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Increasing Artemisinin Content on Artemisia Plants Through Endophytic Bacteria Inoculation as An Effort to Support the Availability of Malaria Drugs

Peningkatan Kandungan Artemisinin pada Tanaman Artemisia Melalui Inokulasi Bakteri Endofit Sebagai Upaya Mendukung Ketersediaan Obat Malaria

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ABSTRACT

In 2020, malaria cases worldwide increased from 219 million cases to 241 million cases with 627 thousand deaths. This creates problems in terms of the availability of malaria drugs. In addition, resistance to Plasmodium sp. against the commonly used malaria drug chloroquine is another problem. Therefore, in the treatment of malaria, combination-based treatment with artemisinin is highly recommended. However, there are obstacles in the production of artemisinin because its content in plants is relatively low. Therefore, this research aims to increase artemisinin content using endophytic bacteria. The method used is a factorial design with 2 factors, namely Accessions (Green & Purple) and endophytic bacteria concentration (0%, 1%, and 2%). Endophytic bacteria isolated from artemisinin plants were first cultured in a nutrient-rich medium to increase their population. Then, they were inoculated by spraying them twice a week for two months with each accession in each treatment combination. The results showed that inoculation with endophytic bacteria had the best concentration in increasing artemisinin content, namely 1% in green and purple Artemisia accessions. Artemisinin inoculation of 1% was able to increase artemisinin by 27.85% compared to control plants in green Artemisia and purple Artemisia accessions with an increase of 32.89% compared to control plants. The best concentration of endophytic bacteria in increasing artemisinin content is 1%.

Keywords: *Artemisia annua* L., *endophytic bacteria, malaria*

ABSTRAK

Pada tahun 2020, kasus malaria di seluruh dunia meningkat dari 219 juta kasus menjadi 241 juta kasus dengan 627 ribu kematian. Hal ini menimbulkan masalah dalam hal ketersediaan obat malaria. Selain itu, resistensi *Plasmodium sp*. terhadap obat malaria klorokuin yang umum digunakan menjadi permasalahan lain. Oleh sebab itu, dalam pengobatan malaria, pengobatan berbasis kombinasi dengan artemisinin sangat dianjurkan. Namun terdapat hambatan dalam produksi artemisinin karena kandungannya dalam tanaman relatif rendah. Oleh karena itu penelitian ini bertujuan untuk meningkatkan kandungan artemisinin menggunakan bakteri endofit. Metode yang digunakan adalah rancangan faktorial dengan 2 faktor yaitu Aksesi (Hijau & Ungu) dan konsentrasi bakteri endofit (0%, 1% dan 2%). Bakteri endofit yang diisolasi dari tanaman artemisinin diperbanyak dan diinokulasikan dengan cara disemprot sebanyak dua kali seminggu selama dua bulan dengan ke masing-masing aksesi pada setiap kombinasi perlakuan. Hasil menunjukkan bahwa inokulasi bakteri endofit konsentrasi terbaik dalam meningkatkan kandungan artemisinin yakni 1% pada aksesi Artemisia hijau maupun ungu. Inokulasi artemisinin sebesar 1% mampu meningkatkan artemisinin sebesar 27,85% dibandingkan tanaman kontrol pada Artemisia hijau dan pada Artemisia aksesi ungu dengan peningkatan sebesar 32.89% dari tanaman kontrol. Konsentrasi bakteri endofit terbaik dalam meningkatkan kandungan artemisinin adalah 1%.

Kata kunci: *Artemisia annua* L., bakteri endofit, malaria

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INTRODUCTION

Malaria is a deadly disease caused by parasites from the genus *Plasmodium*, transmitted through the bite of female Anopheles mosquitoes, which can cause death (Dimi, Adam, and Alim 2020; Supranelfy and Oktarina 2021). According to World Health Organization data, there were 219 million malaria cases and 409 thousand deaths in 2019, and this increased to 241 million malaria cases and 627 thousand deaths in 2020 due to malaria worldwide (Pusparisa 2021; CNN Indonesia 2024). The Central Statistics Agency (BPS 2020) stated that the number of malaria cases in Indonesia in 2019 increased from 0.8% to 0.93% in 2020, dominated by Papua Province, which had the highest rate, namely 63.12% per 1,000 population.

High cases of malaria cause problems with the availability of antimalarial drugs and increased malaria drug resistance. The antimalarial drug commonly used is chloroquine, which was once highly effective against *Plasmodiu*m. However, *Plasmodium* resistance to chloroquine has become a significant obstacle, leading to treatment failure and even death (Nurjanah 2019). This underscores the urgent need for alternative treatments. Based on the Annual Parasite Incidence (API), malaria treatment is now recommended through artemisinin-based combination therapy or ATC (Artemisinin-based combination Therapies) (Subhi Isnaini and Bahrah 2019). Artemisinin, a key component of ATC, is produced from Artemisia annua L., a member of the Asteraceae family. Artemisinin contains a 1,2,4-trioxane ring with an endoperoxide bond that will break when attached to Plasmodium. This reaction releases free radicals that damage the parasite's cell membranes, leading to its death. Artemisinin has proven to be very effective in treating acute malaria from Plasmodium falciparum, which is resistant to chloroquine (Yuliani 2019). However, the main obstacle to the availability of artemisinin in several regions of the world, including Indonesia, is the low levels of artemisinin in plants. This results in low productivity of artemisinin that can be produced (Al Hafiizh et. al., 2016). In fact, according to data from the World Health Organization, around 25-50% of modern medicines or conventional medicines come from medicinal plants, one of which is artemisinin for malaria (WHO 2020). Moreover, natural medicines in developing countries in Southeast Asia are still used as the primary treatment by 80-88% of the population (WHO 2020).

Our research has demonstrated that the inoculation of endophytic bacteria can effectively increase the content of secondary metabolites, such as artemisinin, in Artemisia plants. This method not only promotes plant growth and development but also enhances their resistance to pest and disease attacks. Furthermore, it facilitates the synthesis of secondary metabolite compounds in plants. The inoculation of plant endophytic bacteria, particularly *Burkholderia seminalis* Strain 869T2, has been consistently shown to enhance the growth of various types of plants, including *Arabidopsis*, Pak Choi, Chinese Amaranth, lettuce, oil palm, and others (Hidayat et. al., 2018; Hwang et. al, 2021). These plant endophytic bacteria coexist with their hosts without causing harm and can bolster resistance to biotic attacks and tolerance to abiotic stress by increasing the content of plant secondary metabolites (Eid, 2021; Wu et.al., 2021).

Based on these problems, this study aims to create innovation to solve the problem of artemisinin availability by adding endophytic bacteria in various concentrations to artemisia plants. This differs from natural artemisia plants without endophytic bacterial treatment, so they only contain a small amount of artemisinin. Therefore, it is essential to increase the artemisinin content in Artemisia plants through the use of endophytic bacteria isolate code B5. and can support SDG 2030 goal number 3, related to ensuring a healthy life, encouraging community welfare at all ages and reducing deaths due to malaria, so that this research is a solution appropriate for dealing with health problems simultaneously.

METHOD

The research was conducted in June – October 2023 at the Tissue and Land Culture Laboratory, Faculty of Agriculture, Muria Kudus University. Materials used in this research include green accession and purple accession Artemisia annua L. plants, endophytic bacterial isolate code B5 (Figure 1), soil, manure, burnt husks, Nutrient Agar or NA Conda, tissue, cotton, plastic wrapping, spirit, and alcohol. The materials used in this research are presented in Figure 1. Meanwhile, the tools used include an autoclave, 1 L glass beaker, test tube, Erlenmeyer flask, tube needle, petri dish, measuring cup, Ohaus four-digit analytical balance, Laminar Air Flow Telststar AH- 100, Eppendorf micropipette, hot plate and magnetic stirrer IKA C-MAG HS-7, bunsen, glass stirrer, autoclave GEA 17L-0543, label paper, rubber bands, polybags, hoe, spade, hand sprayer, tray and solatip. The clean tube-shaped culture tools were covered with aluminum foil, while the other tools were wrapped in paper and put in plastic, then sterilized by autoclaving at 121^{0}C pressure 1.5 atm for 30 minutes. NA media was made by weighing 21 grams of NA media and adding distilled water to a volume of 1 liter. The solution was heated on a hotplate with a magnetic stirrer until it boiled, poured into 10 ml petri dishes each, and sterilized using an autoclave for 15 minutes. The Artemisia planting medium is soil: compost: burnt husks in a ratio of 1:1:1.

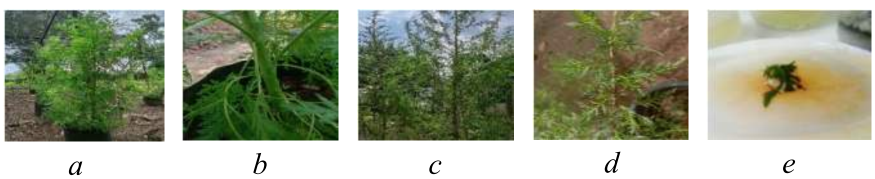

Figure 1. green accession of artemisia plant, b. stem of green accession artemisia, c. purple accession of artemisia plant, d. stem of purple accession artemisia, e. B5 code of endophytic bacteria

The planting material was one-month-old artemisia cuttings from the Center for Research and Development of Medicinal Plants and Traditional Medicine (B2P2TOOT). Bacteria were taken from the culture bottle using a loop needle, then implanted into a bottle with NA media and placed at room temperature. The endophytic bacterial isolate code B5 was multiplied by taking 1 ml of the old culture and placing it in new NA media, incubating for ± 24 hours for bacteria rejuvenation. Then, a 1% and 2% bacterial solution was made and put into a sterile hand sprayer, and the calibration was adjusted to 1 ml per spray. Each plant was sprayed 30 times (30 ml). Control plants were sprayed with the same volume of sterile distilled water. The B5 endophytic bacterial isolate solution was given to cuttings of plants aged 4 WAP 2 times a week until the first flowers appeared. The endophytic bacterial isolate solution code B5 is sprayed through the leaves and roots of artemisia plants (Apriliani, 2018). Artemisia plant cuttings are protected by covering them with plastic for two days after spraying. The care and maintenance include fertilizing once every two weeks, weeding, and controlling pests and diseases. Harvesting is done when the plants begin to appear as flower buds and by cutting the entire Artemisia annua L. plant from the ground surface. Variables observed included accession morphology, plant height, number of branches, number of leaves, the initial appearance of flowers, chlorophyll content, plant fresh weight, plant dry weight, number of bacterial populations, harvest age, and test for artemisinin content using HPLC (High-Performance Liquid Chromatography). and analyzed in the Satya Wacana Christian University laboratory.

This research will use a Complete Randomized Block Design (RAKL) with two treatment factors. The first factor was the administration of a solution of endophytic bacterial isolate code B5 (B), and the second factor was the accession of the Artemisia annua L. (A) plant with four replications. The first factor is the administration of a bacterial solution which consists of 3 levels, namely: B0 for sterile distilled water (control), B1 for the endophytic bacterial isolate solution code B5 (1%) and B2 for the endophytic bacterial isolate solution code B5 (2%) and the second factor are artemisia plant accessions, namely A1 (Accession 1) and A2 (Accession 2), resulting in a combination of six treatment combinations B0A1, B0A2, B1A0, B1A1, B2A0, B2A1. Data were analyzed using variance analysis (ANOVA). If there is a fundamental difference between treatments, continue with the DMRT test at the 5% level. Data analysis was carried out using Minitab software.

RESULT AND DISCUSSION

Plant Morphology of Two Artemisia Accessions After Spraying Endophytic Bacteria

Based on Figure 2 and Figure 3, the observed artemisia plants have characteristics described by the explanation regarding artemisia plants being used as medicinal plants (Konowalik & Kreitschitz, 2012; Hussain, 2020). The green accession Artemisia has a rhombic leaf shape, the leaf stalks are 1:1 in proportion, the leaf tip is tapered (acuminatus) with the leaf edges sharing a pinnate, the base of the leaf is sharp, the stalk is ivory green, and the old leaves will turn yellow from the tip to the middle and dry from the edge of the bone leaf. Young stems are light green, and old stems are green with brown stripes, branching evenly from the base of the stem to the tip of the plant and monopodial. The flowers on the green accession artemisia include composite compound flowers with bunch-shaped bouquets. Young flowers are whitish, while old flowers are bright yellow. Figure 3. shows that the purple accession artemisia has rhombic leaves with very long petioles, leaf edges sharing fingers, tapered leaf tips (acuminatus), young leaves are light green, and old leaves. In monopodial stems, branching is from the base of the stem to the tip of the plant; young stems are green with thin purple stripes, and old stems are flat purple. Purple accession artemisia flowers are compound flowers with panicle-shaped flower bouquets. Young flowers are bright yellow, and old flowers are reddish brown. These two accessions were chosen because they have maximum growth and have been cultivated in gardens and greenhouses at Muria Kudus University at 17 meters above sea level so that they can adapt to agro-climates in the lowlands.

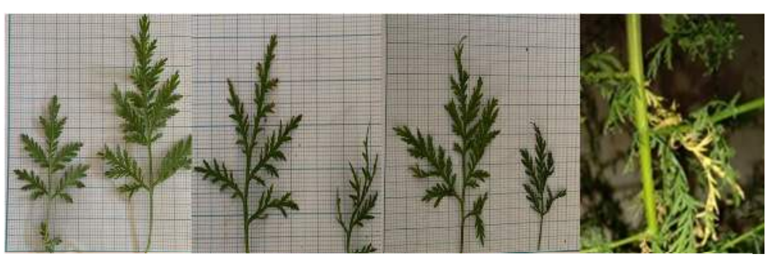

Figure 2. Green accession of artemisia plant

Figure 3. Purple accession of artemisia plant

Based on the observations that have been made, the green accession artemisia and the purple accession can grow well and have the same differences in color and shape of the leaves, stems, and flowers in both control plants and plants that have been sprayed with endophytic bacteria. This result is significant because it is hoped that by spraying, there will be no morphological changes in the two accessions, so it will not impact the yield of artemisia leaves as raw material for artemisinin. The expected differences are not due to environmental factors but to spraying treatment with endophytic bacteria. Plants have epigenetic mechanisms to adapt and overcome the impact of environmental changes, both from biotic factors such as pest attacks and disease and abiotic factors such as changes in weather and the amount of water in the form of puddles or drought. This mechanism is known as plant plasticity, namely the ability of a plant gene to respond to environmental differences through various phenotypes, which are studied through the influence of the environment on changes in genotype or genotype by the environment, GxE (Ashapkin et al. 2020; Napier, Heckman, and Juenger 2023). The phenotypic differences that occur in green and purple accessions of Artemisia annua plants are only caused by genetic differences between the two accessions, which play a more significant role in the morphological diversity of the two plants (Hasanah, Yaya Mawarni, Lisa Hanum 2021). The cause of the diversity in plant appearance is the difference in genetic makeup. Genetic differences are expressed in the form and function of plants, giving rise to diversity in plant growth, which is also due to interactions between genetics and the environment (Casacuberta et al. 2016). In addition, endophytic bacteria are beneficial for plant growth and development without changing the general morphology of the plant. Changes caused by endophytic bacteria include leaf area, specific root length, carbon fixation, and chlorophyll content (Henning et al. 2016).

Artemisinin Content

HPLC test results represented in figure shows an increase in artemisinin levels after being treated with endophytic bacteria spraying. Giving a solution of endophytic bacteria isolate code B5 increased the artemisinin content in artemisia plants, artemisinin (Table 1). In treatments B1A1 and B1A2 it was 0.3429% w/w and 0.202% w/w, which in treatment B1A1, 1% bacterial concentration on green accession artemisia plants, was 27.85% compared to control plants and B1A2, 1% bacterial concentration on purple accession artemisia plants was 32.89% of control plants has been carried out, the artemisinin content in Artemisia plants increased in the treatment of B1A1 (bacteria concentration of 1% on green accession Artemisia) by 27.85% of control plants and B1A2 (bacteria concentration of 1% on purple accession Artemisia) by 32.89% of control plants forming a mutualistic symbiotic relationship with Artemisia plants which can stimulate plants to produce growth hormones or growth regulators (ZPT) and obtain nutrients from the metabolic results of their host plants, so that endophytic bacteria can provide additional nutrients needed for artemisinin synthesis. Apart from that, it is suspected that endophytic bacteria also have the potential to act as growth-promoting agents for Artemisia plants which can produce various kinds of plant growth hormones (Marwan et al. 2011; White et.al., 2019), so that the ZPT can encourage an increase in secondary metabolite content. This is supported by the results of previous research, which showed that spraying with ZPT GA3 was able to increase the artemisinin content of Artemisia plants (Herawati, M. M. Pudjihartati, E. Kurnia, T. D. Pramono 2019).

Table 1. Artemisinin Content on Artemisia Plant from HPLC Test

Sample	**Artemisinin Content (% of dry weight)**
B0A1	0.2682
B1A1	0.3429
B2A1	0.3023
B0A2	0.1523
B1A2	0.202
B2A2	0.154

Green and purple accession artemisia plants that were not sprayed with endophytic bacteria showed differences in artemisinin levels, where the green accession contained higher artemisinin than the purple accession, namely 0.2682 (green) and 0.1523 (purple), respectively. After being sprayed with endophytic bacteria, artemisinin levels in both accessions increased. In green accessions sprayed with 1% endophytic bacteria, there was an increase in artemisinin content by 27.85% dry weight, from 0.2682% to 0.3429% dry weight. In those sprayed with a concentration of 2%, there was an increase in artemisinin content by 32.89% dry weight to 0.3023% dry weight. In purple accessions sprayed with 1% endophytic bacteria, there was an increase of 32.89%, from 0.1523% dry weight to 0.202% dry weight. However, for purple accession artemisia plants that were sprayed with 2% endophyte, there was relatively no increase in artemisinin content.

Endophytic bacteria can produce plant hormones that influence the artemisinin biosynthesis pathway in plants, which results in interactions between compounds produced by endophytic bacteria and biochemical processes in plants, which lead to increased artemisinin production (Anggara, Yuliani, and Lisdiana 2014). BEndophytic bacteria can stimulate the plant defense system, which can withstand stress in plants so that plants allocate more energy and resources for the production of secondary metabolites, including artemisinin (Aziez 2022; Kumari et al. 2023; Narayanan and Glick 2022).

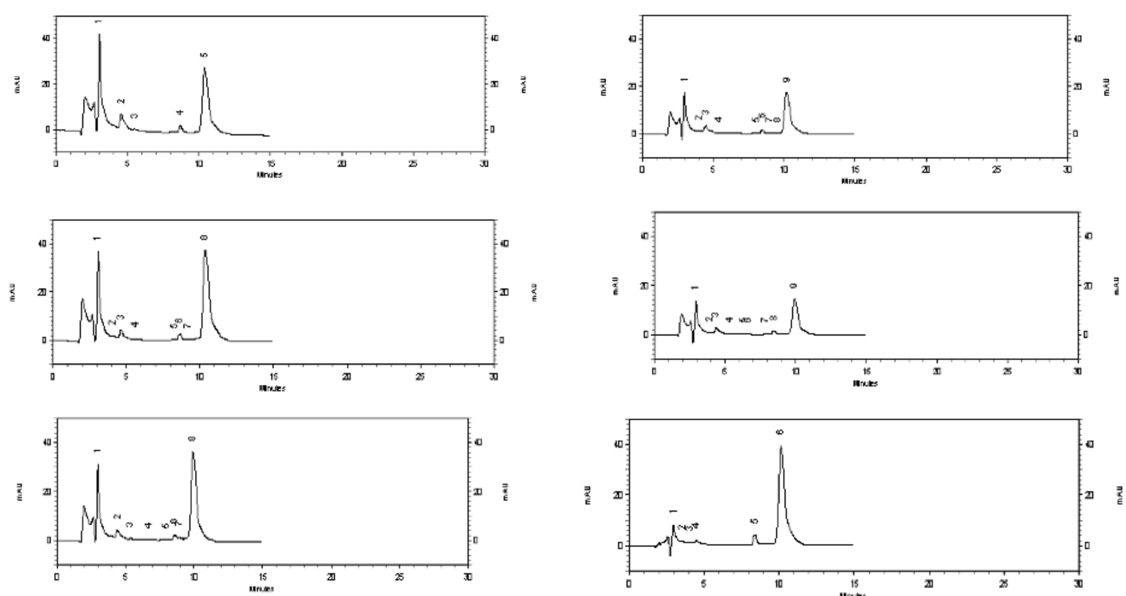

Figure 4. Kromatogram hasil uji kadar artemisinin dengan HPLC, lajur kiri atas ke bawah (A0B0;A0B1;A0B2), lajur kanan atas ke bawah (A1B0;A1B1;A1B2).

The results of the artemisinin content test using HPLC (Table 1, Figure 4) show that the accession treatment significantly increased the artemisinin content. The test results showed that the green accession artemisia had an artemisinin content of 0.2682%, and the purple accession artemisia had an artemisinin content of 0.1523%. According to (Ermayanti et al. 2017) artemisinin content in artemisia plant parts varies; the highest content is in the leaves before the flowering phase. According to (Yuliani 2019) the artemisinin content is different for different accessions due to genetic factors that cause differences in the secondary metabolite content of a plant.

Duncan's multiple distance test at 5% level (Table 2) showed an interaction between bacteria and accessions on leaf number parameters 6 and 8 weeks after planting. The interaction between accessions and bacteria can increase the number of leaves on green accessions of Artemisia plants, which is happening because it involves several mechanisms, namely mutualistic symbiosis between bacteria and plant accessions, such as additional nutrition or protection against pathogens and plant accessions providing shelter and carbohydrate nutrition for endophytic bacteria, which results in an increase in the number of leaves at 6 and 8 weeks after planting. The difference in the increase in the number of leaves can occur because the diversity of physiological structures, metabolites, and growth behavior in different plant types affects their ability to capture various endophytic bacteria (Wu et al. 2021). The results of Duncan's multiple distance test at the 5% level can be seen in Table 2.

Table 2. Effect of Endophytic Bacteria Concentration and Accession Types to The Leaf Number

Treatment	**Leaf Number (Sheet)**			
	2 WAP	**4 WAP**	**6 WAP**	**8 WAP**
Bacteria				
B0	64.34 a	81.5375 a	90.31 a	89.363 a
B1	56.425 a	75.638 a	78.13 a	77.95 a
B2	59.7 a	73.05 a	75.725 a	74.175 a
Accession				
A1	102.53 d	129.1 d	137.25 d	136.3 d
A2	17.783 c	24.37 c	25.525 c	24.69 c
Treatment combination				
B0A1	106.8 e	140.75 e	157.7 h	156.58 h
B1A1	96.325 e	124.18 e	127.7 g	128.4 g
B2A1	104.5 e	122.425 e	126.35 g	123.925 g
B0A2	21.93 e	22.325 e	22.9 e	22.15 e
B1A2	16.525 e	27.01 e	28.58 e	27.5 e
B2A2	14.9 e	23.675 e	25.1 e	24.425 e

Note: numbers followed by the same letter indicate that they are not significantly different in the Tukey comparison test at the 5% level, WAP = week after planting.

CONCLUSION

Inoculation of endophytic bacteria on Artemisia plants at concentrations of 1% and 2% were proven to significantly increase the artemisinin content in two accessions of Artemisia plants, namely green and purple accessions. The highest artemisinin content was obtained from green accessions sprayed with bacteria at 1%. Endophytic bacteria effectively increased artemisinin in green accession artemisia by 27.85% and purple accession artemisia by 32.89% of the artemisinin concentration in control plants. Apart from that, bacterial treatment at concentrations of 1 and 2% also significantly increased plant growth in the number of leaves in green accessions. Inoculation of Artemisia endophytic bacteria code B5 through a spraying technique has other advantages, namely that it can be done quickly and does not change the morphology of the plant, so it does not have the potential to reduce the yield of Artemisia plant leaves, which are the source of artemisinin, so it can support increasing the availability of malaria drug sources.

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