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Antagonistic Activity of Endophytic *Bacillus* Species Against *Colletotrichum Gloeosporioides* for the Control of Anthracnose Disease in Black Pepper (*Piper Nigrum L.)*

[1*]Yap Chin Ann, [2]Awang Ahmad Sallehin, [2]Hairul Azman Roslan, [2]Mohd Hasnain Md Hussain, [2] Samuel Lihan

[1]Research and Development Division, Malaysian Pepper Board, Lot 1115 Jalan Utama, Pending Industrial Area, 93916 Kuching, Sarawak , Malaysia.
[2]Faculty of Resource Science and Technology, University Malaysia Sarawak, 93400 Kota Samarahan, Sarawak, Malaysia.
*Corresponding Author

Abstract

Anthracnose caused by *Collectotrichum gloeosporioides* is a serious disease of black pepper. The antagonistic effect of three *Bacillus species*, i.e. *Bacillus strain CBF, YCA0098 and YCA5593,* were tested against *Collectotrichum gloeosporioides in vitro* and *in vivo*. *In vitro* test showed that all *Bacillus* species significantly reduce the mycelia growth and spore germination of the C. *gloeosporioides.* Scanning electron microscopy revealed significant inhibition of *C. gloeosporioides* spore germination on pepper leaves surface. Combination of bacterial strain CBF, YCA0098 and YCA5593 maintained but not increased the inhibitory effect on spore germination of *C. gloeosporioides* when cells were co-incubated with *C. gloeosporioides* and when the pathogen was incubated in mixture of cell-free culture extracts. Combination of strains maintained efficacy in control of *C. gloeosporioides* in pepper vines compared to individual strains, but reduced variability and improved consistency between experiments, especially mixture of strain CBF, YCA0098 and YCA5593.

Keywords: Anthracnose, Antagonistic, Black pepper, *Bacillus, Collectotrichum*

1.0 INTRODUCTION

Black pepper (*Piper nigrum L.)* is one of the important export commodities in Malaysia, with production area of approximately 16,021 hectare (MPIC, 2013). Leaves anthracnose disease (also known as black berries disease), caused by *Collectotrichum gloeosporioides*, is a worldwide disease of black pepper and caused up to 50% yield losses and reduction of berries quality (Wong, 2002). *Collectotrichum gloeosporioides* overwinter as conidia in plant debris in the soil and generally infects plants as mycelia originating from these conidia or airborne ascospores that directly penetrate host leaves or berries tissue (Agrios, 2005).

Several approaches have been used to prevent, mitigate or control plant diseases. Currently, although the uses of chemical are comparatively suitable to control the fungal diseases, but continuous and abusive application has lead to the apparition of environment and human health problems. Also, traditional pepper breeding program for disease resistance has been hampered by limited gene pool (Lau et al., 2013) and inconsistent results produced (Chen, 2014).

Biological control using antagonistic bacteria has been considered as an alternative disease management strategy due to its potential to provide safe and environmentally compatible disease control (El-Kot, 2008). However, the major problem of biological control is the lack of consistency due to variable efficacy of the biological control agent dependent on the soil environment where the biocontrol agent is applied, the moment and the method of application, the host plant or the pathogen species. In addition, appropriate formulations would also be another challenges for successful implementation of biological control. Published data on this area remain sparse perhaps because they may involve industry secret in comparison with the considerable volume of literature describing selection procedure, mechanism of action or genetics of biological control agents. One approach to overcome inconsistent performance by biological control agent is through integration of multiple microbes into individual biological control formulations (Asghar and Pessarakli, 2010), and a second approach is through diversified the application method (Zhang et al., 2010). With regards to *Collectotrichum gloeosporioides on* black pepper, application of microbial biological control agent may improve disease suppression as these pathogens can infect the host near the soil line and in the foliar canopy.

Species belonging to *Bacillus* is frequently used as biocontrol agents, since they excrete hydrolytic enzymes that are able to degrade cell walls (Chernin and Chet, 2002), iron-chelating siderophores, several cyclic lipodepsipeptides (LDP) (Dalla *et al*., 2003), as well as a great variety of antibiotics such as, iturin (Joshi and Gardner, 2006; Tsuge *et al*., 2001; Phister *et al*., 2004), surfactin (Peypoux *et al*., 1999; Ajlani *et al*., 2007 and Huszcza & Burczyk, 2006), fengycin, (Loeffler *et al*., 1986 and Liu *et al*., 2011), bacillomycin (Athukora et al., 2009, Liu et al., 2011 and Ramarathnam *et al*., 2007) and mycosubtilin (Leclere *et al*., 2005). The objective of the present study were to determine the efficiency and efficacy of *Bacillus strains* CBF, YCA0098 and YCA5593 to control anthracnose disease in black pepper under both greenhouse and field experiments.

2.0 MATERIAL AND METHODS

2.1 Bacteria, fungi and plant materials

The bacterial identifications and the origins of the 3 strains of *Bacillus* spp used in this study are presented in Table 1. Pure cultures of each bacterial cells were maintained in Luria-Bertani broth, amended with 20% glycerol (Fisher Scientific) and stored at -80^{o}C. The bacterial cells were routinely cultured in Luria-Bertani (LB) broth supplemented with

streptomycin (50µg ml^{-1}) and rifampicin (50µg ml^{-1}). All the bacterial cells were resistant to these antibiotic concentrations. The bacterial cells were then streaked onto LB agar, and a single colony was inoculated into LB broth (100 ml in 250 ml Erlenmeyer flask) with constant shaking at 150 rpm for 48 hour at 30^0C. The bacterial culture was centrifuged at 10,000 rpm for 20 min to get cell-free filtrate solution and bacterial cells were suspended in sterilized distilled water and the concentration was adjusted to 1 x 10^9 colony forming units (CFU) per micro liter. Fresh prepared cell-free filtrate solutions and bacterial cell suspension were used for each experiment.

Collectotrichum gloeosporioides was isolated from necrosed leaves and berries of black pepper vines. *Collectrichum gloeosporioides* were grown on PDA medium for 30 days and filtrate was taken. Conidial suspension of C*ollectotrichum species* were added to the different concentration of biocontrol agents so as to make final count adjusted to 8000-12,000 conidial/ml by using haemacytometer.

The pepper cutting of cultivar (Kuching) was sown in pots filled with soil mixture containing peat: sand: soil: (1:1:1) mixture and maintained in greenhouse at 30 ± 5^0C throughout the experiments. The pepper cuttings with 7 new leaves stage were used for experiments in the greenhouse.

Table1: A list of bacterial isolates and their origins

Bacterial isolates	Origin	References
Bacillus subtilis, CBF	Pepper farm, Sg Regit, Johor	Yap, 2012
Bacillus vallismortis, YCA0098	Pepper farm, Sg Udang, Melaka	Yap, 2012
Bacillus firmus YCA 5593	Pepper farm, Mersing, Johor	Yap, 2012

2.2 Determination of in vitro antagonistic activity of Bacillus species

Two independent experiments were conducted to determine the in *vitro* antagonistic activity of *Bacillus* species against *Collectotrichum gloeosporioides.* The first experiment was performed by using dual cultures techniques. One 10 mm disk of a pure culture of *C. gloeosporioides* was placed at the center of a Petri dish containing PDA. A circular line, made with a 6 cm diameter Petri dish dipped in a suspension of bioantagonistic bacteria (5×10^9cfu ml^{-1}), was placed surrounding the fungal inoculum (Figure 1). Plates were cultured for 72 h at 30°C and growth diameter of the pathogen (fungal growth) was measured and compared to control growth where the bacterial suspension was replaced by sterile distilled water. Each experiment considering a single pathogen isolate was run in triplicate and was repeated at least three times. Results obtained are expressed as means % inhibition ± S.D. of the growth of the corresponding pathogen isolate in the presence of any of the bacterial isolates. Percent inhibition was calculated using the following formula:

$$\% \text{ inhibition} = [1 - (\text{Fungal growth} / \text{Control growth})] \times 100$$

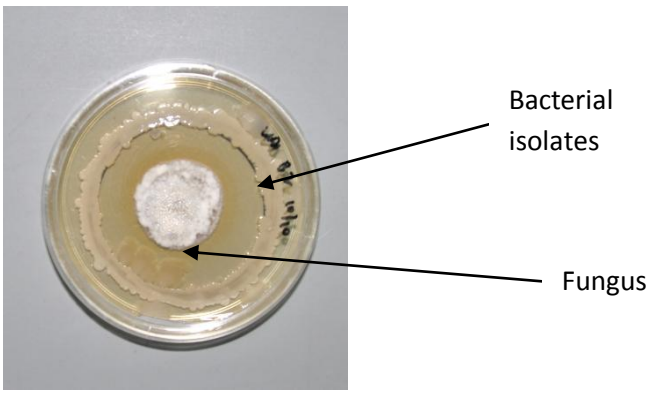

Figure 1: Dual Cultures

In the second experiment, the effects of *Bacillus* species on conidial germination were determined. *Collectrichum gloeosporioides* were grown on potato Dextrose agar for 30 days and further grown in Czapeks medium for 7 days and filtrate was taken. *Bacillus* isolates were grown on LB broth for 48 hours and culture filtrate were taken. The culture filtrate of biocontrol agent were prepared in four concentrations (0.5, 1.0, 1.5, 2.0,) using sterile distilled water. Conidial suspension of *Collectotrichum species* were added to the different concentration of biocontrol agents so as to make final count adjusted to 8000-12,000 conidial/ml by using haemacytometer. Conidial germination studies were carried out in cavity slides. Three replicate slides for each pathogen-antagonist combination were used. For control, conidial suspension was added with distilled water. Slides were incubated at 30^oC for 24 hour. Percentage spore germination was determined by dividing the number of germinating spore by the total number of spore presented per slides as observed under a Nikon Inverted Microcope (IM). Three counts per microscopic field (40 x magnification) were made per slide. A spore was considered to have germinated when the germ tube length was half of the length of the spore (Sariah, 1994). The experiment was repeated three times and plates were arranged in a completely randomized design with five replicates.

2.3 Determination of in vivo antagonistic activity of Bacillus species against *Collectrichum gloeosporioides* under greenhouse condition

Two independent experiments were conducted on cv. Kuching pepper vines to test the efficiency and efficacy of *Bacillus* strains CBF, YCA0098 and YCA5593 for suppression of *C. gloeosporioides* on pepper vines. In both experiments, a total of five bacteria applications were performed. First treatment was performed at the moment of planting by spraying 10 ml of bacterial suspension adjusted to 10^9 cfu ml^{-1}. Four additional bacteria treatment were sprayed on the pepper vines 7, 21, 32 and 56 days after the first treatment by spraying with 10 ml of 10^9 cfu ml^{-1} suspensions. Non-treated control vines were sprayed with distilled water.

C. gloeosporioides was inoculated on pepper vines 28 days after the first treatment. In the first experiment, pepper vines were inoculated by spraying with 20ml of a *C. gloeosporioides* suspension adjusted to 2 x 10^5 zoospores per ml. In

the second experiment, pepper vines were inoculated with the pathogen by spraying 40 ml of an infested substrate on the leaves surface (approximately 5 x 10^5 propagules per plant). The *C. gloeosporioides*-infested substate was prepared by placing mycelium plugs of a 15-days old *C. gloeosporioides* culture grown on modified V8 agar in a sterilized 10-Liter mixture of LB broth. The inoculates substrate was incubated at 25±5^{0}C as described by Kurze *et al*., 2001 reaching approximately 5 x10^5 propagules per ml of inoculums.

Disease levels were assessed 45 days after inoculation by observation of the level of necrosis on pepper leaves (Figure 2) according to the following scale: 0= no necrosis, 1= up to 1/3 of the leaf necrosed, 2= up to 2/3 of the leaf necrosed, and 3= up to 3/3 of the leaf necrosed. Disease severity was calculated using formula described below.

$$S = \frac{\sum_{i=1}^{n} I_i}{n \cdot 3} \cdot 100$$

where S is the disease severity per repetition, *Ii* is the necrosis index per plant, and n is the number of plant per set. In the first experiment design, two sets of five plants per treatment were arranged in a randomized experimental design, whereas in second experiment, three sets of five plants per treatment were used.

Figure 2: Disease severity of black pepper cutting cultivar Kuching, 45 days after *C. gloeosporioides* inoculation in the treatment of spraying with strain *Bacillus species*

Additionally, the effect of individual and combined application of *Bacillus* strains CBF, YCA0098 and YCA5593 on biological control of *C. gloeosporioides* was assessed on pepper vines of cultivar Kuching. Two independent experiments were conducted and in both experiments, a total of 5 bacteria applications were performed as described above. First application treatment was performed at the moment of planting and four additional treatments were performed 15, 23, 38 and 53 days after the first treatment. Non-treated control plants were sprayed with distilled water. 30 days after the first treatment, each plant was inoculated by spraying with 20ml of a *C. gloeosporioides* suspension adjusted to 2 x 10^5 zoospores per ml. In the second experiment, pepper vines were inoculated with the pathogen by spraying 40 ml of an infested substrate on the leaves surface (approximately 1 x 10^6 propagules per plant). At the end of the assay (45 days after pathogen inoculation) necrosis levels of leaves were assessed as described above. Three sets of five plants per treatment were arranged in a randomized experimental design and the experiment was repeated twice.

2.4 Determination of the biocontrol effect of Bacillus species on C. gloeosporioides in the field

Field trials on black pepper (*Piper nigrum L.*) to evaluate the efficacy of *Bacillus* species against *Colletotrichum gloeosporioides* were conducted in Serian district, Sarawak, Malaysia. The fields were known to be naturally infested by *Colletotrichum gloeosporioides.* A completely randomize block design (RCBD) was used. Pepper vines of the variety Kuching were planted in rows with spacing of 2.1m x 2.1m between and within rows. The sites were divided into three blocks or replicates. Each block contained four treatment and 50 vines of pepper vines were planted for each treatment.

The five treatments consisted of (T1): untreated control; (T2): *Bacillus* strain CBF; (T3): *Bacillus* strain YCA0098 ; (T4): *Bacillus* strain YCA5593 ; (T5): fungicide Tebuconazole (43% a.i) 750 g ai ha^{-1}. The development of the pepper berries to maturity took 8 months from flowering to full ripeness. Therefore, the experiment consisted of 8 treatment (monthly basis) based on recommended culture practices. No treatments were applied during harvesting season to prevent accumulation of pesticide residue in pepper berries.

Disease incidence was recorded about one week before harvesting. For each plot, 50 vines were visually examined for the presence or absence leave anthracnose symptoms. Disease incidence of each plot was calculated as the percentage of diseased plants. Disease severity was assessed according to the following scales: 0= no diseased, 1= <5%, 3= 6-15, 5= 16-30%, 7= 31-50%, 9= >50% disease leaves or berries. Disease index and control efficacy were calculated as follows (Luo and Zhou,1994):

$$\text{Disease index} = \frac{\Sigma\,(\text{disease severity score} \times \text{number of leaves with the scale})}{(\text{the highest severity score} \times \text{the total number of leaves examined}} \times 100$$

$$\text{Control efficacy} = \frac{(\text{The mean disease index of the control} - \text{The mean disease index of the control})}{\text{The mean disease index of the control} \times 1000\%}$$

2.5 Scanning electron microscopy of the interaction C. gloeosporioides -bacterial cells on SCRE and leaves

Two co-inoculation methods were performed in order to observe the interaction between *Bacillus* strains CBF, YCA0098, YCA5593 and *C. gloeosporioides in vitro* and on pepper leaves.

To observe the interaction between bacterial strains and *C. gloeosporioides in vitro*, a co-culture was performed, which consisted of co-culture of bacterial cells of each strain and *C. gloeosporioides* propagules in liquid medium (SCRE). In tubes containing 1.8 ml of SCRE, 0.9 ml of a *C. gloeosporioides* suspension (10^5 zoospores per ml) and 0.3

ml of bacterial suspension (10^8 cfu ml^{-1}) were added. Cultures were incubated 24h at 25^0C. Preparation of co-culture suspension for scanning electron microscopy (SEM) was performed as following. In each step, suspensions were centrifuged at 7000 x g for 10 min and pellets were subjected to fixation and dehydratation. For standard fixation, pellet were fixed for 3h at room temperature in glutaraldehyde (2.5% v/v. in 0.1M cacodylate buffer, pH 7.2) and rinsed two times in 0.1 cacodylate buffer and finally in demineriliz ed water. Samples were dehydrated by a series of ethanol rinses (50% to 100%), subjected to critical point drying and mounted on metal stubs (12mm diameter) with double-sided adhesive tape.

To observe the interaction between bacterial strains and *C. gloeosporioides* on the pepper leaves surface, the following procedure was performed. A healthy pepper vines (cv Kuching) leaf was removed from the vines. The leaves were washed with tap water and surface disinfested by immersion in a diluted solution of sodium hypochlorite (2% active chlorine) for 1 min, rinsed three times with steriled distilled water, and placed on sterile filter paper under an air stream to remove excess water. Leaves were split and immersed in tubes containing a 10^8 cfu ml^{-1} suspension of each bacterial strain for 1h. The non-treated control consisted of leaves immersed in sterilized distilled water. Then, leaves were immersed in a *C. gloeosporioides* suspension adjusted to 10^5 zoospores per ml and incubated for 24h at 25^0C in a controlled environment chamber. After this, leaves were prepared for SEM. Leaves were gently removed from tubes and sectioned into 1cm cube. Leaves sections were fixed and dehydrated as described for culture samples, but without the centrifuge steps. Afterwards, leaves sample were subjected to critical point drying and mounted on metal stubs with double-sided adhesive tape.

After being coated with a thin gold layer with a SEM coating unit (Emitech K550 sputter coater, Quorum Technologies, UK), culture and leaves samples were directly examined with a scanning electron microscope (Zeiss DMS960A, Carl Zeiss Inc., Germany) operating at 15-20kV.

2.6 Statistical Analysis

Data were statistically analyzed using procedures of the Statistical Package for Social Sciences (SPSS, version 10·0 for Windows, SPSS Inc., Chicago, IL, USA). All data were analyzed by analysis of variance (ANOVA) and the treatment means were separated by using Duncan's multiple range test at $P \leq 0.05$.

3.0 RESULTS

3.1.1 Determination of in vitro antagonistic activity of Bacillus species against Colletotrichum gloeosporioides

Dual culture assay:

There was no physical contact between antagonists and pathogen (Figure 1). An inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria. All the bacterial strains significantly reduced *Colletotrichum gloeosporioides* growth in comparison with the control. Table 2 summarized the antagonistic activity of *Bacillus species* against *Colletotrichum gloeosporioides*. From the data collected, *Bacillus* strain CBF were found to be the most effective isolates that are able to suppress the growth of *Colletotrichum gloeosporioides*, followed by *Bacillus* strain YCA5593 and *Bacillus* strain YCA0098 with the inhibition percentage of 50.1%, 45.9% and 44.7% respectively.

Table 2: Antagonism of *Bacillus species* against *Colletotrichum gloeosporioides* using dual culture techniques

Pathogenic fungus	**% inhibition**			
	YCA0098	**YCA5593**	**CBF**	**Control (distilled water)**
Colletotrichum gleospeorides	44.7±2.2	45.9±1.8	50.1±2.3	0

3.1.2 Conidial germination studies

The effect of *Bacillus* species on the conidial germination of *Colletotrichum gloeosporioides* are presented in Table 3. The results obtained reveal that the maximum inhibition of conidial germination was brought out by 1% *Bacillus subtilis, CBF* (86.2% for *C. gleospoiridioes*), followed by 1.5% *Bacillus vallismortis, YCA5593* (61.4% for *C. gleospoiridioes*) and 1% *Bacillus firmus, YCA0098* had the lowest inhibition activity over control with inhibition activity was 36.3% against *C. gleospoiridioes* (Table 3).

All concentrations of culture filtrate of *Bacillus* species were found to inhibit conidial germination of *Colletotrichum gloeosporioides* at varying concentration. In this study, it was found out that all the concentration of *Bacillus* species inhibited the germination of *C. gleospoiridioes* in the range of 28.8%-86.2% (Figure 3).

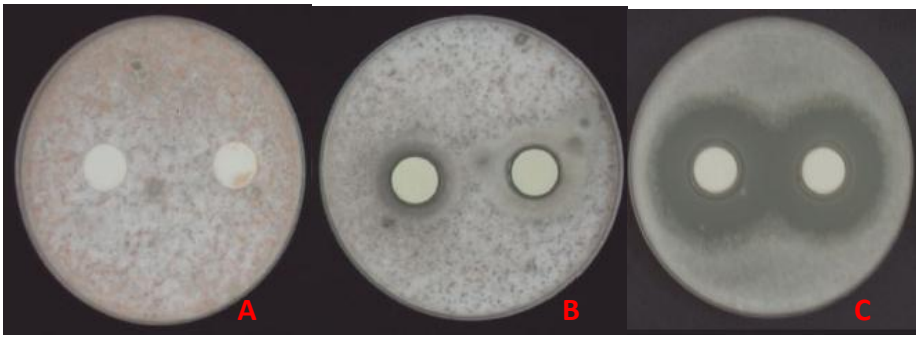

Figure 3: Inhibition of *Colletotrichum species* spore by *Bacillus subtilis.* Plate A (control plate with no inhibition zone were observed. Plate B, Inhibition of *Colletotrichum gleospoeridioes* spore with *Bacillus strain* YCA5593, and plate C: Inhibition of *Colletotrichum gleospoeridioes* spore with *Bacillus* strain CBF

Table 3: The effect of antagonistic microorganism on the conidial germination of *Colletotrichum gloeosporioides*

Antagonists	**Concentration**	***C. gleospoiridioes***	
		% of germination	% of inhibition
Bacillus subtilis (CBF)	0.5	29.0	69.9
	1	13.3	86.2
	1.5	15.6	83.8
	2.0	16.2	83.2
	0 (control)	96.4	/
Bacillus vallismortis (YCA5593)	0.5	46.3	51.3
	1	40.1	57.8
	1.5	36.7	61.4
	2.0	38.3	59.7
	0 (control)	95.0	/
Bacillus firmus (YCA0098)	0.5	68.4	30.8
	1	62.9	36.3
	1.5	65.1	34.0
	2.0	70.3	28.8
	0 (control)	98.7	/

3.3 Determination of in vivo antagonistic activity of Bacillus species against *Collectrichum gloeosporioides* under greenhouse condition

Bacillus spp strains CBF, YCA0098 and YCA5593 were tested individually for biological control of *C. gloeosporioides* on pepper vines (Figure 4). Strains CBF and YCA0098 significantly (P=0.007) reduced disease severity level of leaves anthracnose of first experiments (98% and 95% respectively) and in second experiment (99% and 96% respectively). Strains YCA5593 was only effective in experiment 2 with the disease reduction of 91% and 83% respectively in first and second experiments. Disease severity in non-treated control plants was low and ranged from 25% to 28% in both experiments.

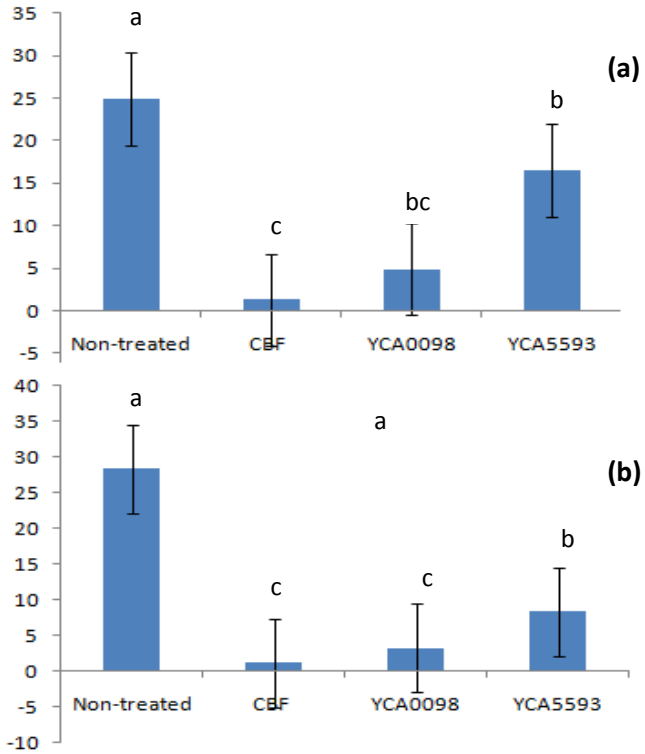

Figure 4: Effect of Bacillus species strains CBF, YCA0098 and YCA5593 on *C. gloeosporioides* disease severity on potted pepper vines of cv. Kuching. Values are the mean of two sets of five plants in experiment (a) and three sets of five plants in experiment (b). Error bar represent the mean standard error, Bars headed by different letter are significantly different (P≤0.05) according to Duncan's multiple range test.

Strains CBF, YCA0098, and YCA5593 and their combination were further tested for *C. gloeosporioides* biocontrol efficacy on pepper vines (Figure 5). Strain CBF produced a reduction of disease severity of 88% and 83% in experiment 1 and 2 respectively, compared to the non-treated control. A higher variability was observed in plants treated with strain YCA5593, which reduce disease severity level 77% and 88% in experiment 1 and 2 respectively, compared to non-treated controls. Application of strain YCA0098 obtained a lower reduction of disease severity in both experiments (70% and 73%), compared to non-treated control. The combination of strains CBF with YCA0098 and CBF with YCA5593, YCA0098 with YCA5593 and the triple combination of strain CBF, YCA0098 and YCA5593 produced a reduction of disease severity that ranged from 56% to 78%, in both experiments.

Although combinations of bacterial strains did not improve efficacy of disease suppression compared to single strains, they reduced variability between experiments. The coefficient of variation between experiments of treatment with single strains was rather high, being 58.6%, 55.6% and 108% for strains CBF, YCA0098 and YCA5593, respectively. The coefficient of variation between experiments of plants treated with mixture of CBF with YCA0098 and CBF with YCA5593 was lower than in treatment with single strains (30.6% and 35%, respectively), whereas the coefficient of variation between experiments of plants treated with the combinations of YCA0098 and YCA5593 and the triple combination was higher (63.3% and 63.2%, respectively). Non-treated control plants reached disease severity levels of 40.5% and 41.8% in both experiment, and a coefficient of variation between experiments of 26.0%.

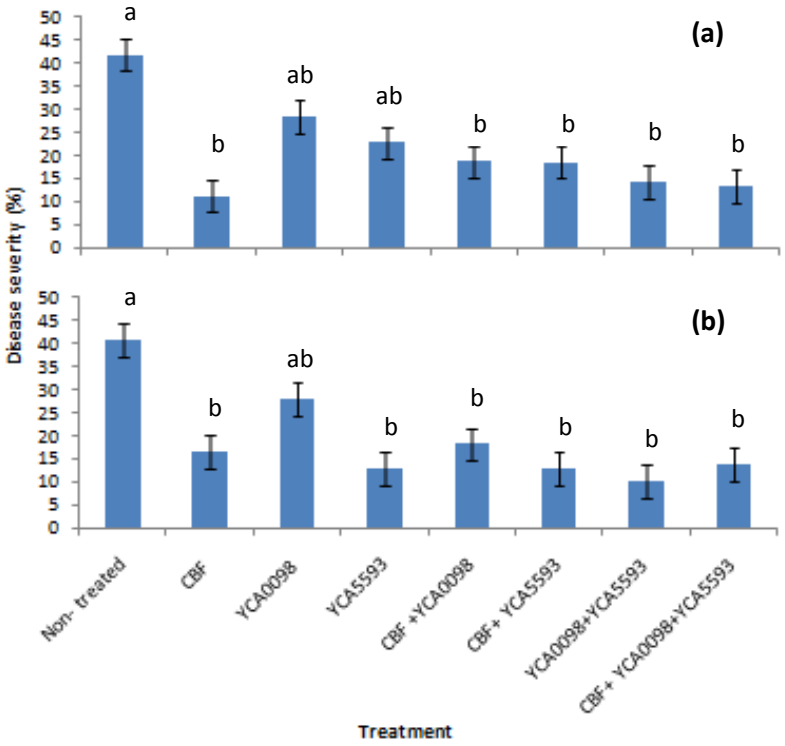

Figure 5: Effect of *Bacillus* species strains CBF, YCA0098, YCA5593 and their combination on *C. gloeosporioides* disease severity on potted pepper vines of cv. Kuching. Values are the mean of three sets of five plants and correspond to two independent experiment (a and b). Error bar represent the mean standard error, Bars headed by different letter are significantly different (P≤0.05) according to Fisher's protected least significant different (LSD) test.

3.4 Determination of the biocontrol effect of Bacillus species on C. gloeosporioides in the field

The results of the field trial indicated that the application of *Bacillus* species against *C. gloeosporioides* significantly suppressed the occurrence of leave anthracnose disease of black pepper. From the results obtained, the disease incidence and disease index of *Bacillus* species against *C. gloeosporioides* were significantly lower than non-treated control (Table 4). The biocontrol efficacy of *Bacillus* strain CBF was the highest (79.45%) with the disease incidence and disease index value of 6.01% and 2.25% respectively. This was followed by the strain YCA0098, having 70.25% biocontrol efficacy value with disease incidence and disease index value of 6.85% and 2.66% respectively. Strain YCA0098 having the lowest value of biocontrol efficiency (72.21%) with disease incidence and disease index value of 7.02% and 2.73%. Although the biocontrol efficiency value is lower than those of the fungicide treatment, Tebuconazole (97.95%), but the all the *Bacillus* species were able to suppressed the disease incidence for >70%.

Table 4: Biocontrol efficacy of *Bacillus* species against leave anthracnose caused by *Colletotrichum gloeosporioides* in comparison with fungicide tebuconazole tested in the field

Treatment	Disease		Biocontrol efficiency ± SE (%)
	Incidence ± SE (%)	Index ± SE (%)	
No treated control	14.90±3.20	8.26±1.20	0.00
Bacillus strain CBF	6.01±0.87	2.25±0.39	80.45±5.12b
Bacillus strain YCA0098	7.02±0.56	2.73±0.35	72.21±3.26b
Bacillus strain YCA0115	6.85±0.30	2.66±0.26	76.25±3.05b
43% Tebuconazole	2.25±0.75	1.78±0.29	97.95±4.26a

3.5 Scanning electron microscopy of the interaction C. gloeosporioides -bacterial cells on SCRE and leaves

Bacillus species strains CBF, YCA0098 and YCA5593 appeared attached to the surface of *C. gloeosporioides* when samples from *in vitro* liquid suspensions were observed with scanning electron microscopy (SEM) (Figure 6). Bacterial cells were observed attached on the surface of hyphal. The cell suspension of *Bacillus* strain CBF inhibits the *C. gloeosporioides* and invasion of pepper leaves. The observation revealed that the normal hyphae had a relatively even width (Figure 6A), while the hyphal treated with CBF, YCA0098 and YCA5593 became shrink (Figure 6B), irregularly swellness at the tips (Figure 6C) and with cytoplasm leaked (Figure 6D).

Scanning electron microscope observations of pepper leaves treated with strains CBF, YCA0098 and YCA 5593 inoculated with *C. gloeosporioides* are shown in Figure 7. The observations shown that the treatment of strain CBF cell suspension reduced the hyphal growth on the surface of pepper leaves and slowed down the formation of infection cushions and thus suppressed infection. The SEM observations showed that the running hyphal growing from the tips of

the leaves and formed abundant infection cushions on the leaves surface in non treated control (Figure 7A). Whereas poor hyphal development was observed on all bacterial-treated leaves (Figure 7B). A magnification of a leaves treated with bacterial strain CBF showed the presence of bacterial cells close to pathogen in epidermis creases, Bacterial cell were also observed embedded in a mucilaginous matrix on the leave surface forming micro-colonies (Figure 7C and 7D). All of these observations may explain how the bacterium is able to reduce *C. gloeosporioides* infection.

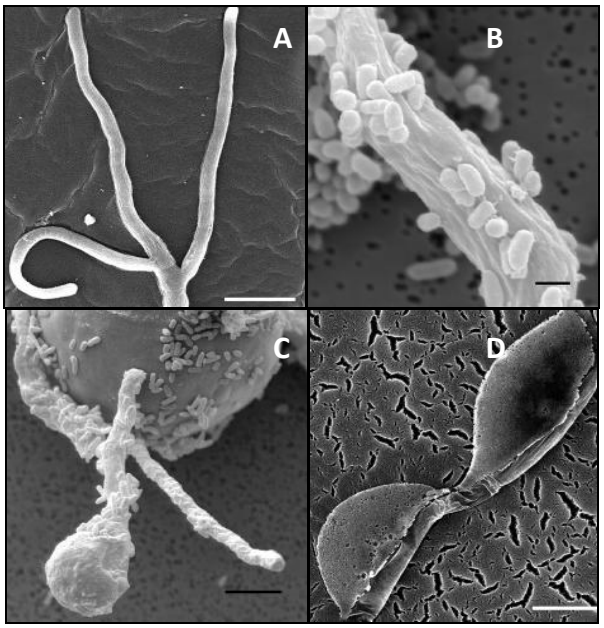

Figure 6: Scanning electron micrograph of *B. subtilis* strains CBF interacting with *C. gloeosporioides* propagules induced to germinate in vitro. Micrographs were taken 24 h after inoculation. (a) Non-treated control showing mycelia branches in relatively uniform width (scale bar: 20µm). (B-D) Treated with *Bacillus* strain CBF showing abnormal hyphae. (b) Hyphae was shrinking after bacterial treatment (Scale bar: 10µm), (c) The tip of the hyphae was irregular swelling (scale bar: 10µm) and (d) cytoplasm of hyphae was leaking (scale bar: 50µm).

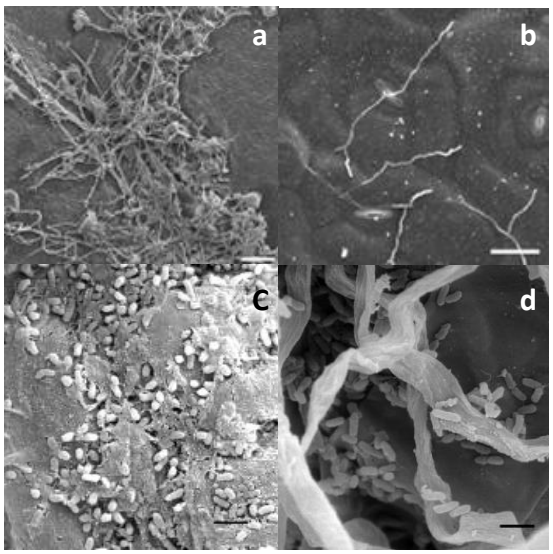

Figure 7: Scanning electron micrograph of *B. subtilis* strains CBF interacting with *C. gloeosporioides* propagules germinating on pepper leaves. Micrographs were taken 24 h after inoculation. (a) The extension of hyphae on the surface of leaves in the non-treated control, also showing the abundant infection cushions formed on the leave surface (scale bar: 200µm). (b) The formation of few hyphae on the surface o pepper leaves after bacterial treatment (Scale bar: 20µm), (c) Bacillus strain CBF cells on the leaves surface (scale bar: 2µm) and (d) Hyphae and bacterial cells on the surface of the CBF cells-treated leaves (scale bar: 2µm).

4.0 Discussion

At present, there are several bio-control products from rhizobacteria which have been developed and many plants' disease bio-control products that contain *Bacillus* species have been used (Gardener, 2004). Among these agents, there is an increasing interest in the introduction of *Bacillus* spp for managing fungal infection. *Bacillus* spp such as *B. subtilis, B. licheniformis, B. amyloliquefaciens and B. cereus* have been reported effective against foliar and soil-borne fungal pathogens (Hassan et al., 2010 and Carneiro et al., 1998). This might probably be due to their ability to induce growth and the defense response in host plant. In addition, *B. subtilis* non-ribosomally synthesizes several kinds of small antibiotic peptides that have antifungal activities, such as iturin (Joshi and Gardener, 2006), surfactin (Peypoux, 1999) and fengycin (Loeffler, 1986). *B. subtilis* also secretes protease enzymes in abundance (Liu *et al.*, 2007). Despite a wealth of new formulation on the genetics and physiology of *Bacillus* and related species, little is known about the efficacy of *Bacillus* spp against leave anthracnose and black berries disease in black pepper. Increased understanding of the antifungal mechanism of antagonist could potentially enhance the value of these species as effective bio-control agent.

In this study, Dual culture assay showed that strains CBF was the most efficient bioantagonistic bacteria with 50.1% inhibition against *Colletotrichum gloeosporioides*, followed by strain YCA5593 with inhibition percentage of 45.9%. The strain YCA0098 showing the lowest inhibition activity with the inhibiton percentage of 44.7%. The presence of antagonistic effect among the bacterial isolates might probably due to the presence of certain fungistatic metabolites secreted by bacteria CBF, *(Bacillus subtillis)*. This finding is similar to the research report by other researcher (Yap, 2012) who reported that many pathogenic fungi are sensitive to *Bacillus* cells suspension or it culture filtrate. This finding is in agreement with Korsten and Jager (1995) who reported that *Bacillus subtilis* was detected as antibiotic producer. Beside, this finding was further support by the conidia germination study. From the results obtained, Inhibition

of germination of pathogenic fungi *in vitro* was attributed to the antifungal properties of volatile and non-volatile antibiotics, as reported earlier (De Costa and Erabadupitiya, 2005). The failure of studied pathogens spore to germinate indicated that the metabolites or antibiotics produced by *Bacillus species* were not only fungistatic but also fungicidal to the spore of the tested fungus. Similarly, supression of spore germination by *Bacillus* spp has also been reported (Smilanick and Denis-Arrue, 1992; Janisiewicz and Roitman, 1988).

In the greenhouse experiments, the effect of the co-incubation of mixture of strain CBF, YCA0098 and YCA5593 and the triple combination on hyphal gemination and growth was not different or was slighly less effective than strain applied individually. This phenomenon might probably due to the competition between bacterial strains. The similar study has also been reported by Purivirojku et al., 2006 who suggested that the growth inhibition among *Bacillus* species in due to nutrient competition. Besides, the incompatibilities of synthesis regulation of different antibiotic between bacterial strains also contribute to the less effective of bacterial mixture as compared to single strain culture. Some studies of *in vitro* incompatibilities between strains have also been reported (Lutz et al., 2004).

The combination of bacterial strain did not improve biocontrol efficacy of *C. gloeosporioides* in pepper vines, but reduce variability between experiments compared to application of individual strains. The coefficient of variation between experiments observed in plants treated with YCA5593 was rather high, compared to the variation observed in treatments with YCA0098 and CBF applied individually. Dual mixtures of CBF with YCA0098 and CBF with YCA5593 were the best in reducing variability. However, the combination of strains YCA0098 and YCA5593 and the triple combination did not produce an important reduction of variability.

Generally, application of mixture of strains may results in higher biocontrol and lower variability of biocontrol, as it has been reported in other studies on different pathosystems. Guetsky et al., 2001 suggested that application of more than one antagonist with different ecological requirements would increase the reliability and decrease the variability of biocontrol. In this study, the combination of biocontrol agents on detached pepper leaves and whole plants increased biocontrol since the effect of their biocontrol mechanism was cumulative. Similar study was reported by Roberts et al., 2005 who reported that an increase of biocontrol effectiveness with strains combinations of *Trichoderma virens, Serratia marcescens* and *Burkholderia spp* against soilborne pathogens *Rhizoctonia solani* on cucumber plants.

The field experiments showed that the *Bacillus* species able to reduce leave anthracnose and black berries disease of black pepper. The control efficacy of *Bacillus* species were more than 70%. Altough the biocontrol efficacy of the *Bacillus* species is lower than fungicide (Tebuconazole), but this finding is similar to the results reported by other researcher, who reported that the biocontrol efficacy of most biocontrol agent ranged from 70-80% (Devasahayam, S. (1996). This indicated that the biological agents cannot completely eliminate the disease occurrence, but they can prevent the disease outbreak from happening. Besides that, the application of biological control agent also able to increase the quality of berries produced as compared to chemical fungicide. This is because no pesticide residue can be detected in the berries applied with biological control agent. The lower level of disease suppression of *Bacillus* species as compared to chemical fungicide was presumable due to the fact that variable efficacy of the biological control agent dependent on the soil environment where the biocontrol agent is applied, the moment and the method of application, the host plant or the pathogen species. In addition, appropriate formulations would also be other factor affecting the efficacy of the successful implementation of biological control.

Eventhough there are many studies has been conducted to determine the mechanism of action of *Bacillus* strains against *Collectotrichum spp*, however, It remains unclear how *Bacillus* strain CBF, YCA0098 and YCA5593 suppresses the incidence and severity of leave anthracnose caused by *C. gloeosporioides* on pepper leaves and berries. In this paper, the results shown that the cell-free filtrate solution was able to inhibit the fungal germination and growth which means the bacterium secrets antimicrobial substances. The SEM results showed that the strain CBF, YCA0098 and YCA5593 treatment caused the shrink, leakage of cytoplasm and irregular tip swelling of fungal hyphae. These indicated that mechanisms responsible for biocontrol exhibited by these bacteria are through the production of hydrolytic enzymes as well as antibiosis.

In conclusion, Bacillus strain CBF, YCA0098 and YCA5593 significantly reduced *C. gloeosporioides* hyphal germination and growth *in vitro* and on pepper leaves presumably by different mechanism of action, being antibiosis one of these mechanisms, at least *in vitro*. Mixture of these bacterial strains reduced variability of biocontrol in pepper vines and seemed to improved consistency of disease suppression compared to application of single strains.

5.0 Conclusion

The coherence of results by *in vitro* and *in vivo* analysis has further strengthened the hypothesis that the mechanism responsible for biocontrol exhibited by *Bacillus* spp is though antibiosis and production of hydrolytic enzymes. On the basis of the results, it is concluded that these bacterial strains are valuable candidates for the development of biopesticides for fungal pathogen. The present study clearly indicated the potential of *Bacillus* spp as biocontrol agents which are able to suppress the fungal pathogens of black pepper. However, further studies *in planta* are required to formulate these strains in suitable carrier material and explore their potential under field conditions.

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