Endophytic bacteria (genus: *Pseudomonas* spp.) isolated from Aceh bamboo root as biological agent against nematode *Meloidogyne* spp.

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**Abstract.** Endophytic bacteria are bacteria that associated with plant tissues without causing any disease. Endophytic bacteria able to produce enzymes that act as nematicide against root knot disease in celery plants caused by *Meloidogyne* spp. Bamboo roots were isolated, obtain 26 isolate by screening of mortality test, and founded 14 isolates were potential as biological control agents with 100 percentage. Furthermore, 14 endophytic bacterial that has been isolates were detected their protease enzyme activity and 9 isolates were shown the protease enzyme as qualitatively result. Aplication of endophyte bacteria on Celery seedling detected that the treatment of bacteria (AB12) had the lowest number of gall, the lowest number of nematode populations in gall compared to other isolates. This result indicates the AB12 isolate similar characterize from genus *Pseudomonas* spp. have good potential as a biological control agent for *Meloidogyne* spp. in celery plants. Some endophytic bacteria in other treatments did not affect the inhibition of nematodes while were able to increase plant growth such as plant height, number of stems and higher root length (AB03 isolate).

1. Introduction

Celery (*Apium graveolens*) is an economically valuable vegetable commodity that often used as a spice in the kitchen. Plant root parasitic nematodes have seriously disease problem in the cultivation of celery plant an. One of the root parasitic nematodes caused this disease from species of *Meloidogyne* spp. [1]. Celery plants are hosts of *M. incognita*, *M. javanica*, and *M. Thamesi* [2]. Parasitic nematodes have been reported an associated with celery plants in group of *Helicotylenchus*, *Longidorus*, *Meloidogyne*, *Trichodoros* and *Xiphnema* reported by [3].

Root knot nematode (*Meloidogyne* spp.) could attacked the root cortex tissue of plant, especially fibrous roots that actively absorb nutrients and water then cause the presence of gall in the roots. The presence of gall can disrupt the system of distribution of water and minerals from the soil through the roots of all parts of the plant, new roots do not emerge, plants wilt easily, become stunted and cause chlorosis [4]. Control strategy of *Meloidogyne* spp. will be more effective if could be done in an integrated system manner by combining several control components, one of which is the use of
biological agents. Endophytic bacteria as a component of biological control and reduce the use of synthetic pesticides [5].

Endophytic bacteria can produce metabolites compound as antifungal [6], antibacterial, antiviral, growth regulators, and producing enzymes such as amylase, cellulose, xylanase, lignose and chitins [7]. Endophytic bacteria as biocontrol agents for nematodes through several mechanisms such as producing toxins, enzymes (protease enzymes, lipases and chitins), nutritional competition, growth promoters and inducing plant resistance by stimulating the formation of salicylic acid, peroxides, phytoexin, PR-proteins and phenol compounds [8].

Bamboo plants growing wild in the province of Aceh used for property and have a variety of other benefits. In addition to being a conventional object, scientists have also found that several species of endophytic bacteria are associated with the roots of a bamboo plant that benefits the host [9]. The purpose of this study was to determine the potential of endophytic bacteria from the roots of bamboo plants as effective biological control agents in suppressing the development of Meloidogyne spp.

2. Materials and Methods

2.1. Materials

The materials used in this research are isolate bacteria endophytic, root knot nematodes, purified water, skim milk agar as media for detected enzym protease.

2.2. Methods

2.2.1. Propagation of Endophytic Bacteria. Isolation of endophytic bacteria was carried out using a combination of the [10]. The roots were weighed 1 g and crushed until crushed with a sterile mortar. Plant extracts were added 9 ml of sterile water. Furthermore, serial dilution was carrying out up to 10^{-3}. Dilution was carried out by taking 1 ml plant root extract then put in 9 ml of sterile water. 100 μl of 10^{-3} dilution suspension was grown on sodium agar as media by the scatter method using a stalk and incubated at room temperature 24-48 hours. An endophytic bacterium has propagated on NA media for 48 hours at room temperature. The results of a single colony culture was taken 1 full loops (ose) and suspended in 8 ml of sterile water, and stirred then the suspension was homogeneous, dilution was made for further testing

2.2.2. Extraction of Parasite Nematodes from Root Samples. Nematodes was extracted from field samples of celery roots infected with root gall nematodes using Baermann funnel then plating on test tube. Before putting on Baermann funnel, the roots were cleaned from the soil root contain, then cut into pieces ± 1 cm and weighed as much as 1 g. The cuttings root were soaked in a test tube containing 5 ml of sterile water, then stored in a laminar airflow chamber and left for 6 hours [11].

2.2.3. Endophytic Bacteria Test against Meloidogyne spp. In Vitro.

2.2.3.1. Nematode Mortality Test. Nematode mortality has determined by the inhibitory test. Endophytic bacteria that has grown on NA (Nutrient Agar) media for 48 hours, then dissolved into sterile water with added 9 ml of water. Then a bacterial suspension of 5 ml with a density of 10^{3}-cfu ml^{-1} was inserted 5ml of nematode extract or ± 100 larvae and deep for 24 hours [5]. Observation of nematode mortality was carried out at 24 hours after treatment using a Nikon type 102 microscope with magnification 4 x.

2.2.3.2. Proteolytic Activity. Proteolytic activity was tested using skim milk media at pH 6.5. Media composition consisted of 10 g of skim milk, 15 g of TSB, 7.5 g of agar and 500 mL of distilled water. Agar, TSB (Tryptic Soy Agar) and distilled water were sterilized by autoclaving at 121°C for 15 minutes. After sterilization was finished, skim milk was further added when the media was still hot. Bacterial cultures were streaked on the media added to skim milk. Proteolytic activity was indicated by a clear zone around the colonies of bacteria, 48 hours after treatment.
2.2.3.3. Characterization of Endophytic Bacteria Isolates. Endophytic bacterial isolates was characterized including gram staining and biochemical tests consisting of citrate, catalyses, motility, indole test, TSIA (Triple Sugar Iron Agar) test, MR-VP test (Methyl Red-Vogues Proskauer) and gram test. A biochemical test was carrying out to determine the ability of bacteria to use macromolecules and macromolecules to produce energy for bacteria and to know the ability to synthesize certain enzymes.

2.2.3.4. Endophytic Bacteria Test Against Meloidogyne spp. In Vivo. Endophytic bacterial isolates (14) from bamboo plants that were potential in inhibiting the development of nematodes in the laboratory were tested on celery plants to see their potential in the field. Celery seed varieties of Amigo were soaked for 30 minutes into a suspension of endophytic bacteria with a population density of 10⁸-cfu mL⁻¹. Seed was plant in pole bags that has been filling with soil medium and manure (1: 2). Furthermore, after the seedlings were 1 week old, the seedlings of the plant had inoculated with Meloidogyne spp. Nematode larvae come from the root knot celery plants that had attacked. Planting has carried out following the RAL (Randomized Block design) pattern with two replications. Some parameters observed are:

2.3. Analysis
2.3.1 Plant Height. Plant height observed at ages 6, 12, 18, 24 and 30 DAA (Day after application) by measured height of plants from ground level to top, using a ruler.

2.3.2 Number of Plant. Calculation of the number of stems has observed at the age of 6, 12, 18, 24, and 30 DAA by counting the whole celery plants.

2.3.3. Root Length. Measurements has made at the base of the stem to the tip of the longest root by harvesting the plant first. Measurements has made on plants that have been aged 30 days using a meter.

2.3.4. Number Root Galls. After the plant was 3 months old, polybag were remove from soil and the roots of the celery plant was counting the number of root gall.

2.3.5. Number of Nematodes from Gall. After the plants were 3 months old, the roots of the plants that formed the gall were counting and extracting the number of nematodes population.

3. Results and Discussion
3.1 Endophytic Bacteria Test against Mortality of Meloidogyne spp. by In Vitro
Based on the nematode mortality observation shown that application of 27 endophytic bacterial isolates from Aceh bamboo plants could inhibited the development of nematodes in celery plants. The results of the analysis indicated P<5% (2.528) this shows that the suspension treatment endophytic bacteria from the roots of bamboo plants significantly affected to mortality of Meloidogyne spp.

In vitro test have been finished to determine the direct effect of endophytic bacteria from the roots of bamboo plants on parasitic nematodes (Meloidogyne spp). 26 isolates were used which had been tested in negative mark for hypersensitivity test [12]. The lowest mortality result occurred in the control treatment. The nematodes that have not treated with endophytic bacteria did not significantly decrease in inhibitory mortality. The treatment of endophytic bacteria AB01, AB02, AB03, AB04, AB05, AB06, AB07, AB08, AB09, AB11, AB12, AB14, AB17, and AB25, rate of mortality increased due to the influence of metabolite compounds produced by bacteria containing toxic. This result indicate the endophytic bacteria have potential effect as nematicidal. The same result have been reported, that the filtrate culture of the Bacillus subtilis B38, Pseudomonas fluorescence B103 and S.
marcescens produced metabolite compounds that caused mortality of *M. Incognita* (90.6% to 92.9%) [13].

| No. | Treatment | Name of Treatment | Nematodes Mortality (%) |
|-----|-----------|------------------|------------------------|
| 1   | Control   | Without endophytic bacteria | 85.96 a |
| 2   | AB01      | Bamboo root 01    | 100.00 d |
| 3   | AB02      | Bamboo root 02    | 100.00 d |
| 4   | AB03      | Bamboo root 03    | 100.00 d |
| 5   | AB04      | Bamboo root 04    | 100.00 d |
| 6   | AB05      | Bamboo root 05    | 100.00 d |
| 7   | AB06      | Bamboo root 06    | 100.00 d |
| 8   | AB07      | Bamboo root 07    | 100.00 d |
| 9   | AB08      | Bamboo root 08    | 100.00 d |
| 10  | AB09      | Bamboo root 09    | 100.00 d |
| 11  | AB10      | Bamboo root 10    | 99.58 ed |
| 12  | AB11      | Bamboo root 11    | 100.00 d |
| 13  | AB12      | Bamboo root 12    | 100.00 d |
| 14  | AB13      | Bamboo root 13    | 99.71 cd |
| 15  | AB14      | Bamboo root 14    | 100.00 d |
| 16  | AB15      | Bamboo root 15    | 97.90 bcd |
| 17  | AB16      | Bamboo root 16    | 95.28 ab |
| 18  | AB17      | Bamboo root 17    | 100.00 d |
| 19  | AB18      | Bamboo root 18    | 98.23 bcd |
| 20  | AB19      | Bamboo root 19    | 97.85 bcd |
| 21  | AB20      | Bamboo root 20    | 99.63 cd |
| 22  | AB21      | Bamboo root 21    | 99.13 cd |
| 23  | AB22      | Bamboo root 22    | 97.34 bcd |
| 24  | AB23      | Bamboo root 23    | 99.15 cd |
| 25  | AB24      | Bamboo root 24    | 95.41 bc |
| 26  | AB25      | Bamboo root 25    | 100.00 d |
| 27  | AB26      | Bamboo root 26    | 97.50 bcd |

BNT 0.05 6.799

Note: The different superscript letters in the same column indicate significantly different values (P<0.05)

Metabolite compounds produced by endophytic bacteria include phosphate solvents, and hydrolyzing enzymes such as chitins, protease, cellulose, lipase and pectin’s [14]. In the same statement, the use of endophytic bacteria can be through several biological control mechanisms such as producing toxins, enzymes, nutritional competition, for plant growth and induce plant resistance [15].

3.2. The Ability of Selective Endophytic Bacteria to Produce Protease Enzymes (Proteolytic Activity)

Fourteen endophytic bacterial isolated from the roots of bamboo plants succeeded in inhibiting the mortality of *Meloidogyne* spp. in vitro. Further selection was using skim milk media to test the ability of endophytic bacteria that produce protease enzymes as a protein-splitting biocatalyst into oligopeptides or amino acids [5]. Test results on skim milk media showed 9 isolates of endophytic bacteria possessing proteolysis activity with proteolysis ability including isolates AB01, AB03, AB04, AB07, AB08, AB09, AB12, AB14, and AB17 (Table 2.). The production of bacterial extracellular
enzymes causes protein content in NA media and 1% skim milk hydrolyzed into peptides and amino acids as indicated by clear zone as an indicator that endophytic bacteria are able to utilize protein in the media as a nutritional source [16][17]. Protease enzymes play an important role in degrading intestinal and nematode cuticles [14]. Endophytic bacteria that using under this research had the percentage of Meloidogyne spp. mortality of 100% indicated them be able to control the development of pathogenic nematodes that cause root disease in plants celery.

| No. | Treatment | Name of treatment | Result |
|-----|-----------|------------------|--------|
| 1   | AB01      | Bamboo root 01   | +      |
| 2   | AB02      | Bamboo root 02   | -      |
| 3   | AB03      | Bamboo root 03   | +      |
| 4   | AB04      | Bamboo root 04   | +      |
| 5   | AB05      | Bamboo root 05   | -      |
| 6   | AB06      | Bamboo root 06   | -      |
| 7   | AB07      | Bamboo root 07   | +      |
| 8   | AB08      | Bamboo root 08   | +      |
| 9   | AB09      | Bamboo root 09   | +      |
| 10  | AB11      | Bamboo root 11   | -      |
| 11  | AB12      | Bamboo root 12   | +      |
| 12  | AB14      | Bamboo root 14   | +      |
| 13  | AB17      | Bamboo root 17   | +      |
| 14  | AB25      | Bamboo root 25   | -      |

Note: (+) = reacts positively to the tests conducted; (-) = react negatively to the tests conducted

Protease enzymes one of the mechanisms of a biological control agent of Meloidogyne spp. [18]. These enzymes can be use directly by bacteria to degrade nematode cell walls. According to research [5] endophytic bacteria from sweet potato plants namely Enterobacter sp. EAS (1a), Enterobacter sp. EAS (3a), Enterobacter ludwigii. EAS (4), and Burkholderia cepacia EAS (6) produce lipase and protease enzymes. The enzymes produced by endophytic bacteria were known to play an important role in degrading the extracellular layer, it can inhibit the development of Meloidogyne spp. Pseudomonas fluorescens CHA0 which produces extracellular enzymes (Protease enzymes) were antagonistic to Melodydogne incognita [18]. Bacillus firmus MPB04 produces protease and cellulose enzymes, which cause higher mortality of Meloidogyne incognita than Bacillus sp. MPB115 that does not produce protease enzymes [19].

3.3. The Characterizations of Endophytic Bacteria Producing Protease Enzymes

The fourteen endophytic bacteria from the roots of bamboo plants that were able to inhibit the mortality of nematodes Meloidogyne spp. Biology chemical testing and gram staining was done (in Table 3). The results of gram staining test for fourteen endophytic bacterial isolates, (four isolates were round, nine isolates were rod and one isolate was comma). All bacteria tested were Gram-negative bacteria, which have colour look like pink, contain lipids and fat in a higher percentage than gram-positive bacteria [20], have thinner peptidoglycan than gram-positive bacteria [21].

A Biochemical test was carried out based on the results of metabolism due to the working ability of enzymes and by-products of secondary metabolites. Table 2 shown that the endophytic bacterial isolate as a whole have flagella. Flagella are one of the main structures outside bacterial cells that cause motility in bacterial cells. The fourteen isolates showed different morphological and colony cell characterizations. Isolation and characterization of a bacterium that is cell morphology (cell shape and cell structure) is an important step for identification. Based on the results of biochemical tests that have been carried out it can be known that all endophytic bacterial isolates from bamboo plants are
positive catalyze. Most endophytic bacteria use citrate as their carbon source and some are able to ferment glucose and lactose [21].

### Table 3. Biochemical tests on 14 endophytic bacterial isolates

| No. | Isolates | H$_2$O$_2$ | Motility | Indol | MR | VP | SC | TSIA | Gram |
|-----|----------|------------|----------|-------|----|----|----|------|------|
|     |          |            |          |       |    |    |    |      |       |
| 1   | AB01     | +          | +        | +     | +  | +  | +  | +    | Coccus |
| 2   | AB02     | +          | +        | +     | -  | +  | -  | -    | Coccus |
| 3   | AB03     | +          | +        | +     | -  | +  | -  | -    | Coccus |
| 4   | AB04     | +          | +        | +     | +  | +  | -  | -    | Diplobacil |
| 5   | AB05     | +          | +        | +     | -  | -  | -  | -    | Coccus |
| 6   | AB06     | +          | +        | +     | +  | +  | -  | -    | Diplobacil |
| 7   | AB07     | +          | +        | +     | +  | +  | -  | -    | Bacil |
| 8   | AB09     | +          | +        | +     | +  | +  | -  | -    | Diplobacil |
| 9   | AB11     | +          | +        | +     | -  | +  | -  | -    | Bacil |
| 10  | AB12     | +          | +        | +     | -  | -  | +  | +    | Bacil |
| 11  | AB14     | +          | +        | +     | +  | -  | -  | +    | Spiritum |
| 12  | AB17     | +          | +        | +     | -  | +  | -  | +    | Bacil |
| 13  | AB25     | +          | +        | +     | -  | +  | +  | -    | Bacil |

**Description:** H$_2$O$_2$= Hydrogen Peroxidise test, MR = *Methyl Red*, VP = *Vogues Proskaurt*, SC = Simmons Citrate, TSIA = Triple Sugar Iron Agar, G = Glucose, S + L = Sucrose + Lactose

Isolates that have cell morphological and biochemical similarities show that these bacteria belong to the same genera. So far, it appears that the similarity possessed by one endophytic bacterial isolate from the results of gram staining and biochemical tests that have been carried out are identified as belonging to the genus *Pseudomonas* spp. (AB 11 isolates). There were several endophytic bacteria that are symbiotic with the roots of bamboo plants, one of which is a bacterium from the genus *Pseudomonas* [9]. Endophytic bacteria from bamboo plants in the Chanting (China) region after being bred and analyzed for diversity and colony characteristics, found the dominant genera namely *Alcaligenes*, *Pseudomonas*, *Staphylococcus* and *Bacillus* [22]. In a study he conducted stated that *Pseudomonas* are rare and only found in bamboo forests [23]. However, its position is very important associated with plants as a microbial group because it is able to infect / attack plant pathogens and increase plant growth.

#### 3.4. Selective Test of Endophytic Bacteria Against Meloidogyne spp.

*In vivo*

The fourteen endophytic bacterial isolates from bamboo plants were tested qualitatively for the activity of the protease enzyme so that 9 isolates were obtained. The isolate is then tested *in vivo* to see its potential.

**3.4.1. Plant Height.** The results observation of the application endophytic bacteria to plant height on a weekly basis could see in (Figure 1) The results of analysis of variance shows that the application of endophytic bacteria has a very significant effect on plant height 12, 18, 24 and 30 DAA but does not significantly affect plant height 6 DAA. Base on the Figure 1, the average plant height in each
treatment has increased except for AB17 isolates observed at 12 HSI. The different things were shown in the treatment of bacterial isolates AB03, AB12 and AB14. A treatment it was suspected that endophytic bacterial isolates applied to celery plants could increase plant growth by showing the highest average of 6.5-7 cm.

**Figure 1.** Average height of celery plants due to the treatment of endophytic bacteria isolates

3.4.2. **Number of Plant.** The results of observing the application endophytic bacteria to the number of plant on each week could see in Figure 2. The results of analysis show that the application endophytic bacteria to the number of plants have a very significant effect every week. Results of analysis variance showed that the highest number of plants were found in AB03 isolates that were not significantly different from controls (+).

**Figure 2.** Average number of stems of celery plants due to the treatment of endophytic bacteria isolates

On Figure 2, showed that endophytic bacteria have the ability to increase plant growth by producing indole acetic acid (IAA) which can act as PGPR (*Plant Growth Promote Rhizobacteria*). Endophytic bacteria isolated from forestry plants could increase the growth of rubber seedlings, which was thought to be due to their ability to produce growth hormones such as auxin (IAA), cytokines and gibberellins [24].

3.4.3. **Root Length.** The results of the application of endophytic bacteria for root length in celery plants could see in Figure 3. The results of analysis variance showed that the application of endophytic bacteria to root length had a very significant effect with a value of P>0.05% (63.62). In the figure, it
could see that bacteria isolate AB04 (4.65 cm) has the highest average among other isolates and it was not significantly different from AB01 (4.10 cm) isolate treatment.

In addition to being able prevent plant diseases, antagonistic bacteria also play a role as a growth booster because it has protection against PGPR for plants (*Plant Growth Promoting Rhizobium bacteria*). Endophytic bacteria as PGPR increase crop production because its activity helps dissolve inorganic materials in the soil that can be used for growth. In Figure 3 isolate AB04, can increase root growth compared to control and other isolate treatments. However, there are no different from isolates AB01 and AB03. The root system is very important in the release of nutrients because a good root system will shorten the distance needed for nutrients to start plant roots.

![Figure 3](image-url)

**Figure 3.** Average root length of celery plants due to the treatment of endophytic bacterial isolates

### 3.4.4. Number of Gall

The results of application endophytic bacteria to the number of gall in celery plants could see in Figure 4. The results of analysis variance shown the application endophytic bacteria to the number of gall observed in the roots of celery plants have a very significant effect with a value P>0.05% (31.26). In Figure 4. The treatment of AB09 bacterial isolates has the highest average number of gall among other isolates. However, it was not significantly different from AB01 isolate treatment.

From the description of the picture, it appears a difference in the average number of gall caused by nematode attack on the treatment of endophytic bacterial isolates. The results showed that all isolates tested had various effects and could significantly reduce the nematode population when compared to the control (+) and it caused by the different abilities of these isolates endophytic bacteria in suppressing the infection to *Meloidogyne spp.* The treatment of endophytic bacterial isolates from the roots of bamboo plants AB 12 had the lowest average that was not significantly different from AB07 isolates it had the potential as a biocontrol agent for *Meloidogyne spp.* The low number of gall in celery roots due to endophytic bacteria can suppress the penetration and reproduction of nematodes and produce secondary metabolites as nematicide [6].

AB 17 isolates had the same average as control (+) because the plants were dead and could not observe. The results also showed that the number of gall observed in the roots of celery plants was still high and same as the treatment of isolates AB 01 and AB 09.

The decline of nematode populations due to endophytic bacteria have the ability as an antagonistic agent in inhibiting the development of *Meloidogyne spp.* suppressive mechanisms include colonizing cortical tissue and producing metabolite compounds that can suppress the development of pathogenic nematodes and induce plant resistance [5].
3.4.5. Nematode Population in Plant Roots. The results showed that the application of endophytic bacteria on the celery plants affected the population number of nematodes and showed significant differences in control (+). That shows endophytic bacteria can do a mechanism of inhibition of pathogenic infections, which were characterized by the low number of nematode populations found in AB12 treatment. The decline in nematode populations due to endophytic bacteria has the ability as an antagonistic agent in inhibiting the development of *Meloidogyne* spp. These suppressive mechanisms include colonizing cortical tissue and producing metabolite compounds that can suppress the development of pathogenic nematodes and induce plant resistance [25].

Effectiveness of biological agents in suppressing disease is determined by their ability to adapt to the environment so that the agency was able to induced disease resistance. Several types of endophytic bacteria have been identified as potential biological agents against the parasitic nematode Pratylenchus brachyurus on patchouli plants [26][27]. The effect of bionematicidal endophytic bacteria was also report to have suppressed the development of *Meloidogyne* spp. by 74% in coffee plants [25].
4. Conclusions
Endophytic bacterial isolates obtained from the roots of bamboo plants showed potential results in inhibiting the growth of *Meloidogyne* spp. of 100% in the laboratory, among the 26 endophytic bacterial isolates as many as 14 isolates have the highest percentage of mortality. From the results of an enzyme, detection in 14 isolates tested with selective media obtained nine endophytic bacterial isolates that showed positive reactions to a test enzyme (protease) namely AB04, AB07, AB08, AB09, AB12, AB14, and AB17. Variance test showed that the treatment of endophytic bacterial isolates from the roots of bamboo plants (AB12) had the lowest number of gall, the lowest number of nematode populations in gall and compared to other isolates so that it had the potential as a biocontrol agent for *Meloidogyne* spp. in celery plants. Meanwhile, some endophytic bacteria in other treatments did not affect the inhibition of nematodes but were able to increase plant growth such as plant height, number of stems and higher root length (AB03 isolate). It was assumed that each endophytic bacterium interacts with plants to give different responses to nematodes and plants. Further research needs to carry out a combination of several potential endophytic bacterial isolates to be a formula.

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