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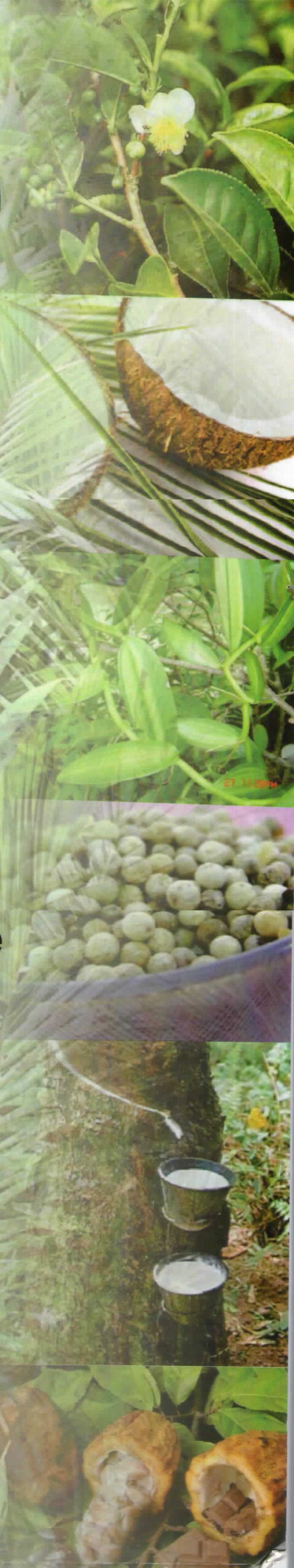

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Address:
Faculty of Agriculture, Bogor Agricultural University
Jln. Meranti Dramaga, Bogor Indonesia 16680
Tel. +62 251 8629354, +62 251 8629350
Fax. +62 251 8629352
Email.faperta@ipb.ac.id

Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA
40450 Shah Alam
Selangor Darul Ehsan, Malaysia
Tel: +603-5543 5583 /5539 /5567
Fax. +603-5543 5563

Editor team :
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Influence of endophyticbacteria and organic materialson the*Meloidogyne incognita* infection of black pepper

Abdul Munif[1*]and Rita Harni[2]
[1]Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University (IPB)
Jl. Kamper,Kampus IPB Darmaga, Bogor 16680, Indonesia
*Corresponden: abdulmunif@ipb.ac.id
[2]Research and Development Agency, Ministry of Agriculture, Indonesia

ABSTRACT

Plant parasiticnematodes*Meloidogyneincognita*is stilla major problem and cause significant demage and losses to black pepperinIndonesia. The need to control and manage nematode population to acceptable levels remains a big concern.Endophyticmicroorganisms resident within plant tissues have attracted attention due to their interesting features related to plant growth and biocontrol activity against plant pests and diseases. The objective of this study was to evaluate the effect of selectedendophytic bacteria*Bacillus* sp AA2 and MER7 in combination with organic materials onplant parasitic nematodes *M. incognita*infection of black pepper.Endophytic bacteria that used in this study were isolated from roots of black pepper using a surface sterilization method. The results of the researchshowed that application of selectedendophytic bacteria in combination with organic materials were able to reduce the infection of*M.incognita*in the rootsplant. Application of endophytes and organic material was also ableto increase the growth of black pepper.

Key words: endophytic bacteria, *Meloidogyneincognita*, black pepper, root gall

INTRODUCTION

Black pepper (*piper nigrum* L) is one of the important export commodities in Indonesia. However, the production of black pepper is now threatened by pests and diseases. One of the main disease on black peper is “yellow diseases” caused by plant parasitic nematodes *Meloidogyne* sp. and *Radopholus similis*(Mustika 1990).The symptom expression of “yellow diseases”of black pepper showed yellow leaves with stiff droop and noticeable defoliation around the vines then plant die. Several control methods have been developed to combat the nematodes, but they are still a serious problem of black pepper especially in Bangka Island. The use of chemical pesticides with persistent pesticides can result in negative impacts on the environment, pathogens become more resistant, disruptive presence of beneficial microbes in the soil, and human health. Use of Pesticides also provide residual effects on pepper which is currently as one obstacle for consumers. In connection with the need to develop a system of agricultural production, including plant disease control systems are environmentally sound one of them with biological control.

Biological control of nematodes has emphasized the use of antagonistic microorganisms and is mediated through mechanisms such as predation, competition, antibiosis and hyperparasitism (Stirling 1991). Antagonist is a term usually used for parasites, predators, pathogens, competitors or other organisms that repel, inhibit or kill plant parasitic nematodes. Management of the soil antagonistic potential generated by complex interactions between plant-health promoting rhizobacteria, obligate bacterial parasites, fungal egg pathogens/parasites, predacious or trapping fungi, endoparasitic fungi, fungal and bacterial mutualistic endophytes and endomycorrhizal fungi has been discussed(Sikora 1992).

Recently the use of endophytic microorganisms for the protection of plant diseases and pests has been exploited and studied. Endophytic bacteria are bacteria that live inside plant tissues without doing symptoms on these plants. As the internal plant habitat, endophytic bacteria provides several advantages as biological control agents namely colonization of an ecological niche also used by plant pathogens, less competition with other microorganisms, sufficient supply wirth nutrients, less exposure to environmental stress factors, and better translocation of bacterial metabolites throughout the host plant. Several studies have shown that endophytic bacteria isolated from various plant tissues are able to suppress plant parasitic nematodes *Meloidogyne incognita* on cotton and tomato plants (Hallmann*et al* 1997; Munif *et al* 2000) as well as to control plant nematode *Pratylenchus*sp on patchouli (Harni*et al.* 2007). In addition, some bacterial endophytes have been reported to enhance plant growth because it can increase the availability of nutrients to induce plant resistance (Backman and Sikora 2008).

The objective of this research was to study the potential of biological agents bacterial endophytes*Bacillus* sp AA2 and MER7 and organic materials, compost commercial and cattle dung, for controlling *Meloidogyneincognita* on black pepper and their effect on the plant growth promotion.

MATERIALS AND METHODS

Isolation of endophytic bacteria

The selected endophytic bacteria that used in this experiment originally was isolated from balck pepper. A total of 10 black pepper plants were uprooted at random from a field site of the farmer pepper plantation in Bangka and West Java. The pepper roots were transported to the laboratory for immediate processing. The isolation procedure for endophytic bacteria followed the method described by Hallmann*et al*. (1997). The pepper roots were washed under running tap water to remove adherent soil particles and then blotted dry on tissue paper. The root material of the two part was weighed and surface sterilized with 3% sodium hypochlorite containing 0.01% Tween 20 for 3 min, followed by four rinses in sterile 0.01 M potassium phosphate buffer (PB) at pH 7.0 (80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4, 2 g KH_2PO_4). To confirm complete surface sterilization, the surface disinfected roots were imprinted on tryptic soy agar (TSA). If bacterial growth occurred within 48 hours, samples were discarded. The pepper roots were then macerated with a sterile mortar and pistil in three times PB (w/v). The macerate was decanted into sterile conical flasks and shaken for 30 second. A dilution series was made and 100 µl of each dilution was plated onto 1/10 strength TSA with a drigaeski spatula. Petri plates were incubated at 24°C for 2-3 days and colony forming units (cfu) were determined. Three replicates were made per dilution. On each Petri plate a zone containing approximately 30 bacterial strains was marked and all bacterial strains from this zone were transferred and purified on full strength TSA. The bacterial strains were stored in tryptic soy broth (TSB) plus 20% glycerol at -80°C.

Nematode extraction and inoculation.

Meloidogyne incognita originally obtained from infected black pepperroots in Bangka Island. The nemetodeswere extracted from heavy infected roots of pepper and cultured in two week old tomato plants to act as source of inoculum. Extraction of nematode was done by using Baermann funnel method. After two months, the nematodes were extracted from the tomato roots and inoculated into potted black pepper plants that already treated with endophytic bacteria and organic materials.

Nematode inoculum was extracted from 2-3 month old tomato plants. The plants were uprooted and nematode eggs were extracted using the technique decribed by HUSSEY and BARKER (1973). Galled tomato roots were washed in water to remove soil debris. The roots were cut into 1-2 cm pieces and macerated in a warring blender for 20 seconds. The macerate was filled into a flask containing 500 ml of a 1.5 % NaOCl solution and manually shaken for 3 minutes to free eggs from the gelatinous matrix. The egg suspension was rinsed over a sieve combination of 250 µm, 100 µm and 25 µm mesh size to remove excess chlorine and the eggs on the 25 µm sieve gently washed under tap water. The eggs from the 25 µm sieve were collected in tap water, then passed again over seaves with 45 µm and 20 µm mesh size. The eggs from the 20 µm sieve were collected, filled in flask containing 600 ml tap water and agitated for 10 days at 24°C supplemented with O^2 to support juvenile hatch. The juveniles in the water suspension were adjusted to 2000 Juveniles/ml and used as inoculum for the experiment.

Bacterial application

Organic material that used in this experiment were commercial compost and cattle dung. The commercial compost was produced by fertilizer company (Petrokimia Ltd.) and the cattle dung was obtained and collected from the farmer.

The bacterial strains *Bacillus* sp AA2 and MER7 were pre-cultured on TSA for two days at 28°C. For experimental use, a loop of bacteria was transferred into tryptic soy broth (TSB) and shaken at 100 rpm for two days at 24°C in the dark. The bacterial suspension was adjusted to OD_{560}=2.0 using a spectrophotometer (Pharmacia, Ultrospec III, USA) representing 10^9-10^{10}cfu/ml depending on the bacterial strain used. Ten ml of bacterial suspension was applied as soil drench by poured onto the soil surface around each pepper plant per pot. Control plants were treated with 10 ml steriloe water.

Twomonths-old black pepper plants were planted in pots filled with an unsterile sand/compost commercial or cattle dung (1:1 v/v). Two weeks after transplanting, seedlings ofblack pepper were drenched with 10 ml of a bacterial suspension. Four days later, plants were drenched with 2000 juveniles of *M. incognita*. The inoculation of nematodes was carried out by drenching with the juveniles into the potted soil arround the roots. Control plants received sterile water. Plants were fertilized biweekly with a 0.2% nutrient solution. Three months after nematode inoculation, the experiment was terminated and number of galls, population of larvae, shoot fresh weight, and total root weight were recorded.

RESULT AND DISCUSSION

The effect of treatments on *M. incognita*

The treatment of endophytic bacteria *Bacillus* sp AA2 and MER7 in the present experiment showed high effect in suppression of the number of root galls caused by *M. incognita*on black pepper compared with control. Root gall was suppressed by all teratments. Application of bacterial endophytes *Bacillus*sp AA2 and MER with organic materials was higher significant in suppression root galls than if endophytes applied alone (Table 1). Even the application of *Bacillus* sp AA2 and MER7 alone (without organic materials) showed significant effect in suppression of root galls. The most effective treatment in suppression of root galls were Bacillus sp MER7 with cattle dung, Bacillus sp MER7 with commercial compost and Bacillus sp AA2 with cattle dung. The application of organic material, compost commercial and cattle dung was able to promote the effectivity of endophytic bacteria.

The effect of treatments on the plant growth

Endophytic bacteria *Bacillus* sp AA2 and MER7 in this study showed positive effect in promoting of the plant growth. Almost all of the treatments were able to increase of the plant height except *Bacillus* AA2 with commercial compost. The data indicated that number of branches and leaves varied depending on different treatments and Bacillus sp MER7 was the most effective in increasing of the number of brances and leaves. The data also shows that fresh root and shoot weight varied and most effective in increasing fresh root weight was Bacillus sp AA2 and the most effective in increasing fresh shoot weight was Bacillus sp MER7 (Table 1.).

Table 1. The influence of endophytic bacteria *Bacillus*sp AA2 and MER7 and organic material on the number of root galls and the growth of black pepper in the greenhaouse after 3 months of nematode inoculation

Treatments	Number of root galls	Plant height (cm)	Number of branches	Number of leaves	Fresh root weight (gr)	Fresh shoot weight (gr)
Bacillus sp AA2	48.8 ab	42.4 a	2.8 a	15.2 a	5.8 a	25.1 a
Bacillus sp MER7	70.4 a	44.0 a	5.6 a	18.4 a	5. 8 a	29.5 a
*Bacillus*spAA2+commercial compost(cc)	40.6 ab	38.8 a	5.0 a	16.8 a	5.9 a	22.3 a
*Bacillus*spAA2+cattle dung (cd)	31.2 b	44.6 a	4.2 a	16.4 a	5.9 a	20.7 a
*Bacillus*sp MER7 + commercial compost(cc)	25.6 b	46.2 a	2.6 a	15.2 a	5.1 a	21.4 a
*Bacillus*spMER7+cattle dung (cd)	4.4 b	44.2 a	3.8 a	17.0 a	4.9 a	22.4 a
Control	111.4 a	41.6 a	2.8 a	15.8 a	5.0 a	22.0 a

Figures followed by same small letters on the same column are not significantly different at 5% Duncan test.

Biocontrol mechanisms by which endophytic bacteria may protect plants against pathogens are multifarious. The bacteria may compete for space and nutrients in the internal tissue (Backman and Sikora 2008). The steady release of organic nutrients such as sugars, amino acids, fatty acids, nucleotides, vitamins, plant hormones and other substances into the plant tissue and rhizophere presents the matrix for growth and activity of microorganisms (Hallmann *et al* 1997; Buchenauer 1998). Evidence has been accumulated that antibiotics produced by rhizobacteria and endophytic bacteria constitute an important factor in suppression of root diseases. The degree of disease control by endophytic bacteria producing antibiotics depends on the sensitivity of the pathogen population to the antibiotics and the quantities produced (He *et al*. 2009; Sikora *et al*. 2007))

Antagonistic activity against plant parasitic nematodes is also a common phenomenon reported for rhizosphere bacteria. Bacterial strains belonging to the genera *Pseudomonas*, *Bacillus*, *Agrobacterium* and others are known to reduce *M. incognita* on white clover and cucumber (Becker *et al*. 1988), *Heteroderaschachtii* on sugar beet (Oostendorp and Sikora 1989), *Heteroderaglycines* on soybean (Kloepper*et al.* 1999), *Globoderapallida* on potato (Hasky-Gunther*et al*. 1998), *Meloidogyne incognita* on bhendi (Vetrivelkalai*et al*. 2010) and

*Pratylenchus brachyurus*on patchouli (Harni *et al.* 2007). The mechanisms of endophytes involved in the control of plant parasitic nematodes vary greatly.

The mechanisms by which endophytic bacteria increase plant growth still needs to be explored. However, the mechanisms might be similar to those reported for rhizosphere bacteria, which include inhibition of deleterious microorganisms (Kloepper*et al.* 1991), production of plant growth regulating substances, such as ethylene, auxins, or cytokinins (Arshad and Frankenberger 1991) or release of biologically fixed N_2 (Bashan and Holguin 1997).Mechanisms by which plant growth promoting rhizobacteria and endophytic bacteria may stimulate plant growth are either by suppressing deleterious microorganisms or pathogens or by producing plant growth regulators as well as by lowering ethylene levels in plants (Hallmann *et al.* 1997; Kloepper*et al.* 1999).Fot the conclusion, this research indicate that the close interaction between endophytic bacteria and the plant would favor this mechanisms as a preferred choice for biological control strategies of plant parasitin nematodes.

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