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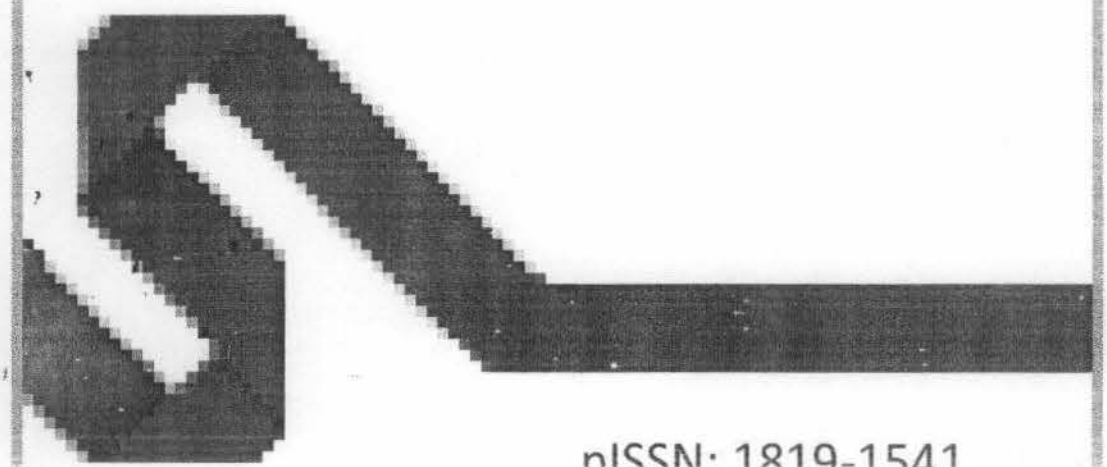

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Bioformulation of Antagonistic Bacterial Consortium for Controlling Blast, Sheath Blight and Bacterial Blight Diseases on Rice

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ABSTRACT

Among diseases affecting rice production, blast, sheath and bacterial leaf blight causes significant losses. This study was aimed to control multiple rice diseases using beneficial bacterial consortium as an alternative biocontrol strategies. *In vitro*, greenhouses and field test were carried out to study antagonistic effect of bacterial consortium against rice pathogens. The bacterial isolates used in single or in mixture combination were apparent to reduce sheath blight, neck blast and bacterial leaf blight under *in vitro* test. The suspension formulation mixture of A6 (*B. firmus* E 65, *P. aeruginosa* C32b, *B. cereus* II 14), A8 (*B. firmus* E65, *Serratia marcescens* E31, *P. aeruginosa* C32b, *B. cereus* II 14), talc based-A8 and animal compost granule-A8 had percentage inhibition to *P. oryzae* of 71.04, 79.19, 63.87 and 66.68%, respectively. *P. aeruginosa* C32a produced glucanolytic index of 2.26 and specific activity of 0.448 U mg^{-1}. In greenhouse test, the lowest intensity of neck blast was shown by A6 treatment. Bentonite formulation showed good effect in suppressing bacterial leaf blight lesion length in greenhouse test. The cell viability decrease was ranged from 2.39 to 18.30% among different bioformulations. Talc-A8 based formulation was stable at period of storage showing no viability lost. Talc-A5 (*Bacillus firmus* E65, *Pseudomonas aeruginosa* C32b) formulation was effective against sheath and bacterial leaf blight but showed lower effect on neck blast disease in the field. Further studies should focus on the evaluation of different carriers other than talc-based powder as stable and cheaper media, then scale-up possibilities for further applications.

Key words: Rice, bacterial antagonist, *R. solani*, *P. oryzae*, *X. oryzae* pv. *oryzae*, consortium, bioformulation

INTRODUCTION

Rice is susceptible to several bacteria and fungal diseases. Rice sheath blight (ShB) caused by *Rhizoctonia solani*, blast caused by *Pyricularia oryzae* and bacterial leaf blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) are the most serious diseases that affect rice production. Fungal infections may affect grain discoloration, change grain chemical and nutritional

characteristic and reduce germination (Gnanamanickam, 2009). Pathogenic fungi may grow rapidly through the production mycelium and spores. ShB and blast can attack rice plants at seedling stage until generative stage, causing poor crop growth and yield loss. Grain yield losses due to ShB and blast are estimated in the range of 5.2-69 and 11-50%, respectively (Akita *et al.*, 2005; Baker *et al.*, 1997). The total area infected by blast in Indonesia was estimated of 1,254 milion Ha or covering area of 12% (Utami, 2005). Bacterial leaf blight (BB) was known as a major bacterial rice disease throughout rice growing countries, including Indonesia. It was reported that BB disease severity was ranged from 10 to 95% (Kadir *et al.*, 2007). The symptom start at leaf tips then increases in length and width with a wavy margin, making lesions turn to whitish as the disease advances, then severely infected leaves tend to dry quickly. The bacterial pathogen infects the host plant at the maximum tillering stage resulting in 20-40% yields reduction in China (Ji *et al.*, 2008) and up to 50% in severe infection in lowland rice in Indonesia (Suparyono *et al.*, 2004).

Disease control may be accomplished with the use of cultural practices, resistant varieties as well as fungicide application (Vikal *et al.*, 2007). Current effort to control rice disease mostly is emphasized on the use of resistant varieties, however; the occurrence of pathogen strains that differ among seasons and among locations can cause resistance rice varieties cannot survive much longer. The use of chemicals were effective but still expensive and the overuse of chemical pesticides had caused serious environmental problems and thus the need for the development of non-chemical alternative methods to control plant diseases is therefore necessary (Cook and Baker, 1983; Gnanamanickam, 2009).

Among the biocontrol agents, *Pseudomonas* spp. and *Bacillus* spp. had shown activities in suppressing fungal infection. Use of different bacterial species of *P. aeruginosa, S. marcescens, Bacillus* spp., *B. subtilis, P. fluorescens* and *B. firmus* E65 in biocontrol of plant pathogens have been reported by several workers (Someya *et al.*, 2000; Asaka and Shoda, 1996; Kim *et al.*, 2009; Suryadi *et al.*, 2011). *B. subtilis* and *P. fluorescens* have been successfully formulated and some formulations are available commercially for biological control of crop diseases (Jayaraj *et al.*, 2005; Mathivanan *et al.*, 2005). The formulations are very stable due to the ability of *Bacillus* bacterium to from spores (Emmert and Handelsman, 1999). The spores are long-lived and are resistant to heat or desiccation. To date, bacterial agent containing several additives such as peat and talc had been commercially developed as dry bioformulations (Mathivanan *et al.*, 2005; Gnanamanickam, 2009).

The use of several antagonist with different mode of action may improve biocontrol efficacy under wide range of environmental conditions (Grosch *et al.*, 2011); however; the success of biological control of plant diseases depends upon the availability of effective formulations of biocontrol agents, survival during storage as well as rapid multiplication and colonization after inoculation (Ashofteh *et al.*, 2009). In most of horticulture crops, the efficacy of biocontrol agent in suppressing foliar plant pathogen often less effective as compared to soil-borne plant pathogen. The inconsistent performance of the introduced agents on aerial plant parts need to modify the delivery system or supplementation of additives (Guetsky *et al.*, 2002; Ting *et al.*, 2009). At present, the most economical method for producing most economical biocontrol agents is using liquid culture fermentation (suspension) (Ashofteh *et al.*, 2009). However, this bioformulation need to be improved in terms of its efficacy as well as viability under field condition. *Bacillus* is a gram-positive bacterium producing endospores, resistant to dry conditions and heat. Thus, *Bacillus* suitable for applications in the field as a biological control agent (Mubarik *et al.*, 2010); while Gram-negative

bacteria *Pseudomonas* and *Serratia* can grow in simple media and easily colonizes in rhizosphere and phylosphere of rice plants. Carrier material formulations are widely used in biological control such as a solid form (granules), flour and suspension (Sridhar *et al.*, 1993; Ardakani *et al.*, 2009). As pointed out by Jayaraj *et al.* (2005), the use of talc or bentonite combined with bacterial antagonist may improve its efficacy in suppressing tomato damping off disease.

Aside of direct antagonistic activity against several soil-borne fungal pathogens, these bacteria were reported to promote plant growth and yield (Gnanamanickam, 2009). When the isolates are mixed with some other strains or other bacterial antagonists, the biocontrol efficacy is increased (Duffy and Weller, 1995). Multiple strain mixtures of microbial agents such as mixtures of fungi, mixtures of bacteria and combination of bacteria with fungi had been employed to enhance the consistency of control (Duffy and Weller, 1995; Schisler *et al.*, 1997; Nandakumar *et al.*, 2001). Karthikeyan *et al.* (2008) reported that application of three antagonist *P. fluorescens*, *B. subtillis* and *Trichoderma viride* tested alone and in combination (mixture) for suppression of onion leaf blight under greenhouse and field conditions showed disease suppression of 24.81 and 42.44%, respectively. The antagonistic activities of bacteria were mainly attributed to the production of antibiotic substances, most of which were dipeptides or cyclic peptide and also capable of producing certain volatile extracellular metabolites which have an antifungal activity (Fravel, 1988; Lisboa *et al.*, 2006). The potential use of bacterial strains could be attributed to their effect to secrete hydrolytic enzymes such as chitinase and β-1,3 glucanase in supernatant. The extracellular complex of β-glucanases was able to hydrolyze glucanes of microorganism cell wall biopolymers (Thrane *et al.*, 1997; Singh *et al.*, 1999; El-Katatny *et al.*, 2001). There was also evidence indicating that glucanase of several bacteria could be involved in induction of systemic resistance in plants (Collins and Jacobsen, 2003). In the previous study, the use of mineral carriers such as talc or bentonite together with antagonistic bacteria have been reported effective to control seedling damping-off disease on cotton plant (Ardakani *et al.*, 2009).

In large scale application, the formulation should allow maximum concentration of biomass and active products to be produced at a low price. The benefit use of bioformulations in this study may provide reasonable level of disease protection, particularly in organic or semi organic farming system where disease controls are limited.

The objectives of this study were to screen some antagonistic indigenous bacterial isolates (*Pseudomonas aeruginosa*, *Bacillus cereus*, *Seratia marcescens* and *B. firmus*) capable of suppressing rice pathogens, evaluate their bioformulation (talc, bentonite, animal compost granule) and efficacy against major rice diseases such as ShB, neck blast and BB.

MATERIALS AND METHODS

This study was carried out at Laboratory Microbiology and Green house of Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), field trials at Pusakanagara Experiment Station and Cianjur-West Java farmer's field during Dry Season (DS) of 2012. The bacterial isolates and pathogens used in the present study were obtained from the Biogen-Culture Collection (BiogenCC)-ICABIOGRAD (Table 1).

The bacterial antagonist culture was maintained on nutrient agar (NA) and King's B (KB) medium. These isolates were sub-cultured once a month and maintained until the end of the experiment. The whole culture of bacteria that had been incubated for 24 h on liquid media was counted using serial dilutions on 0.85% physiological saline and the bacterial cells number was calculated using total plate count method.

Table 1: List of bacterial and pathogens used in the study

Code of isolates	Species	Host/location	Sources origin	Date collection	IAA* (ppm)
Bacterial antagonist					
E 65	*B. firmus*	Rice Sukabumi, W. Java	BiogenCC	2004	21.0
E 31	*Seratia marcescens*	Rice Sukabumi, W. Java	BiogenCC	2004	20.2
C32b	*Pseudomonas aeruginosa*	Soil Mud Sidoarjo, East Java	BiogenCC	2004	12.1
II.14	*B. cereus*	Chili pepper Bogor, W. Java	IPBCC	2007	70.8
Pathogen					
Rs-SK	*R solani*	Rice Sukamandi, W.Java	BiogenCC	2006	
Po-Kn	*P. oryzae*	Rice Kuningan, W.Java	BiogenCC	2010	
Xoo-IV	*Xoo*	Rice Sukamandi, W. Java	BiogenCC	2006	

*IAA: Indole acetic acid, Biogen CC: Biotechnology of Sumberdaya genetic culture collection, IPBCC: Institute pertanian bogor culture collection

R. solani, *P. oryzae* and *Xoo* pathogen isolates used in this study were grown under Room Temperature (RT) 22°C and Relative Humidity (RH) of 80%. The fungus was maintained on Potato Dextrose Agar (PDA) medium or Oat Meal Agar (OMA) incubated for one week at room temperature; whilst *Xoo* bacterium was cultured using semi synthetic media Wakimoto agar.

***In vitro* test and preparation of bacterial cultures for bioformulations:** *In vitro* test was performed using dual culture method. Each bacterial consortium was grown on NA medium incubated for 24 h. One loopful of bacterial colony was streaked onto PDA plate, then pathogen tests were placed 3 cm away at the opposite of bacterial colony. The treatments were arranged with three replications, consisted of treatments labeled A1 (*B. cereus* II.14), A2 (*B. firmus* E65), A3 (*P. aeruginosa* C32b), A4 (*S. marcescens* E31), A5 (*B. firmus* E65+*P. aeruginosa* C32b), A6 (*B. cereus* II.14+*B. firmus* E65+*P. aeruginosa* C32b), A7 (*B. cereus* II.14+*P. aeruginosa* C32b+*S. marcescens* E31) and A8 (*B. cereus* II.14+*P. aeruginosa* C32b+*S. marcescens* E31+*B. firmus* E65). Untreated control and chemical standard were prepared using water and 2% Copper sulphate ($CuSO_4$), respectively.

The bacterial cells were harvested and centrifuged at 6000 rpm for 15 min and resuspended in phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted to approximately 10^8 Colony Forming Unit CFU mL^{-1} or Optical Density $(OD)_{595}$ = 0.3 using a spectrophotometer. The isolates were kept at -20°C until used. The inoculums was produced by transferring one loopful from that culture to 100 mL of Nutrient Broth (NB) or King's B (KB) media in a 250 mL Erlenmeyer flask and incubated at room temperature (28±2°C) on a rotary shaker at 150 rpm for 48 h. Later on, the broth containing±10^8 CFU mL^{-1} was used for the preparation of formulations. To the 400 mL of bacterial suspension, 1000 g of a purified talc, animal compost granule and bentonite powders, calcium carbonate ($CaCO_3$) 15 g (adjusted to neutral pH) and carboxymethylcellulose (CMC) 10 g were mixed under sterile conditions following the method described by Ardakani *et al.* (2009). The product was air dried to reduce the moisture content (less than 20%), then packed and sealed in polypropylene bags. The bacterial alone (without carrier) was prepared as suspension treatment. The bioformulations was stored in room temperature under continuous controlled room temperature and relative humidity (RT = 20°C; RH 80%).

The dual culture test against *R. solani*, *P. oryzae* and *X. oryzae* pv. *oryzae* (*Xoo*) was performed with three replications. Each formulation was suspended in NB medium, then 1 mL of suspension was centrifuged at 10.000 rpm for 5 min. A total of 100 mL supernatant was mixed with solid agar

medium on petri dishes. The pathogen was placed on medium containing bacterial supernatant and incubated for one week, then counted for pathogen growth. The inhibition growth was compared with the untreated controls. The inhibition of pathogenic growth was measured after 7 days incubation at room temperature. The inhibition percentage was determined following the equation Udomsilp *et al.* (2009) as follow:

$$\text{Inhibition (\%)} = \frac{C\text{-}T}{C} \times 100$$

where, C is the colony diameter of the mycelium on the control plate and T is the colony diameter of the mycelium on the treatment plate.

Characterization of glucanase activity: Glucanase assay was done by culturing bacteria on solid media in petri dishes containing glucan substrate (Wood and Weisz, 1984). Glucan substrate was derived from oat flour which was extracted with 20% disodium carbonate (Na_2CO_3). Bacterial isolates that had been prepared in 2 mL of Luria Broth (LB) media was taken and put into four wells (0.5 cm diameter) in a petridish containing glucan media. After incubation at room temperature for 3 days, the clear zone formed around the bacterial colony. Clear zone surroundings the wells indicated the presence of glucan hydrolysis produced by bacterial glucanase enzymes. Glucanase activity test was performed by growing cultures in LB medium and crude enzyme extract was obtained by centrifuging the culture at 5000 rpm for 15 min. A total of 0.2 mL crude enzyme extract was reacted with 1.8 mL of glucan substrate. The mixture was incubated at 50°C for 5 min. The reaction was stopped by adding 3 mL of dinitrocalicylic acid (DNS) reagent. The tube was heated in boiling water for 15 min and added with 10 mL of distilled water. Absorbance was then measured at a wavelength of 550 nm (Miller, 1959). The enzyme activity was determined based on β glucanase activity which expressed in units per mL. One unit (U) of glucanase activity was defined as the amount of enzyme that produces one μmol of glucose in one min. Total protein concentration was assayed by the method of Bradford (1976) using Bovine Serum Albumin (BSA) as the protein standard.

Cells viability of bioformulation: The bioformulation was checked for its viability using total plate count method for period of storage from initial formulation to two month storage. The viability lost was determined by comparing with initial concentration (CFU mL^{-1}).

Greenhouse testing of bioformulation against sheath blight, neck blast and bacterial leaf blight of rice: Various formulations were assessed for their efficacy in controlling ShB, neck blast and BB severity under greenhouse condition. For ShB disease, rice cv. IR 64 was used in the assay. Rice seedlings was immersed in 100 mL of bioformulation for overnight, then 14 days-old rice was planted on plastic pots 15×30 cm^2 containing 3 kg of Ultisol soil fertilized at rate of recommended NPK (1:1:1). The assay was arranged as factorial expt. using randomized complete design with three replications. Rice plant was sprayed using bacterial formulations at 28 Days after Planting (DAP) and 42 DAP. Artificial inoculation was done using of *R. solani* inoculums grown on PDA which inserted onto basal part of rice tillers at 30 DAP. Disease severity was evaluated weekly until final observation. Untreated control was prepared without formulation treatments except pathogen. Sheath blight disease symptom was observed based on formula Relative Lesion Height (RLH) described by Ahn *et al.* (1986):

$$\text{RLH } (\%) = \frac{\text{Lesion length}}{\text{Plant height}} \times 100$$

where, the RLH value was converted into Area under the Disease Progress Curve (AUDPC) (Shaner and Finney, 1977):

$$\text{AUDPC} = \sum \frac{Y_i + Y_{i+1}}{2} \times (t_{i+1} - t_i)$$

where, n is number of observation, t_i is time of observation and Y_i is ShB lesion length.

Greenhouse test for neck blast assay was performed in factorial experiment using Completely Randomized Design (CRD) with three replications. Rice seedlings cv. Inpari 13 (14 day-old) was immersed in 100 mL of each formulation for 24 h, then planted in small pot containing 3 kg of 'Ultisol' soil and NPK fertilizer (1:1:1). Rice seedling-soaked in sterile distilled water was served as control. Treatment application onto rice plant was done by spraying directly with a total of 100 mL of each formulation three times i.e. at 14, 28 and 42 DAP. Artificial inoculation was done using pathogenic fungi *P. oryzae* (Po-Kn isolate) grown on PDA media. A piece of agar±4 cm^2 containing pathogenic fungi was inserted at rice panicle during panicle initiation stage. Rice panicle was also spray-inoculated with spore suspension containing 5.33×10^6 spore mL^{-1}. Neck blast disease severity was determined using Standard Evaluation System (SES) for rice (IRRI, 1996) at 14 Days after Inoculation (DAI).

In a separate test, treated rice plants were also inoculated with *Xoo* bacterial inoculums by clipping method at maximum rice tillering stage. The BB disease severity was observed using SES (IRRI, 1996). In addition to disease severity, rice dry weight was also observed.

Field testing of bioformulations against sheath blight, neck blast and bacterial leaf blight of rice: The field experiment for bioefficacy against ShB, neck blast and BB of rice was carried out at disease endemic areas of Pusakanagara Experiment Station of ICRR+6 m above Sea Level (ASL); Indramayu (6 m ASL) and Cianjur farmer's field (287 m ASL), West Java during Dry Season (DS) of 2012. The experiment was arranged in Randomized Complete Block Design (RCBD) with six replications. Rice cv. Inpari 13 was planted in 4×6 m^2 plot size using 25×25 cm plant spacing, following the recommended agronomical practices. Bioformulations of Talc-A5, A6 and A8 were applied using knapsack sprayer onto rice plant at 14, 28 and 42 DAP. Plant was artificially inoculated by *R. solani* inoculums at maximum tillering stage. Disease assessment was carried out by observing ShB lesion length based on SES (IRRI, 1996).

For neck blast testing, rice seeds cv Inpari 13 was sown for 18 days in rice nursery. Fungal inoculation was done by spraying *P. oryzae* spores with a density of 5.5×10^7 spores mL^{-1}. Rice plants was treated with the three best formulation prepared in Talc (A5, A6, A8); whilst inoculation of *P. oryzae* alone (without formulation) served as untreated control. The bacterial formulation was applied by three times spraying at 3, 7 and 9 DAI. Observations was done at 14 DAI based on neck blast disease assessment given by SES (IRRI, 1996), using 9 scale basis i.e., 0 = no lesions; 1 = small, brown, specks of pinhead size; 3 = small, roundish to slightly elongated, necrotic, gray spots about 1-2 mm in diameter; 5 = typical blast lesions infecting <10% area; 7 = typical blast lesions infecting 26-50% area; 9 = typical blast lesions infecting >51% area.

Field experiment for BB efficacy with artificial inoculation was conducted at Ciranjang District, Cianjur-West Java. Rice plants cv. Inpari 13 each was planted in a 4×6 m^2 plot size using 30×30 cm plant spacing. The experiment was arranged in a randomized complete block design with six replications. Plots were treated with candidate bacterial consortium and untreated control. Plants were treated with three candidate bacterial consortium (10^7 CFU mL^{-1}) prepared in talc based (A5, A6 and A8), by three time sprays-applications using knapsack sprayer at 14, 28 and 42 DAP. Untreated plot without bioformulation was served as control. Disease severity was evaluated at 70 DAP using scale basis of SES (IRRI, 1996). In addition to disease severity, yield data was recorded by measuring grain weight m^{-2}.

Diseases severities were calculated using the formula:

$$DS = 100\% \times \sum \frac{n \times v}{N \times V}$$

Where:
DS = Disease severity
n = Number of leaves infected
v = Value score of each category attack
N = Number of leaves observed
V = Value of the highest score

Statistical analysis: All data were analyzed for treatment effect differences by Analysis of Variance (ANOVA). Comparison of means was performed using Duncan Multiple Ranged Test (DMRT) ($p = 0.05$) by the SAS statistical package.

RESULTS

***In vitro* test:** The effect of bacterial antagonistic isolates applied singly or in combination mixtures against *R. solani* and *Xoo* was presented in Table 2. Suspension-A2 and consortium A8 showed higher *R. solani* inhibition (41.92%) but it showed weaker when using consortium A5 or A7.

Based on *in vitro* test, some formulations also capable of suppressing the growth of *P. oryzae* causing the formation of inhibitory zones. In the control treatment, *P. oryzae* grew normally to reach the whole petridish (no inhibitory zone). It was shown that suspension formulation-A8

Table 2: Effect of suspension bacterial antagonist (single and mixtures isolates) against inhibition of *R. solani* and *Xoo* at *in vitro* test

Treatment/code	*R. solani* growth inhibition (%)	*Xoo* clearance zone (cm)
A1. *B. cereus* II.14	2.29^{a}	0.00±0.00
A2. *B. firmus* E65	41.92^{bc}	0.62±0.02
A3. *P. aeruginosa* C32b	0.00^{a}	0.33±0.01
A4. *S. marcescens* E31	0.00^{a}	0.00±0.00
A5. *B. firmus* E65+*P. aeruginosa* C32b	18.43^{ab}	0.58±0.03
A6. *B. cereus* II.14+*B. firmus* E65+*P. aeruginosa* C32b	20.93^{ab}	0.50±0.02
A7. *B. cereus* II.14+*P. aeruginosa* C32b+*S. marcescens* E31	0.00^{a}	0.25±0.05
A8. *B. cereus* II.14+*B. firmus* E65+*P. aeruginosa* C32b+*S. marcescens* E31	62.47^{c}	0.52±0.03
Copper sulphate (2%)	55.92^{c}	0.20±0.01
Pathogen alone (untreated)	0.00^{a}	0.00±0.00

Means in columns followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

Table 3: Effect of bacterial formulation on inhibition of *P. oryzae* at *in vitro* test

	Inhibition of *P. oryzae* (%)				
Formulation	Suspension	Talc	Bentonite	ACG	Mean
A2. *B. firmus* E65	45.92^{ab}	28.51^{bc}	27.40^{bc}	43.33^{ab}	36.29^{a}
A6. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b	46.29^{ab}	15.92^{c}	18.89^{c}	34.44^{a-c}	28.88^{a}
A8. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	54.44^{a}	40.00^{ab}	31.11^{bc}	42.59^{ab}	42.03^{b}

ACG: Animal compost granule. Means followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

revealed the highest inhibition zone (62.47%) with the significant efficacy similar with that of copper sulphate treatment. The consortium A7 had weaker effect on *R. solani*.

The bacterial antagonistic isolates produced *Xoo* inhibition zones in dual culture test, ranging from 0.0 to 0.62 cm in diameter. The highest inhibition zone was observed using *B. firmus* E65 (A2), whilst consortium A5 (*B. firmus* E65+*P. aeruginosa* C32b) and A6 (*B. cereus* II.14+*B. firmus* E65+*P. aeruginosa* C32b) showed similar inhibition zone of 0.5 and 0.52 cm, respectively (Table 2).

Table 3 showed the efficacy of four formulations to *P. oryzae* at *in vitro* test. The inhibitory zone at 14 DAI was ranging from 15.92 to 54.44%. The suspension-A2 treatment using single isolates of *B. firmus* E65 significantly reduced growth of *P. oryzae* compared with other carrier formulation treatments (Table 3). The mean average of bacterial formulation was shown by A8 (*B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31) treatment (42.03%).

Effect of bacterial isolates producing glucanase and their activity against *R. solani* and *P. oryzae*: The glucanolytic index was ranged from 1.08 to 3.40. The mechanisms of bacterial isolates particularly *B. firmus* E65, *B. cereus* I.21 and *P. aeruginosa* C32a produced glucanase enzymes that could degrade cell walls of fungi, with varying effect with the glucanolytic index of 1.34, 1.75 and 3.40, respectively. *S. marcescens* E31 showed the lowest glucanolytic index (1.08), whilst *P. aeruginosa* C32 b (2.50) and *B. cereus* II. 14 (2.50) had almost similar glucanolytic index compared to that of *P. aeruginosa* C32a.

When compared with control treatment, C32a was more effective than other isolates in suppressing *P. oryzae* at 14 DAI (Fig. 1a). This was probably due to the isolate increase its antagonistic activity and also producing more effective hydrolytic enzymes such as glucanase (Fig. 1b). Culture filtrate collected from the exponential phase causing percentage reduction of *P. oryzae* and *R. solani* of 59.11±5.02 and 37.33±14.87, respectively.

Qualitative assay using total plate count method to the crude enzyme activity of 24 h-old culture *Pseudomonas aeruginosa* C32a was shown in Fig. 2. It was shown that the *P. aeruginosa* C32a produced extracellular glucanase activity during the exponential phase (24 h) with the specific glucanase activity of 0.045 U mg^{-1}. The enzyme activity was stable until 48 h, then decreased after 60 h incubation.

A morphological change such as hyphal lysis was observed in fungi growing in PDA plates containing culture filtrate of *P. aeruginosa* C32a. Based on microscopic observation, it was revealed that cytoplasmic leakage of mycelium occurred, resulting in deformation of hyphae (Fig. 3). Morphological changes such as abnormal hyphal shapes were also observed in fungi grown with culture filtrate of bacterial consortium.

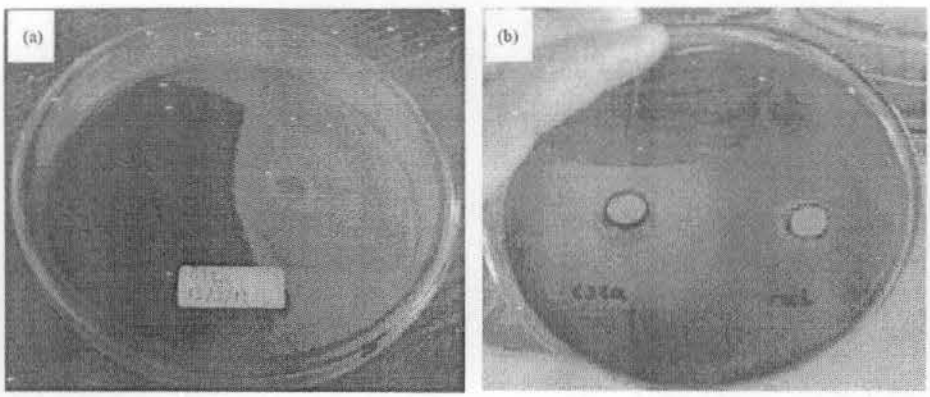

Fig. 1(a-b): Biocontrol activity of (a) *P. aeruginosa* C32a isolate against and (b) *P. oryzae*. Qualitative assay of glucanase producing bacteria (C32a and C32b) showing inhibition zone

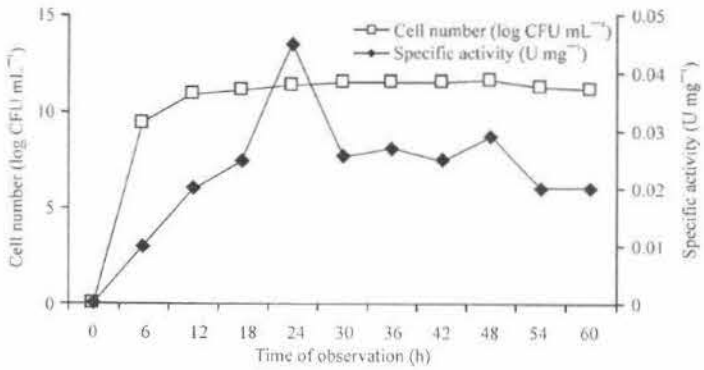

Fig. 2: Glucanase activity during exponential growth of *P. aeruginosa* C32a

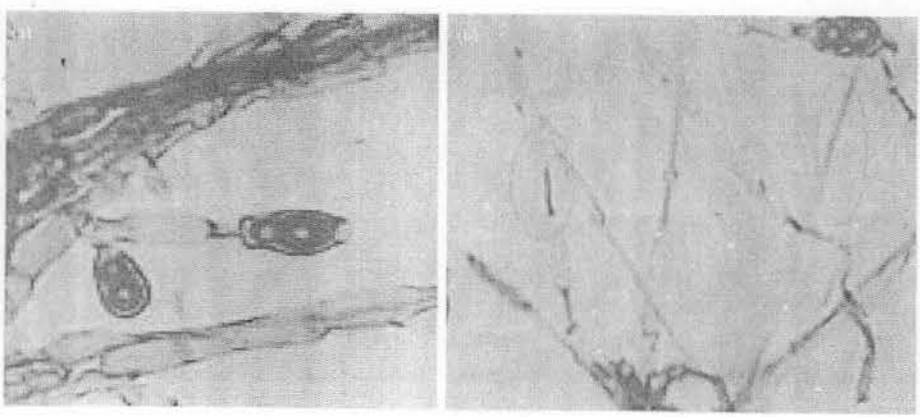

Fig. 3(a-b): Hyphal observation (a) Control and (b) Formulation treatment showing lysis of hyphae (400x)

Cells viability of bioformulation in storage condition: Single cultures of *B. cereus* II. 14, *B. firmus* E65, *P. aeruginosa* C32b and *S. marcescens* E31 had viability of 2.5×10^9, 2.6×10^9, 5.1×10^9 and 6.0×10^9 CFU mL^{-1}, respectively. Means viability of bacterial isolates prior mixing with other carrier was approximately of 1.4×10^9 CFU mL^{-1}. At initial application, the formulation had an average cell count of 4×10^9 CFU mL^{-1}. During storage formulations at room temperature, the number of bacterial cells formulations had an average of 4.17×10^8 CFU mL^{-1} in the first month of storage, while in the second month, the number of bacterial cells formulation obtained of 2.8×10^7 CFU mL^{-1}. The overall cells viability was slightly decreased during period of storage, ranged from 2.39 to 18.30% among different bioformulations (Table 4). A8-talc based formulation was stable at period of storage showing no viability lost. The talc formulated product in this study indicated its shelf life up to 2 month storage with adequate population.

Effect of bioformulation against sheath blight, neck blast and bacterial leaf blight of rice in greenhouse test: The result of greenhouse study on the effect of bioformulation against sheath blight was presented in Table 5. Area under disease progress curve was ranged from 225.49 to 349.98, whilst ShB disease suppression ranged from 12.69 to 35.57%. Under greenhouse study talc and bentonite A2 formulation showed effective against the disease with the ShB suppression of 25.61 and 35.57%, respectively.

Neck blast observation at 14 DAI showed that artificial inoculation at panicle initiation stage produced more neck blast symptoms on rice (Table 6). An effective formulation to inhibit the growth of neck blast disease was the animal compost granule-A6 formulation and suspension-A2 formulation, whereas the least-effective formulation was Talc-A8 formulation.

Bentonite-A6 formulations treatment showed 25.84% neck blast intensity with percentage inhibition of 63.75% compared with that of untreated control, while the suspension-A2 formulation had an inhibition of 73.1%. Result of this study indicated that after 14 DAI bacterial formulations

Table 4: Viability of bacterial isolates in different formulas after mixing with other carriers at 1 and 2 months storage

	No. of bacterial cells (CFU mL^{-1})		
Formulation	1 month	2 month	Viability loss (VL)* (%)
Suspension			
A2. B. firmus E65	3.7×10^8	2×10^7	11.28
A6. B. cereus II.14+*B. firmus* E65+*P.aeruginosa* C32b	1.6×10^9	9.5×10^8	2.39
A8. B. cereus II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	2.9×10^9	1.2×10^9	4.00
Animal compost granule (ACG)			
A2. *B. firmus* E65	7×10^7	4.5×10^7	2.55
A6. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b	5.9×10^8	4.5×10^7	12.80
A8. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	5.2×10^8	1.9×10^8	5.00
Talc			
A2. *B. firmus* E65	5.4×10^8	1.5×10^7	17.90
A6. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b	1.6×10^8	1.5×10^7	12.60
A8. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	1.0×10^7	1.0×10^7	0.00
Bentonite			
A2. *B. firmus* E65	2×10^7	5×10^6	8.60
A6. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b	6×10^8	1.5×10^7	18.30
A8. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	3.3×10^8	1.0×10^7	17.80

*Calculated based on formula: VL: (Vf-Vi)/Vf x 100%; where VL= Viability loss, Vf: Viability at final observation, Vi: Viability at initial observation. CFU: Colony forming unit

Table 5: Greenhouse test on the effect of formulations to sheath blight (*R. solani*) on rice cv. IR 64

Formulation	AUDPC*	ShB disease suppression (%)
Suspension		
A2. *B. firmus* E65	269.15	23.10
A8. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	305.59	12.69
Talc		
A2. *B. firmus* E65	260.37	25.61
A8. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	238.63	31.82
Bentonite		
A2. *B. firmus* E65	225.49	35.57
A8. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	257.43	26.45
Control (Untreated)	349.98	0.00

*AUDPC: Area under disease progress curve, ShB: Sheath blight

Table 6: Green house test on the effect of bioformulation to neck blast (*P. oryzae*) disease severity and biomass dry weight of rice cv. Inpari 13

	P. oryzae disease severity (%)				
Formulation	Suspension	Talc	Bentonite	ACG	Mean
A2. *B. firmus* E65	24.05^{bc}	36.46^{ab}	26.06^{bc}	49.17^{a}	33.93^{B}
A6. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b	44.58^{ab}	33.60^{bc}	22.29^{c}	40.42^{ab}	35.22^{B}
A8. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	21.63^{c}	42.70^{ab}	35.95^{ab}	22.56^{bc}	30.71^{A}
Mean	30.08	37.58	28.10	37.38	-
	Biomass dry weight (g)				
Formulation	Suspension	Talc	Bentonite	ACG	Mean
A2. *B. firmus* E65	73.45	62.23	92.16	43.16	67.75
A6. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b	51.47	69.84	64.7	71.57	64.39
A8. *B. cereus* II.14+*B. firmus* E65+*P.aeruginosa* C32b+*S. marcescens* E31	65.93	66.14	80.36	70.47	70.72
Mean	63.62	66.07	79.07	61.73	-

ACG: Animal compost granule. Means followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

treatment reduced neck blast intensity by 39.5% compared with that of the untreated control (79.47%). Bentonite-A2 and animal compost granule-A8 treatment were effective in controlling neck blast, their efficacy was 55.2 and 69.9%, respectively. Only formulation suspension-A2, animal compost granule-A2, Bentonite-A6 and animal compost granule-A8 had lower neck blast intensity, compared with that of untreated control. The bentonite-A2 and A6 treatments showed neck blast reduction by more than 50%. Talc-A2, A6 and A8 treatments showed reduction of blast severity less than 50%; whilst the animal compost granule (ACG)-A2, A5 and A6 could only reduce neck blast by less than 20% compare with that of untreated control.

The effect of bioformulation to the dry biomass of plant weight was not significant, with the average of plant dry weight ranging from 43.16 g to 92.16 g (Table 6). The average biomass of dry weight was highest for bentonite-A2 formulation treatment with an average of 79.07 g; whilst the highest biomass dry weight was shown by bacterial formulation A8 (70.72 g).

The results demonstrated the ability of A5 formulation containing two isolates of *B. firmus* E65 and *P. aeruginosa* C32b in suppressing BB at 14 DAI with the mean average of BB lesion length of 7.91 cm (Table 7). A8 formulations which was a consortium of isolates of *B. firmus* E65, *B. cereus*

Table 7: Greenhouse test on the effect of bacterial formulation to BB lesion length and grain dry weight on rice cv. Inpari 13

	BB lesion length (cm)				
Formulation	Suspension	Talc	Bentonite	Oil	Mean
A5. *B. firmus* E65+*P.aeruginosa* C32b	7.58	7.80	7.78	8.47	7.91[a]
A6. *B. cereus* II.14+*B. firmus* E65+*P. aeruginosa* C32b	8.50	10.30	10.24	7.92	9.24[a]
A8. *B. cereus* II.14+*B. firmus* E65+*P. aeruginosa* C32b+*S. marcescens* E31	12.88	9.93	12.38	11.02	11.55[b]
Mean	9.66	9.34	10.14	9.13	
	Grain dry weight (g pot^{-1})				
Formulation	Suspension	Talc	Bentonite	Oil	Mean
A5. *B. firmus* E65+*P.aeruginosa* C32b	41.75[a]	36.38[ab]	30.62[bc]	32.72[b]	35.36[a]
A6. *B. cereus* II.14+*B. firmus* E65+*P. aeruginosa* C32b	25.19[cd]	20.10[de]	19.89[de]	17.78[de]	20.74[b]
A8. *B. cereus* II.14+*B. firmus* E65+*P. aeruginosa* C32b+*S. marcescens* E31	41.79[a]	20.85[de]	16.50[e]	17.04[e]	24.04[b]
Mean	36.24	25.78	22.34	22.51	

BB: Bacterial leaf blight. Means followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

Table 8: Effect of talc-based formulation against sheath blight, neck blast and bacterial leaf blight disease severity and yield on rice cv. Inpari 13 (Pusakanagara, Experiment Station; Indramayu and Cianjur farmer 's fields, West Java. 2012 dry season)

Formulation	Sheath blight severity (%)	Grain weight m^{-2} (g)	Neck blast severity (%)	Grain weight m^{-2} (g)	Bacterial leaf blight severity (%)	Grain weight m^{-2} (g)
Talc-A5. *B. firmus* E65+*P.aeruginosa* C32b	37.33[bc]	783.83[a]	11.04[a]	471.67[a]	37.59[b]	656.6[a]
Talc-A6:*B. cereus* II.14+*B. firmus* E65+ *P. aeruginosa* C32b	35.83[c]	765.33[a]	9.87[a]	488.5[a]	41.07[b]	626.6[a]
Talc-A8. *B. cereus* II.14+*B. firmus* E65+ *P. aeruginosa* C32b+*S. marcescens* E31	40.33[b]	587.67[b]	10.81[a]	486.67[a]	37.62[b]	700[a]
Control (untreated)	46.33[a]	517.67[b]	9.52[a]	481.97[a]	65.99[a]	645[a]
CV(%)	8.72	11.76	18.26	3.08	25.36	9.86

Means in column followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT) at p = 0.05

II.14, *S. marcescens* E31 and *P. aeruginosa* C32b showed varying results in suppressing BB disease. Aside from suppressing the growth of *Xoo*, the formula A5 (*B. firmus* E65+*P. aeruginosa* C32b) with bentonite formulation was also able to affect on dry weight (35.36 g).

Field testing of bioformulations against sheath blight, neck blast and bacterial leaf blight of rice: In Pusakanagara experiment station, the talc-A6 treatment affected a lower ShB disease severity compare with that of untreated plot (Table 8), ranged from 37.33 to 40.33%, which were significantly different compared with that of untreated plot (control). The bacterial formulation of the mixtures (talc-A8) had a weak effect on grain yield; however talc-A5 and A6 through foliar applications significantly increased grain weight m^{-2} compared to that of untreated plot (control). The application of bioformulation talc-A5 and A6 increased yield up to 220-240 g m^{-2}.

The effect of talc carrier formulations against neck blast disease at 21 DAI showed spots with brown edges, white or grayish center patches. Results of the field test under farmer's field, showed that talc based formulation of bacterial mixtures had lower effect on neck blast severity (Table 8). The treated plot showed no significant advantage on rice yield. Based on this study it was indicated that talc based formulation reduced neck blast disease under greenhouse conditions, however; it exhibited lower efficacy against neck blast in the field.

Under field test experiment in Cianjur farmer's field application of bacterial formulation reduced BB severity ranging from 37 to 65% (Table 8). Higher BB disease severity was observed on control plot (without bacterial formulation). Talc-A5 formulation had significant effect on BB disease severity, with the disease suppression of 45.76%. However, the bacterial consortium had no significant effect on grain yield of which the grain weight ranged from 626.6 g to 700 g m^{-2}.

DISCUSSION

In the present study, bacterial antagonistic bioformulation against rice diseases were studied under laboratory, greenhouse and field test. The use of carrier material at *in vitro* test showed lower effect than that of suspension formulation. This presumably due to decreasing bacterial viability due to mixing process; hence the variability effect of bioformulations in this study needed to be elucidated.

Talc-based combined with A5 (*B. firmus* E65+*P. aeruginosa* C32b), A6 (*B. cereus* II.14+ *B. firmus* E65+*P. aeruginosa* C32b) and A8 (*B. firmus* E 65, *Serratia marcescens* E31, *P. aeruginosa* C32b, *B. cereus* II 14) formulation were effective against ShB and BB disease suppression in the field test. However, application of this bioformulation had no significant effect on reduction of neck blast disease, compared with that of the control treatment; this probably was caused by unfavorable conditions, such as high temperature and humidity during cropping season that affected neck blast disease progression. This study was supported by Nandakumar *et al.* (2001) using similar talc based formulation, who reported that the application of bioformulation containing *P. fluoresence* isolates could increase the reduced ShB disease and increase rice yield in the field test. Karthikeyan *et al.* (2008) also reported that talc based formulation of mix cultures containing *Bacillus subtilis, P. fluorescence, Gliocladium virens* and *Trichoderma* spp. could also significantly reduced leaf blight incidence and increased growth of onion both in glasshouse and field trials. Anand *et al.* (2010) reported that use of talc-based formulation of *P. fluorescence* might increased the survival of bacteria in phylloplane of chili plant and thus suppressed growth of fruit rot caused by *Colletrotichum capsicii* and powdery mildew caused by *Leveillula taurica*. Recent study also pointed out that talc-based formulation of *Pseudomonas* strains (Talc-B1 and Talc-B2) were effective against sugar beet disease (Jorjani *et al.*, 2012).

The present study indicated that the use of more than three different isolates are promising in reducing rice diseases such as ShB and BB of rice and further increased yield. This support hypothesis that use of combined application of two or more bacterial antagonist as bioconrol agent could enhance level of plant disease control (Raupach and Kloepper, 1998).

In general the isolates combinations mixed with animal compost granule and bentonite carriers showed moderate inhibition against *P. oryzae*.

It had been reported that antifungal mechanism of bacterial antagonist was influenced by production of hydrolytic enzyme such as chitinase and glucanase (El-Katatny *et al.*, 2001). In the present study, exponential phase culture filtrate *P. aeruginosa* C32a inhibited growth of both fungal tested, indicating that the growth suppression was due to extracellular metabolite, such as glucanase, present in the cultures. Similar study reported that *Streptomyces hygroscopicus* produced extracellular metabolite that effective to suppress phytopathogenic fungi (Prapagdee *et al.*, 2008). Lee and Kobayashi (1989) also reported the deformation of *R. solani* hyphal growth was due to effect of antifungal metabolite secreted by *Burkholderia cepacia*.

Bentonite and talc-A5 formulation showed good effect in suppressing BB lesion length on rice plants. Ardakani *et al.* (2009) reported that use of talc-based formulation of *P. fluorescens* could

increased healthy plant more than 3 fold compared with that of untreated plant. Bentonite (a montmorillonite clay) is a very smooth powder, lightweight and easy to absorb fluids due to the high absorption capacity so that cells bound more bacteria than other formulations (Vidhyasekaran *et al.*, 1997; Ting *et al.*, 2009). In addition, the formulation of bentonite readily soluble in water. Thus, this formulation is an appropriate carrier material for the biocontrol of rice diseases. Talc formulation showed similar effect in suppressing BB disease. Talc powder is also lightweight and easily soluble in water but the formulation is a little difficult to mix with other material; however from an economic point of view, talc is cheaper than bentonite formulation. Further test is needed to increase cell viability using suitable carriers among different bioformulations.

In this study, the bioformulations prepared for BB biocontrol were less effective in terms of BB disease reduction under greenhouse conditions; however, talc-based formulation of A5 treatment showed the lowest BB disease severity under BB field disease pressure during 2012 dry season. Previous study also indicated in this area that the incidence of BB was observed severely on rice cv. IR 64. Results of the above-mentioned studies clearly indicated that development of stable bioformulations of antagonistic bacteria is the importance and offers a powerful and eco-friendly alternative to replace synthetic chemicals and minimizes the dependence on pesticides. The results of this study may have practical application in establishing of plant disease management strategies especially in controlling rice diseases. Nevertheless, use of bacterial formulation depends upon the development of formulations in which the bacteria can survive for considerable length of time. The number of bacterial cells formulations had an average of 2.8×10^{7} CFU mL^{-1} and no viability loss using talc-formulation during storage of formulations at room temperature. This condition was also reported by Anitha and Rabeeth (2009) who pointed out that talc-based formulation were also stable up to 3 month with cell viability of 122×10^{7} CFU g^{-1}.

Further study should evaluate other carrier material that may improve its effectiveness under unfavorable field conditions.

CONCLUSION

From the above result, it may concluded that the use of bacterial isolates used singly or in mixture combination were apparent to reduce ShB, neck blast and BB of rice under *in vitro* test. The formulations suspension-A6 (*B. firmus* E65, *P. aeruginosa* C32b, *B. cereus* II 14), suspension-A8 (*B. firmus* E65, *S. marcescens* E31, *P. aeruginosa* C32b, *B. cereus* II 14), Talc-A8 and ACG-A8 showed inhibition to *P. oryzae* by 71.04, 79.19, 63.87 and 66.68%, respectively.

Talc-A8 based formulation was stable at period of storage showing no viability lost. This indicated that talc carrier should be developed for reason of cheaper delivery. Although the formulations Talc-A5 (*B. firmus* E65, *P. aeruginosa* C32b) was effective against ShB and BB of rice but revealed lower effect on neck blast disease reduction under field test; hence its effect on neck blast should be reevaluated under similar field conditions in endemic areas.

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