Microbial diversity of shallot plantation in peat-lands applied with three types of botanical pesticides

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Abstract. One method of controlling disease that is environmentally friendly is the use of plant-based pesticides also known as botanical pesticides. The use of botanical pesticides has proven to be effective in controlling several species of pests and plant diseases, because they contain active secondary metabolite compounds. These compounds affect pathogenic microorganisms, so it is feared will also affect antagonistic microorganisms. This research will find out the impact of the application of several botanical pesticides on shallots to microbial biodiversity. The study was conducted in Peat-lands, at South Kalimantan. Microbial identification was carried out at the Phytopathology Laboratory and the Laboratory of Biological Control of the Department of Pests and Plant Diseases, Lambung Mangkurat University, Banjarbaru. The results showed that the application of plant-based pesticides from Kepayang fruit extract and Galam leaf extract had an influence in decreasing the population of microorganisms, respectively by 80.44% and 75.26%. Chirinyuh were increased the population by 36.60%, as well as the control treatment, the population of microorganisms increased by 17.77%. Meanwhile the application of synthetic pesticide Dhitane M-45 reduced the population of microorganisms by 95.73%. Types of microbes found in the soil and onion rhizosphere are Pseudomonas flourescens, Bacillus sp., Fusarium sp., Aspergillus sp., Curvularia sp., Scopulariopsis sp., Stachybotrys bisbyi, and Penicillium sp.

Keywords: biodiversity, botanical pesticides, microbes.

1. Introduction
The increasing productivity of onion crops outside of Java is faced with constraints of sub-optimal land conditions such as peat-lands with humid nature that can induce the development of disease; in addition to mineral content beyond the limits of plant requirements that can cause plant toxicity.

One of the causes of the low productivity of shallots in South Kalimantan comes from biotic factors such as a plant disease, because it is almost always found in every area of planting shallots [1].

Some pathogens that cause onion-plant disease are Alternaria porri, Colletotrichum gloesporiodes, Cercospora duddiae, Peronospora destructor, Phytophthora porri, Fusarium oxysporum, Sclerotium cepivorum, Bortytis allii, Pseudomonas allicola, and Erwinia carotovora [2].

To control plant diseases, many methods have been applied. One method of controlling disease that environmentally safe is the use of botanical pesticides.
The use of botanical pesticides is proven to be able to control the pathogens that cause plant diseases, but there is a concern that the active ingredients of botanical pesticides will also affect the diversity of antagonistic agents as controlling plant pathogens.

This research will look at the impact of the application of three types of botanical pesticides on shallots to microbial biodiversity, both of which are detrimental (as pathogens of onion plants) and which function as natural enemies.

2. Materials and Methods

2.1. Materials

Three types of botanical pesticides, namely kirinyuh, kepayang, and galam, Shallots of Batu Ijo variety, aquades, 90%, methanol, Potato Dextrose Agar Media, Nutrient Agar Media, King's B Media, Cling wrap, Aluminum foil, Label paper and Sample bags.

2.2. Methods

The study was conducted in Peat-lands, Tegal Arum village, Landasan Ulin District, Banjarbaru City, South Kalimantan. Microbial identification was carried out at the Phytopathology Laboratory and the Laboratory of Biological Control, Department of Plant Pests and Diseases, Lambung Mangkurat University, Banjarbaru. The study was conducted from June to September 2019. The shallot variety used was Batu Ijo. Microbial identification was using literature that contained identification [3-5].

The experimental design used was a Randomized Block Design (RBD) with the following treatments: t0: Control; t1: Chirinyuh (Chromolaina odorata) extract; t2: Kepayang (Pangium edule) extract; t3: Galam (Melaleuca leucadendra) extract; t4: Dithane M-45 (synthetic pesticide). Each treatment had replicated 5 times, so that 25 unit experiments were formed.

2.2.1. Botanical pesticides extraction

Each ingredient: Chirinyuh (Chromolaina odorata), Kepayang (Pangium edule) and Galam (Melaleuca leucadendra) weighed as much as 100 g, mixed with water as much as 100 ml. Then, a solution was made by blending with a blender. After refining, the mixture was added with 400 ml of water again, so that the volume became 500 ml. The solution was left for 24 hours at room temperature. After that it could be used as botanical pesticides.

2.2.1.1. Preparation of extracts

The preparation of extracts was carried out by sorting the raw materials, which were in the forms of plant parts. The materials were the parts of the leaves and fruit that were sorted in order to separate them from dirt or unneeded materials. The materials were then cut into small pieces before dried using an oven at 50°C for 48 hours and after the drying the materials were mashed using a blender. The raw materials that had been mashed into coarse powder were sifted using intertwined gauze. After being smoothed and powdered they were weighed for ± 600 grams and then soaked with 96% ethanol for 72 hours at a ratio of 1:10 (w / v). The results of soaking were filtered again using filter paper. Afterwards, the filtered results were evaporated using a rotary evaporator at a temperature of 60-70˚C with a pressure of 400-450 mmHg in order to obtain the extracts. The remaining ethanol was used to rinse the filter paper 3-4 times. The extracts were then stored in a refrigerator.

The extracts were ready for application. The extracts were then measured using a measuring cup as much as one milliliter per a replicate plot. There were 24 milliliters available. After they were measured, the extracts were put into the beaker, added with 0.4 ml of Tween adhesive and stirred for five minutes. They were then stirred using a magnetic stirrer at a speed of 700 rpm for five minutes. The extracts were ready to be applied to each plant with a spray volume of 500 l / ha.
2.2.2. **Microbial isolation**

Sampling was carried out before and after the application of botanical pesticides to see whether there was a good or detrimental effect on the microbial biodiversity in the onion crop. Diseased plants were taken to figure out the effect of botanical pesticides application on reducing the intensity of disease.

About Five samples of soil for each treatment were collected. Sampling time was after tillage, before lime and fertilizer were applied, one week after the first application of botanical pesticides and the last was one week after the last application of botanical pesticides.

2.2.2.1. **Isolation from plant tissues**

Parts of diseased plants were cut into small pieces at the boundary between diseased and healthy plants. After that, it was sterilized by dipping the symptomatic plant parts with 70% alcohol once and the distilled water three times then dried on sterile tissue. Furthermore, the cuttings of plant parts were grown in the media and were placed in an incubator at 25°C to observe the growth. After that, purification was carried out from the isolation of pathogens which were overgrown with several types of pathogens in new culture media to obtain pure culture.

2.2.2.2. **Isolation from the soil**

Soil which was taken from around the roots of diseased plants, weighed on an analytical balance of 10 gr. Then put into a used glass bottle containing 90 ml of sterile water and homogenized for 15 minutes in an orbital shaker with a speed of 150 rpm and dilution of 10^-6 dilutions. After a single colony was obtained, purification was continued before identification.

2.2.3. **Pathogen Identification**

2.2.3.1. **Identification of Fungi**

The Fungi would be identified by observing the colonies with macroscopic and microscopic structure. Observation of the colony was carried out by observing the color of the colony in a petri dish, the color changes seen from the age of the culture, shape, and state of hypha’s growth. As for the observation of the microstructure, it was done by observing under a microscope. The fungus mycelium was grown on a sample that has been isolated by using a device called needle ent on the flame of a lamp called bunsen. Put over the object glass that had been dripped with sterile-water solution. Had it closed using a glass cover to be observed under a stereo microscope. Given the nail polish preparations were observed under a stereo microscope on the side of the glass cover. Then the sample was identified using the guide book Fungi Identification [4,6,7].

2.2.3.2. **Identification of bacteria**

This identification was carried out to figure out the type of bacteria would be obtained, by going through several tests. Testing could be done as follows:

2.2.3.2.1. **Gram reaction testing**

This test was an initial step in identifying a yet unknown species of bacteria. This test was performed using 3% KOH, where in this test if there was a reaction (if there was removed mucus that did not break following the needle called ose) then it was rolled up in a gram negative reaction because the lipid content was very high. Otherwise, if there was no reaction, it was included in the gram-positive group. If the test showed gram negative or gram positive reactions, the isolate could be included in the appropriate genus.

2.2.3.2.2. **Yeast dextrose calcium carbonate agar (YDC) media Test**

In this test, if the results of the isolates turned out yellow, they were included in the genus Erwinia or Xanthomonas, and if they were not yellow they were belonged to the genus Pseudomonas, Erwinia, or Agrobacterium. The next step was testing with the Miller-Schoth agar (MS) if the colony previously obtained was yellow and testing with the Kings'B (KB) agar if it did not turn yellow. If on this medium
emitted fluorescent pigments, then it was included in the genus Pseudomonas. Those who did not have fluorescent could be included as the genus Pseudomonas, Erwinia, or Agrobacterium. Further testing was carried out to non-fluorescent using MS agar media. If it grew, it belonged to the genus Erwinia and what was growing could be included as the genus Pseudomonas, or Agrobacterium. In addition to those listed in the scheme, this identification was also done by observing and seeing the shape of bacteria.

2.2.4. Isolation and Purification of Antagonistic Microbes

2.2.4.1. Isolation of Pseudomonas Fluorescence group
A total of 10 g of soil taken from the onion plant rhizosphere was put into an Erlenmeyer flask containing 90 ml of sterile-water solution and shaken for 15 minutes at a speed of 150 rpm. Then, the solution was diluted with distilled water until $10^{-7}$. At $10^{-7}$ dilution, 0.5 ml was taken and spread on King’s B media. Growing bacterial colonies were seen on UV lamps. The greenish-yellow colony was moved to the new King’s B to be purified.

2.2.4.2. Isolation of Bacillus spp.
Isolation of Bacillus spp. was carried out by heating as much as 10 gr the roots of onion plant which had been dissolved with 90 ml of distilled water at 80°C for 30 minutes in a water bath. After that, took as much as 0.5 ml and spread on TSA media. TSA media containing bacterial suspensions were incubated in a temperature of 27°C for 48 hours. Brownish-colored bacterial colonies that arise and beige were transferred into a petri dish containing TSA media for purification.

2.2.4.3. Isolation of Antagonistic Fungi
Soil samples obtained from the onion-plant rhizosphere were weighed as much as 10 gr and then suspended in 90 ml of distilled water and were shaken for 15 minutes at a speed of 150 rpm after which a suspension was taken as much as 1 ml and put in 9 ml of distilled water and then the vortex to be homogeneous. The dilution was carried out until a $10^{-5}$ dilution obtained. The result of dilution was taken as much as 0.5 ml and was transferred to the PDA media. Then was incubated and purified.

2.3. Observation

2.3.1. Antagonistic Microbial Identification

2.3.1.1. Identification of Antagonistic Fungi
The materials used for identification were sterile water and pure fungus isolates from healthy onion-plants. By forming a 1 x 1 cm rectangle on a dense PDA media, took a piece of rectangular PDA media using a spatula, placed it on a slide glass, took the isolate that would be identified using a needle ent, placed it on the edge of the PDA media piece that was rectangular shaped above the slide glass then covered with glass cover, wet the tissue under the slide glass in a Petri dish using a dropper pipette, covered the Petri dish and wrapped with a cling warp. The growth should be observed. After growing, took the cover glass, placed it on a slide glass and observed the shape of the spores under a microscope, photographs and identification were done by comparing existing literature from references.

2.3.1.2. Identification of antagonistic bacteria
Identification of antagonistic bacteria was carried out by observing six characteristic items, namely: colony shape, optical properties, colony color, colony size, edge / colony edge shape, and gram bacteria. The ingredients used for the identification of gram bacteria were pure bacterial isolates from healthy onion-plants, sterile water, KOH 3%. Tests were conducted to classify bacteria into groups of fluorescence and non-fluorescence. The incubated media was then observed by placing it under a UV lamp to see the luminescence came from bacteria. Bacterial isolates that fluoresced under UV light indicated that these bacteria were the fluorescence group, whereas non-fluoresced under UV light indicated that the bacteria were non-fluorescence group. Then gram testing was conducted to classify the bacteria
into two big groups namely gram positive and negative bacteria by taking one ose of sterile bacterial isolates and placed on a glass slide that has been given 1 drop of 3% KOH solution.

Bacterial mass was mixed and changes were observed. If mucus was formed, it showed that the bacteria were Gram negative. On the other hand, the mass of bacteria that did not form mucus indicated that the bacteria were Gram positive. Then, observed the colonies in each media, photos and identification by comparing existing literature either from books or from the internet.

2.3.1.3. Observation of diversity index, index of species richness, and index of domination
To find out what species dominates and how diverse the types of microbes in plantations and the onion rhizosphere are, the Simpson diversity index and Shannon dominance index will be calculated.

Diversity Index (H') from Shannon-Wiener [8,9]:

\[ H' = \sum_{i=1}^{n} p_i \ln p_i, \text{ where } p_i = \frac{n_i}{N} \]

H' = Shannon's-Wiener diversity index
\( p_i \) = number of individuals of a species/total number of species
\( n_i \) = number of individuals of a species i
\( N \) = Total number of individuals of all types

The value of H’ is defined as follows:
H’<1: Low diversity; H’1-3: Medium diversity; H’> 3: High diversity

Index of species Richness [9]:

\[ R = \frac{(S - 1)}{\ln N} \]

\( R \) : Index of species richness
\( S \) : Number of species
\( N \) : Number of individual species

Index of Domination (D) according Simpson [8,9]:

\[ D = \sum_{i=1}^{n} \left( \frac{n_i}{N} \right)^2 \]

\( D \) = Index of Domination
\( n_i \) = Number of individual per species
\( N \) = Number of individuals of all species

3. Results and Discussion
According to the research result, the application of botanical pesticides extracted from Chirinyuh, Kepayang, Galam affected to microbial population of Shallot rhizosphere (Table. 1).

Shallot corps without the treatment of both botanical and synthetic pesticides increased its number of microbial population about 17.77%. The land with chemical pesticide Dithane M-45 showed the decreasing of a huge number of microbial population 95.73%. However, botanical pesticides had varied effects to microbial population [10]. Chirinyuh extract application caused 36.60% increasing population. Meanwhile, extract Kepayang and Galam caused the decrease in microbial population about 80.44% and 75.26%.
The increasing or decreasing number of microbial population around the shallot’s rhizosphere plant were not positively correlating with the increasing or decreasing of major disease attacks on shallot plant (Table 2).

Table. 2 shows that although the microbial population increased in the control land, the intensity of the main onion disease pathogen attack remained the highest compared to the other treatments. This is presumably happening because among the microbes found in the onion rhizosphere there is a main cause of the onion disease. One of the main diseases of red-onion plant found in South Kalimantan is a malignant disease called moler caused by *Fusarium* spp. [1].

**Table 1.** The total of microorganism population before and after the application of botanical pesticides.

| Treatment          | Before the application of botanical pesticides (× 10¹⁰) | After the application of botanical pesticides (× 10¹⁰) | The Increasing (+) or decreasing (-) of microorganism population caused by botanical pesticides (%) |
|--------------------|--------------------------------------------------------|------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| Control            | 492.5                                                  | 580                                                  | 17.77                                                                                           |
| Dithane M-45       | 609                                                    | 26                                                   | -95.73                                                                                           |
| Chirinyuh Extract  | 509.5                                                  | 696                                                  | 36.60                                                                                           |
| Kepayang Extract   | 542                                                    | 106                                                  | -80.44                                                                                           |
| Galam Extract      | 578                                                    | 143                                                  | -75.26                                                                                           |

**Table 2.** The correlation between the microbial population and pathogen attack on shallot plant

| Treatment          | Type of microbe                  | The Increasing (+) or decreasing (-) of microbial population caused by botanical pesticides (%) | The intensity of disease attack (%) | Isolated from |                     |          |
|--------------------|----------------------------------|-----------------------------------------------------------------------------------------------|-----------------------------------|----------------|-------------------|----------|
| Control            | *Fusarium* sp., *Curvularia* sp., | 17.77                                                                                         | 37.40                             | Soil           | Plant tissue      |          |
|                    | *Penicillium* sp., *Aspergillus* |                                                                                               |                                   |                |                   |          |
| Dithane M-45       | *Aspergillus* sp.                 | -95.73                                                                                         | 15.91                             |                |                   |          |
| Chirinyuh Extract  | *Fusarium* sp.                    | 36.60                                                                                         | 12.32                             |                |                   |          |
|                    | *Aspergillus* sp.                 |                                                                                               |                                   |                |                   |          |
| Kepayang Extract   | *Fusarium* sp.                    | -80.44                                                                                         | 18.48                             |                |                   |          |
|                    | *Aspergillus* sp, *Stachybotrys*  |                                                                                               |                                   |                |                   |          |
|                    | *bisbyi*                          |                                                                                               |                                   |                |                   |          |
| Galam Extract      | *Fusarium* sp.                    | -75.26                                                                                         | 16.26                             |                |                   |          |
|                    | *Scopulariopsis* sp.              |                                                                                               |                                   |                |                   |          |
|                    | *Curvularia* sp.                  |                                                                                               |                                   |                |                   |          |

Onion cropping land applied with botanical pesticides shows a decrease in microbes in the soil except for Chirinyuh extract. The decrease in microbial population is assumed to be caused by the presence of secondary metabolite compounds contained by botanical pesticides which are thought to be toxic to microbes in shallot’s rhizosphere. Kepayang extract contains high cyanide acid, which is toxic [10]. Kepayang fruit flesh contains saponins, flavonoids, and polyphenols [9,10,11]. This compound is proven effective in controlling pests and some plant pathogens.

Table. 2 shows that in the corps applied with Kepayang extract, the intensity of *moler* disease attack as the main disease of onion is still high (18.48%) if compares to the onion crops that has been given
Chirinyuh extract which is 12.32%. This is presumably due to microbes in the soil, some of which function as natural control agents, as the population decreases dramatically (80.44%) because of the Kepayang extract. Some antagonistic microbes have properties as *Plant Growth Promoting Rhizobacteria* (PGPR), produce antibiotics that can inhibit the growth of pathogens, especially from soil infectious groups, and have the ability to colonize plant roots [11]. Decreasing antagonistic microbial population provides an opportunity for increased intensity of pathogenic attack.

The application of Galam leaf extract has an effect in reducing the microbial population in the soil by 75.26%. Galam leaf contains methyl eugenol, as an attractant for pollinating insects, but there are some methyl eugenol that have evolved and have anti-fungal activity. The compound may have evolved in response to pathogens, as methyl eugenol has some antifungal activity.

The application of Chirinyuh extract produced the lowest intensity of *moler* disease (12.32%). Chirinyuh-extract application did not cause a decrease in microbial population in the shallot rhizosphere. On the contrary, Chirinyuh-extract application caused an increase in the microbial population by 36.60%. The increase in microbial population is thought to make a significant contribution in reducing the attack of *moler* disease in this land. Antagonistic microbes or biological control agents of plant diseases are microorganisms obtained from nature, whether in the form of bacteria, fungi, actinomycetes or viruses that can suppress, inhibit or destroy plant-disturbing organisms [12,13]. The types of microbes obtained from the isolation results of onion cultivation are presented in Table 2.

To figure out whether the onion crop is dominated by one particular species or it has a high species richness index, four indexes such as the diversity index, species richness index, dominance index, and microbial balance index are calculated on the shallot plants which are applied with botanical pesticides made of Chirinyuh, Kepayang and Galam extract (Table 3).

**Table 3.** The average diversity index, species richness index, dominance index, and microbial balance index on shallot plants applied with botanical pesticides made of Chirinyuh, Kepayang and Galam extracts.

| Treatment          | Species Index (H') | Diversity Index (R) | Richness Index (D) | Microbial Balance Index (E) |
|--------------------|--------------------|--------------------|--------------------|-----------------------------|
| Control            | 1.33               | 1.86               | 0.28               | 0.96                        |
| Dithane M-45       | 1.15               | 1.54               | 0.39               | 0.83                        |
| Chirinyuh Extract  | 0.99               | 1.54               | 0.44               | 0.71                        |
| Kepayang Extract   | 1.73               | 2.40               | 0.19               | 0.97                        |
| Galam Extract      | 1.75               | 2.57               | 0.18               | 0.98                        |

Table 3 shows that the application of botanical pesticides results a very low species diversity (0.99) because, the value of species diversity is very low if it has a range of values <1. However, the application of other botanical pesticides (Kepayang extract and Galam extract) gives a low level of species diversity, respectively 1.73 and 1.75. Similar case happens with the application of the chemical pesticide Dithane M-45, it is resulting in a low level of species diversity of 1.15. This is presumably because at the time of the study, the condition of temperature was very hot, so it was less favorable for the development of microbes that preferred humid condition.

The species richness level of shallot plantation applied with both plant-based pesticides and synthetic pesticides is quite low (all species richness values are below 3.5), and no single species dominates because the dominance index values are close to 0 (below 0.5).

**4. Conclusion**

The results showed that the application of plant-based pesticides from Kepayang fruit extract and Galam leaf extract had an influence in decreasing the population of microorganisms, respectively by 80.44% and 75.26%. Chirinyuh plant pesticide extract treatment increased the population of microorganisms by 36.60%, as well as the control treatment without given plant pesticides, the population of
microorganisms increased by 17.77%. While the application of synthetic pesticide Dhitane M-45 reduced the population of microorganisms by 95.73%.

Types of microbes found in the soil and onion rhizosphere are *Pseudomonas fluorescens*, *Bacillus* sp., *Fusarium* sp., *Aspergillus* sp., *Curvularia* sp., *Scopulariopsis* sp., *Stachybotrys bisbyi*, and *Penicillium* sp.

The results of this study indicate that in the selection of botanical pesticides to control plant pathogens must be done carefully because some botanical pesticides will reduce the total number of microorganism populations as a whole, even though the type of microbes in the soil does not affect the application of botanical pesticides.

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References
[1] Safitri YA, Hasanah U, Salamiah, Samharinto, and Pramudi MI 2019 *Asian Journal of Agriculture* 3(2)
[2] Semangun H, 2004, *Indonesian Horticulture Plant Diseases*, (Yogyakarta: Gadjah Mada University Press).
[3] Alexopoulos CJ and Mims CW 1979 *Introductory Mycology* (New York: John Wiley and Sons).
[4] Barnett HL and Barry BH 1998 *Illustrated Genera of Imperfecti Fungi*, 4th edn., (Minnesota: APS Press).
[5] Booth, C 1971 *The Genus Fusarium*, (Key Surrey: Commonwealth Mycological Institute).
[6] Agrios GN 2005 *Plant Pathology*, 4th ed., (United State of America: University of Florida).
[7] Sinaga M S, 2003, *Fundamentals of Plant Disease*, (Jakarta: Penebar Swadaya).
[8] Southwood TRE, 1978, *Ecological Methods*, vol. 2, (New York: Champman and Hall).
[9] Ludwig JA & Reynolds JF, 1988, *Statistical Ecology. A Primer on Methods and Compling* (New York: John Wiley and Sons).
[10] Heyne K 1987, *Indonesian Useful Plants*, 3rd edn., (Jakarta: Forestry Department)
[11] Sesanto L, 2008, *Introductory of Plant Diseases Biological Control*, (Jakarta: PT. Raja Grafindo Pesada).
[12] Harborne, J.B., 1996, *Phytochemical Methods*, (Bandung (ID): Bandung Institute of Technology).
[13] Anonymous, 2010, *Natural Pesticides*, Indonesia.