Management of endophytic bacteria and organic material for the biological control of yellowing disease on pepper

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Abstract. Yellowing disease caused by plant-parasitic nematodes have been recognized as potentially serious constraints to crop productivity on pepper. The use of chemical pesticide in agriculture in Indonesia for controlling plant diseases are still the most preferred way by farmers. Therefore, alternative control strategies for plant pest and diseases that are cheap and environmentally friendly needs to be developed. It is widely recognized that organic matter can improve soil quality and ecosystem health. In many cases, organic matter and microorganism can reduce plant losses caused by soil borne pathogens. However, little information is available about the influence of combination of organic material and bacterial endophytes on the severity of yellowing disease in pepper. The objective of this study was to evaluate the effectiveness of formulation of endophytic bacteria in combination with organic material against yellowing disease on pepper. Selected endophytic bacteria isolated from pepper and organic material were used in this experiments. The results showed that biological agents of endophytic bacteria and organic material are effective in controlling plant parasitic nematodes and stimulate the plant growth of pepper. Combination of endophytic bacteria and organic material may be one component for nematode control in pepper.

Keywords: Meloidogyne spp, root gall, soil quality, ecosystem health

1. Introduction
Most of the pepper plantations in Indonesia are smallholder plantations with narrow land ownership and poor management due to limited capital, facilities and knowledge or skills. The main problems of pepper production in Indonesia are low levels of crop productivity and production, inefficient farming, low product quality and diversification, and high yield losses due to plant pests and disease.

Yellowing disease has become a major obstacle in the production of pepper in Bangka which is the center of white pepper. Crop losses in the cultivation of pepper due to yellowing diseseas can reach up to 30-40%. Yellowing disease in pepper is a complex disease caused by the nematodes Meloidogyne incognita and Radopholus similis and in some cases associated with the fungus Fusarium sp. [1]. Various attempts have been developed to control this disease including fertilization, mulch use and pesticide, but they have not able to overcome this disease and remain a major problem in several areas. The use of chemical pesticides can cause negative impacts on the environment, pathogens become more resistant and kill beneficial natural enemies. At present the existence of pesticide residues in agricultural products has become a major issue for pepper consumers in developed countries. It is necessary to
develop a method for controlling plant parasitic nematodes which is environmentally friendly, including by using biological control agents and organic material.

Endophytic bacteria within plant tissues have attracted due to their interesting features related to plant growth and for the bioprotection of plants against plant pests and diseases, including plant parasitic nematode on tomato, cucumber, coffee, black pepper [2, 3, 4, 5, 6]. Organic materials is the most importance component of soil quality and ecosystem health. The soil quality is characterized with the soil’s physical, chemical, and biological properties that are affected by organic matter content. Adding organic matter to soil influences biological activities and improve also soil chemical, physical, and parameters [7]. Application of organic material in many cases can increase the activity of beneficial microorganisms, including biological control agents that able to suppress plant-parasitic nematodes and soil borne plant pathogens [8, 9]. It is importance to evaluate the effects of organic matter application on activities of plant-parasitic nematodes and soil borne disease in the plant production system. The objective of this research was to study the potentiality of endophytic bacteria and organic material in yellowing disease controlling yellowing disease caused by plant-parasitic nematodes on pepper and to obtain a parasitic nematode.

2. Material and Method

2.1. Production of inoculum of Meloidogyne incognita
Nematode M. incognita was extracted from the infected pepper root by yellowing disease from Bangka. Furthermore, M. incognita nematodes are propagated on tomato plants that are sensitive to these nematodes for 2 months. After 2 months the plant was removed, the roots were extracted by the extraction method to obtain active nematode larvae, the extraction results were made as the source of M. incognita nematode inoculum.

2.2. Isolation of endophytic bacteria
Endophytic bacteria are explored from pepper plants taken from Bangka and Sukabumi (West Java). Root samples are washed with running water until clean, then the roots are weighed as much as 2-3 g of the wet weight of the roots. The root samples were surface sterilized in stages by immersing it in 70% alcohol solution for 30 seconds, then immersed in 2% NaOCl solution for 1-2 minutes, then rinsed with sterile water three times. As the surface sterilization control is successful, then the roots that have been sterilized (before being mashed) are grown by placing them on a petri dish with TSA (Tryptic Soy Agar) medium. If within 24-48 hours of growing bacteria, contamination or surface sterilization occurs which is not successful and must be repeated. Samples of sterilized roots are then homogenized using sterile mortar. Furthermore, dilution was carried out 4 time serial dilution. A total of 1 ml of suspension was taken using a micro pipette and then put into a test tube containing 9 ml of sterile water to get a dilution of $10^{-4}$. Furthermore, from each dilution 0.1 ml was taken and grown on 10% TSA media, then incubated for 24 hours at room temperature. Endophytic bacterial isolates were then subjected to differentiation and purification. Storage of bacterial isolates was done by growing each bacterial isolate on TSB (Tryptic Soy Broth) media mixed with 20% glycerol then stored in temperature of -4°C.

2.3. Organic material
The organic materials were used in this study cow dung (CD) and commercial organic fertilizer (OF). Cow dung is an organic material that is available quite a lot in the community and is easily obtained because it is almost available in many places. Cow dung is taken from a dairy farm that has been dried and separated from the garbage or the remnants of grass. The organic fertilizer used is commercial fertilizer in the form of granules which are also widely sold in the market or in the community.

2.4. Greenhouse experiment
A total of seven potential endophytic bacterial isolates namely isolates AA2, MER7, ANIC, TT2, MER9, HEN1, EH11, and TT2 were tested in the greenhouse experiment. Bacterial isolates were grown
on TSA media for 48 hours at room temperature. Furthermore, each petri dish containing bacteria was suspended in sterile water up to $10^9$ cfu / ml or OD600 = 1. LDL type 3-month-old pepper seedling variety Natar were gently removed from the nursery and soaked in suspension of endophytic bacteria for 2 hours later planted in polybags containing mix soil and sand media and organic material (3-1-1). Two weeks after treatment with endophytic bacteria, the pepper seedlings were inoculated with 1000 *M. incognita* nematode larvae. Three months after inoculation of plant nematodes, the pepper plants were harvested for observation. The parameters observed were numbers of root gall, nematode populations in the soil, pepper plant growth, numbers of branch and leaves.

3. Result and Discussion

The results showed that endophytic bacteria were able to reduce root gall on pepper in the greenhouse experiment. The effectiveness of biological agents endophytic bacteria against *M. incognita* in pepper plants was vary. In this study, generally showed that the incorporation of biological agents endophytic bacteria and organic materials were able to suppress the root galls caused by *M. incognita* compared to control. The application of biological agents and organic matter, whether applied alone or in combination with organic materials, were able to suppress the number of root galls in pepper and nematode population in the soil (Table 1).

**Table 1.** The influence of endophytic bacteria and organic material on the infection of *Meloidogyne incognita* of Pepper in the greenhouse experiments

| Treatment | Number of gall/ root system | Percent in reducing root gall (%) | Nematodes population/ 100 g soil | Percent in reducing nematode population (%) |
|-----------|----------------------------|----------------------------------|----------------------------------|---------------------------------------------|
| Control   | 101 a                      | 0                                | 1432 ab                          | 0                                           |
| MER7      | 70.4 ab                    | 30.6                             | 1212 b                           | 15.4                                        |
| ANIC      | 66.0 ab                    | 34.6                             | 312 cde                          | 78.2                                        |
| MER 9     | 57.4 ab                    | 43.5                             | 504 c                            | 64.8                                        |
| AA2       | 48.8 bc                    | 51.7                             | 352 cde                          | 75.4                                        |
| HEN1      | 42.2 bc                    | 58.2                             | 13 f                             | 99.0                                        |
| TT2       | 32.6 bc                    | 67.7                             | 50 ef                            | 96.5                                        |
| EH11      | 9.0 c                      | 91.1                             | 23 ef                            | 97.0                                        |
| TT2 + CD  | 16.0 bc                    | 84.2                             | 184 hi                           | 87.2                                        |
| MER9 +CD  | 17.6 bc                    | 82.6                             | 416 defghi                       | 70.9                                        |
| EH11 +CD  | 28.8 bc                    | 71.5                             | 388 fghi                         | 72.9                                        |
| ANIC +CD  | 39.2 bc                    | 61.0                             | 278 fghi                         | 80.6                                        |
| HEN1 + CD | 18.0 bc                    | 82.2                             | 130 hi                           | 90.9                                        |
| MER 7 + CD| 12.0 c                     | 89.0                             | 120 hi                           | 91.6                                        |
| AA2 + CD  | 7.8 c                      | 93.2                             | 47 i                             | 96.7                                        |
| HEN1+OF   | 24.6 bc                    | 76.4                             | 316 fghi                         | 77.9                                        |
| AA2 +OF   | 15.0 bc                    | 86.0                             | 300 fghi                         | 79.1                                        |
| EH11 + OF | 23.6 bc                    | 76.6                             | 1548 a                           | -8.1                                        |
| MER 7 + OF| 25.0 bc                    | 75.2                             | 200 ghi                          | 86.0                                        |
| TT2 + OF  | 19.0 bc                    | 82.0                             | 608 def                          | 57.5                                        |
| ANIC +OF  | 26.0 bc                    | 74.2                             | 396 efghi                        | 72.3                                        |
| MER 9 +OF | 25.0 bc                    | 75.0                             | 486 defghi                       | 66.1                                        |
This study showed that endophytic isolates were able to reduce the number of root gall caused by *Meloidogyne* spp whereas the reducing was ranged from 30.6% until 91.1%. The highest suppression of root galls was shown by isolate EH11 with 91.1%. The isolate of bacterial endophytes could also reduce the population of nematodes in the soil with range from 15.4% to 99.0%. The highest reducing of nematode population was shown by isolate HEN1 with 99% (Table 1). The influences of endophytic bacteria in suppressing of root galls caused by plant parasitic nematode *Meloidogyne* spp on vegetable plants and perennial plants have been reported by several researchers [10, 11, 12, 13, 14, 15].

The result showed that combination of endophytic bacteria and cow dung (CD) were able to suppress the numbers of root gall caused by *Meloidogyne* spp with the percent effectiveness from 61.0% until 93.3%. The highest suppression of root gall was shown by combination between isolate AA2 and cow dung with 93.3%. The combination of bacterial endophytes and cow dung could also reduce the population of nematodes in the soil with range from 70.9% to 96.7%. The highest reducing of nematode population was shown by the combination between isolate AA2 and cow dung with 96.7% (Table 1). Combination of endophytic bacteria and commercial organic fertilizer (OF) were also able to suppress the numbers of root gall caused by *Meloidogyne* spp from 74.2% until 86.0%. The highest suppression of root gall was shown by combination between isolate AA2 and commercial organic fertilizer with 86.0%. The combination of bacterial endophytes and commercial organic fertilizer could reduce the population of nematodes in the soil up to 86.0%, except combination of isolate EH11 and commercial organic fertilizer with negative (-8.1%). The highest reducing of nematode population was shown by the combination between isolate AA2 and cow dung with 86.0% (Table 1). Adding of organic matter influences important biological activities of soils, including biological agents. The incorporation of organic materials in the soil will provide energy source for soil organisms and increase diversity and activities of beneficial soil microbes [7]. The incorporation of Sudan grass increased populations of total bacteria, fungi, and natural enemies of plant parasitic nematode nematodes [9].

The potential use of endophytic bacteria isolated from cucumbers, tomatoes and cotton such as *Aerococcus viridans*, *Bacillus megaterium*, *B. subtilis*, *Pseudomonas chlororaphis*, *P. vasicularis*, *Serratia marcescens* and *Sphingomonas panceioplastis* can reduce the population of *M. incognita* in cucumbers and tomatoes up to 50% [16]. Application of endophytic bacteria through seed treatment can reduce 30-50% of the amount of swelling (gall) of *M. incognita* in cotton plants [2]. Application of endophytic bacteria isolated from patchouli roots was reported to suppress *P. brachyurus* population by 73.9% in patchouli plants in greenhouses [17].

The biological agents of endophytic bacteria and organic material, cow dung and commercial organic fertilizer, can increase the growth of pepper seedlings as indicated by the increase in plant height, number of branches and number of leaves. Application of endophytic bacteria were able to stimulate the plant growth of pepper. Three isolate, EH11, MER7 and MER9 were able to increase the plant growth. The highest plant height have been shown by isolates MER7 and EH11. All isolate of endophytic bacteria were able to stimulate the number of branches and leaves. The highest number of branches was shown by isolate MER7 and MER9. The highest number of leaves was shown by isolate TT2 (Table 2). Some endophytic bacteria are reported to be able to stimulate plant growth by producing plant hormones such as IAA, plant growth regulators such as cytokinin, gibberellin, and activity of 1-aminosiklopropan-1-carboxylic (ACC) deaminase and producing phosphates that support plant growth [18].

The incorporation of endophytic bacteria and organic material were able to increase numbers of branches and leaves of pepper. The highest number of branches were shown by combination between isolate AA2 with cow dung (CD) and isolate MER9 with commercial organic fertilizer (OF). The highest number of leaves was shown by combination between isolate EH11 with commercial organic fertilizer (Table 2). Importantly, that endophytic bacterial species and the diversity was significantly higher by incorporation of organic matter. It has been reported that application of organic matters in the soil potentially may increase endophyte presence and diversity. The effect of these endophytes was able to increase the plant growth by reintroducing endophyte into tomato plants in a greenhouse. Adding of organic materials in the soil was able to promote the
majority of bacterial isolates to promote tomato plant growth and to enhance biomass accumulation [19].

Table 2. Effect of endophytic bacteria and organic material on the growth of pepper in the greenhouse experiment

| Treatments       | Plant height (cm) | Increase in the number of branches | Increase in the number of leaves |
|------------------|-------------------|------------------------------------|---------------------------------|
| Control          | 19.5 abc          | 0.4 b                              | 6.8 ab                          |
| EH21             | 27.0 a            | 1.2 ab                             | 9.8 ab                          |
| AA2              | 18.5 abcd         | 2.4 ab                             | 9.8 ab                          |
| MER7             | 27.0 a            | 3.2 a                              | 9.4 ab                          |
| TT2              | 17.0 abcd         | 3.0 ab                             | 10.0 ab                         |
| MER9             | 23.6 ab           | 3.2 a                              | 9.8 ab                          |
| HEN1             | 16.0 bcd          | 3.0 ab                             | 9.4 ab                          |
| ANIC             | 12.6 cd           | 1.5 ab                             | 7.8 ab                          |
| MER 7 + CD       | 23.2 ab           | 1.4 ab                             | 7.8 ab                          |
| TT2 + CD         | 22.6 abc          | 2.6 ab                             | 8.2 ab                          |
| HEN 1 + CD       | 22.4 abc          | 1.8 ab                             | 11.2 ab                         |
| ANIC + CD        | 20.2 abc          | 2.2 ab                             | 10.2 ab                         |
| MER9 + CD        | 8.7 d             | 2.6 ab                             | 6.4 ab                          |
| EH11 + CD        | 19.0 abcd         | 2.8 ab                             | 10.2 ab                         |
| AA2 + CD         | 18.4 abcd         | 3.0ab                              | 9.0 ab                          |
| HEN 1 + OF       | 17.8 abcd         | 2.2 ab                             | 10.0 ab                         |
| ANIC + OF        | 17.8 abcd         | 2.0 ab                             | 6.0 ab                          |
| AA2 + OF         | 16.7 abcd         | 2.0 ab                             | 8.6 ab                          |
| TT2 + OF         | 16.4 bcd          | 2.4 ab                             | 9.4 ab                          |
| EH11 + OF        | 15.2 bcd          | 2.6 ab                             | 12.4 a                          |
| MER 9 + OF       | 13.8 bcd          | 3.0 ab                             | 10.4 ab                         |
| MER 7 + OF       | 19.6 abc          | 2.4 ab                             | 9.0 ab                          |

Management of organic matter by addition and maintenance them, will improve the soil agro-ecosystem, including physical, chemical, and biological characteristic of soil. The impact will increase plant production. However, various sources of organic materials have different effects on the general biological activities in soil, especially plant growth promoting rhizobacteria and microbial endophyte population and diversity. The impact of these microbes will be importance for the biological control against specific pathogens, including plant parasitic nematodes [7, 19]. This research indicated that the total population plant-parasitic nematodes are especially affected by organic materials added in the form of composts or soil amendments.

4. Conclusion

The Application of endophytic bacteria and organic material on pepper seedlings are able to increase plant growth and reduce root galls caused by M. incognita at 70-90%. The results of this study indicated that incorporation of endophytic bacteria and organic material can be an alternative strategy in controlling yellowing disease caused by plant parasitic nematodes on pepper.

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