IDENTIFICATION OF BACTERIAL MICROFLORA OF THE DISEASED BANANA LEAFROLLER (*Erionota sp.* Linn.)

Honey Jane C. Mascariñas, Ph.D.
College of Technology and Allied Sciences, Bohol Island State University, Calape, Bohol, Philippines.
E-mail: hjmascarinas@yahoo.com

ABSTRACT

Although banana production grew to a more profitable yield, insect pests became one of the most common problems for the farmers. This study is a survey of bacterial microflora isolated from the diseased banana leafroller (*Erionota sp.* Linn.) that were collected from different sampling sites. Characterization of bacterial isolates was performed using cultural tests, morphological tests, and physiological tests. Seven different bacterial isolates were identified from the diseased insects. These were *Bacillus cereus, Bacillus thuringiensis, Corynebacterium species, Methylococcus species, Micrococcus luteus, Planococcus species,* and *Pseudomonas aeruginosa.* Of the seven identified bacteria, *Micrococcus luteus* was the most abundant and was almost present in all diseased insects collected. There was variability within the insect host as to the occurrence of the specific bacterial microflora. *Bacillus cereus, Corynebacterium species, Methylococcus species,* and *Planococcus species* were considered host-specific while *Bacillus thuringiensis, Micrococcus luteus* and *Pseudomonas aeruginosa* were isolated from both larval and pupal stages of the host insects. The findings of this study provide a possible tool to combat insect pests of the banana plantations. However, it is important to undertake more research on the bacterial microflora isolated from the diseased banana leafroller and determine the degree of pathogenicity of this microbial flora on the infected host.

Key words: Banana, Leafroller, Biocontrol, Entomology, Microflora

INTRODUCTION

One of the most prevalent issues that destroyed agricultural products and resources, including rice, corn, coconuts, various fruits, and others, was parasitic insects. These pests consume not just the valuable fruits but also the foliage, stems, and even roots of the plants. The crop itself became seriously infected and eventually perished. They grow so quickly as a

result of this and reproduce avariciously that they pose a threat to the entire plantation and the sizable community of consumers who depend on these products (Baltazar, 1991; David, 2004; Agrios, 2005).

Researchers, however, conducted some studies on how to eliminate these varieties of pests. The application of pesticides, herbicides, and other chemical-based insecticides had become widely accepted by farmers, agricultural industries and other consumers to solve these miserable problems. Even though it has great effects on pest population, it has lethal or toxic substance not only to the detrimental insects but also to the environment as well as to the beneficial or harmless one (Ali et al., 2021). Recently, the use of microbes such as bacteria, fungi, viruses and protozoans in controlling pests and parasitic insects has great advantages-can often be cultured in large numbers economically, they are generally fairly specific in action and usually not harmful to other plants animals and also live no toxic residues. Like the effects of insecticides, the biological control on pernicious insects has also numerous drawbacks include dependency in certain weather conditions, difficulties in mass producing certain microbes such as viruses, killing hosts too slowly, and potential hazards to other animals (Rosomer, 1981; Mahmood et al., 2016; Damalas and Koutroubas, 2016).

Most agriculturists and even farmers now use biological management extensively (van Lenteren et al., 2018; Bale et al., 2008). According to Hajek (2018) and Lacey et al. (2015), biological control involves the introduction of insect populations using living creatures that are encouraged by humans. Chemical pesticides have very sometimes been used to successfully eradicate invasive pests. The use of microbes or their by-products in the control of insect pest species is known as microbial control, and it is one of the most effective biological pest control methods (Pfadt, 1985; Metcalf and Luckman, 1994).

Microbial control will play an important role in many pest management programs, while in others it may be totally impractical, either because it does not give the degree of control desired or because it is simply not economically feasible. However, where microbial control can be used as a food in pest management programs it conforms nicely to the objectives of pest-management. Even though many insect pathogens have been successfully used in insect-control programs, there are many problems yet to be solved if microbial control is to reach its full potential (Metcalf and Luckmann, 1994). As these problems are solved, insect pathogens will play an increasingly important role in pest-management programs.

This study focuses on a particular crop pest, banana leafroller, *Erionata sp*. that infested on the banana leaves and survey for possible presence of pathogens. The results of

this study aims to develop a possible measure or tool against insect pest. The applications of bacterial pathogens on affected resources are very important to the economy as well as to human population.

MATERIALS AND METHODS

Collection of Samples

Collections of samples were conducted in 5 different sampling sites. A total of 30 diseased insects were gathered using a simple method by unrolling the infected leaves and picked the insects with the use of pre-cleaned forceps. The insect samples were stored inside sterile sampling vials and were brought to Biological Science Laboratory for bacterial isolation, characterization and identification (Bergey, 1994).

Extraction and Isolation of Bacteria

The samples were macerated and ground thoroughly using a sterile mortar and pestle where 2ml sterile distilled water was added into. An inoculum of each homogenate was then streaked on the surface of the agar medium and the plates were incubated at 30°-35°C in an upside-down position. Before the molten nutrient agar medium was transferred into plates, an anti-fungal drug was added at a dosage of 200 parts per million (ppm) per liter of NA (nutrient agar) medium.

Purification of Bacterial Culture

Exhibition of different bacterial colonies were seen after 24 to 48 hours of incubation. Bacterial colonies were isolated in pure cultures before identification and characterization was administered. Different colony types were re-inoculated and re-streaked three to four times on nutrient agar plates to obtain pure bacterial isolates.

Characterization and Identification of Bacterial Isolates

Cultural Tests

Only NA medium was used to describe the appearance or growth characterization of a particular bacterial culture such as texture on agar medium and turbidity. The molten medium was poured into sterile plates and then, cooled to room temperature. The isolate was streaked into the plates and incubated for 24 hours in upside-down manner. The following cultural characteristics were studied such as colonial formation, appearance, color, size, and shape.

Morphological Tests

These tests consider the cell's reaction to gram staining, size, shape, cell, arrangement and motility of the bacterial isolates.

a. Gram Stain Reaction

The isolates were transferred to NA slant and incubated at 25° to 30°C for 16 to 24 hours. A bacterial film was spread thinly with a drop of distilled water on a clean slide and air dried for a few minutes in a tilted position. Then the underside of the slide was lightly flamed for fixation. The smear was flooded with crystal violet solution for one minute, then washed with water for few seconds and drained off. The smear was then flooded with iodine solution for one to two minutes, rinsed with water, and air dried. Decolorization was administered to the smear by dripping 95% ethanol over the tilted slide for 10 to 15 seconds then washed immediately with water. Counterstaining was done using safranin solution for 45 seconds, after which, the smear was rinsed with water, then blot-dried. Microscopic examination was then, followed.

b. Shaeffer-Fulton Spore Staining

A known test organism (*B. subtilis*) and an unknown culture were inoculated on NA slants and were incubated at 30°C for 36 to 48 hours. Smears were prepared on separate pre-cleaned slides, air dried and fixed by heat. The prepared smears were covered with absorbent paper and were flooded with malachite green solution. The prepared smears were then steamed for 8-10 min in a boiling water bath after which, the absorbent paper was removed. The slides were cooled and washed thoroughly with water. Counterstaining was followed using safranin solution for 30 to 60 sec, rinsed and blot dried. Microscopic examination was then administered. Endospores were indicated by a green color, while vegetative cells were observed red. The position, shape and distention of the spores were likewise noted.

c. Anthony's Method on Capsule Formation Test

The isolates were grown on NAG (nutrient agar + 0.1% glucose) and NAS (nutrient agar + 0.1% sucrose) and were incubated at 30°C for 48 hours. A small amount of bacterial growth was emulsified in a loopful of glucose solution at the opposite ends of the slide. Using the sliding technique on preparing a smear, the bacterial mixture was spread at one side of the slide and, then air dried. It was stained with crystal violet solution for about two minutes after which it was washed with copper sulfate solution, drained, and blot dried. The smear was then examined under the oil immersion objective. Capsules appeared light blue to colorless, while cells were observed dark purple.

d. Hanging Drop Technique Test for Bacterial Motility

The isolates were inoculated on NA slants and incubated at 30°C for 24 to 48 hours. Sterile distilled water about one to two ml was added to each slant and loosened the bacterial growth by gently tapping the bottom of the tube. A loopful of the suspension was obtained and transferred in the precleaned cover slip. A small drop of water was also deposited on each corner of the cover slip. Then the slide was lowered down onto the cover slip until it touches the drop pf water. The slide was turned right side up quickly so that the suspension would not be spread over the cover slip. It was then examined under oil immersion objective.

e. Intracellular Lipid staining Method

Bacterial culture was transferred to N-limited GYEA (Glucose Yeast extract agar) at 30° to 35°C for 24 to 48 hours. A smear was made, then, air dried and fixed by heat. The smear was flooded with Sudan black and stained for 15 minutes. The excess stain was drained off and blot dried. Counterstaining was done by using safranin solution for 10 seconds, then, washed with water, blot dried and was examined under oil immersion objective. Lipid inclusions were observed blue to black, while cells were pale red.

f. Glycogen and Starch Granules Staining Method

After conducting intracellular lipid staining method, the same cultures were used in this method. A small amount of growth was mixed with a drop of Lugol's iodine on a clean slide. Then, a precleansed cover slip was placed over the mixture and was examined under oil immersion objective. Starch granules appeared dark blue while glycogen appeared reddish brown.

Physiological Tests

Physiological observations include oxygen requirement, substrate utilization, products formed, ability to grow under various conditions, and ability to grow in the presence of certain inhibitory and biochemical compounds.

a. Oxygen requirement test

The isolate was stabbed in Thioglycollate agar tubes and incubated for 24 hours. After which, the growth was observed. If the growth is at the surface, the organism is strictly aerobes; facultative anaerobes if growth is observed throughout the medium, and anaerobic if growth is confined only at the bottom.

b. Nutrient Agar catalase test

The isolate was inoculated in the nutrient slant and after 24-hour incubation; a small amount of growth was transferred to a glass slide. To it, one to two drops of 3% H_2O_2 was added. Formation of bubbles is indicative of a positive result for the presence of catalase.

c. Oxidation/Fermentation Test

In the oxidation/fermentation of carbohydrates, the isolates were inoculated in Hugh and Leifson medium and incubated at 30°C for 48 hours. Two sets for each isolate were prepared. The first was covered in 2cm vaspar and the second set was not covered. There were two sets of sterile carbohydrates – the glucose and lactose solutions. After incubation period the following was observed: if acid is produced in the open tube it is an oxidative metabolism and, fermentative if acid is produced in both test. The change of color from green to yellow indicates acid fermentation. Bubble formation or breakage of the Hugh and Leifson agar indicates gas formation with carbohydrates.

d. Indole Production from Tryptophan

The indole test was performed in a tryptone broth in which the isolates were inoculated and then, incubated at 35°C for 36 to 48 hours. Indole was detected after incubation by adding a solution containing p-dimenthyl aminobenzaldehyde. Kovac's solution contains the reagent together with amyl alcohol that extracts the indole compound and brings it to the surface in a thin layer indicated by a red color.

e. Nitrate Reduction Test

The isolate was inoculated into the nitrate broth and incubated at 37°C for 48 hours. After incubation period, one ml of nitrate reagent A and one ml of nitrate reagent B was added. At distinct color which turns to brown easily indicates that NO_3 is reduced to NO_2. If the result is negative, a pinch of powdered zinc was added. If the red color appeared, nitrate was reduced by zinc to nitrate was reduced further to NO or N_2.

f. H_2S Production Test

The isolate was stabbed in Lead Acetate agar slants and incubated at 30°C for 48 hours. After two-day incubation, browning on the surface and along the line of puncture indicates that there was hydrogen sulfide production from amino acid.

g. Gelatin Liquefaction Test

The isolate was stabbed in nutrient gelatin tubes and incubated at 33°C for 48 to 72 hours. After incubation period, tubes were placed in an ice bath for 15 minutes. If the

medium in the ice bath is liquid, it indicates that gelatin is liquefied; otherwise, it is a negative result.

h. Starch Hydrolysis

The isolate was inoculated in starch agar plates and incubated for 24 hours. After incubation period, growth was flooded with iodine solution. A clear area along the line of growth is positive for starch hydrolysis, otherwise it is negative.

i. Methyl Red (MR) and Voges-Proskauer (VP) Test

The isolate was inoculated in nutrient broth with 0.5% glucose (NBG) and incubated at 33°C for 48 to 60 hours. After incubation period, one ml of culture was transferred to a tube in which one to two drops of methyl red indicator was placed for MR test tube. For the other tube (VP test), five drops of 5% alpha-naphthol was added and mixed then 7 to 10 drops of 40 % KOH added, mixed and stood for 15 to 20 minutes. Red color is positive for MR test indicating acid test for glucose; yellow is negative. For VP test, pink to red color was positive indicating acetoin production, otherwise, it is negative.

j. Citric Acid Utilization

The isolate was inoculated in Simmon's Citrate Agar slants by stabbing the butt and streaking on the surface and incubated at 33°C for 48 hours. Growth or blue color means positive utilization of citrate.

k. Phenylalanine Deamination Test

The isolate was streaked in PA slants and incubated at 33°C for 18 to 24 hours. About 4 to 5 drops of 10% $FeCI_3$ solution was added and a slight rotation was done to loosen growth. The immediate appearance of an intense green color indicates positive phenylalanine deamination.

RESULTS AND DISCUSSION

Identification of the bacterial microflora isolated from the diseased banana leafroller collected from different sampling sites is shown in Table 1.

Table 1. Bacterial Microflora of the Diseased Banana Leafroller (*Erionota sp.* Linn.) Collected from Different Sampling Sites.

Insects Life Stage	Bacteria
Larva	*Bacillus thuringiensis* *Methylococcus species* *Micrococcus luteus* *Pseudomonas aeruginosa*

Pupa	*Bacillus cereus* *Bacillus thuringiensis* *Corynebacterium species* *Micrococcus luteus* *Planococcus species* *Pseudomonas aeruginosa*

The result of the study showed variability within specific insect host, in particular, to the developmental stage of the host as the occurrence of specific bacterial microflora. The bacteria *Bacillus cereus, Corynebacterium species, Planococcus species* and *Methylococcus species* were found to be host-specific which infect insects at certain stage of development. However, the latter were considered to be less dispersed since its presence is detected in only to few sampling sites. Meanwhile, *Bacillus thuringiensis, Micrococcus luteus,* and *Pseudomonas aeruginosa* and were found to be non-host specific since these bacteria were isolated from different insect host at no particular stage of development. Of the three bacteria, *Micrococcus luteus* was observed to have the widest range of infestation as it was isolated in almost all insect samples collected from different sampling sites.

The development and spread of the disease caused by these bacterial species within an insect population is dependent on the interaction of the pathogen, the host and the environment (Feldhaar and Gross, 2008; Boucias and Pendland, 2012). The presence of more than one bacterial species isolated from a single diseased insect is said to be transmitted by ingestion of either pathogenic or nonpathogenic bacteria by the insect host. The dispersion of this bacterial species is possible by transovarian transmission in which the adult insect migrated along with the infectious egg to her offspring. Some immature insect such as larvae obtained such kind of bacterial species from the maternal secretions from its accessory glands or fecal excretions. On the other hand, the pupa is usually infected by means of aerial contamination due to its immobility or caused by secondary infection (Metcalf and Luckmann, 1994).

Such characteristics of a pathogen that influence its ability to spread throughout a host population are its virulence and infectivity, its capacity to survive, and its capacity to disperse (Tanada and Kaya, 2012). As they exhibit host specificity, these bacterial microflora are said to be effective source of microbial insecticides for controlling a specific pest problem without upsetting the ecological relationships of other animals in a particular area (Gillott, 2005). Thus, insect pathogens may play an increasingly important role in pest-management programs.

CONCLUSION

This study provides the identification of the bacterial species isolated from the diseased banana leafroller collected from different sampling sites. A total of seven different bacterial isolates were characterized and identified namely: *Bacillus cereus, Bacillus thuringiensis, Corynebacterium species, Methylococcus species, Micrococcus luteus, Planococcus species,* and *Pseudomonas aeruginosa.* Of the seven identified bacteria, *Micrococcus luteus* exhibited the widest range of host-specificity having isolated in almost all samples collected. Some bacterial isolates, however, showed host-specificity such as *Bacillus cereus, Corynebacterium species,* and *Planococcus species* having isolated only in the pupa of the insect host while *Methylococcus species* was associated only within the larva of the leafroller.

It is recommended that further studies should be conducted on the isolation and identification of bacterial microflora from the diseased banana leafroller. A larger number of infected leafrollers must be considered in order to observe greater variability between insect host and its pathogens. Likewise, more sampling sites must be involved for better comparisons of results. Pathogenicity test on the different isolates is also recommended to assess the degree of pathogenicity of this microbial flora on the infected host.

REFERENCES

Agrios, G. N. 2005. *Plant pathology*. Elsevier.

Ali, S., Ullah, M. I., Sajjad, A., Shakeel, Q., & Hussain, A. 2021. Environmental and health effects of pesticide residues. *Sustainable Agriculture Reviews 48: Pesticide Occurrence, Analysis and Remediation Vol. 2 Analysis*, 311-336.

Bale, J. S., Van Lenteren, J. C., & Bigler, F. 2008. Biological control and sustainable food production. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1492), 761-776.

Baltazar, C. R. 1991. An inventory of Philippine Insects. Los Banos: UPLB Publishing Office. P. 398.

Bergey, D. H. 1994. *Bergey's manual of determinative bacteriology*. Lippincott Williams & Wilkins.

Boucias, D. G., & Pendland, J. C. 2012. *Principles of insect pathology*. Springer Science & Business Media.

Damalas, C. A., & Koutroubas, S. D. 2016. Farmers' exposure to pesticides: toxicity types and ways of prevention. *Toxics*, *4*(1), 1.

David, B. V. 2004. *General and applied entomology*. Tata McGraw-Hill Education.

Feldhaar, H., & Gross, R. 2008. Immune reactions of insects on bacterial pathogens and mutualists. *Microbes and infection*, *10*(9), 1082-1088.

Gillott, C. 2005. *Entomology*. Springer Science & Business Media.

Hajek, A. E., & Eilenberg, J. 2018. *Natural enemies: an introduction to biological control*. Cambridge University Press.

Hall, I. M. (2012). Microbial control. *Insect pathology an advanced treatise*, 477-511.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., & Goettel, M. S. 2015. Insect pathogens as biological control agents: Back to the future. *Journal of invertebrate pathology*, *132*, 1-41.

Mahmood, I., Imadi, S. R., Shazadi, K., Gul, A., & Hakeem, K. R. 2016. Effects of pesticides on environment. *Plant, soil and microbes: volume 1: implications in crop science*, 253-269.

Metcalf, R. L., & Luckmann, W. H. (Eds.). 1994. *Introduction to insect pest management* (Vol. 101). John Wiley & Sons.

Pfadt, R. E. 1985. Fundamentals of applied entomology. *Fundamentals of applied entomology.*, (Edn 4).

Rosomer, W. S. 1981. The Science of Entomology. 2nd ed. NewYork: Mcmillan Publishing Co., pp. 458-459.

Tanada, Y., & Kaya, H. K. 2012. *Insect pathology*. Academic press.

van Lenteren, J. C., Bolckmans, K., Köhl, J., Ravensberg, W. J., & Urbaneja, A. 2018. Biological control using invertebrates and microorganisms: plenty of new opportunities. *BioControl*, *63*, 39-59.