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Full Length Research Paper

A possibility of chitinolytic bacteria utilization to control basal stems disease caused by *Ganoderma boninense* in oil palm seedling

Dwi Suryanto[1,2]*, Rizky Hadi Wibowo[2], Edy Batara Mulya Siregar[3] and Erman Munir[2]

[1]Research Center for Environment and Population Issues, Universitas Sumatera Utara, Jln. Perpustakaan No. 5, Medan 20155, Indonesia.
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Jln. Bioteknologi No. 1, Medan 20155, Indonesia.
[3]Department of Forestry, Faculty of Agriculture, Universitas Sumatera Utara, Medan, Indonesia.

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The utilization of chitinolytic bacterial isolates *Enterobacter* sp. KR05, *Enterobacter cloacae* LK08, *Bacillus* sp. BK13, *Enterobacter* sp. BK15, and *Bacillus* sp. BK17 to control basal stem rot disease caused by *Ganoderma boninense* in oil palm seedling was studied. Antagonistic assay of chitinolytic bacterial isolates to *G. boninense* was conducted in minimum salt medium agar with 2% colloidal chitin as sole carbon source. To examine ability of the isolates in reducing basal stem rot disease incidence, the 3 to 4 months old of oil palm seedlings were treated by pouring oil palm seedling growing media with chitinolytic bacterial isolates a day prior infestation of *G. boninense* spores. The result showed that all chitinolytic isolates inhibited the growth of *G. boninense in vitro*. Hyphal abnormalities that is, dwarf, tiny, curled, twisted and bulby hyphae were observed after antagonistic assay. All chitinolytic isolates were able to reduce the disease incidence on the oil palm seedling to some extent. The isolates might infest into the oil palm seedling root as endophytes.

Key words: Antagonistic assay, chitinolytic bacteria, *Ganoderma boninense*, hyphal abnormality, oil palm.

INTRODUCTION

Basal stem rot (BSR) caused by fungi *Ganoderma boninense* is a major problem in oil palm plantation, especially in Indonesia and Malaysia (Susanto et al., 2005). Previously, *G. boninense* was only found on older trees as basal stem rot disease, but recently it has been found in seedling and in upper stem (Sanderson, 2005) and younger tree where symptoms appear earlier and are more severe, leading to greater replanting (Susanto et al., 2005). BSR spread through contacted adjacent root or by spreading the fungal spores (Sanderson, 2005).

To control BSR, chemical, cultural, and mechanical control was conducted. However, no control practices have been proved satisfactory (Susanto et al., 2005). Chemical control has raised problems such as environmental pollution and resistance of disease-causative organisms to fungicides. Bivi et al. (2010) observed low disease incidence of BSR in some natural stands, suggest that the disease is most likely kept under control by some biological means. Due to these observations, recent control measures to overcome the *Ganoderma* problem are now focused on the use of biological control agents (Bivi et al., 2010; Susanto et al., 2005).

A number of fungi are particularly susceptible to lytic by microorganisms. Investigations on lytic activity of biocontrol agents have focused mainly on the characterization of enzyme systems capable of degrading fungal cell wall components, of which chitinases are

*Corresponding author. E-mail: d.suryanto@lycos.com.

among the most intensively studied (Downing and Thomson, 2000; Anitha and Rabeeth, 2010). Therefore, chitinase is known as one of the antifungal proteins (Wang et al., 2005).

Several biological control agents of chitinolytic microbes have been reported to inhibit the growth of fungal plant pathogen. Chitinolytic bacteria such as *Aeromonas hydrophila*, *Aeromonas caviae*, *Pseudomonas maltophila*, *Bacillus licheniformis*, *Bacillus circulans*, *Vibrio furnissii*, *Xanthomonas* spp., and *Serratia marcescens* play an important role in biological control of plant pathogenic fungi (Gohel et al., 2006). Combined chitinolytic bacteria of *Serratia plymuthica* strain C-1, *Chromobacterium* sp. strain C-61, and *Lysobacter enzymogenes* strain C-3 inhibited the growth of *Phytium capsici*, *Rhizoctonia solani*, and *Fusarium* spp. (Kim et al., 2008). Suryanto et al. (2011) showed that chitinolytic bacterial isolates inhibited the growth of *Fusarium oxysporum*, *G. boninense*, and *Penicillium semitectum*. Furthermore, chitinolytic bacteria reduced the incidence of red pepper seedling wilt caused by *F. oxysporum* (Suryanto et al., 2010). In this study we evaluated the possibility of utilization of chitinolytic bacteria to control basal stem disease caused by *G. boninense* in oil palm seedling.

MATERIALS AND METHODS

Chitinolytic bacterial isolates and fungal isolates

Bacterial isolates *Enterobacter* spp. (KR05, BK15), *Enterobacter cloacae* (LK08), and *Bacillus* spp. (BK13, BK17) used in this study are collection of Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara. The isolates were kept at ±28 to 30°C in a modified salt medium (0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.001 g $ZnSO_4$, and 0.001 g $MnCl_2$ in 1.000 ml) containing 2% (w/v) chitin colloidal (MSMC) agar, with a pH of 6.8. Fruiting body of *G. boninense* was collected from oil palm trunk of oil palm plantation in Marihat- Pematang Siantar, North Sumatra as source of spore for oil palm seedling infection.

Assay of bacterial-fungal antagonism

The ability of the chitinolytic bacterial isolates to inhibit *G. boninense* growth was conducted in vitro. Fungal cultures were grown at the center of MSMC agar. Two pieces of paper discs immersed with ≈ 10^8 cells/ml of bacterial suspension were placed in the opposite direction about 3.5 cm from the fungal culture. Cultures were incubated at ± 28 to 30°C. Inhibitory activity was determined based on the inhibition zone formed around bacterial colonies on medium MSMC. Inhibition zone was measured from 5 to 10 days of incubation as the radius of the normal fungal growth subtracted with the radius of the inhibited fungal growth.

Observation of abnormal fungal hyphae of antagonistic assay

Inhibited hyphae of *G. boninense* of antagonistic assay were cut by 1 cm^2. The hyphae was examined under light microscope and compared with normal ones.

Examination of chitinolytic bacterial isolates inhibition to *G. boninense* in oil palm seedling

Soil samples of oil palm plantation and stable manure were pasteurized for 3 to 5 h at 80°C. The soil was mixed with stable manure with ratio of 3:1 for growing media. The media were put into a 17 × 35 cm polythene bag in which the 3 to 4 month of oil palm seedling grown.

Twenty milliliters of chitinolytic bacterial suspension of ≈ 10^8 cells/ml were thoroughly pour-inoculated into the oil palm seedling growing media. One gram of fruiting body was crushed and suspended with 0.09% NaCl. Spores were counted using a hemocytometer and adjusted to ≈ 2 × 10^5 spores/ml. Spore suspension was thoroughly applied after 24 h of the bacterial application. (+) control was treated similarly but without chitinolytic bacterial inoculation, meanwhile (-) control with no application both of spore and chitinolytic bacterial inoculation.

Observation on disease incidence

Observation was conducted on seedling root on every week for 4 months. The Disease Incidence (DI) was measured using the following equation (Cooke, 2006).

$$DI = \frac{\text{Number of infected plant units}}{\text{Total number of plant units assessed}} \times 100\%$$

Fungal and chitinolytic bacteria reisolation

Reisolation of chitinolytic bacteria was conducted from the oil palm growing media and the seedling root. One gram of the media was suspended with 0.09% NaCl. One ml of the suspension was spread in MGMC agar. Bacterial colonies in MGMC agar with clear zone were counted after 5 days of incubation. As in fruiting body, surface-sterilization was also carried out to pieces of the oil palm seedling root. The root piece was longitudinally cut and pressed onto MGMC agar for chitinolytic bacterial reisolation and onto PDA for fungal reisolation. Fungal colony growing on PDA was compared to colony of *G. boninense*. All cultures were incubated at ± 28 to 30°C.

RESULTS

Disease manifestation in oil palm seedling

Basal stem rot manifestation on oil palm seedling was showed as spotted yellowing on some leaves followed by necrotic symptom or yellowing of most leaves. Fruiting body (Figure 1a) is not produced until late disease infestation in plant (Semangun, 2000). *G. boninense* colony in PDA was whitish cotton-like hyphae with circles around the base of colony (Figure 1b). Spear leaves were smaller with necrotic symptom on the tip (Figure 1c) as also observed by Susanto et al. (2005). Palms yellow and die within a few months of becoming infected (Sanderson, 2005).

In mature tree the symptoms shown as the young

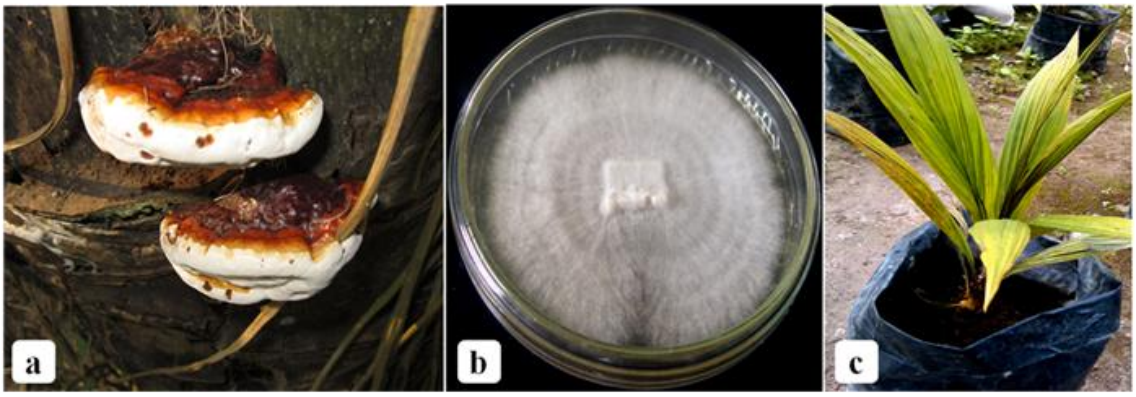

Figure 1. a: Fruiting bodies of *G. boninense;* b: Colony of *G. boninense* on PDA; and c: Basal stem disease symptom on oil palm seedling leaves.

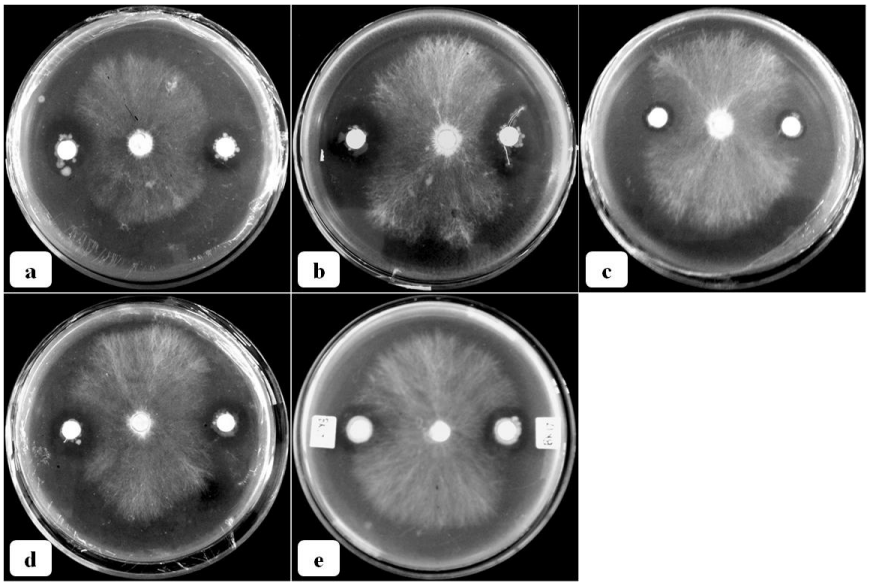

Figure 2. Antagonistic assay of a: *Enterobacter* sp. KR05; b: *Enterobacter cloacae* LK08; c: *Bacillus* sp. BK13; d: *Enterobacter* sp. BK15; e: *Bacillus* sp. BK17, on *G. boninense in vitro*.

frond yellowing can be accompanied by multiple spears, frond die back and collapsed fronds. Fruit and male flower development often ceases, giving infected palms the appearance of having a narrow waist at the canopy. Frond symptoms are accompanied by varying degrees of basal stem rot, which in severe cases can be detected by rotting frond bases that are easily removed. Severe basal rot can result in large areas of the trunk collapsing (Sanderson, 2005).

Assay of chitinolytic bacterial isolates against *G. boninense in vitro*

Chitinolytic bacterial isolate ability to inhibit growth of *G. boninense* hyphae was evaluated by growing chitinolytic isolates next to the fungi in chitin containing media (Figure 2). Chitinolytic bacteria were often characterized by their ability to produce a clear zone around their colony in chitin containing media.

The ability of chitinolytic bacterial isolates to inhibit growth of *G. boninense* hyphae was varied (Table 1). Suryanto et al. (2011) reported different ability of chitinolytic bacteria to inhibit growth of different fungi such as *F. oxysporum*, *G. boninense*, and *P. semitectum*. This variation might be due to species specific, different bacterial chitinase activity, chitin composition of the fungal mycelium, the growth rate of the bacterial and the fungi, and other antifungal metabolites.

Table 1. Inhibition zone of antagonistic assay of chitinolytic bacterial isolates to *G. boninense.*

Bacterial isolates	Inhibition zone (mm) of days					
	5	6	7	8	9	10
Enterobacter sp. KR05	3.95	5.22	6.76	7.58	9.00	9.07
E. cloacae LK08	5.11	6.46	7.83	9.76	10.31	10.16
Bacillus sp. BK13	5.20	7.18	8.40	9.31	9.40	9.45
Enterobacter sp. BK15	3.15	6.96	7.26	7.78	8.02	13.87
Bacillus sp. BK17	7.38	11.51	11.45	12.12	12.27	13.88

Table 2. Hyphal abnormality of *G. boninense* as a result of antagonistic assay with chitinolytic bacterial isolates.

Bacterial isolates	Antagonistic signs
Enterobacter sp. KR05	Inhibited hyphal growth; dwarf hyphae
E. cloacae LK08	Inhibited hyphal growth; dwarf, curled, and twisted hyphae
Bacillus sp. BK13	Inhibited hyphal growth; dwarf, tiny, lytic, and twisted hyphae
Enterobacter sp. BK15	Inhibited hyphal growth; curled and twisted hyphae
Bacillus sp. BK17	Inhibited hyphal growth; tiny, twisted, and bulby hyphae

Table 3. Disease incidence on oil palm seedling caused by *G. boninense.*

Bacterial isolates	Disease incidence (%)/week-											
	1	2	3	4	5	6	7	8	9	10	11	12
Enterobacter sp. KR05	4	4	4	4	4	4	4	4	4	4	4	4
E. cloacae LK08	4	4	8	8	8	8	8	8	8	8	8	8
Bacillus sp. BK13	8	8	8	8	8	8	8	8	8	8	8	8
Enterobacter sp. BK15	4	4	4	4	4	8	12	12	12	12	12	12
Bacillus sp. BK17	4	4	4	4	4	4	4	4	4	4	4	4
(+) control	4	4	4	8	8	12	16	16	20	20	20	20
(-) control	4	4	4	4	4	4	4	4	4	4	4	4

Observation of abnormal fungal hyphae of antagonistic assay

Degradation of cell wall component to some extent results in hyphal abnormality. Observation of abnormal fungal hyphae was conducted to determine the effect of bacteria on morphological structure of *G. boninense* hyphae. Abnormalities in fungal hyphae are due to morphological changes of impaired growth of fungi occurs in the hyphae. Abnormal hyphae of *G. boninense* that is, dwarf, tiny, tiny, curled, twisted and bulby hyphae were observed after antagonistic assay of the fungi with chitinolytic isolates (Table 2).

Assay of chitinolytic bacterial isolates to *G. boninense* in oil palm seedling

To know chitinolytic isolate ability in reducing disease incidence caused by *G. boninense*, infestation both chitinolytic bacterial isolates and *G. boninense* spores into growing media of oil palm seedling was conducted. All chitinolytic isolates showed to reduce basal stem rot disease incidence in oil palm seedling to some extent compared to that of (+) control, even could reduce the incidence to the level of (-) control (Table 3).

Disease incidence was still occurred in (-) control as a possible result of contamination of the seedling from contaminated seedling or through the air. Fungus may spread by strong wind currents that dislodge spores from agitated leaves, by nursery workers handling diseased plants, and by movement of slugs or other pests (Uchida and Kadooka, 1997). However, basal stem disease incidence was still weak rather than of (+) control, in which fungal infestation was purposely inoculated.

Fungal and chitinolytic bacteria reisolation

Many chitinolytic bacteria were isolated from soil. Hence,

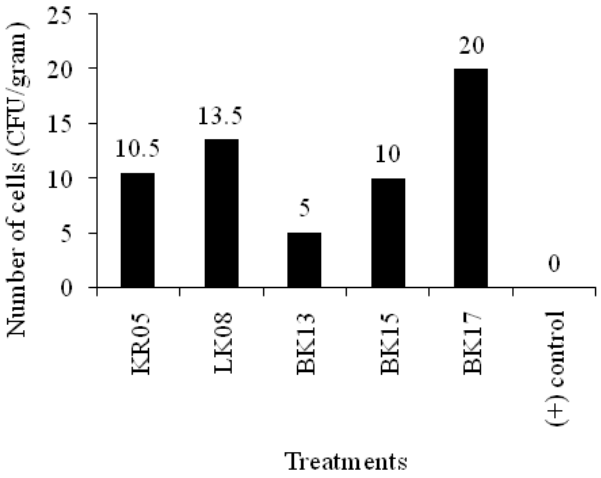

Figure 3. Number of reisolated bacterial isolates (cfu × 10^5) from the oil palm seedling growing media treated with chitinolytic bacterial isolates at 5 incubation days in MSMC agar.

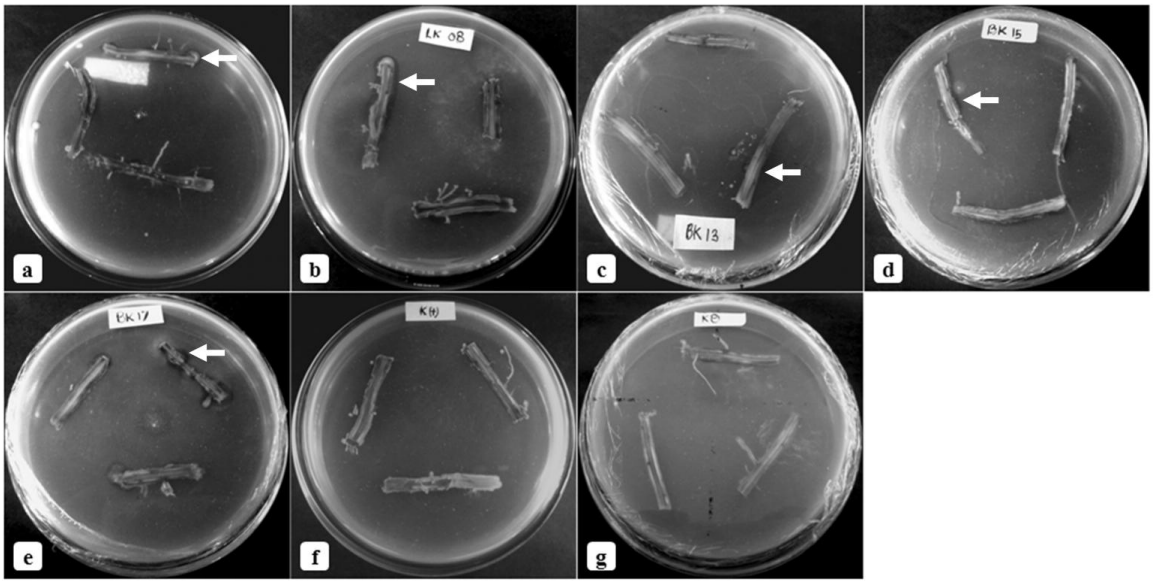

Figure 4. Reisolation of bacterial isolates from oil palm seedling treated with (a). *Enterobacter* sp. KR05, (b). *Enterobacter cloacae* LK08, (c). *Bacillus* sp. BK13, (d). *Enterobacter* sp. BK15, (e). *Bacillus* sp. BK17, (f). (+) control, and (-) control from oil palm seedling root of last day of observation of bacterial-fungus infestation in MSMC agar. Clear zone is pointed out with white arrow.

in the present study isolates were also screened from soil samples. Reisolation of the chitinolytic bacterial isolates from the treated growing media with chitinolytic bacterial isolates showed that the chitinolytic bacteria were survived and spread within the growing media (Figure 3).

Reisolation of the oil palm root in MSMC agar found that growing chitinolytic bacteria showed clear zone around the root surface (Figure 4). This result suggested that the chitinolytic isolates might penetrate into the oil palm root. It was not known whether the chitinolytic isolates would permanently be in the root tissue or not. It is interesting to know their role and contribution to the health of the plant. Factor affecting the cell penetration was not examined in this study. Further study should be undertaken.

DISCUSSION

Basal stem rot caused by *G. boninense*, is a devastating

disease that attacks oil palm plantation. Many efforts have been attempted to eliminate the disease, but no control scenario is successful to control the disease (Bivi et al., 2010; Susanto et al., 2005). In some natural stands, some biological means may suppress BSR (Bivi et al., 2010), suggest an effort to seek proper biological control agent (Bivi et al., 2010; Susanto et al., 2005). Bacteria as biological control agents typically use their metabolites such as enzymes and antibiotics to suppress the growth of other microorganisms. We evaluated the possibility of chitinolytic bacterial isolate utilization to control basal stem rot in oil palm seedling.

Biological control of plant disease using bacteria and fungi is based on the ability of microbes to produce mycolytic enzymes such as chitinase and β-1, 3-glucanase that lyse fungal cells (Patel et al., 2007; Kucuk and Kivanc, 2004; Gohel et al., 2006; El-Katatny et al., 2000), and through parasitic mechanism enable the parasite to enter the hyphae of the pathogen (Ozbay and Newman, 2004; Alabouvette et al., 2006). The presence of other metabolites in addition to chitinase is also responsible for inhibiting fungal growth (Getha and Vikineswary, 2002). Investigations on lytic activity of biocontrol agents have focused mainly on the characterization of enzyme systems capable of degrading fungal cell wall components, of which chitinases are among the most intensively studied (Downing and Thomson, 2000; Anitha and Rabeeth, 2010).

Crude chitinase extracted from *Streptomyces griseus* showed to inhibit growth of *F. oxysporum*, *Alternaria alternate*, *R. solani* and 2 isolates of *Aspergillus flavus* (Anitha and Rabeeth, 2010). The isolate degradation products, mainly GlcNAc, are then taken up by the cells as a carbon and nitrogen source (Tsujibo et al., 2002). Therefore, fungi and bacteria are important degraders of chitin in the soil ecosystem and contribute to the recycling of vital carbon and nitrogen resources (Metcalfe et al., 2002). In this study chitin colloidal was used as a sole C-source. Chitin colloidal is one of the substrates commonly used to induce chitinase as in the case of *Aeromonas caviae* (Inbar and Chet, 1991).

Inhibition zone increased during incubation time, and was observed on 5 days of incubation and continued to 10 days of incubation. Watanabe et al. (1997) showed that chitin hydrolysis occurred within 24 to 72 h after incubation in chitin colloidal as C-source. However, Kim et al. (2008) showed that chitinase activities of the bacterial strains reached the maximum level between 10 and 20 days after bacterial inoculation. This indicated that chitinase was still produced and diffused to the media to degrade fungal hyphae.

Antagonistic mechanisms of all isolates to *G. boninense* seemed to be similar to inhibit hyphal growth and to lyse hyphal cell wall. Microscopic observation of fungal hyphae after antagonistic assay showed the occurrence of hyphal abnormality. Getha and Vikineswary (2002) also reported that hyphal distortion like lytic of hyphal ends, swollen hyphae, abnormal branching of hyphae and the formation of hyphal protuberances was observed after contacted *F. oxysporum* f.sp. *cubense* hyphae with *Streptomyces violaceusniger* strain G10. Harjono and Widyastuti (2001) reported that endochitinase caused necrotic of hyphal tip and hyphal lytic. The lytic activity of bacteria is one of the mechanisms that have been implicated in biocontrol for several years (Alabouvette et al., 2006).

The maximum reduction of the disease incidence was shown by *Enterobacter* sp. KR05, *Bacillus* sp. BK17, following by *E. cloacae* LK08, *Bacillus* sp. BK13, and *Enterobacter* sp. BK15 respectively after 12 weeks of fungal infestation. Higher disease incidence was found in (+) control, in which the spores were infested without chitinolytic bacterial inoculation. Susanto et al. (2005) reported that *Trichoderma koningii* and *Trichoderma harzianum* could be used as biological control of *G. boninense*. *Burkholderia cepacia* genomovar I has antagonistic activity against *G. boninense* based on *in vitro* dual culture and poison food tests (Azadeh et al., 2010). Endophytes *P. aeruginosa* and unknown bacterial isolate EB4 can be effective biological control agents of *G. boninense* (Bivi et al., 2010).

It was understandable that the chitinolytic isolates were survived and capable of colonizing in the growing media of oil palm seedling since huge chitin sources were available in soil. Chitin is second abundant biopolimer (Tsujibo et al., 2002), in which fungi and arthropods contributed the most (Singh et al., 2008; Tsujibo et al., 2002). However, *Bacillus* sp. BK17 was more capable of propagating within the media. This indicated that ability to survive and colonization of the isolates varied, in which cell motility and substrate bioavailability limited colonization of the chitinolytic isolates. The movement of isolates was stimulated by chitin availability in the media (Suryanto et al., 2011). Furthermore, swarming activity (motility) in solid media was observed as indication of the colonization ability of the bacteria in the environment. The swarming ability is considered as one useful step in selecting biological control agent. Swarming therefore, is thought to be a successful strategy developed by flagellated microorganisms to ensure their rapid expansion in the natural environment, where microbial activities are often associated with solid surfaces (Senes et al., 2002).

Association between plant and microbe is a complex web relation. It evolves both sides, not only from the microbe but also from the plant (Rubini et al., 2005). Many plant-microbe associations are very tigh and species based such as rhizobia with their legumes, or mycorhiza with their host. The plant-associated habitat is a dynamic environment in which many factors affect the structure and species composition of the microbial communities that colonize roots, stems, branches and leaves. Endophytic species that were common in the host plants under natural conditions often are good colonizers

and grow fast *in vitro* (Mejía et al., 2008). Endophytic bacteria as internal colonizers may compete in the vascular system depriving plant pathogen for nutrients and space (Azadeh et al., 2010; Bivi et al., 2010).

Introducing endophytic bacteria to the roots to control plant disease is to manipulate the indigenous bacterial communities of the roots in a manner, which leads to enhanced suppression of soil-borne pathogens. The use of endophytic bacteria should thus be preferred to other biological control agents as they are internal colonizers, with better ability to compete within the vascular systems (Bivi et al., 2010). Understanding which microbial species are involved, how and when they occur and what are the advantages of these plant interactions, it is possible to use this approach to control several plant disease in cacao (Rubini et al., 2005).

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