

Effectiveness of Endophytic Bacteria *Rhizophora* sp. from Mangrove Plants to Control Root-Knot Nematodes (*Meloidogyne* spp.).

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ABSTRACT

Meloidogyne spp. is a root-knot nematode that can cause damage to plants up to 68%. The common control is the use of pesticides, but in the long term, it can damage the soil, so it is necessary to use biological agents that can control *Meloidogyne* spp. without causing environmental damage. The purpose of this study was to determine the ability of endophytic bacteria to control root-knot nematodes *Meloidogyne* spp.. The method used in this study was the exploration of endophytic bacteria from the Wonorejo mangrove ecotourism area, Surabaya using purposive sampling method. The five best endophytic bacteria were tested further with antagonistic tests. This test was carried out in vitro using 3 concentrations, namely 10⁶, 10⁸, and 10¹⁰ CFU/ml with 3 repetitions. The obtained results were tested using probit analysis to obtain the LC₅₀ values. It was found that LC₅₀ values for the most effective concentration to kill 50% of the *Meloidogyne* sp population is 10⁹ and 10¹⁰ CFU/ml. It was also revealed that the isolate that is most capable of controlling *Meloidogyne* spp. nematodes is isolate A43.

1. INTRODUCTION

Meloidogyne spp. or better known as the root-knot nematode (RKN) is a type of parasitic nematode that has many hosts or is called polyphage or cosmopolitan. RKN has characteristics in the form of posterior parts, the ends are pointed, wavy and there is a hyaline tail terminus. The anterior part is characterized by set off and flat. The stilet type is a stomastylet equipped with a knob. The digestive tract has a pharynx that connects the median bulb with the pharyngeal gland lobe. The pharyngeal gland overlaps with the intestines.

Root-knot nematodes (RKN) are regarded as major pests that inflict significant economic damage on various crops and lead to decreased agricultural yields (Subedi *et al.*, 2020). Losses due to RKN attacks are very significant and vary according to plant types. Mustika (2005) summarizes the loss of some plants due to RKN, including potato 32-71%, patchouli 45%, pepper 32%, ginger 65%, and coffee 56,84%. For potato it was 15%, tomato 27%, green bean 20%, and 4-90% for soybean (Raihana *et al.*, 2018). This damage depends on the density of the nematode, differences in the structure of the root tissue, the compounds that the host plant has. The intensity of damage due to RKN causes plants to become dwarfed and interfere with production because plant roots are damaged and cannot drain the source of nutrients contained in the soil to all plant tissues (Khotimah *et al.*, 2020). Nematode is generally controlled by using synthetic nematicides. This method is highly discouraged to be carried out for long term because it can pollute the environment, kill other organisms, and leave residues on agricultural products. The use of biological agents such as endophytic bacteria can be another environmentally friendly alternative (Harni, 2016).

Plants have the concept of holobiont in their growth. Holobiont is a plant tissue that can be overgrown by various other types of microorganisms without causing harm to the plant (Lyu *et al.*, 2021). These microorganisms are called

endophytic microorganisms. Endophytic bacteria and fungi are widely used as biological agents to control plant pathogens. Pathogen control using biological agents is one of the efforts made to ensure that the environment is not polluted and maintain the concept of sustainable agriculture.

Mangrove plants are commonly found on the coast of Surabaya. The significant increase in mangrove plant area between 2015-2018 was caused by the increasing availability of nutrient sources and environmental suitability for mangrove plants in Surabaya (Irawanto, 2020). Mangrove plants require an environment with salinity characteristics of 30-38‰, environmental temperature between 27-28°C and an average mud thickness of 1.39 m (Poedjirahajoe *et al.*, 2017). The increase in mud thickness is the main factor that causes mangrove plants in Surabaya to increase. The increase in mangrove area in Surabaya can be used by utilizing endophytic bacteria contained in it.

Endophytic bacteria of mangrove plants are able to survive in conditions with high salinity, so it can be assumed that these endophytic bacteria are able to become a formidable biological agent. Bacteria that are able to survive in high salinity are called halophiles. These bacteria have a mechanism by controlling osmotic pressure inside the cell with the aim of preventing the entry of negative salt ions into the cell that cause the cell to become excessively dehydrated. Another mechanism that endophytic bacteria use is to increase acidity residues, decrease hydrophobic residues and the insertion of halophilic peptides (Reed *et al.*, 2013).

Lethal concentration 50 or LC₅₀ is defined as an effective concentration to kill 50% of the test organism population in this case it is RKN (Jelita *et al.*, 2020). The calculation of LC₅₀ was carried out using probit analysis. Prophytic analysis is a type of regression used to respond to binary variables. Binary variables are variables that have 2 possibilities such as; Yes or no, dead or alive. This analysis uses the death response of test organisms from various concentrations used to determine the effective concentration to kill 50% of the population. This analysis can be done automatically using the IBM SPSS Statistic 24 application. The calculation of LC₅₀ needs to be known so that the application of the concentration of endophytic bacteria from mangrove plants does not kill the entire population of nematodes in the soil, because there are still many free-living nematodes that are feared to be killed so that they can disrupt the ecosystem in the soil. The purpose of this study is to determine the ability of endophytic bacteria of mangrove plants to control *Meloidogyne spp* root nematode.

2. RESEARCH MATERIALS AND METHODS

The tools and materials needed in this study are a petri dish 100×15 mm (Iwaki), Erlenmeyer 50 ml (Herma), a test tube (Iwaki) measuring 18×150 mm, an ose needle, an L rod, a petri dish, Autoclave Wall american Model No. 50x, Laminar Air Flow (SV 900 SS), analytical scales (Kern PCB), orbital shakers (IKA Werke Yellowline), bunsen, baermann funnel sets, 500 g white cotton (Onemed), plastic wrap (cling wrap) size 3700×38 cm cut with a length of 3 cm to cover the edges of the cup, sterile aquades, 96% alcohol, 70% alcohol, Himedia brand nutrient media (NA), Himedia brand nutrient broth (NB) media, 5 nematocidal tested endophytic bacterial isolates and IBM SPSS statistic 24 application.

The experimental design was carried out using Factorial Completely Randomized Design using the 5 best endophytic bacteria based on nematocidal tests using 3 concentrations, namely 10⁶, 10⁸ and 10¹⁰ and replicated 3 times. The results obtained were then tested using probit analysis in the SPSS program.

In toxicology, this probit analysis is commonly used to measure the toxicity of chemicals to live animals. This analysis is done by examining the reaction of organisms to various concentrations and then comparing the concentration with the reactions that occur (Singh & Zahra, 2017). LC₅₀ (Lethal Concentration 50) is an effective concentration that makes 50% of the test animal population killed (Mullo *et al.*, 2022). The probit analysis using the relationship between dependent and independent variables is modeled using the cumulative distribution function of the standard normal distribution (Mahmudah, 2024).

2.1. Research location

Exploration of endophytic bacteria of mangrove plants was carried out in the Wonorejo Mangrove Ecotourism area, Surabaya with coordinates 7°18'25.376" S and 112°49'18.520" E; 7°18'25.996" S and 112°49'20.298" E; 7°18'26.150" S and 112°49'21.727" E; 7°18'26.363" S and 112°49'25.860" E; and 7°18'26.168" LS and 112°49'27.480" E (Figure 1a).

Whereas, exploration of *Nematode Meloidogyne* spp. was carried out in Sumberkembar Village, Pacet District, Mojokerto Regency with coordinates 7°36'58,266" LS and 112°32'50,467" E (Figure 1b). In vitro research was carried out at the plant health laboratory 1 UPN "Veteran" East Java.

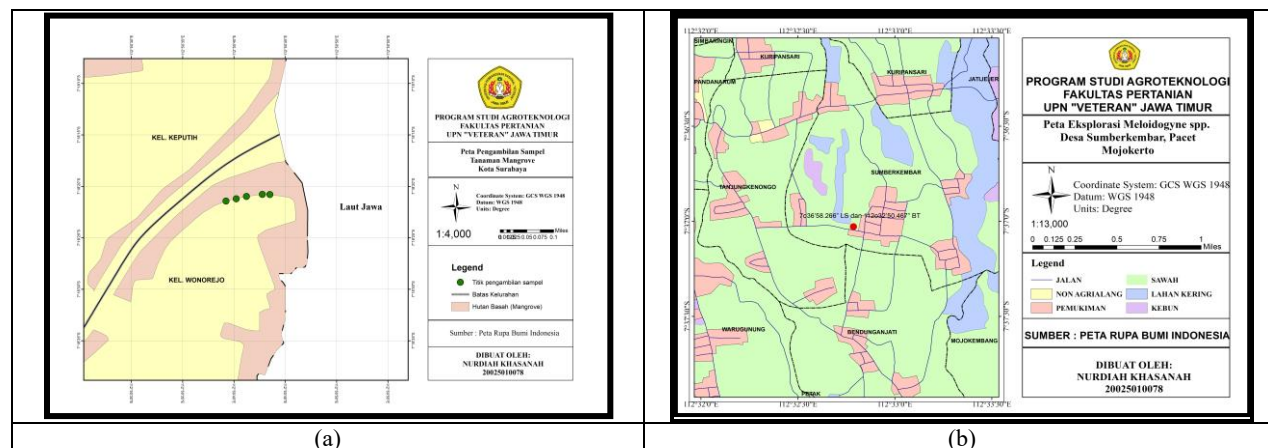


Figure 1. (a) Map of endophytic bacteria uptake, and (b) Map of the exploration of the nematode *Meloidogyne* spp.

2.2. Research Implementation

2.2.1. Research Preparation

The research preparation carried out includes the manufacture of NA and NB media and sterilization of all research tools to be used. The manufacture of NA media is carried out by weighing 28 grams of instant media then dissolved in 1000 ml of sterile aquades then sterilized by autoclave with a temperature of 121°C with a pressure of 1 atm. The manufacture of NB media is carried out by weighing 8 grams of liquid NB media then dissolved in 1000 ml of sterile aquades and sterilized by autoclave with a temperature of 121°C with a pressure of 1 atm. Sterilization of research tools is carried out using ovens for tools made of iron and autoclaves for tools made of glass.

2.2.2. Exploration and Identification of Root Pure Nematodes (NPA)

Exploration of root-knot nematodes (RKN) was carried out by taking soil 10-20 cm deep from the ground surface around plants suspected of being affected by RKN attacks. The symptoms of NPA attack on the top of the plant are that the plant looks dwarfed, leaves turn yellow and look wilted, while at the bottom of the plant there is excessive formation of puru on the roots of the plant (*Gall*).

The results of the exploration were then extracted using the baermann funnel method. The baermann funnel method was chosen because by using this method, more nematodes can be obtained in living conditions and get the population close to reality in the field (Tintori *et al.*, 2022). This method is carried out by weighing soil and root samples of 100 and 10 gr respectively, then each sample is placed on a Baermann funnel set strainer and filled with water until it stagnates. Suspensions that have been left for 24-48 hours are taken to be identified.

2.2.3. Rejuvenation of Endophytic Bacterial Isolate

The isolates used were 5 isolates that had been nematocidal tested. The five isolates are 3 isolates from the roots (A23, A42, and A43), 1 isolates from the stem (B33) and 1 isolate from the leaves (D31). The five isolates when nematocidal tested showed mortality results above 40%. Rejuvenation is done by transferring bacteria from the old NA media to the new NA media within the LAF to prevent contamination from unwanted bacteria from growing.

2.2.4. Antagonist Test

The antagonist test of endophytic bacteria with RKN was carried out by following a modified method (Oktafiyanto *et al.*, 2018). Endophytic bacteria with concentrations of 10^6 , 10^8 and 10^{10} were cultured on NB media as much as 20 ml, then placed on an *orbital shaker* at a speed of 150 rpm for 24 hours. The bacteria that had grown were then added to 10 NPAs using dropper pipettes. Mortality observations were made after 24 hours.

2.2.5. LC₅₀ Calculation

The LC₅₀ calculation was carried out by probit analysis using the SPSS program. The data entered were the concentrations used, the number of nematodes that died and the overall number of nematodes.

3. RESULTS AND DISCUSSION

3.1. Antagonist Test Results

Figure 2 shows a comparison of healthy tomato roots (2a) and tomato roots affected by root knot disease (2b) by nematodes *Meloidogyne* spp (2c). This pathogen is transmitted through infected soil, manure, and infected seed tubers. Generally, nematodes cause damage to the roots, because nematodes suck the root cells, so that the tissue vessels are disrupted and the translocation of water and nutrients is hampered. Plants attacked by *Meloidogyne* spp. develop galls or knots on their roots (Raihana *et al.*, 2018). The size and shape of the galls depend on, the number of nematodes in the roots, the species of nematode, and the plant age. Root infection by nematodes in generative stage plants causes reduced production of flowers and tomato fruits. Severe attacks on the roots cause obstruction of water and nutrient transport, plants wilt easily, especially in hot and dry conditions, stunted or stunted plant growth, and chlorosis of the leaves due to nutrient deficiency. As a result of this root knot disease, flowers and fruits will be reduced or their quality will be low. High levels of nematode attacks cause root damage and disrupt nutrient absorption, so that plant growth is inhibited and plant weight becomes dwarf.



Figure 2. (a) Healthy tomato plant roots, (b) Swelling of tomato roots due to RKN attack, and (c) Root-knot nematodes found from exploration.

The results obtained at the time of the antagonist test were mortality from NPA after 24 h after application. The results of ANOVA showed that the isolate type factor and its interaction with concentration did not significantly affect nematode mortality with p values of 0.507 and 0.816, respectively. While the single concentration factor had a very significant effect with a p value = 0.000. Table 1 presents the results of the DMRT further test about the effect of treatment combinations on the mortality of *Meloidogyne* spp at a 95% confidence level. The A43 isolate with a concentration of 10^6 CFU/ml has a lowest mortality value of 10%. Isolates with a density of 10^{10} CFU/ml can produce the highest mortality compared to densities of 10^6 CFU/ml and 10^8 CFU/ml, this is because the higher the density of bacteria, the more bacteria that secrete antitoxin compounds so that they can kill nematodes (Harni *et al.*, 2020). The high mortality of NPA at this concentration is due to the higher the concentration, the more bacterial colonies are

contained in it, so that more antitoxin compounds can be released (Yudantari *et al.*, 2015). Endophytic bacteria in mangrove plants in India such as *Rhizopora apiculata* and *Avicennia marina* are reported to be producers of antibiotics, producers of auxin hormones, capable of fixing nitrogen and as producers of the enzymes pectinase, protease, chitinase and lipase (Gayathri & Muralikrishnan, 2013). Endophytic bacteria that act as producers of various enzymes can increase the mortality value of NPA in vitro. The cytoplasmic membrane of NPA can be damaged due to various metabolic activities of NPA, such as the outer layer of nematodes can be damaged by the presence of chitinase enzymes in endophytic bacteria (Pratiwi *et al.*, 2015), the lipid layer of nematodes can be degraded by lipase enzymes found in endophytic bacteria (Huzni & Tri Rahardjo, 2015). Endophytic bacteria of mangrove plants are also able to resist the development of *Salmonella* sp. so it can be said that mangrove endophytic bacteria can be anti-microbial materials (Ramadhanty *et al.*, 2021).

Table 1. Effect of isolate types and endophytic bacteria concentration on the mortality (%) of *Meloidogyne* spp.

Isolates	Endophytic bacteria concentration (CFU/ml)			Average
	10^6	10^8	10^{10}	
Isolate A23	16.67±5.77	33.33±11.54	56.67±11.54	35.56 a
Isolate A42	16.67±5.77	30.00±10.00	56.67±25.10	34.44 a
Isolate A43	10.00±0.00	23.33±5.77	60.00±17.32	31.11 a
Isolate B33	16.67±5.77	16.67±5.77	56.67±5.77	30.00 a
Isolate D31	13.33±5.77	20.00±0.00	50.00±10.00	27.78 a
Average	14.67 A	24.67 B	56.00 C	

Note: The numbers followed by different letters show a significant difference on the DMRT test at $\alpha = 0.05$

3.2. Probit and LC₅₀ Analysis

3.2.1. Probit of A23 Isolate

The probit graph on the A23 isolate shown in Figure 3a has an equation $y = 0.3845x - 3.5944$ which shows a positive reaction, where every increase in x (log concentration) will increase the value of y (log probit) by 0.3845. Based on Figure 3. It can be seen that the probit value starts with a negative number, this is due to the very small probability result. This probability value is then converted into a log probit value into a negative value. The value also affects the coefficient of c (-3.5944) to be negative. Negative coefficient values do not affect the relationship between the probit value and the concentration log. The yield of LC₅₀ of A23 isolate is 9.34 which means the most efficient concentration to control 50% of the NPA population using A23 isolate is 10^9 CFU/ml.

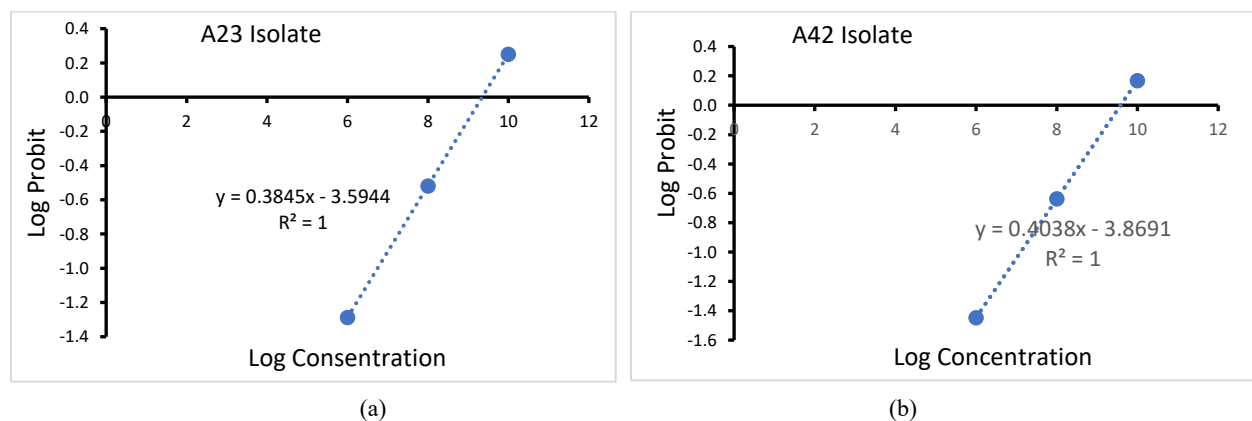


Figure 3. Prophytic analysis: (a) A23 isolate, and (b) A42 isolate

3.2.2. Probit of A42 Isolate

The probit graph on the A23 isolate shown in Figure 3b has the equation $y = 0.2797x - 2.6123$ which shows a positive reaction, where every increase in x (log concentration) will increase the value of y (log probit) by 0.2797. Based on

Figure 4, it can be seen that the probit value starts with a negative number, this is due to the probability results obtained are very small. This probability value is then converted into a probit value into a negative value. This value also affects the coefficient of c (-2.6123) to be negative. Negative coefficient values do not affect the relationship between the probit value and the concentration log. The yield of LC_{50} of A42 isolate is 9.34 which means the most efficient concentration to control 50% of the nematode population using A42 isolate is 10^9 CFU/ml.

3.2.3. Probit of A43 Isolate

The probit graph on the A43 isolate shown in Figure 4a has the equation $y = 0.4038x - 3.8691$ which shows a positive reaction, where each increase in x (log concentration) will increase the value of y (log probit) by 0.4038. Based on Figure 4a, it can be seen that the probit value starts with a negative number, this is due to the probability result obtained is very small. This probability value is then converted into a probit value into a negative value. This value also affects the coefficient of c (-3.8691) to be negative. Negative coefficient values do not affect the relationship between the probit value and the concentration log. The yield of LC_{50} of A43 isolates is 9.57 which means the most efficient concentration to control 50% of the nematode population using A43 isolates is 10^9 CFU/ml.

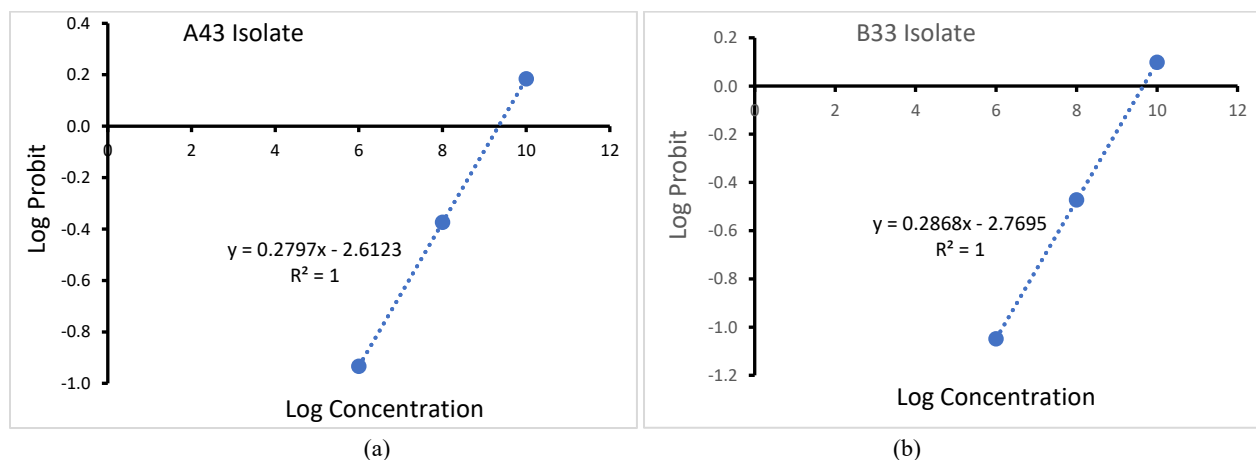


Figure 4. Prophytic analysis: (a) A43 isolate, and (b) B33 isolate

3.2.4. Probit of B33 Isolate

The probit graph on the A43 isolate shown in Figure 4b has the equation $y = 0.2868x - 2.7695$ which shows a positive reaction, where each increase in x (log concentration) will increase the value of y (log probit) by 0.2868. Based on Figure 4b, it can be seen that the probit value starts with a negative number, this is due to the very small probability result. This probability value is then converted into a probit value into a negative value. This value also affects the coefficient of c (-2.7695) to be negative. Negative coefficient values do not affect the relationship between the probit value and the concentration log. The yield of LC_{50} of B33 isolate is 9.65 which means the most efficient concentration to control 50% of the population of nematodes using B33 isolate is 10^9 CFU/ml.

3.2.5. Probit of D31 Isolate

The probit graph on the A43 isolate shown in Figure 5 has the equation $y = 0.3321x - 3.3709$ which shows a positive reaction, where every increase in x (log concentration) will increase the y value (log probit) by 0.3321. Based on Figure 5, it can be seen that the probit value starts with a negative number, this is due to the very small probability result. This probability value is then converted into a probit value into a negative value. This value also affects the coefficient of c (-3.3709) to be negative. Negative coefficient values do not affect the relationship between the probit value and the concentration log. The yield of LC_{50} of D31 isolates is 10.53 which means the most efficient concentration to control 50% of the nematode population using D31 isolates is 10^{10} CFU/ml.

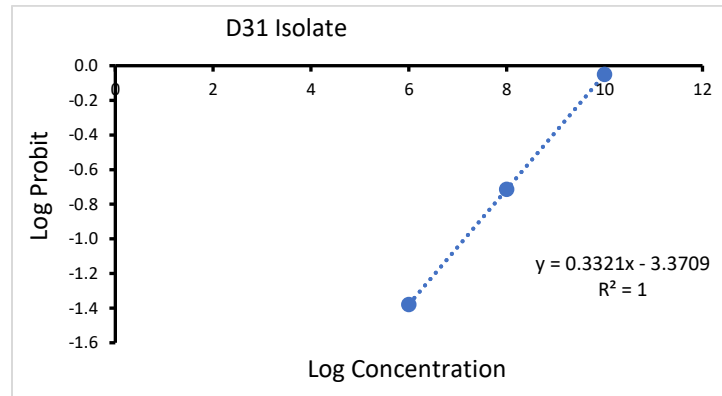


Figure 5. Prophytic analysis on D31 isolate

4. CONCLUSION

The conclusion that can be drawn is that endophytic bacteria *Rhizophora* sp. from mangrove plants are effective to control *Meloidogyne* spp. Mangrove endophytic bacteria with isolate codes A23, A42, and A43, B33 and D31 are able to control RKN in vitro with concentrations of 10^9 to 10^{10} CFU/ml. The higher the concentration of endophytic bacteria, the more toxins that can be released to kill *Meloidogyne* spp. Based on probit analysis, each increase in the log concentration value will increase the probit values.

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