Journal of TROPICAL BIODIVERSITY E-ISSN : 2774-4116

Endophytic Fungus from Soursop (*Annona Muricata* L) and Potential Antibacteria

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Submission	:	January, 09th 2023
Revision	:	February 08th 2023
Publication	:	April 30th 2023

Abstract

The aim of this research was to isolate endophytic fungi from different parts of soursop plantations in Cikareo Village, Sukabumi, West Java, and to test their antibacterial activity. The researchers obtained six isolates from the soursop plant's leaves, stems, and fruits. These isolates displayed varying growth abilities, with only two (Le-1 and St-2) found to inhibit the growth of Escherichia coli bacteria. Overall, this study highlights the potential of endophytic fungi from soursop plantations as a source of antibacterial agents.

Keywords: Antibacterial, Endophytic fungi, Macro-algae, Soursop (*Annona muricata* L)

INTRODUCTION

Endophytes are microorganisms that live in association with plant tissues(Frank et al., 2017; Rai et al., 2021; Santoyo et al., 2016). The association of endophytic microorganisms in plant tissues is neutral to symbiotic. In plant tissues, microorganisms produce secondary metabolites that help plant defense, while plants provide a food source for the growth and proliferation of endophytic microorganisms(Bolívar-Anillo et al., 2020; Raimi & Adeleke, 2021; Stępniewska & Kuåniar, 2013; Strobel et al., 2004). One example of an endophytic microorganism is a fungus. These endophytic fungi can be found in various types of plant tissues, such as leaves, fruit, twigs, or plant roots (Nouh, 2019). Secondary metabolite compounds produced by endophytic fungi can function as antibiotics, antivirals, anticancer, antidiabetic, antimalarial, and antioxidants (Strobel & Daisy, 2003). Several experts have isolated and studied endophytic microorganisms from various plants, including; medicinal plants, plantation plants, and plants in the forest (Maysarah, 2009). There are approximately 300,000 plant species spread over the earth, each containing one or more endophytic microorganisms, including fungi (Strobel & Daisy, 2003). Soursop (*Annona muricata* L) is an ea plant speciesely to contain endophytic fungi(Hridoy et al., 2022).

Soursop (*Annona muricata* L) is widely used as medicine because it contains active compounds in the form of secondary metabolites in its tissues(Berumen-Varela et al., 2019). Soursop plants have a tree habit, with a height of 3–8 m (Leiss et al., 2013).

The shape of soursop leaves is inverted (*obovatus*), 6-18 cm long (Tjitrosoepomo, 2013; Steenis, 2013). The upper surface of the leaves is smooth, smooth and shiny (*nitidus*). Flat leaf edges (integer), rather thick and stiff leaves. Tapered leaf tip (*acuminatus*), pointed leaf base (*acutus*). Pinnate leaf reinforcement (*penninervis*) (Am Zuhud, 2011). The fruit is compound egg-shaped, measuring 15-35 x 10-15 cm. The fruit skin has fine scales. Young fruit is green, while old soursop fruit has a slightly blackish skin. Black seeds and white flesh (Am Zuhud, 2011; Leiss et al., 2013). Soursop plants come from tropical areas in the Americas, namely the Amazon Forest (South America), the Caribbean and Central America. Currently, soursop is spread in various parts of the world, especially areas with tropical climates (Am Zuhud, 2011).

Soursop contains acetogenins, tannins, steroids, alkaloids, saponins and flavonoids which are antitumor, anticancer, antibacterial and antiparasitic. Solomon (2016), soursop leaf extract can inhibit the growth of *Staphylococcus aureus* bacteria with an inhibition zone diameter of 17 mm, *Salmonella typhimurium* of 15 mm, *Escherichia coli* of 13 mm, and *Streptococcus pyogenes* of 15 mm.

Staphylococcus aureus is a cocci-shaped Gram-positive bacterium, 0.8-1.0 µm in diameter. On solid media, it shows an irregular cell arrangement, whereas on broth media it shows a separate arrangement of cells or arranged as short chains(Arientová & Holub, 2021; Cheung et al., 2021; Shrestha et al., 2021). *S. aureus* does not have flagella and does not have spores(Westerlund-Wikström et al., 1997). On the skin, respiratory tract, and human digestive tract, *S. aureus* is found as normal flora. *S. aureus* is the most common cause of pyogenic skin infections and is also the most pathogenic type (Nickerson et al., 2009).

Escherichia coli is a short rod-shaped Gram-negative bacterium (cocobacil), 0.4-0.7 µm in size, does not have spores and some strains have capsules. *E. coli* grows well on almost all media commonly used in Microbiology laboratories; On the media used for the isolation of enteric bacteria, most of the *E.coli* strains grow as lactose-fermenting colonies. *E. coli* is facultative anaerobe (Kanungo, 2002).

Based on this background, this study aims to: 1). Obtain endophytic fungal isolates from soursop plant organs (Annona muricata L.), 2). To determine the ability of the endophytic fungi isolates isolated from the organs of the soursop plant.

The hypothesis tested in this study are:1). Soursop plant organs (leaves, stems and fruit) contain several types of endophytic fungi, 2). Isolates of endophytic fungi from the three plant organs have different antibacterial properties against the test bacteria (Escherichia coli and Staphylococcus aureus).

METHOD

A. Place

This research was conducted at the Laboratory of Microbiology and Genetics, Faculty of Biology, Universitas Nasional, Jl. Bambu Kuning, Pejaten, Pasar Minggu, South Jakarta.

B. Equipment and materials

Equipment used in this study include: oven (WTB Binder), autoclave (Delixi), laminar air flow, rotary shaker (Model VRN-210), refrigerator, incubator (Memmert), centrifuge (Hittech), centrifuge tube, digital scales , electric cooker, pH universal, beaker, Erlenmeyer (Pyrex) flask, Petri dish (Pyrex), test tube, tube rack, Bunsen burner, measuring cup, filter cloth, volumetric pipette, tweezers, spatula, scissors, knife, core borer (cork hole punch), vortex mixer, bulb, scalpel, loop, and ruler.

The materials used in this study were various healthy plant organs (leaves, stems and fruit) of soursop (Annona muricata L.), *Escherichia coli* and *Staphylococcus aureus* battery isolates, 70% ethanol, Chlorox, Medium MEA (Malt Extract Agar) (Scharlau), dextrose, Yeast extract, MHA Medium (Mueller Hinton Agar) (Oxoid), Mc Farland 0.5, paper discs 6 mm in diameter (Scheeur), 0.9% physiological NaCl solution, 30 µg chloramphenicol antibiotic as positive control, filter paper, plastic wrap, aluminum foil, swabs, newspapers, plastic, cotton, label paper and markers.

Procedure

1. Equipment sterilization

Equipment and materials used for research such as Petri dishes, test tubes, volumetric pipettes, paper discs, filter paper, core borers are sterilized using an autoclave at 121 ^{0}C.

2. Preparation of Microbial Growth Media

a. Media MEA (Malt Extract Agar)

The medium used for the growth of endophytic fungi in this study was MEA. MEA media was prepared by weighing 3.55 g of MEA, adding 0.5 g of Bacto agar, then dissolving it with distilled water in 100 mL and heating to boiling. The media was then sterilized by autoclaving for 15 minutes at 121^{0}C (1-2 atm pressure).

b. PDB Media (Potato Dextrose Broth)

This media is a liquid medium consisting of potato extract liquid. 200 g of potatoes are boiled with sufficient water until the potatoes are soft, then filtered to obtain liquid potato extract. Then 10 g of dextrose was added, then distilled water was added to a final volume of 500 mL and heated while stirring continuously until homogeneous. The solution was measured for its pH value with universal pH. Then put 50 mL in a 100 mL Erlenmeyer. The media was sterilized using an autoclave at 121°C for 15 minutes.

c. MHA Media (Muller Hinton Agar)

The medium used for the growth of the test bacteria was MHA. 19 g of MHA media was weighed, 2.5 g of bacto agar, then dissolved in 500 mL of distilled water and heated while stirring until boiling. Then the media was sterilized in an autoclave at 121oC for 15 minutes.

3. Isolation of Endophytic Fungi

a. Sampling

Samples were taken from plantations in Cikareo Village, Sukabumi, West Java. Plant parts taken were leaves, fruit and healthy stems of the soursop with scissors.

b. Surface Sterilization

Isolation of endophytic fungi from plants begins with sterilization of each surface of the plant organs obtained. The plant organs are washed with running water to remove dirt on the surface of the plant organs. After the sample is cleaned, it is then drained and divided into several pieces measuring 3 cm.

The plant pieces were soaked in 70% ethanol for 2 minutes. Then followed by immersion in Chlorox solution for 5 minutes, soaked again in 70% ethanol for 1 minute and finally put into sterile distilled water.

c. Endophytic Fungi Isolation

Isolation of endophytic fungi was carried out using the direct planting method, namely after the last soak using sterile distilled water, the sample pieces were dried on sterile filter paper for a few minutes. Each sample piece was then placed on the MEA medium with the surface of the plant organs attached to the agar medium. Sample inoculation was carried out on medium in a Petri dish and duplicated for each plant organ. Each cup contains 5 sample pieces. The process was carried out in laminar air flow, and then incubated until fungal colonies grew at 27-290^{o} C (room temperature).

4. Purification of Endophytic Fungi

Endophytic fungi that have grown on MEA isolation media are then gradually purified one by one. Each pure endophytic fungus isolate obtained was then transferred into MEA medium in a Petri dish. This purification aims to separate endophytic colonies with different morphologies to be isolated. Morphological observations were carried out again after incubation for 5-7 days, and if macroscopically different colony growth was found, they had to be separated again until pure isolates were obtained.

Each pure isolate was then isolated into upright MEA medium and incubated for 3-5 days. After perfect fungal growth, sterile paraffin was given with a height of 1 cm above the surface of the fungal isolates. The mushroom culture is used as a stock culture. For working cultures, isolates were not treated with paraffin. Each isolate was made in duplicate (2 times).

5. Endophytic Fungus Fermentation

Endophytic fungal fermentation was carried out by liquid fermentation using PDB media. Pure colonies of endophytic fungi in MEA Petri dishes which had been incubated for 5 – 7 days, were then cut using a core borer as many as 5 pieces. The mushroom pieces were then inoculated into the PDB liquid fermentation medium as much as 50 mL in a 100 mL Erlenmeyer flask.

Furthermore, rocking fermentation was carried out on the Erlenmeyer flask using a rotary shaker 130 rpm (shaking/minute) at room temperature for 7 days. Each culture that had been shaken was put into a 15 mL centrifuge tube which was previously sterilized, then centrifuged at 3000 rpm for 20 minutes. The supernatant obtained was then used to test the antibacterial activity against *Staphylococcus aureus* and *Escherichia coli bacteria*.

6. Preparation of test bacteria

One ose of test bacterial colonies was suspended in 0.85% physiological NaCl solution. The turbidity was homogenized using McFarlan standard 0.5 (bacterial density 1.5x108) on a black background and bright light. McFarlan turbidity standard 0.5 was prepared by mixing 0.5 mL of 1% BaCl2 solution with 9.5 mL of 1% H2SO4.

The sterile swab is dipped into the test bacterial suspension, then drained by pressing the tip of the swab and rotating it on the inner wall of the tube to remove excess liquid. Furthermore, the swab is applied to the surface of the media in such a way that the growth of bacteria is evenly distributed.

7. Bacterial Activity Testing

Antibacterial activity testing was carried out using the Kirby-Bauer method, known as the paper disc method or the "paper disc method" (Lay, 1994). Each blank paper disc was heated in an oven at 700C for 15 minutes, then the paper disc was dipped into the endophytic fungus supernatant obtained. The disc containing the supernatant was left in a sterile Petri dish for 15 minutes, and then placed on the surface of the media containing the test microbes. As a positive control, chloramphenicol 30 µg was used, while as a negative control, paper discs containing sterile PDB medium were used.

The number of paper discs placed in one Petri dish is 5 discs and the distance between the discs is adjusted so that they are not too close. Incubation was carried out at room temperature for 18 hours. If there is inhibition of the growth of the test bacteria, a clear zone will be seen around the paper disc. The diameter of the inhibition zone formed around the disc was measured using a ruler. Paper discs have a diameter of 6 mm, so an inhibition zone that has a diameter of $\leq$ 6 is said to have no inhibition zone. Concentrations that have a diameter of > 6 mm are said to have an inhibition zone.

8. Identification

Identification was carried out by observing macroscopic and microscopic morphological characteristics. Then a comparison was made using a mushroom identification book, namely Illustrated Genera of Imperfect Fungi and A Guide To Tropical Fungi.

D. Research Design and Data Analysis

The antibacterial activity of each isolate was tested for the significance of the difference in the inhibition zones produced for each test bacteria, using RAL-F (Completely Randomized Factorial Design) with two treatment levels and three replications. The first treatment level consisted of 6 isolates of endophytic fungi, positive control and negative control. The second treatment level is the test bacteria consisting of *Staphylococcus aureus* and *Escherichia coli*. The data obtained were analyzed by software statistical analysis using the two way ANOVA method of the Statistical Product and Service Solution (SPSS) program version 22

RESULT

A. Endophytic Fungus Isolate from Soursop (*Annona muricata* L.)

The results of the isolation of endophytic fungi from parts of the soursop plant totaled 6 isolates. On the leaves there was 1 isolate, on the stem 2 isolates of endophytic fungi and on the fruit there were 3 isolates of endophytic fungi. The number of isolates from each part of the plant can be seen in table 1.

Table 1. Number of Endophytic Isolates from Soursop Plants

No	Part of Plant	Isolate Code	Number of Isolates
1	Leaf	L1	1
2	Stem	St1, St 2	2
3	Fruit	Fr1, Fr2, Fr3	3
	Total		6

Each isolate of endophytic fungi obtained from soursop plants was observed macroscopically to determine the characteristics of each species of isolate. Macroscopic observation of endophytic fungi morphology can be observed from the color of the surface and reverse side of the colony, colony texture, topography and observation of radial and concentric lines. The process of macroscopic observation of fungal colonies can help identify the type of isolate, because each type of endophytic fungus has different characteristics. The colonies of each endophytic fungus are shown in the following figure.

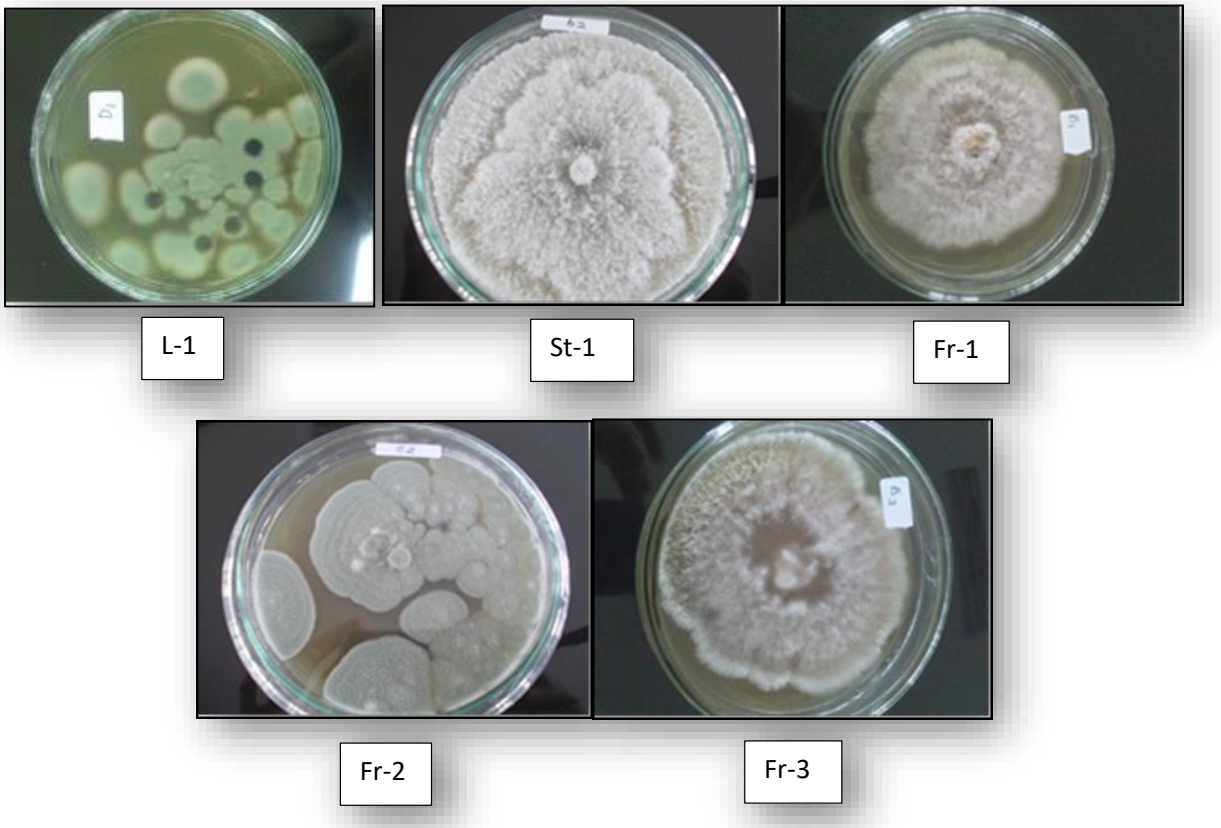

Figure 1. Colonies of Endophytic Fungi Isolated from Soursop (*Annona muricata* L.)

The surface of the colony isolate Le-1 is light green, the back side of the colony (reverse side) is yellow, the texture of the colony is velvety, the topography is verrugose and has concentric circles. The surface of isolate St-1 is green, the reverse side is yellow, the texture is velvety, the topography is verrugose and has concentric circles.

Isolate St-2 has white color, yellow reverse side, woolly texture, rugose topography and has radial lines. Fr-1 isolate is white, reverse side yellow, woolly texture, umbonate topography and has concentric circles. The endophytic fungus Fr-2 isolate is dark green in color, the reverse side is yellow, the texture is velvety, the topography is verrugose and has concentric circles. Fr-3 isolate has white color, white reverse side, woolly texture, verrugose topography and has radial lines.

B. Antibacterial Power of Endophytic Fungi Isolates against *Staphylococcus aureus* and *Escherichia coli*

The antibacterial activity of six endophytic fungal isolates against *S. aureus* and *E.coli* bacteria can be seen in Table 2.

Table 2. Average Diameter of Inhibition Zones of Endophytic Fungus Extracts Against Test Bacteria

No	Endophytic fungi Extract	Average Inhibition Zone Diameter (mm)	
		Staphylococcus aureus	*Escherichia coli*
1	Le-1	6,00	6,33
2	St-1	6,00	6,00
3	St-2	6,00	6,67
4	Fr-1	6,00	6,00
5	Fr-2	6,00	6,00
6	Fr-3	6,00	6,00
7	chloramphenicol 30 µg	31,00	27,67
8	Negative control	6,00	6,00

DISCUSSION

The results of the isolation of endophytic fungi found in each organ of the soursop plant were different. On the leaves there was 1 isolate, on the stem there were 2 isolates and on the fruit there were 3 isolates. This shows that each plant tissue can be occupied by several different types of endophytic microorganisms. According to Petrini *et al.*, (1993), differences in the type and number of endophytic microorganisms are influenced by the adaptability of these endophytic microorganisms to microecological influences and specific physiological conditions of their host plants.

When observed from the nature of growth, each of these isolates has a different ability to grow. Isolate St-2 was able to grow faster than the other isolates, and isolate Le-1 was the isolate that grew the longest. The difference is due to the different adaptability to the growing environment.

The results of the antibacterial activity test showed that endophytic fungal isolates were only able to inhibit the growth of *Escherichia coli* which represented Gram negative bacteria, and were unable to inhibit the growth of Staphylococcus aureus bacteria which represented Gram positive bacteria. This is caused by differences in the peptidoglycan layer found in Gram-positive bacteria which cannot be penetrated by the bioactive compounds produced by these endophytic fungal isolates.

Of the 6 isolates tested, only 2 isolates showed inhibition of the growth of *Escherichia coli* bacteria, namely isolates Le-1 and St-2. Isolate Le-1 was only able to inhibit the growth of *E. coli* bacteria with an average inhibition zone of 6.33 mm, while

isolate St-2 had an inhibition zone of 6.67 mm. The diameter of the inhibition zone determines the potency of an antibacterial compound (Jawetz, 2001).

CONCLUSION

Obtained as many as 6 isolates. It consisted of 3 isolates from fruit, 1 isolate from leaves and 2 isolates from stems. Endophytic fungal isolates were only able to inhibit Escherichia coli. Isolates that inhibit the growth of Escherichia coli bacteria are isolates Le-1 and St-2

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