = percent control reduction, a = AUDPC in control (untreated), b = AUDPC of treated. Data were analysed using analysis of variance (ANOVA) and significance different was compared using Duncan multiple range test (DMRT) at $P=0.05$.

RESULTS AND DISCUSSION

Several strains of the genus *Bacillus* received much attention for biocontrol agents (Jamalizadeh *et al.*, 2009). In this study used of mixed cultures or consortium containing bacterial genera of *B. firmus* E 65, *Bacillus* sp. E 66, *Bacillus* sp. E 76 as well as other genera such as *Citrobacter* sp. C 1A, *Klebsiella* sp. C 29D and *Pseudomonas aeruginosa* CPKKP 25 may inhibit bacterial blight disease progress on rice cv. IR 64.

A total of seven endophytic bacterial isolates were evaluated for biocontrol effectiveness of *Xoo* and some isolates that showed higher inhibition zones on agar plates were chosen for bacterial consortium development. Five bacterial consortiums showed *Xoo* clearance zone on WA media suggesting the presence of antibacterial activity (Table 3).

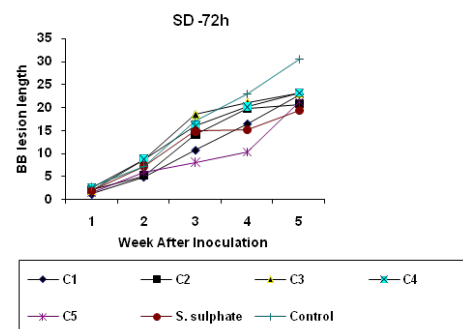
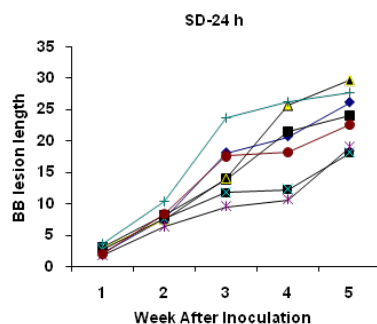
Table 3. Effect of bacterial consortium against *Xoo* under *in vitro* test

Treatment	clearance zone
C 1 + <i>Xoo</i>	+
C 2 + <i>Xoo</i>	++
C 3 + <i>Xoo</i>	++
C 4 + <i>Xoo</i>	++
C 5 + <i>Xoo</i>	++
Chemical control (Streptomycin sulphate) + <i>Xoo</i>	+++
Untreated control (sterile water) + <i>Xoo</i>	-

Notes: -= no inhibition zone, += inhibition zone 1-2 mm; ++ = inhibition zone 3-5 mm, +++ = inhibition zone 6-10 mm.

Results of the *in vitro* test showed that all consortium could inhibit *Xanthomonas oryzae* pv. *oryzae* growth ranging from 1.5 mm to 4.0 mm. The inhibition zone was less than that of standard chemical control. The presence of inhibition zone has been used as the evidence of antibiotic production by bacteria (Crawford *et al.*, 1993). This inhibition zone was presumably due to several mechanisms produce by bacterial metabolites. Rate of inhibition effect could increase if more bioactive compound presence in filtrates (Nalisha *et al.*, 2006).

Under *in vitro* test, consortium treatments C 2, C 3, C 4, and C 5 were effective in suppressing *Xoo* growth causing diameter of inhibition zone ranging from 1.5 mm to 8.0 mm. Among the consortium tested using seed-dipping application for 24 h and 48 h; consortium C 1 and C 2 were the most effective treatment to reduce *Xoo* under screen house condition. In this study, disease severity as indicated by lesion length was ranging from 1.0 cm at initial observation to 31.0 cm at final observations. The mixture of bacterial isolates treated as bacterial consortium, can reduced disease progress of bacterial blight severity as shown by lower lesion length (Figure. 1).

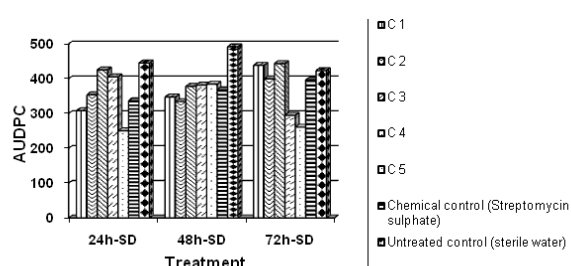
**Figure 1.** Effect of seed-dipping application on bacterial blight lesion length at weekly observation

The effectiveness of bacterial consortium to reduce bacterial blight lesion length ranged from 1.0 cm to 29.6 cm, whilst control (unprotected) showed bacterial blight lesion length ranged from 2.0 cm to 30.5 cm. The efficacy results of endophytic bacterial consortium against reduction of bacterial blight disease development on rice cv. IR 64 was shown in figure 2. Disease progress curve for bacterial blight of rice as indicated by AUDPC value was ranging from 250.25 to 491.75. In case of the combination C 1 and C 2 consortium treatment, applied as seed-dipping 24h and 48h showed relatively stable in reducing bacterial blight AUDPC compared to other treatments, whilst C 4, C 5 consortium only effective reduce bacterial blight AUDPC using seed-dipping 72h. The presence of the inhibition or reduction of bacterial blight disease progress might be attributed to metabolite activity produced by consortium bacterial community that complemented to each other, or by producing competitive mechanism.

Table 4. Effect of seed-dipping application of bacterial consortium to rice agronomical characters.

Treatment	Plant height (cm)*			Seed dry weight (gr)		
	SD-24 h	SD-48 h	SD-72 h	SD-24 h	SD-48 h	SD-72 h
C 1 + Xoo	95 ^{ns}	101 ^{ns}	110 ^{ns}	45 ^{ns}	45 ^{ns}	50 ^{ns}
C 2 + Xoo	95	110	95	55	45	50
C 3 + Xoo	95	96	90	50	50	55
C 4 + Xoo	105	105	100	50	52	50
C 5 + Xoo	95	99	105	50	50	60
Chemical control (Streptomycin sulphate) + Xoo	110	100	103	52	50	51
Untreated control (sterile water) + Xoo	107	110	105	52	50	56

Notes: * means from 10 replications, ns= not significant based on DMRT (P=0.05).

**Figure 2.** Effect of seed-dipping application using bacterial consortium on bacterial blight area under disease progress curve (AUDPC) on rice cv. IR 64.

Control efficacy was calculated in order to evaluate its protective application compared to that of untreated control. Two combinations consortium C 1 (containing isolates *E 64,*E 65,*E 66,*C 1A,*C 29D) and C 2 (containing isolates *E 64,*E 65,*E 76,*C 1A,*CPKKP 3.5) were found more effective in inhibiting bacterial blight disease development compared to other treatments. The preventive application of C 5 consortium was higher using seed-dipping of 24 h and 72 h, respectively; even higher than that of chemical control.

Plants produce chemical substances in response to pathogen infection that induce defense responses and prevent of potential pathogens attacks; for instance it was reported that rice leaves excrete phenolic compounds with antimicrobial activity that may ultimately reduced pathogen infection (Horino and Kaku, 1988). The lower bacterial blight disease infection at later stage of rice maturity might also due to synergistic effect of each endophytic isolates that complement to each other. The bacterial endophytes were reported to produce several mechanisms such as antibiotics, enzyme biodegradation, secondary metabolites (hormones) as well as competition in terms of nutritional factors (Cook and Baker, 1996; Hwang *et al.*, 2001; Neeno-Eckwall, 2001; Jacobsen *et al.*,

2004). Several microbial populations acting alone or together was reported inhibit activity of *Fusarium* wilt pathogen (Alabouvette *et al.*, 1998).

Results of antagonistic test in this study under screen house condition showed that culture filtrates of bacterial endophytes labeled as consortium C 1 and C 2 could reduce bacterial blight disease development on rice cv. IR 64. In another biocontrol experiment, it was reported that use of biomass rather than culture filtrates was more effective against *Pythium ultimum* (Yuan and Crawford, 1995).

The evidence disease reduction caused by C 1 and C 2 consortium may categorized effective and could be applied further for biocontrol agents against bacterial blight of rice. The effectiveness of biocontrol agents required several factors i.e. capability in colonizing the pathogen, capability to reduce disease symptoms as well as capable of interacting with other microorganisms (indigenous isolates) (Cook and Baker, 1996).

A higher degree of biocontrol can be expected when co-metabolic activities within a microbial community complement to each other. In such consortium the microorganism can act synergistically, or one microorganism may be able to cause competition or stimulated metabolite synthesis of antagonist (Wilson and Backmann, 1999). An example of using a mixed culture containing several isolates for biocontrol of *Fusarium* wilt has been reported by Akhtar *et al.* (2010). It was also reported that several secondary metabolites such as streptomycin, neomycin, tetracycline, and nistatin can act as antibacterial substances (Madigan *et al.*, 2000; Mc-Manus *et al.*, 2002). In Indonesia, frequently streptomycin was used to control plant pathogens. It was shown from this study that streptomycin sulphate showed similar effect with some of consortium treatments.

This study indicates that consortium of bacterial isolates have demonstrated protective activity against bacterial blight of rice caused by *Xanthomon-*

as *oryzae* pv. *oryzae*. The *Bacillus* isolates varied in biocontrol potential as inline with other worker who found similar variation in effectiveness when *Bacillus* spp was sprayed onto apple fruits (Jamalizadeh *et al.*, 2008).

The effect of seed-dipping application of bacterial consortium towards agronomical characters is shown in Table 4. It was shown that none of the treatment showed significant effect on plant height and seed dry weight of rice.

The seed-dipping applications were proven to be a simple effective treatment for controlling bacterial blight of rice. Overall, the present study indicates that the advantages of using mixed cultures such as C 1 and C 2 consortium are apparent, and further can be selected and exploited as bacterial endophytes consortium which will be beneficial for controlling bacterial blight infection in the field. Such experiment required more attention and need to be evaluated further in the field.

CONCLUSION

Evaluation of the bacterial consortium in reducing *Xoo* under limited control environment in the screen house showed various effectiveness. Consortium labeled as C 1 (containing isolates *E 64,*E 65,*E 66, *C 1A, *C 29D) and C 2 (containing isolates *E 64,*E 65,*E 76, *C 1A, *CPKKP 3.5) exhibited the highest effectivity in reducing bacterial blight infection by two seed-dipping treatments (24 h and 48 h incubation time), whilst C 4 and C 5 only effective by seed-dipping- 72 h incubation. None of these consortium affected plant height nor yield of rice.

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