BIOSCIENCE JOURNAL

ANTAGONISTIC ACTIVITY OF BACTERIA FROM WILD HONEY AGAINST *Colletotrichum musae*, AND TESTING OF WILD HONEY AS BIOPESTICIDE SPRAY TO CONTROL BANANA ANTHRACNOSE

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How to cite: RIVAREZ, M.P.S., et al. Antagonistic activity of bacteria from wild honey against *Colletotrichum musae*, and testing of wild honey as biopesticide spray to control banana anthracnose. *Bioscience Journal*. 2021, **37**, e37089. https://doi.org/10.14393/BJ-v37n0a2021-49921

Abstract

Anthracnose is a foliar and fruit disease caused by *Colletotrichum* spp. affecting a wide range of crops. Infection occurs early followed by quiescence in fruits, such as in banana, where chemical-based pesticides are used as a dependable fungal control for many years. There is an increasing need for a safe control and as implicated in the Organic Agriculture Act of 2010 (RA 10068) in the Philippines. This scenario drove the use of alternative pest control such as the use of biologicals and natural products. In this study, seven bacteria were isolated from wild honey, produced by *Apis mellifera*, wherein four (BC2, BC3, BC6 and BC7) were found to be an effective antagonist against *Colletotrichum musae* in *in vitro* conditions. These bacteria were identified to belong to the genus *Lactobacillus* spp. (BC2, BC3, BC7) and *Bacillus* spp. (BC6) based on sugar utilization tests, morphological and cultural growth in PDPA. For the *in vivo* test, different dilutions of wild honey were used and it was found out that lower concentrations were effective as biopesticide spray to prevent anthracnose infection. Lastly, we report herewith the first isolation of bacteria with biological control potential from wild honey, and to apply the raw or natural product as biopesticide in postharvest fruits.

Keywords: *Bacillus*. Banana Anthracnose. Biological Control. *Colletotrichum musae. Lactobacillus*. Wild Honey.

1. Introduction

Banana is one of the most important crops in the Philippines having a production of 8.9 million tons in 2014 valuing to 130 billion Pesos (Alvindia et al. 2006). Banana belong to the genus *Musa* and widely distributed from the Pacific to West Africa but are mainly found in the South-East Asian-New Guinea region (Jones 2000). Although production has been increasing over the years, threats such as plant diseases pose a hindrance to sustained production. Banana (*Musa* spp.) is attacked by numerous fungal, bacterial, virus and nematode diseases. One of these diseases is the anthracnose disease that infects banana at the postharvest stage. Anthracnose is a foliar and fruit disease caused by *Colletotrichum* spp. present in a wide host range including banana. Anthracnose is the name given to a disease that appears as sunken, brown to black lesions found on the peel of bananas during transport, storage and ripening (Jones 2000). The disease arises when dormant infections of the dormant fungus in the green peel is activated as fruit ripens, a phenomenon called quiescence. Banana is a highly perishable fruit that suffers severe postharvest losses both in terms of quality

and volume. Anthracnose caused by the fungus *Colletotrichum musae* (Berk. and Curt.) Arx highly contributes to this conundrum in the banana industry. In the Philippines, *Colletotrichum* spp. is highly implicated in the diseases and in the plantations of banana for export (Alvindia et al. 2000; Lim et al. 2002; Alvindia et al. 2006). Banana anthracnose is one of the most important diseases of banana globally and is one of the major constraints to the banana postharvest industry. Infection on the banana plant usually starts during the development of the fruit but remains quiescent until the fruit is picked and allowed to ripen. At the yellow ripe stage of the fruit, it causes deterioration of the appearance, color and nutritive quality and renders them unfit for marketing and consumption. *Colletotrichum musae* may form lesions on fruits without skin bruising but produces larger lesions when fruits are damaged (Su et al. 2011). The anthracnose damage translates to postharvest losses that is greater than any other postharvest diseases in banana, thereby causing severe economic losses to farmers and traders.

Numerous studies were reported regarding biological and organic approaches to control the occurrence of the banana anthracnose disease. Major banana importing countries like Japan and China promulgate zero tolerance to pesticide residue to incoming agricultural produce including fruits such as banana. However, the most common control method for the banana anthracnose disease is the postharvest treatment of banana fruits with synthetic fungicides, such as benomyl and thiabendazole (TBZ) (Xiangchun et al. 2011). In the Philippines and in Ethiopia, hot water-treatment at 49-52°C for 15-20 min is recommended to control postharvest diseases in banana such as anthracnose and finger rot (Acedo et al. 2001; Bazie et al. 2014). However, the effect of such fungicides is potent but ephemeral thereby resulting fungicide resistance by *C. musae*. Moreover, fungicide toxicity and harmful effects on human health and the environment is growing, and consumers are demanding alternative treatments to reduce the use of synthetic fungicides for postharvest disease control in perishable fruits such as bananas.

In a certain study, fruit-coating materials composed of oleic, palmitic and lauric acids significantly inhibited growth of *C. musae*. Moreover, malic, citric, oxalic and maleic acids all significantly reduced growth of *C. musae* and complete inhibition of growth was achieved with potassium sorbate and sodium benzoate at 0.125% and oxalic and maleic acids at 0.5% (w/v). These organic acids also increased the lag time prior to growth initiation, which implies effective control even at the early infective stage (Al Zaemey et al. 1993). Similarly, the use of microbials and microbial extract to control banana anthracnose also gained attention. Such as is the case of yeasts (Chuang and Yang 1993), bacterial antagonists and actinomycetes (Postmaster et al. 1997; De Costa and Erabadupitiya 2005; Lassois et al. 2008; Williamson et al. 2008; Fu et al. 2010; Peeran et al. 2014).

Honey is made by bees (*Apis* spp.) from nectar or honeydew. The process of honey making starts when forager bees fly out in the field and search for pollen and nectar. When they get back to the hive, honey is made through the mechanism of food exchange from bee to bee also known as trophallaxis. It is therefore important to understand that the color and composition of honey may be different from one location to another because different nectars are used by different colonies of bees (Rokop et al. 2015). In the field of chemistry, pharmaceutical companies are trying to exploit the antioxidant and the antimicrobial capability of different honey to treat cardiovascular and gastrointestinal tract diseases by analyzing its chemical content (Bouhlali et al. 2016). On the other hand, the field of microbiology studies the antimicrobial potential of honey due to the presence of symbiont Lactic Acid Bacteria (LAB) specifically the *Lactobacillus spp.* and *Bifidobacterium spp.* in the intestine of the bees. There are studies suggesting these LAB's produce bioactive compounds that are antagonistic to other microorganisms (Olofsson et al. 2016; Sandi and Salasia 2016). In addition, there are also studies which shows that *Fructobacillus spp. and Bacillus spp*. are present in the microbial hubs which may aid in the increase of the population of LAB and have inhibitory effect to other microorganisms (Wang et al. 2015; Swain and Ray 2016). In agricultural production, plant pathogens are major threats to agricultural productivity due to the losses that it causes. Losses are caused by pathogens like *Colletotrichum* species which infects major exported products like mango and banana. In order to satisfy the yield demand of the increasing population, chemical-based fungicides against anthracnose were used against as a dependable agricultural management. Chemical fungicides are highly proven as fast and effective method for inhibiting these pathogens. For that reason, biological control agents were promoted as an alternative to chemicals. Commonly used biological control agents are *Bacillus*-based agents extracted in various source which are proven effective in controlling numerous plant pathogens. According to studies,

Bacillus has the ability of promoting plant growth, contains systemic resistance and widely used in production of antimicrobial compounds like lipopeptides, antibiotics and enzymes, and competitors for pathogenic growth of pathogens by colonization. Lastly, antagonism against fungal pathogens of plants was found out to be mainly due to its ability to produce lactic acid and some antibiotic metabolites (Van den Bosch 1982; Campbell 1989; Bellows and Fisher 1999; Gnanamanickam 2002).

This study evaluated the antimicrobial properties and associated bacteria of wild honey from Mindoro. Biological control using bacterial antagonists is governed by the fact that bacteria produce substances that are harmful to other microorganisms such as fungi as other bacteria. These substances are organic acid, antibiotic metabolites among others. The study revolved on the hypothesis that bacterial antagonists are present in wild honey that originated from the gut of the honeybees and produce certain antifungal compounds. It was the main goal of the study to determine whether our native honey that are sold cheap and is readily available in the province of Occidental Mindoro can also be used in biological and organic disease management in the Philippine setting. Thus, the study generally aimed to evaluate the biological control potential of isolated bacteria from honey against *Colletotrichum musae* in *in vitro* conditions and to determine whether wild honey can be used as a biopesticide spray to prevent banana anthracnose. Moreover, the study specifically aimed (1) to characterize and identify associated bacteria from wild honey, (2) to test the possible antagonism of the isolated bacteria from wild honey against the anthracnose pathogen, *Colletotrichum musae*; and (3) to evaluate the efficacy of wild honey as biopesticide spray to prevent anthracnose disease in banana.

2. Material and Methods

Acquisition of wild honey from Occidental Mindoro

Samples of honey was obtained from wild sources in Sablayan, a town in the province of Occidental Mindoro. The honey was harvested from local sources around local forests in Sablayan, not from a man-made apiary. Thus, the sources of nectar for the bees to feed on came from random sources in the wild. The wild honey was transported from Occidental Mindoro to the College of Agriculture and Food Science (CAFS), UP Los Baños through a courier. It was stored at room temperature in a sealed jar, until it was used for the succeeding parts of the experiment.

Isolation of the bacteria associated with wild honey

PDPA (Potato dextrose peptone agar), a general culture medium was used to isolate bacteria associated with wild honey from Sablayan, Occidental Mindoro. Honey was prepared in diluted suspensions following the serial dilution technique. Ten-fold dilutions up to the 10^{-5} were prepared. An aliquot (0.1 mL) of the 10^{-3} to 10^{-5} dilutions were surface plated onto PDPA plate with three replications each. The isolation plates were incubated for 3 da at room temperature and single colonies observed from the plates with colony count were transferred on PDPA slants. These slants were duplicated with one set stored on the refrigerator while the other set was stored under room temperature for the preliminary test of antagonism.

Characterization of the isolated bacteria

Bergey's manual was used in the tentative identification of the bacterial isolates (Brenner et al. 2004). Gram staining, endospore staining, catalase test, test for oxygen requirement and sugar utilization tests were done according to the steps indicated from the Introductory Phytobacteriology (PPTH 103) laboratory manual (Natural (undated)).

Characterization of the isolated bacteria: gram staining and spore staining

Bacterial cells from a 24-hr culture and cells from a 4-da old culture were used in the Gram staining and spore staining, respectively. A smear of bacterial cells was prepared by air drying and heat-fixing. In Gram staining, Lugol's iodine served as the stain while safranine as the counterstain. In endospore staining, malachite green served as the stain. Red or pink indicates that the bacteria is Gram negative while Blue or

violet indicates that it is Gram positive. Lastly, the presence of green circular bodies adjacent to or within a bacterial indicates a positive test for bacterial endospores.

Characterization of the isolated bacteria: catalase test

Toxicity to the bacteria brought about by the production of reactive oxygen species (*i.e.* hydrogen peroxide, peroxide) by the plant can be overcome by the enzymatic action of catalase. The enzyme catalyzes the degradation of hydrogen peroxide to oxygen gas and water. Catalase is produced by anaerobic bacteria, but greater in amount in obligate anaerobe than in facultative anaerobe. Strictly aerobic bacteria do not produce catalase because the degradation product, particularly oxygen gas, is toxic to them. To evaluate whether the bacterium produces catalase or not, a thin smear of the bacterium was prepared on a sterile glass slide. One drop of 3% aqueous hydrogen peroxide solution was dispensed onto the smear. A few minutes of observation was dedicated until bubbling occurs. The formation of bubbles is indicative of the production of hydrogen gas as a result of the action of catalase on the hydrogen peroxide molecules. Thus, bubbling indicates a positive reaction for the presence of catalase.

Characterization of the isolated bacteria: test for oxygen requirement

Various species of bacteria have different requirement for oxygen. Aerobic bacteria require the supply of oxygen for its metabolic processes and survival. The oxygen they require is used as the terminal electron acceptor in the electron transport chain of cellular respiration. Anaerobic bacteria, on the other hand, make use of organic compounds as terminal electron acceptor. Facultative aerobic bacteria can survive either in the presence or absence of oxygen. In this test, an agar seal was used to prevent oxygen in reaching the bacteria grown in the culture medium in a test tube. The culture medium used in this experiment contains bromcresol purple which turns yellow if there is significant decrease in the pH of the medium. The low pH indicates that the bacteria undergone lactic acid fermentation under anaerobic conditions. Thus, yellowing of the medium indicates that the bacterium is able to perform anaerobic fermentation. Moreover, if the bacterium grows on the surface of both sealed and unsealed then it is most probably a facultative anaerobe.

Characterization of the isolated bacteria: acid production from carbohydrates as a test for sugar utilization

Different bacteria have varying ability to utilize sugars as food source. Medium C was used in this experiment to indicate the successful production of acids from the degradation of various sugars. The dye basically contains bromcresol purple in ethanol solution (0.15% w/v). Test tube slants containing various sugars namely, cellobiose, glucose, lactose, maltose, mannitol, mannose, sorbitol, were prepared. A change in color from purple to yellow indicates successful lactic acid formation due to lactic acid fermentation of sugars. Nevertheless, yellowing will just occur in sugars that are compatible with the enzymes produced by the bacterium. Thus, different genera of bacteria or even different species would have a different set of sugars utilized. Thus, this test can be very useful in taxonomy and can be used for differentiating and identifying different bacterial isolates.

Isolation of *Colletotrichum musae* (Berk. and Curt.) Arx from diseased banana fruit specimens

Banana fruits with conspicuous symptoms of the anthracnose disease caused by *C. musae* such as sunken dark lesions were obtained from the local market of Batong Malake, Los Baños, Laguna. The fruits came from nearby towns, thus are locally produced. Tissue planting technique in potato dextrose agar (PDA) was done in order to isolate the causative fungal pathogen. A 3x3 cm section of the banana peel was excised from the diseased specimen which contains half infected and half healthy tissue. The tissue portions were surface sterilized using 0.5% sodium hypochlorite solution for 30 sec, rinsed with sterile water for 1 min and then blot and air dried in a laminar flow hood. The tissue portions were placed in PDA and mycelial growth was observed after 5-7 da of incubation at room temperature. An agar block containing the actively growing mycelium of putatively *Colletotrichum* species was obtained and transferred to a fresh PDA culture plate. Pure culture was made by transferring advancing margin of the 5-day old subculture growth of the pathogen

into new PDA plates and slants with three replications. The fungal pathogen was stored at refrigerating conditions at 4°C until future use. Microscopic observation and identification of the pathogen was also done. The pathogen was identified up to the species level using taxonomic keys and related literature.

Pathogenicity test of the isolated fungal pathogen

In order to test whether the isolated fungus is certainly *C. musae* and whether it can cause anthracnose in banana, pathogenicity testing was done. A seven-day-old pure culture of the fungus in PDA slant was prepared and 10 ml of sterile distilled water was added to suspend the conidia. The number of conidia was standardized using a haemocytometer in order to obtain 50,000 conidia/mL final concentration. The conidial suspension of the fungus was sprayed onto the surface of wounded and unwounded banana fruits and was incubated at room temperature for one week. Positive results for the pathogenicity test are the appearance of the anthracnose symptom characterized by brown to black sunken lesions.

In vitro* test for antagonism against *Colletotrichum musae

In order to test for possible antagonistic effect of the isolated bacteria against *C. musae,* the agar co-cultivation technique was employed. Each bacterium was streaked in the middle of PDPA plate and a mycelial disc (0.8 cm diameter) of a seven-day old pure culture of the pathogen was placed on opposite sides of the plate serving as the subsamples (Figure 1). Five replicates were prepared for the seven bacterial isolates. The assay plates were incubated at room temperature with ambient lighting. After 72 hours of incubation, zone of inhibition was measured and percent inhibition was computed as follows (adapted from Peeran et al. 2014): % growth inhibition = $[(R_C - R_A) / R_C] \times 100$, where R_C is the radial growth (in cm) in the control setup (no co-cultivation with bacteria) and R_A is the radial growth (in cm) in the co-cultivation assay setup.

***In vivo* test for efficacy of wild honey as biopesticide spray**

Blemish-free 'more yellow than green' banana fruits (variety *Lakatan*) were bought from the local public market of Barangay Batong Malake, Los Baños, Laguna. The fruits were disinfected using 0.5% sodium hypochlorite for 2 minutes followed by submerging twice in sterile distilled water for one minute, then blot and air dried. The surface disinfected fruits were sprayed with different concentrations of wild honey suspensions in sterile water.

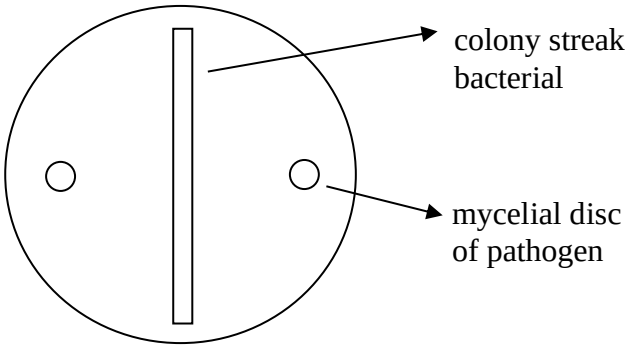

Figure 1. Set up for agar co-cultivation test of antagonism.

The following are the treatments prepared in this study: Treatment 1 (T1) is the negative control (water only); T2 is the positive control (pathogen only, wounded); T3 is the positive control (pathogen only, unwounded); T4 is spraying of 10^{-1} v/v wild honey suspension only; T5 is spraying of 10^{-3} v/v wild honey suspension only; T6 is spraying of 10^{-5} v/v wild honey suspension only; T7 is spraying of 10^{-1} v/v wild honey suspension with spray-inoculated pathogen; T8 is spraying of 10^{-3} v/v wild honey suspension with spray-inoculated pathogen; and T9 is spraying of 10^{-5} v/v wild honey suspension with spray-inoculated pathogen.

Dilutions of wild honey was applied to the fruits through the use of atomizer then air dried under laminar flow hood for 15 min. Spore suspension of the pathogen was standardized at 50,000 conidia/mL

using hemocytometer and was sprayed on the fruit surface also by atomizer followed by air drying at room temperature for 30 min. After air drying, each treatment of fruits was incubated on a moisture-saturated (almost 100%) chamber for 24 hours. After the incubation period, the plastic used to cover the fruit tray were removed and setup was observed for disease severity and disease incidence after 7 days. Disease severity was rated as follows: 1 = no observable anthracnose lesions in the fruit surface, 2 = 1-25% surface area with lesions, 3 = 26-50% surface area with lesions, 4 = 51-75% surface area with lesions and 5 = 76-100% surface area with lesions (Sivakumar et al. 2002). Five replicates for each treatment were prepared with two subsamples each. The average disease severity was calculated for each treatment after seven days.

Statistical analysis

The experiments were laid out and implemented following the Completely Randomized Design (CRD). All data gathered in the co-cultivation assay for antagonistic activity (percent inhibition) were analyzed using One way analysis of variance (ANOVA). On the other hand, Two-way analysis of variance (ANOVA) in CRD was implemented for *in vivo* test for efficacy of wild honey as a biopesticide spray. All the data gathered in this study will undergo statistical analysis through analysis of variance (ANOVA at $\alpha = 0.05$ ($^{\#}$)), provided that all assumptions are satisfied. If there is significant difference among the treatments, their means will be subjected to pairwise mean comparison using Tukey's test. All tests are considered significant at $\alpha = 0.05$ ($^{\#}$) or $P < 0.05$ (*) or < 0.01 (**). All data analyses were carried out using the Virtual Studio of the Statistical Analysis Software (SAS) University Edition (SAS Institute, Inc., Cary, NC, USA, 1976).

3. Results and Discussion

Bacillus spp. and *Lactobacillus* spp. were isolated from wild honey

Bacteria associated with wild honey were isolated in pure culture and were characterized for proper identification up to the genus level. The seven bacterial isolates were tagged as BC2, BC3, BC4, BC5, BC6, BC7 and BC8. Based on the results of the different diagnostic tests conducted, the isolates were tentatively identified to belong to the genera *Bacillus* spp. (BC4, BC5 and BC6) and *Lactobacillus* spp. (BC2, BC3, BC7 and BC8). All isolates were Gram positive while only BC4, BC5 and BC6 are all endospore-forming, which is characteristic of the genus *Bacillus*. *Lactobacillus* spp. isolates (BC2, BC3, BC7 and BC8) did not produce any endospores. Morphologically, all are long rods, characteristic of the *Bacillus* and *Lactobacillus* genera (Brenner et al. 2004; Jense 2005). Figure 2 shows the three-day-old pure cultures of the four potentially antagonistic bacterial isolates in PDPA plates.

Colletotrichum musae (Berk. and Curt.) Arx was isolated and was proven to cause anthracnose disease in banana fruit

The causative fungal pathogen of the anthracnose disease was isolated, characterized, identified, and tested for pathogenicity in healthy banana plants. The colony of the fungus in PDA is light red to pink in color and it also gives off a light red pigment to the culture medium, which is PDA. When viewed under the microscope, conidia are characterized by brown to light brown color and is cylindrical to ellipsoid in shape. The conidia are also slightly smaller than the conidia of *C. gloeosporioides* when viewed under 1000X magnification. Appressoria were also slightly smaller than *C. gloeosporioides* and characterized as dark brown and irregularly lobed (Sutton and Waterston 1970; Lim et al. 2002). Additionally, the pathogenicity test proved that the fungus indeed causes anthracnose disease in healthy and surface sterilized banana fruit, as observed after seven days of incubation. All observed cultural, morphological and pathogenicity characteristics led to the identification of the fungal pathogen as *Colletotrichum musae* (Berk. and Curt.) Arx as similarly described in literatures (Zakaria et al. 2009; Su et al. 2011).

In vitro tests proved that four bacterial isolates have significant antagonistic activity against *C. musae*

Bacteria isolated from wild, unprocessed honey was tested for possible antagonistic effect on *Colletotrichum musae.* These four isolates are tagged BC2, BC3, BC6, and BC7, three of which belong to genus *Lactobacillus* (BC2, BC3 and BC7) and one from genus *Bacillus* (BC6). Figure 3 shows the photos of the dual

culture assay plates for antagonism against *C. musae.* The four bacterial isolates exhibited significant inhibitory effect to the growth of the banana anthracnose pathogen. Based on the results of the *in vitro* assay, inhibition of the radial growth of the pathogen was around 40% to 55%. BC7 and BC6 exhibited the highest percent inhibition at around 45% to 50%. Individual efficacy of the four isolates is not significantly different from each other based on the Tukey's pairwise mean comparison at $\alpha = 0.05$ as shown in Figure 4.

In this study, inhibition of colony growth is highly indicative that the isolated bacteria from honey produce metabolites that inhibit fungal growth or competes with the fungus for nutrients and resources. Several studies have been reported wherein *Bacillus* species such as *Bacillus licheniformis, B. amyloliquefaciens* and *B. subtilis* were able to control conidial germination of the *C. musae* up to 98% (Mahadtanapuk et al. 2007). The results of the assay clearly indicate that BC2, BC3 and BC6 produced antifungal metabolites, thus inhibiting *C. musae* through antibiosis. On the other hand, BC7 inhibited *C. musae* either through competition or through direct parasitism. Antifungal proteins from *B. amyloliquefaciens* were already isolated and purified. These proteins were proven to have antagonistic activity against the grown of *C. gloeosporioides* that cause anthracnose disease in watermelon in Korea. The antifungal protein was found out to exhibit a β-1,3-glucanase activity (Kim and Chung 2004).

Spray application proved that diluted wild honey helps prevent the occurrence of anthracnose and perhaps, growth of the pathogen in *in vivo* conditions

Dilutions of wild honey were applied through spraying to test for its protective potential to prevent anthracnose disease in banana. In the *in vivo* test (Figure 5), application of diluted honey alone did not cause disease to the fruits which means that the microorganisms present in wild honey are not pathogenic to the banana fruits. But as the pathogen was amended to the sprayed honey, there was a higher disease severity with lower dilution of the honey as shown in Figure 6. Although there is the presence of the antagonistic bacteria in the honey, the disease progress was not delayed. Among the dilutions applied, spraying of 10^{-5} v/v wild honey suspension with spray-inoculated pathogen (T9) resulted to decreased disease severity (2.5) as compared to the positive control (3.0). This may be explained by the finding of Madras-Majewska et al. (2016) wherein they detected the presence of *Bacillus* spp. in all their honey samples from Thailand and Poland, but the bacterial population is very minimal at 10 cfu/g. Lastly, the amount of bacterial propagule is relatively lower compared to the 50,000 spores/ml of the pathogen.

Results of the *in vivo* test for potential biopesticidal activity of wild honey showed promising results, especially when honey is prepared at lower concentrations (10^{-5} v/v). Preparations of higher concentration renders the medium crowded while lower concentrations result to even spreading of the antagonistic microorganisms present in wild honey. In addition, honey is composed mainly of about 38.2% fructose and 31.3% glucose. Presence of this sugars in high amount would suggest that the spore of the pathogen has an abundant carbon source thus enhances their growth capacity leading to a higher disease severity. Preparing wild honey in lower concentrations delivers less sugars to the surface of the fruit, thus less favoring pathogen growth while evenly spreading the beneficial microbes.

Postharvest biological control of fruit is already a fast-developing field in plant pathology. Most plant extracts, oils and microbial antagonists are applied directly to wounds, which are often rich in nutrients and sugars that is needed by the pathogen for successful establishment (Janisiewicz et al. 2001). Therefore, competition for nutrients and space can be an effective mechanism of biological control in postharvest fruits. Possible biocontrol mechanisms have been suggested to operate on fruits (Jijakli and Lepoivre 1993, 1998) including antibiosis, parasitism, induced resistance and competition for nutrients and space.

Numerous studies were conducted on the postharvest biological control of diseases in banana fruits. The flourishing research in banana postharvest is mainly due to the fact that banana fruits are a good candidate for biological control. However, controlling crown rot on banana is challenging because of the complex of fungi associated with the disease and the cut crown tissue allows for a large area of entry for pathogens (Williamson et al. 2008). Moreover, once the antagonistic activity has been established, it is essential to perform tests under natural conditions of infestation to assess the real protection afforded by the biocontrol strategy (Lassois et al. 2008).

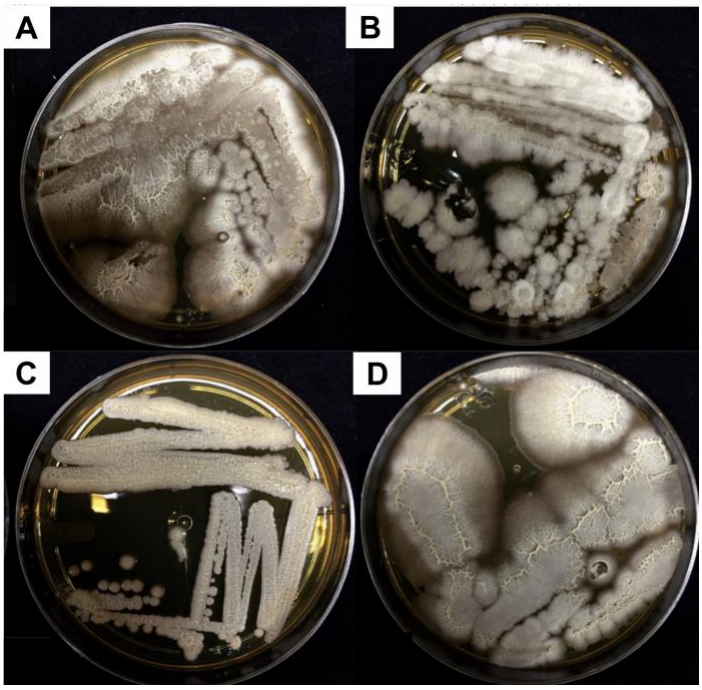

Figure 2. Three-day old pure cultures in PDPA of the four promising bacterial antagonists isolated from wild honey. A – BC2; B – BC3; C – BC6; D – BC 7.

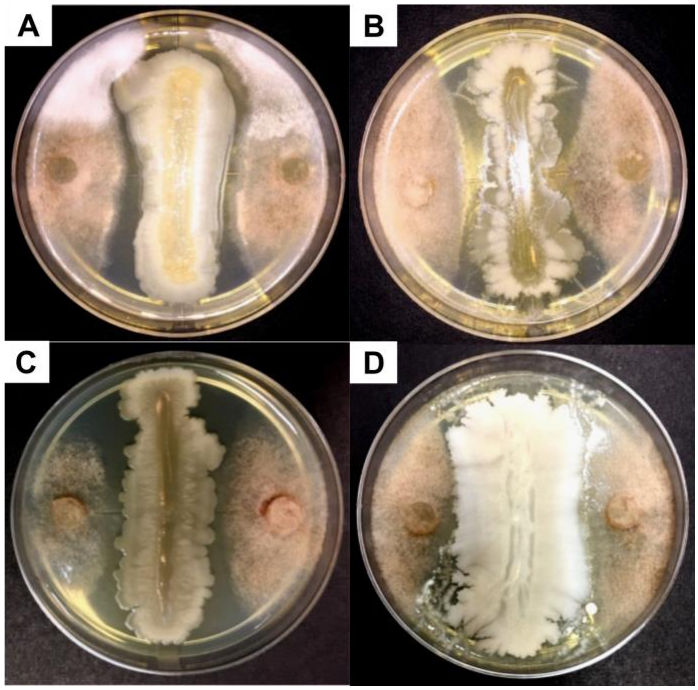

Figure 3. Photographs showing the results of the dual culture assay to test for possible antagonism of the bacterial isolates against *C. musae.* Culture media used is PDA and photos were taken after 72 hrs of incubation. A – BC2; B – BC3; C – BC6; D – BC 7.

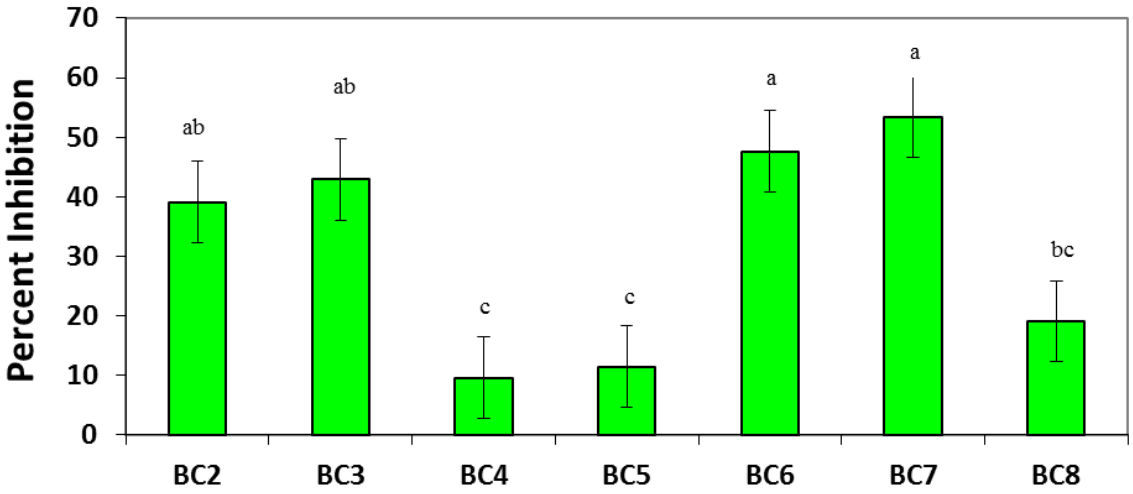

Figure 4. Percent inhibition radial growth inhibition of *C. musae* co-cultivated with different bacteria isolated from honey. Bars with the same letters are not significantly different at α=0.05.

Figure 5. Photographs showing the different treatments done in the *in vivo* biopesticidal spray test using dilutions of wild honey to prevent banana anthracnose disease. A – treatment 1 (T1) is the negative control; B – T2 is the positive control (pathogen only, wounded); C – T3 is the positive control (pathogen only, unwounded); D – T4 (10^{-1} v/v wild honey suspension only); E – T5 (10^{-3} v/v wild honey suspension only); F – T6 (10^{-5} v/v wild honey suspension only); G – T7 (10^{-1} v/v wild honey suspension with pathogen); H – T8 (10^{-3} v/v wild honey suspension with pathogen); I – T9 (10^{-5} v/v wild honey suspension with pathogen).

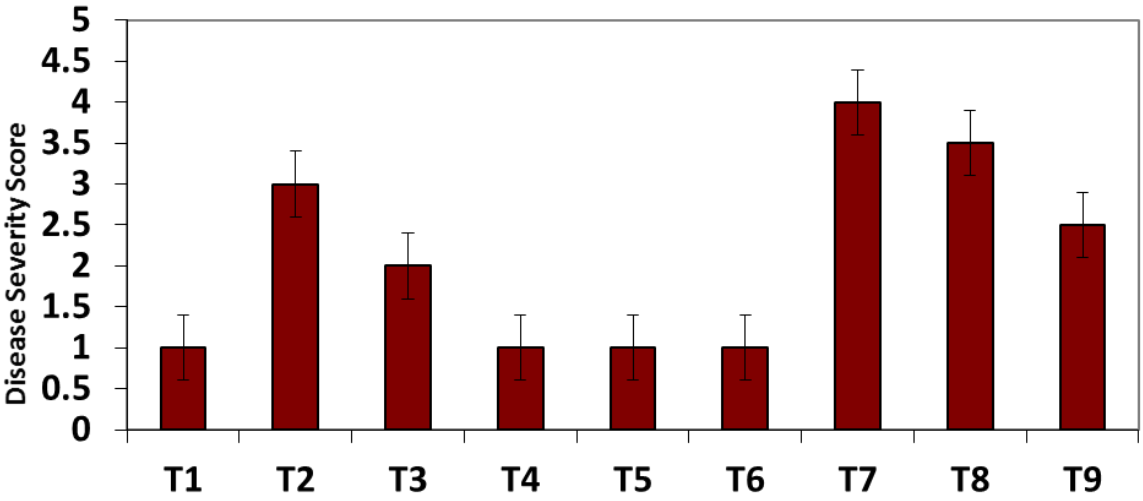

Figure 6. Disease severity scores of banana. Note: (1 (0%), 2 (1-25%), 3 (26-50%), 4 (51-75%), 5 (76-100%)). Treatment 1 (T1) is the negative control; T2 is the positive control (pathogen only, wounded); T3 is the positive control (pathogen only, unwounded); T4 (10^{-1} v/v wild honey suspension only); T5 (10^{-3} v/v wild honey suspension only); T6 (10^{-5} v/v wild honey suspension only); T7 (10^{-1} v/v wild honey suspension with pathogen); T8 (10^{-3} v/v wild honey suspension with pathogen) and; T9 (10^{-5} v/v wild honey suspension with pathogen).

4. Conclusions

The associated bacteria in honey were associated and its antagonistic property against *Colletotrichum musae* was evaluated. Wild honey was also tested for its potential application as a biopesticidal spray against the banana anthracnose disease. Honey contains low population of bacteria. These bacteria are non-pathogenic to banana. Microorganisms isolates such as BC2, BC3, BC6, and BC7 that was identified as Lactobacillus spp. and Bacillus spp. have antagonistic potential against *Colletotrichum musae.* Usage of honey alone is recommended at lower concentrations to be used as a biopesticide spray for banana because its sugar content enhances the growth of the pathogen. The inoculation experiment in this study was carried out as an approximation of the natural conditions. Such situations are seldom encountered in practical conditions because estimation of natural conditions is rather challenging. The assay for protection level could be better under natural field conditions and infestation. The researchers suggest testing the efficacy of this isolates by formulating them individually. It also of interest to identify the bacterial isolates up to the species level using molecular approach. Lastly, it is also recommended to further test honey as a biopesticide spray for postharvest produce. Formulations and fabrication of composite coating materials is highly recommended. Not only honey is very safe for humans, it also has a very high potential in the field of crop and food protection.

Authors' Contributions: RIVAREZ, M.P.S.: conception and design, acquisition of data, analysis and interpretation of data, drafting the article; PARAC, E.P.: drafting the article; LAUREL, N.R.: conception and design, acquisition of data, analysis and interpretation of data; CUNANAN, B.V.: conception and design, acquisition of data, analysis and interpretation of data; MAGARRO, A.B.: conception and design, acquisition of data, analysis and interpretation of data; DIMASINGKIL, S.F.M.: drafting the article. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

Acknowledgments: The authors would like to thank Demetrio Alvarez and Ramon Cortez of the University of the Philippines Los Baños in Laguna, Philippines, for their technical support during the conduct of the experiments.

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Received: 5 August 2019 | **Accepted:** 2 October 2021 | **Published:** 29 December 2021