Wheat bran soil inoculant of sumatran nematode-trapping fungi as biocontrol agents of the root-knot nematode *meloidogyne incognita* on deli tobacco (*nicotiana tabaccum l*) cv. deli 4

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Abstract. A pot experiment was carried out to test the effectiveness of nematode-trapping fungi (NTF) isolated from Sumatera for controlling infection by the root-knot nematode (RKN) on Deli tobacco plant. Wheat bran soil containing $10^9$ conidia of *Arthrobotrys. oligospora*, *Candellabrella musiformis* and *Dactylella eudermata* was added to the soil as a dry inoculum. Carbofuran was also applied as chemical agent and comparison treatment. Seedling tobacco (*Nicotiana tabacum L.*) cv. Deli 4 was inoculated with root knot (*Meloidogyne incognita* Chitwood.) seven days after the plant were transplanted to the pots. *A. oligospora*, *C. musiformis* and *D. eudermata* were found to be reliable as biocontrol agents, reducing the number of vermiform nematodes, swollen root, sausage shaped and galls in tobacco plant after 7, 15 and 30 days of infection with *M. incognita*. Treatment with NTF produced results that were comparable with Carbofuran® as a control agent in the reduction of the number of infections in tobacco plant caused by *M. incognita* in *Nicotiana tabacum* var. Deli 4. They also optimize the growth of the tobacco plants especially up to 15 days after infection.

Keywords: *A. oligospora*, *C. musiformis*, *D. eudermata*, *M. incognita*, Solid State Fermentation

1. Introduction

Recently, it has been reported that plant parasitic nematode infestations were spreading widely not only in West, Central, and East Java provinces, but also to the North Sumatera province [1]. They infect almost all species from the family of *Solanaceae* including tobacco and cause 32%-71% crop losses with a cost of approximately of 2 trillion rupiah or about 149,835,200 USD [1].

*Nicotiana tabacum* var. Deli (Deli tobacco) is the preferred leaf used for wrapping cigars, with famous names like Black Sumatera. As one of the most economically important commodities in Indonesia, Deli tobacco also has a problem with RKN. Infection of RKN *Meloidogyne* species on tobacco plants leads to young plant mortality at the age of 30-45 days, and mortality can reach more
than 50% of the plant population per hectare. In addition, nematode attack can also cause growth retardation, decreased chlorophyll content, stunt growth and caused the death of the plant before it enters the generative phase [2]. Furthermore, RKN infestations can act as agents of predisposition for infection by other pathogens, tobacco infected by RKN will easily infected by *Ralstonia solanacearum* or *Phytophthora nicotianae* causing disease complexes.

The long term protection of an economically important plants such as tobacco and other crop plants as hosts against *Meloidogyne* spp. (*M. hapla*, *M. incognita*, *M. javanica* and *M. arenaria*) will be achieved more effectively when other management strategies are also applied. In addition to the application of antagonists, other techniques such as crop rotation, soil solarisation and use of nematicide should form an integrated pest management program.

Fungi as the biocontrol agent to control disease caused by plant nematode has only been reported in experiments under laboratory condition. Some fungi such as *Beauveria* sp., *Paecilomyces* sp., *Verticillium* sp, *Trichoderma harzianum* and some endophytic fungi have been known as entomopathogens with potential in nematode control. They have been tested as bio-insecticides for control of diseases caused by pests such as insect pathogen, also for plant parasitic nematode [3,4]. However, they have not been widely applied by farmers or growers in a farming area. Fungi as the biocontrol agent to control disease caused by plant nematode has only been reported in experiments under laboratory condition. Some fungi such as *Beauveria* sp., *Paecilomyces* sp., *Verticillium* sp, *Trichoderma harzianum* and some endophytic fungi have been known as entomopathogens with potential in nematode control.

There is very little research using NTF as biocontrol agents to control nematode pathogen such as RKN in Indonesia. As is well known, some fungi such as *Arthrobotrys* spp. (*Arthrobotrys dactyloides*, *A. brochopaga*, and *A. oligospora*) and *Monacrosporium* spp. (*Monacrosporium geophyropagum*, *M. cianopagum*, *M. doedycoides*), *Dactylaria* spp. and other *Orbitalesiae* have potential in the control nematode in the soil. [5] has been testing some media for production of inocula of nematophagous fungi *Dactylella* sp and *Duddingtonia flagrans* using ingredients available in Indonesia e.g. corn flour, cassava flour, rice flour and rice bran and CMA+ dung with some success. There has been research using such NTF to control plant parasitic nematodes in Indonesia; however, the experiments have only been carried out on laboratory scale. The creation of fungal inoculum by using water as a suspension medium to create a conidial suspension for either injection or spray in laboratory or field conditions is a common method used by researchers and growers [6]. It must be considered, that if a bio-pesticide such as NTF inoculum is going to be used in large quantities for large-scale farming, mass production methods for industrial purposes. There are two current methods used for large scale biocontrol agent production, submerged fermentation (SMF) and solid-state fermentation (SSF) [7]. According to [8], NTF as biocontrol applied with SSF have an advantage over those produced by submerged fermentation: aerial fungal spores are more tolerant to UV radiation; have a higher spore stability; a higher spore resistance to drying; and higher spore germination rates for longer storage periods.

The objectives of this research are:

1. Use of wheat bran as a substrate for Indonesian NTF conidia production.
2. Testing efficacy of wheat bran inoculum as a biocontrol agent for *Meloidogyne incognita* and their effect on the growth of *Nicotiana tabacum* var. Deli 4.

2. Methods

2.1. Host Preparation

Seeds of Deli 4 tobacco were spread on moist white cloth, placed on glass lids and allowed to germinate. Three days old seedlings of Deli 4 tobaccos were removed to a squared tray containing sterile compost soil and incubated at 15-25°C in daylight. At 15 days of age tobacco plants were transplanted to the treatment pots containing 100g of soil and inoculum of NTF or Carbofuran. Soil around rhizosphere of host plant was injected with 1000 J2 of *M. incognita* seven days after receiving
15 days of seedling of Deli 4 tobacco. Deli tobacco plants in the treatments pots were maintained in the growth cabinet in a randomized pattern. The treatment pots were moved or rotated each two or three days regarding to obtain the same condition of light and temperature in the growth cabinet.

2.2. Inoculant Preparation
Three strains of NTF (Arthrobotrys oligospora, Candelabrella musiformis, Dactylaria eudermata) have been selected for further testing on the basis by proceeding experimental results reported in previous experiment. Wheat bran soil medium was used to make a dry inoculum. Each strain of NTF was cultured on Potato Dextrose Agar at 25°C for 14 days. Five agar disks (5 mm in diameter) of each strain were added to 30 ml of wheat bran medium (1 unit wheat bran Bergin Fruit and Nut Company); to 4 units compost in 50-ml Erlenmeyer flasks, and the cultures were then incubated for 14 days in darkness at 25°C [9].

Numbers of conidia were counted by a dilution series method where the 1 gram of wheat bran/compost taken from flask contain conidia was added into 9 ml of distilled water and number of conidia was count from the 10⁻¹ dilution. A supernatant was obtained by pouring the suspension through sterile gauze into a sterile 15ml test tube. The conidial concentration was measured by the dilution method by counting the number of conidia in a 5 µL-drop 20 times under the microscope (100X) and multiplying the number counted by 100 to estimate the total number of conidia/ml. Each experimental pot containing 100g of sterile soil received 1g of wheat bran NTF inoculum. Inoculated pot soil was incubated in the growth cabinet (Bud Box Cabinet) 25ºC for three days before receiving ornamental tobacco plants.

2.3. Chemical Agent Preparation
Carbofuran was used as a chemical control of nematodes as it is widely used in agriculture. The pesticide was diluted to a working concentration by dissolving 2 mg/10ml of solid Carbofuran in methanol to 200µg/ml or 2 mg/10ml liquid/suspension [10,11]. Carbofuran solution (10 ml) was poured into each pot, and controls received 10 ml of distilled water. The Carbofuran was poured into the pot (3 pots) together with fungal treatments three days before receiving a seedling tomato plant.

2.4. Root Knot Nematode Inoculant Preparation
Infected roots with galls were washed gently and cut into 1 cm section. Roots were vigorously shaken for 4 minutes in sodium hypochlorite solution (100 ml NaOCl 5-10%). Roots were poured and washed through a 250 µm sieve. Roots containing galls in the sieve were placed in a crystallizing dish, some petroleum jelly was spread on the borders of crystallizing dish to prevent the escape of nematodes and it was covered with a glass lid. The crystallizing dish containing nematodes was incubated at 25ºC temperature room for 4-7 days. A piece of tomato root was examined under the microscope to confirm infection, and the infected tomato root was used as a source of inoculum. Mature nematode egg masses were selected, and three galls/egg mass (contain 400-600 per egg mass) were picked up with a pair of forceps. The egg mass was transferred into 15 ml tube containing sterile tap water. The 15 ml tube containing egg mass was left at room temperature to allow the juveniles (J2) to hatch. Most temperate species can be kept at 18-22ºC and tropical and sub-tropical species at 25-30ºC. Once hatched, the J2 was inoculated on to a previously transplanted tomato host. Preparation J2 of root-knot nematode used the combined technique described by Davies (Rothampstead Research) [12,13].

2.5. Soil Preparation
Soil was dried for four days and sieved, and after air drying and sieving, soil (100 grams per 8.5 cm pot) was sterilized with dry sterilization program (121°C at 1 atm. for 15 mins with a drying cycle) in 2000 ml beaker glass covered by aluminium foil. This stage was repeated once. Under sterile conditions, sterile soil was transferred to 8.5 cm pots so that each pot contained 100 grams of sterile soil. The soil was then treated with Carbofuran and/or a conidial suspension of nematode-trapping fungi, and distilled water (10 ml) was used as a control.
There were three replicates of each treatment (Table 4.4). In order to avoid contamination, all treated pots were covered with para film on the top and bottom end of each pot. All treated pots were kept in the 25°C room for three days before receiving a tobacco seedling.

2.6. Harvesting and Root Assessment
The invasion and development of J2 stages, swollen root, sausage stage, pear-like (kidney-shape) of mature female [14] and galls was assessed at 7 and 15 days and 30 days.

3. Results
3.1. ASSESSMENT I: The effect of NTF and Carbofuran on the number of vermiform nematode and swollen roots.
On the first assessment, two stages *M. incognita* infection were observed as vermiform nematodes and swollen roots. Analysis of variance with Kruskal Wallis ANOVA on the data after seven days of infection with *M. incognita* showed that all treatments were statistically significant difference with the significant value less than 0.05 (Table 1).

Table 1. Analysis variance for the effect of NCA on the number of vermiform nematode and the swollen root of Deli tobacco after seven days infection.

| Test Statistics | vermiform | swollen |
|----------------|-----------|---------|
| Chi-Square     | 11.999    | 13.528  |
| df             | 5         | 5       |
| Asymp. Sig.    | .035      | .019    |
| a. Kruskal Wallis Test | Significant | Significant |
| b. Grouping Variable: Nematode Control Agent |

The tobacco plants treated by *C. musiformis* had significantly fewer vermiform nematode roots in comparison with the infected control (Fig. 3.1). Furthermore, all fungally treated plants showed lower numbers of vermiform nematode roots, followed by Carbofuran, than the positive control plants after seven days infected.

The fungal and Carbofuran treatments also had lower numbers of swollen roots than positive control plants. Tobacco plants treated by *D. eudermata* have the lowest number of swollen roots and positive control plants had the highest number of swollen roots. This is also support by analysis of variance by Kruskal Wallis ANOVA in Table 1. with a value P<0.05.
3.2. ASSESSMENT II, The Effect of NCA on the number of infections 15 days after infected by *Meloidogyne incognita*

On the second assessment, four stages of infection were observed. There were a number of immature females and mature females, and a number of swollen roots and sausage shaped roots on the root of the positive control Deli tobacco treatment. Analysis of variance by Kruskal Wallis ANOVA show all treatments were statistically different with a significant value lower than 0.05 (Table 2).

**Table 2.** Analysis of variance for the effect of NCA on the number of immature female, mature female, swollen and sausage shaped of Deli tobacco after 15 day infection.

| Test Statistics a,b | Immature female | Mature female | Swollen | Sausage shaped |
|---------------------|-----------------|---------------|---------|---------------|
| Chi-Square          | 10.568          | 12.598        | 12.547  | 13.840        |
| df                  | 5               | 5             | 5       | 5             |
| Asymp. Sig.         | .061            | .027          | .028    | .017          |
| Significant         | Sig.            | Sig.          | Sig.    | Sig.          |

a. Kruskal Wallis Test

b. Grouping Variable: Nematode Control Agent

The effect of the addition of NCA on the roots of tobacco plant was significant in reducing the number of *M. incognita* infections after 15 days post inoculation. If compared with infective control plants, the numbers of infections were suppressed by the addition of chemical treatment and also fungal treatment (Figure 2). The lowest number of immature female was on tobacco plants treated by *A. oligospora*, while the maximum was on the infected control plants. The lowest number of mature females was found on tobacco plants treated by Carbofuran and *A. oligospora*. Tobacco plants treated by *D. eudermata* had the lowest number of swollen roots, and tobacco plants with the lowest number of sausage shaped roots were on tobacco treated by Carbofuran followed by *D. eudermata* treated plants (Figure 3).
Figure 2. The effect of NCA on the number of immature female and mature female of *M. incognita* after 15 days. Significantly different treatments are shown by an annotation letter above the bars.

Figure 3. The effect of NCA on the number of swollen root and sausage shaped of *M. incognita* after 15 days. Significantly different treatments are shown by an annotation letter above the bars.

3.3. ASSESSMENT III, The Effect of NTF and Carbofuran on the number of infection after 30 days after infected by *Meloidogyne incognita*

On the third assessment (30 days after infection), four stages of infection were observed, these were immature females, mature females, sausage shaped roots of tobacco plants and galls. Analysis of variance by Kruskal Wallis ANOVA of data taken at 30 days post infection with nematodes, showed that all treatments were statistically significantly different in terms of the number of immature females, mature females, sausage shaped root and also galls (Table 3).
Table 3. Analysis of variance for the effect of NCA on the number of immature and mature female, sausage shaped and galls on Deli tobacco after 30 days of infection.

| Test Statistics<sup>ab</sup> | immature | mature | Sausage shaped | Galls |
|-------------------------------|----------|--------|----------------|-------|
| Chi-Square                    | 12.074   | 10.610 | 12.403         | 12.611|
| df                            | 5        | 5      | 5              | 5     |
| Asymp. Sig.                   | .034     | .060   | .030           | .027  |
| Significant                   | Sig.     | Sig.   | Sig.           | Sig.  |

a. Kruskal Wallis Test

b. Grouping Variable: Nematode Control Agent

Based on the data presented in Figure 4 the maximum number of immature, mature, sausage shaped roots, and galls were found on infected control plants. The lowest number of immature females was found on tobacco treated by *D. eudermata*, and there were no immature females found on roots after 30 days, following by the tobacco plants treated by *A. oligospora*. The lowest numbers of mature females were found on tobacco plants treated by Carbofuran followed by *C. musiformis* and *D. eudermata*. The lowest number of sausage shaped roots were found on tobacco plants treated by *D. eudermata*, and the lowest number of galls was on tobacco plants treated by *C. musiformis* (Figure 5).

Figure 4. Effect of NCA on the number of immature female and mature female galls of *M. incognita* after 30 days infection. Significantly different treatments are shown by an annotation letter above the bars.
Figure 5. Effect of NCA on the number swollen and sausage shaped and galls development by *M. incognita* after 30 days. Significantly different treatments are shown by an annotation letter above the bars.

3.4. The effect of wheat bran inoculum of Nematode Control Agent to the Growth of *Nicotiana tabacum* var. Deli 4 over 30 days of infection.

The effect of NCA on the growth of Deli tobacco (*Nicotiana tabacum* var. Deli 4) is presented in the Fig. 3.6 below. Based on the line chart in Figure 6, either Carbofuran or fungal treatment plants showed an increase in the growth on the plant in terms of plant height, root depth, stem fresh weight, stem dry weight and root fresh weight parameter until the end of assessment. However, if compared with negative and positive control plants, only Carbofuran treated plants grew better in terms of all growth parameters.
All fungal treatments plants were better only in terms of the plant height parameter (Figure 6A) and stem dry weight parameter (Figure 6D) if compared with positive control plants. However, there is no different between fungal treatment plants with negative control plants in plant height parameter also stem dry weight parameter. C. musiformis and D. eudermata treated plants were better in terms of the root fresh weight parameter (Figure 6E), but there was no different between both fungal treatments plants with negative control plants.

4. Discussion
Analysis of variance to specify the effectiveness of Sumatran NTF in suppress the number of infections showed that all treatments were statistically significantly different throughout the experiment. Effects are first seen at 7 days after infection by J2 of M. incognita, and continue at 15 days and 30 days. C. musiformis is the most effective treatment in reducing the number of vermiform nematodes and D. eudermata is the most effective in reducing the number of swollen roots especially on the first assessment (7 days) after infection. As the nematode life cycle and infections continued, the number of immature and mature female, swollen and sausage shaped root were also reduced at the

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**Figure 6.** The effect of Nematode Control Agent on the growth progression of *Nicotiana tabacum* var. Deli 4 (Deli tobacco) 7, 15 and 30 days after infection *Meloidogyne incognita.*
second assessment (15 days) after infection and also the number of galls at the last assessment (30 days) after infection.

Analysis variance by Kruskal Wallis ANOVA also shows almost none of the treatments supported the growth of *Nicotiana tabacum* var. Deli 4 compared with the uninfected control plants. Even though the growth of Deli tobacco showed increases in all measured parameters; root depth, stem fresh weight, stem dry weight and root fresh weight until the end of assessment (Figure 6). A lack of plant growth stimulation when using NTF to control RKN has been previously reported. [15] attempted to reduce infection of *Meloidogyne mayaguensis* on tobacco *Nicotiana tabacum* L. var. Paraguay x Claro cv. *Mayiguana* using *Arthrobotrys oligospora*, *Arthrobotrys conoides*, *Arthrobotrys* sp. *Dactylaria sahelensis*, *Dactylaria* sp. and *Monacrosporium bembicodes* as BCAs. Numbers of infections were suppressed and growth of tobacco was improved, but most fungal treatments did not show any phyto-stimulant effects.

Similar research using wheat bran/sand inoculum (1:1 v/v) containing *Verticillium chlamydosporium* 1% w/w per treatment pot to control *Meloidogyne arenaria* on tomato plant was conducted by [16]. Research proved the inoculation of *V. chlamydosporium* in wheat bran/sand formula could reduce numbers of infections by *M. arenaria* but there was no stimulant growth effect on the growth of tomato plant.

Experiments on improving formulation and BCA dosage have had varied results. Based on the research done by [17], inoculation conidia of *A. dactyloides* in a gum-arabic pellets formula with several dosage levels of inoculum (0.2, 0.4, 0.6, 0.8 w/w) did not show a stimulant effect to the growth of tomato plant. The results showed that increasing the inoculum dosage application was not significant in increasing the plant height, root depth or stem fresh weight parameter. However, increasing inoculum dosage led to a significant increase in the dry weight of tomato plant until the end of the assessment.

The dosage of 1 g in 100 g growth medium (w/w) containing $10^9$ conidia of NTF proved to work very well in increasing plant dry weight due to the formation of the hormone auxin in the plant host [18]. According to Ahemad and Kibret [18], auxin (IAA= Indole-3-acetic acid) will increase the content of organic and inorganic substances in the cells and at the same time will increase the dry weight and fresh weight of host plant.

Auxin has been shown to be present in high concentrations especially in the rhizosphere area when the microbial activities increase. The population of microbes and metabolic activity is increase in the presence of root exudates caused by RKN infestations [19]. However, the dosage used in this experiment could not stimulate formation of cytokinin hormone that plays a role in cell division, cell growth and leaf buds network so that high growth of plants was not significantly different [20].

In contrast, another work by Faria [20] using several dosage levels of BCA (0, 1x$10^4$, 5x$10^4$, 1x$10^5$, and 5x$10^5$ CFU/gr soil) of *L. lecanii* to control *M. incognita* on tomato plants, showed that 5x$10^5$ CFU/gr soil (the highest dosage used) suppressed infection and also improved plant growth [21].

Further research is needed to establish the levels of dosage (w/w of inoculum/ growth medium) of inoculum needed to achieve biocontrol of NTF and also to better understand the effect of fungal treatment on the growth of host plant in terms of plant growth promotion. It is also important to determine the effect of inoculum in different forms, liquid versus solid state fermented, and differences in formulation. Applications and mass production of nematode-trapping fungi in SSF inoculum using local substrate available in Indonesia such as corn flour, cassava flour, rice flour mix with compost, sand or sow dust to commercial scale remains a challenge that must be solved. The work described in this chapter is a preliminary study which could be a reference for further research especially when using the biological control nematode such as *Meloidogyne* spp. applied in Indonesia.
5. Conclusion

Biological control using fungi is a sustainable alternative to dispose of conventional pesticide containing hazardous chemical compound. The use of Sumatran nematode-trapping fungi to control root-knot nematodes may be developed to protect the workers and environment from the pesticides. SSF inoculant such as wheat bran soil inoculant has some criteria as bio-control. They are easy to use and store, affordable, effective, and suitable solution to protect the crops especially in Sumatera Utara, Indonesia. Isolating indigenous strains of NTF from Sumatera Utara continued by pot trial and micro field trial experiment is need to be done to ensure the effectiveness Sumatran NTF of as biological control agents to control Plant Parasitic Nematode such as Root-Knot Nematode. Formulation of Sumatera Utara isolate should be adapted to ensure both good conservation of the Sumatran NTF and high virulence against root-knot nematode.

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