

# Effect of micronutrient-enriched media on the efficacy of *Bacillus subtilis* as a biological control agent against *Meloidogyne incognita*

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**Abstract.** Adiwena M, Murtikasono A, Egra S, Hoesain M, Asyiah IN, Pradana AP, Izatika ZN. 2023. Effect of micronutrient-enriched media on the efficacy of *Bacillus subtilis* as a biological control agent against *Meloidogyne incognita*. *Biodiversitas* 24: 33-39. The root-knot nematodes *Meloidogyne incognita* poses a serious threat to horticultural food and plantation crops. One of the effective and efficient measures to overcome this nematode is using bacteria as a biological control agent. This study aimed to test the effectiveness of *Bacillus subtilis* bacteria grown on media with additional micronutrients as a biological control agent for *M. incognita*. The test was carried out by growing bacteria in 100 mL of nutrient broth (NB) added with FeCl<sub>2</sub>, MnCl<sub>2</sub>, and CuCl<sub>2</sub>. Each type of micronutrient was administered at concentrations of 35 ppm, 40 ppm, 45 ppm, 50 ppm, and 55 ppm. As a control, NB media was used without the addition of micronutrients. The test results showed that bacteria performed best at 55 ppm MnCl<sub>2</sub>, 45 ppm MnCl<sub>2</sub>, 40 ppm MnCl<sub>2</sub>, 40 ppm CuCl<sub>2</sub>, 50 ppm MnCl<sub>2</sub>, and 35 ppm MnCl<sub>2</sub>. These six treatments were then used in an antagonism test. A total of 150 J2 *M. incognita* in 4 mL of suspension was transferred into a petri dish with a diameter of 5 cm. After that, 1 mL of each bacterial suspension was poured into a petri dish following the treatment, nutrient broth as control, nutrient broth plus 35 ppm MnCl<sub>2</sub>, nutrient broth plus 40 ppm MnCl<sub>2</sub>, nutrient broth plus 45 ppm MnCl<sub>2</sub>, nutrient broth plus 50 ppm MnCl<sub>2</sub>, nutrient broth plus 55 ppm MnCl<sub>2</sub> and nutrient broth plus 40 ppm CuCl<sub>2</sub>. The mortality rate of *M. incognita* in each treatment was 77.5% in 55 ppm MnCl<sub>2</sub>, 74.5% in 45 ppm MnCl<sub>2</sub>, 77.25% in 40 ppm MnCl<sub>2</sub>, 73.25% in 40 ppm CuCl<sub>2</sub>, 78.75% in 50 ppm MnCl<sub>2</sub>, 79% in 35 ppm MnCl<sub>2</sub>, and 53.25% in control. The same six treatments were also used to measure the chemotaxis index of *M. incognita* on tomato roots. The chemotaxis index ranged from 16% to 24% in the treatments, while in control, it was 33%. This study suggests that the addition of micronutrients MnCl<sub>2</sub> and CuCl<sub>2</sub> to the growth medium of *B. subtilis* can increase antagonistic activity against *M. incognita* and suppress its chemotaxis response in tomato roots soaked in the bacterial suspension.

**Keywords:** Biocontrol, chemotaxis, exudate, mortality, pluronic

## INTRODUCTION

Root-knot nematodes (RKNs) are cosmopolitan pathogens that infect more than 2000 plant species worldwide (Tapia-Vázquez et al. 2022). Of the many species of RKNs, the most problematic species in Indonesia is *Meloidogyne incognita* (Chaerani 2022). Nematodes can infect horticultural crops, food crops, plantation crops, and spice plants. In Indonesia, *M. incognita* has been reported to infect tomatoes, celery, pepper, coffee, and eggplant (Taher and Suastika 2012; Kurniawati et al. 2017; Chaerani 2022). Yield losses caused by infection with nematodes vary, depending on environmental conditions, population density, and plant varieties infected. Several studies report that it causes an average yield loss of 35% to 80% (Forghani and Hajihassani 2020). Double infection involving *M. incognita* and other pathogens, such as fungi or bacteria, can cause total yield loss and plant death (Tapia-Vázquez et al. 2022). For example, tomato and tobacco plants infected with *M. incognita* and *Ralstonia solanacearum* simultaneously can

cause crop failure (Furusawa et al. 2019; Asghar et al. 2020). Similarly, when *M. incognita* attacks Solanaceae plants and is followed by infection with *Fusarium oxysporum*, leading to more severe infection and plant death (Hua et al. 2019). Infection symptoms caused by *M. incognita* are generally marked by root gall. The gall disturbs the absorption of water and nutrients from the soil and subsequently inhibits nutrient distribution and plant growth (Elling 2013). Insufficient nutrients directly cause stunted plant growth, yellowing leaves, lower yields, and decreased plant freshness. Moreover, this circumstance indirectly makes plants susceptible to other pathogenic infections (Subedi et al. 2020). The disturbances in plant roots caused by *M. incognita* can also cause plants to wilt despite sufficient water in the soil (Kaloshian and Teixeira 2019).

Multiple control measures have been carried out to suppress *M. incognita* infection in various agricultural commodities. However, to date, *M. incognita* infection remains unresolved. This nematode has a unique life niche, due to its semi-endoparasite and sedentary natures, making it difficult to control (Singh et al. 2019). The use of

agrochemicals such as synthetic nematicides has been reported to effectively control *M. incognita* infection in various horticultural commodities (Hajihassani et al. 2019). However, farmers hardly opt for synthetic nematicides because of the expensive price. Using synthetic nematicides may be a good solution for commodities with high economic value. However, it is not equally beneficial for commodities with low economic value and small-scale farmers (Regmi and Desaegeer 2020).

Using biological control agents is deemed a decent solution to the issue abovementioned. Several biological agents from the bacterial class have been reported to control *M. incognita* (Khanna et al. 2019). Using biological agents is considered effective, efficient, easy to propagate, economical, and flexible due to multiple modes of action (Basyony and Abo-Zaid 2018). A rhizobacterium, *Bacillus subtilis*, has nematocidal activity. It produces protease enzymes and chitinase enzymes, fixes nitrogen, and dissolves phosphate. Furthermore, consortium isolates *Bacillus* SK07, *Bacillus* SK14, *Bacillus* KB14 and *Pseudomonas diminuta* Pd01 can control *Meloidogyne* spp. and *Pratylenchus coffeae* on tomato and coffee, respectively (Asyiah et al. 2020; Asyiah et al. 2021).

In several studies, antagonistic bacteria from the genus *B. subtilis* are also proven effective in controlling RKNs at the laboratory, greenhouse, and field scales (Chinheya et al. 2017). Its effectiveness as a nematode control agent is closely related to its ability to produce secondary metabolites. Protease enzymes produced by antagonist agents can degrade nematode cell walls and nematode egg cell walls (Lee and Kim 2016). Another study reports that biological agents can produce chitinase enzymes for controlling RKNs. This ability is related to the structure of nematode cell walls and nematode eggs, which are mostly composed of protein and chitin (Abdel-Salam et al. 2018). Furthermore, bacteria from the genus *Bacillus* have also been reported to produce HCN, which is toxic to nematodes (Sehrawat et al. 2022). These mechanisms help to directly control RKNs by antagonistic bacteria. Furthermore, antagonistic bacteria can control RKNs indirectly by inducing plant resistance (Messa 2021). Several studies report that *Bacillus* sp. can induce systemic resistance. Systemic resistance is activated due to the induction of antagonistic agents and is closely related to the production of plant-resistance compounds, such as PR-proteins, tannins, saponins, and alkaloid compounds (Kim et al. 2015). Plant systemic resistance suppresses infection by various pathogens, not only nematodes (Pieterse et al. 2014).

Fortifying growth media using micronutrients can increase the activity of *Bacillus subtilis* as an antagonist against *M. incognita*. Micronutrient is one of the microelements needed by *Bacillus* sp. for growth and development (Wiyono et al. 2008). These elements include iron (Fe), copper (Cu), zinc (Zn), molybdenum (Mo), manganese (Mn), magnesium (Mg), and boron (Br) (Yabalak et al. 2022). Iron is one of the essential microelements required by bacteria. Good bacteria require Fe in the growth process, such as in the formation of bacterial biofilms, as a cofactor for several enzymes, to the

formation of antibiotic compounds. Generally, the optimal Fe element used by bacteria is  $Fe^{2+}$ , but the most abundant is  $Fe^{3+}$  (Dos Santos et al. 2021). As such, bacteria require more techniques to obtain  $Fe^{2+}$ . Bacteria synthesize siderophore compounds to compete with pathogens in the rhizosphere to obtain Fe ions (Rangarajan et al. 2012).

To date, studies related to increasing the antagonistic activity of *Bacillus* sp. against plant parasitic nematodes, particularly *M. incognita*, remain underexplored. Therefore, the aim of this study was to examine the effect of micronutrient addition on the antagonistic activity of *B. subtilis* against *M. incognita*.

## MATERIALS AND METHODS

### Research time and site

The research was carried out from August to October 2022 at the Plant Protection Laboratory, Faculty of Agriculture, Universitas Borneo Tarakan, Indonesia and the Plant Pest Organism Control Technology Laboratory, Faculty of Agriculture, Universitas Jember, Indonesia.

### Collection of bacterial isolates

The bacterial isolate used in this study was *Bacillus subtilis* isolated from healthy coffee plant roots cultivated in Kalibendo and Kalimalang of Banyuwangi District, Sumberwringin of Bondowoso District, East Java Province, Indonesia.

### Preparation of *Meloidogyne incognita* inoculum

The inoculum of *M. incognita* was collected from the Plant Protection Program, Universitas Jember, Indonesia. The inoculum was obtained from the continuous propagation of tomato plants from a single egg mass. Tomato plants that had been infested by pure *M. incognita* were then rooted and carefully washed to remove soil particles. The tomato roots were cut to a size of 1-2 cm and put into a tube containing 2% NaOCl. Next, the tube was shaken for 5 minutes to obtain *M. incognita* eggs. The nematode eggs obtained were then filtered using a sieve with a size of 500 mesh. The eggs obtained were rinsed using distilled water from NaOCl (Ehwaeti et al. 1998). Nematode eggs were collected in a petri dish filled with 10 mL of sterile distilled water and incubated in a dark room. After 7 days of incubation, the resultant J2 *M. incognita* was used for further testing.

### Bacterial growth test on micronutrient-enriched media

The test was carried out by growing bacteria in 100 mL nutrient broth (NB) (HiMedia, India). The bacterial suspension formed was used in the next stage. A total of 1 mL of bacterial suspension in NB media was added to 99 mL of new NB media fortified with micronutrients. The micronutrients used were  $FeCl_2$ ,  $MnCl_2$ , and  $CuCl_2$ . Each micronutrient was administered at concentrations of 35 ppm, 40 ppm, 45 ppm, 50 ppm, and 55 ppm. As a control, NB media was used without micronutrients. 1 mL of bacterial suspension was incubated at 30°C for 48 hours. The suspension was calculated for its density using the

total plate count (TPC) method by performing serial dilutions (Townsend and Naqui 1998). The test was conducted in vitro, following a completely randomized design with 20 treatments, and each treatment was repeated 4 times.

#### **In-vitro test of *Bacillus subtilis* antagonism against *Meloidogyne incognita***

The six treatments with the best results were used in this test. Bacteria were grown for 48 hours on NB media with added micronutrients, and as a control, bacteria were grown on NB media without micronutrients. A total of 150 J2 *M. incognita* in 4 mL of suspension was put into a petri dish with a diameter of 5 cm. 1 mL of each bacterial suspension was poured into a petri dish according to the treatment. Furthermore, treated nematodes were incubated for 24 hours at 28°C in a dark room. The test was performed in a completely randomized design with 7 treatments and 4 replications. After 24 hours of incubation, nematode suspension was dripped with 100 L 1 N NaOH to activate the inactive nematodes. The active nematodes showed a wavy phenotype, while the dead nematodes showed a straight and rigid body position after 5 minutes administration of 1 N NaOH. The number of living and dead nematodes was observed for analysis (Wiratno et al. 2019).

#### **The effect of seed treatment on in vitro chemotaxis of *Meloidogyne incognita***

Bacteria were cultured on 100 mL NB micronutrient-enriched media marked with the best results in the bacterial growth test. In control, bacteria were grown on NB media without micronutrients. The test was conducted in a completely randomized design with 7 treatments and 4 replications. Tomato seeds of the Tantyna variety were washed using 2% NaOCl for 2 minutes. The seeds were soaked in a bacterial suspension for 60 minutes. After being soaked, tomato seeds were grown in water agar for 7 days at 28°C with light and dark periods interchanged every 12 hours. Once grown, tomato roots were cut and placed on Biologix 12 well plates containing 5 mL 20% Pluronic F-127 Gel at 10°C, which was infested with 100 J2 *M. incognita*. After 6 hours, the number of nematodes reaching the roots was observed. Nematodes identified within a radius of 2 mm from the roots were counted as nematodes with chemotaxis response to the roots (Wang et al. 2009; Shivakumara et al. 2018).

#### **Data analysis**

The data were analyzed for variance using the DSAASTAT version 1.101. When a difference was identified, Tukey test with a 95% confidence interval would be performed (Munif et al. 2019).

## **RESULTS AND DISCUSSION**

#### **The effect of added micronutrients on bacterial growth**

The added micronutrients on bacterial growth media showed various effects on bacterial growth. *Bacillus*

*subtilis* grown on media with the addition of 35 ppm to 55 ppm FeCl<sub>2</sub> resulted in lower growth compared to the control and other treatments. However, the lower growth rate was not statistically significant. The addition of other micronutrients from MnCl<sub>2</sub> and CuCl<sub>2</sub> groups resulted in better bacterial growth, which was significantly different from the control. Based on the calculation of bacterial cell density, six micronutrients with the best performance were 55 ppm MnCl<sub>2</sub>, 45 ppm MnCl<sub>2</sub>, 40 ppm MnCl<sub>2</sub>, 40 ppm CuCl<sub>2</sub>, 50 ppm MnCl<sub>2</sub>, and 35 ppm MnCl<sub>2</sub>. The bacterial growth on micronutrient-enriched media is presented in Table 1.

Even in small amounts, the presence of micronutrients gives a major effect on microbial metabolism. Excessive administration of micronutrients can inhibit microbial growth and even cell death (Choong et al. 2016). Several types of micronutrients can help the growth of bacterial cells. Mn compounds may serve as activators of many types of enzymes (Liu et al. 2021). Deng and Wang (2016) stated that bacteria grown on media with the addition of Mn have better growth potential due to increased enzyme activity. Another study reported that Cu plays a role in microbial cell respiration, cytochrome c oxidase, and other vital activities (Forte et al. 2016).

The results of the present study are in line with several other studies which also acknowledge increased microbial activity in micronutrient-fortified media. Each microbe has a different sensitivity to several types of micronutrients (Barra et al. 2021). Wei et al. (2004) reported that *Bacillus* sp. could grow better on media fortified with Mn compared to Fe. In the present study, bacteria grown on Fe exhibited a negative growth response compared to the control. This phenomenon does not generally apply to all *Bacillus* isolates. For some bacteria, Fe plays a role in regulating several enzymes, such as catalase, peroxidase, and oxygenase (Hantke 2001).

**Table 1.** The growth of *Bacillus subtilis* on micronutrient-enriched media

Treatments	Bacterial cell density (Log CFU mL <sup>-1</sup> )
35 ppm CuCl <sub>2</sub>	8.54 ± 0.15 c
35 ppm FeCl <sub>2</sub>	7.48 ± 0.21 a
35 ppm MnCl <sub>2</sub>	8.55 ± 0.09 c
40 ppm CuCl <sub>2</sub>	8.59 ± 0.13 c
40 ppm FeCl <sub>2</sub>	7.33 ± 0.29 a
40 ppm MnCl <sub>2</sub>	8.62 ± 0.07 c
45 ppm CuCl <sub>2</sub>	8.47 ± 0.09 c
45 ppm FeCl <sub>2</sub>	7.60 ± 0.26 ab
45 ppm MnCl <sub>2</sub>	8.67 ± 0.05 c
50 ppm CuCl <sub>2</sub>	8.51 ± 0.14 c
50 ppm FeCl <sub>2</sub>	7.36 ± 0.35 a
50 ppm MnCl <sub>2</sub>	8.58 ± 0.12 c
55 ppm CuCl <sub>2</sub>	8.45 ± 0.06 c
55 ppm FeCl <sub>2</sub>	7.38 ± 0.29 a
55 ppm MnCl <sub>2</sub>	8.88 ± 0.04 c
Control	7.99 ± 0.11 b

Note: Values followed by different superscript letters indicate significant difference from the control at  $p \leq 0.05$

The addition of several trace elements can increase the growth of *Bacillus* sp. (Borah et al. 2002). According to Shen et al. (2019), administration of trace elements can affect the viability and sporulation of *Clostridium sporogenes* and has a broad antimicrobial spectrum against other pathogenic bacteria. Some of these trace elements are absorbed and taken to the bacterial cytoplasm, which is necessary for the bacterial metabolic process (Zhang and Gladyshev 2009). Iron can function as an activator of several enzymes in metabolic activities (Kortman et al. 2016). Gumienka et al. (2018) reported that the trace element copper can act as a cofactor in the catalytic process of bacterial enzymes. Bacterial metabolic activities, such as the formation of spores of *Bacillus* sp., requires protease enzyme. Urek and Pazarlioglu (2007) contend that an increase in the production of protease enzymes can be achieved by the addition of manganese. The addition of other trace elements, such as zinc, can foster the growth of bacterial organelles (Kloubert and Rink 2015).

#### The effect of adding micronutrients on *Bacillus subtilis* antagonism against *Meloidogyne incognita*

Six treatments included micronutrients, which enhanced the antagonism ability of *B. subtilis* against *M. incognita*. The mortality rate of *M. incognita* increased by 20% in the treatment with the addition of micronutrients. The analysis results proved that the mortality rate in the treatments was significantly different from the control. The mortality rate of *M. incognita* in each treatment was 77.5% in 55 ppm MnCl<sub>2</sub>, 74.5% in 45 ppm MnCl<sub>2</sub>, 77.25% in 40 ppm MnCl<sub>2</sub>, 73.25% in 40 ppm CuCl<sub>2</sub>, 78.75% in 50 ppm MnCl<sub>2</sub>, 79% 35 ppm MnCl<sub>2</sub>, while in control it was 53.25% (Figure 1). The mortality rates in the treatments were significantly different. The highest mortality rate of 79% was recorded in 35 ppm MnCl<sub>2</sub>, while the lowest mortality at 73.25% occurred in the administration of 40 ppm CuCl<sub>2</sub>.

The addition of micronutrients to bacterial growth media affects the physiology of bacteria (Borah et al. 2002). Several types of micronutrients aid in regulating the production of important enzymes such as proteases (Liu et al. 2021). The protease enzyme is one of the enzymes important in controlling nematodes. Protease can degrade protein, which is one of the constituent compounds of nematodes and nematode eggs (Thongkaewyuan and Chairin 2018). Several studies report that microbes with protease-producing abilities can better control *M. incognita* when compared to microbes without protease-producing abilities (Mostafanezhad et al. 2014; Gomes et al. 2018).

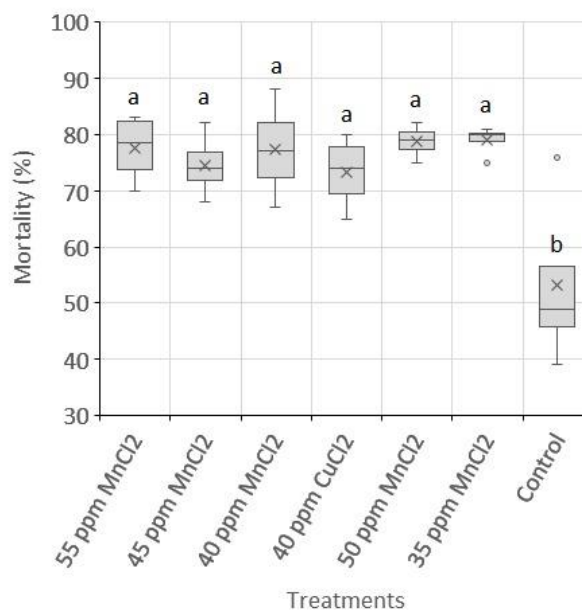
In addition to protease enzymes, the production of chitinase enzymes is also influenced by MnCl<sub>2</sub> and CuCl<sub>2</sub>. A previous study reported that the administration of MnCl<sub>2</sub> and CuCl<sub>2</sub> increases the production of chitinase in *Bacillus*. However, at a concentration above 100 ppm, administration of these two compounds can reduce bacterial growth and reduce chitinase production (Kuzu et al. 2012). Like proteases, chitinase is important in controlling nematodes. The body wall and eggs of nematodes are composed of protein and chitin. The degradation of the bacterial cell wall by proteases and

chitinases can cause nematode mortality (Yoon et al. 2012; Mota and Dos Santos 2016).

In addition to directly affecting enzyme production, the addition of micronutrients also increases bacterial growth. In the present study, it was also observed that the density of bacterial cells treated with MnCl<sub>2</sub> and CuCl<sub>2</sub> was higher than that of the control. This phenomenon was proven in the test of the present study, where a higher density of bacterial cells leads to a higher potential for enzyme production by bacteria. Bacteria also have other modes of action in killing nematodes, where the higher number of bacteria, the higher the probability of nematode mortality (Mota et al. 2017).

#### Nematode chemotaxis against tomato roots treated by soaking Seed using bacterial suspension with the addition of micronutrients

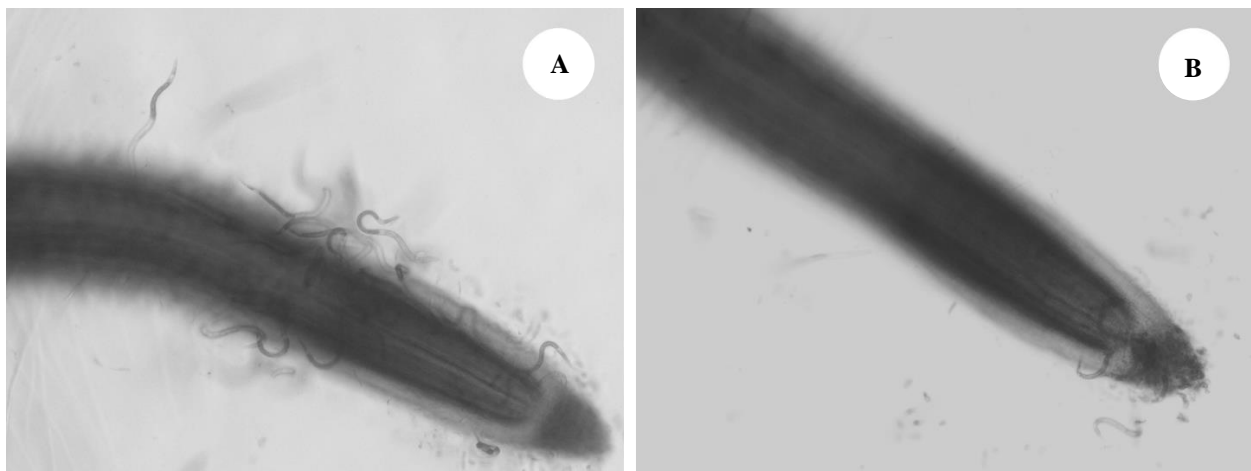
The addition of micronutrients to the growing media of *B. subtilis* decreased the chemotaxis index of nematodes toward tomato roots (Figure 2). The chemotaxis index in treatments ranged from 16% to 24%, while in control, it was 33%. The chemotaxis indices for each treatment were 21.75% (55 ppm MnCl<sub>2</sub>), 23.75% (45 ppm MnCl<sub>2</sub>), 21.75% (40 ppm MnCl<sub>2</sub>), 19.5% (40 ppm CuCl<sub>2</sub>), 16.5% (50 ppm MnCl<sub>2</sub>), 20.25% (35 ppm MnCl<sub>2</sub>) and 33% (in control) (Figure 3). The addition of 50 ppm MnCl<sub>2</sub> was the best treatment for reducing the chemotaxis index of nematodes on tomato roots. The chemotaxis index in the addition of 50 ppm MnCl<sub>2</sub> was the lowest at only 16.5%. The chemotaxis index treatment with the addition of 50 ppm MnCl<sub>2</sub> was not significantly different from the other treatments. However, chemotaxis index of the treatment was significantly different from that of the control (33%).



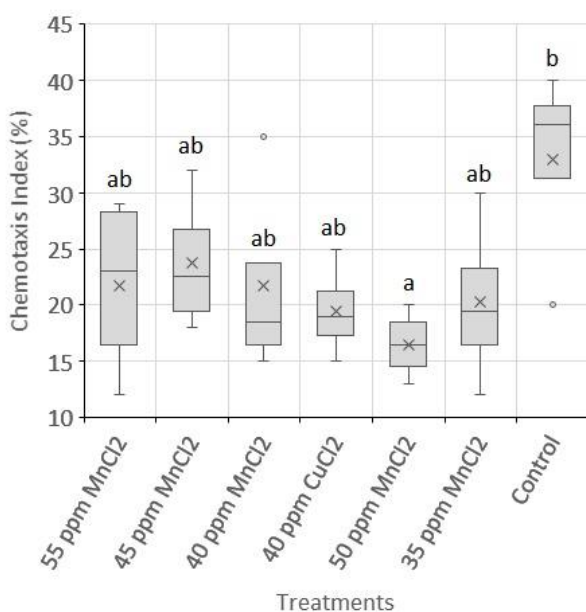
**Figure 1.** The mortality rate of *Meloidogyne incognita* due to *Bacillus subtilis* infection on micronutrient-enriched media

Chemotaxis is a movement influenced by chemical stimuli (Rasmann et al. 2012). In this study, chemotaxis means nematode movement influenced by root exudates. Several studies reported that the movement of nematodes underground is strongly influenced by the diversity and concentration of root exudates (Guerrieri et al. 2019). Root exudates in plants can be in the form of organic acid compounds, carbohydrates, proteins, and other compounds. The type of compounds secreted by roots in the form of root exudates is highly dependent on the type of plant. In addition, environmental factors also play an important role in causing differences in the type and amount of root exudate (Baetz and Martinoia 2014; Vives-Peris et al. 2020).

Wang et al. (2009) noted that *M. incognita* showed a chemotaxis response to several organic acids, such as malic acid, oxalic acid, and lactic acid. The chemotaxis response is also influenced by the concentration of the given compound. For example, *M. incognita* give a positive chemotaxis response to citric acid in small concentrations, but the presence of citric acid in high concentrations gives a negative response. A positive chemotaxis response is indicated by the movement of nematodes toward the compound being tested, while a negative response causes the nematodes to move away from the compound being tested (Wang et al. 2021).



**Figure 2.** The response of nematode chemotaxis in tomato seedling roots. A. Control; B. Seeds soaked in 55 ppm  $MnCl_2$



**Figure 3.** The chemotaxis index of *Meloidogyne incognita*

The type and amount of root exudate secreted by a plant species are strongly influenced by environmental factors (El Zahar et al. 2014). For example, several types of organic acids are absent from tomato root exudates subjected to continuous lighting treatment (Mavrodi et al. 2021). Another study reported that stressful conditions, such as extreme drought, can reduce the number of root exudates (Huang et al. 2014; Sun et al. 2021). This study shows that nematodes have a higher chemotaxis response in control plants than in treatment plants. This is because bacteria grown on micronutrient-enriched media produce different secondary metabolites than bacteria grown on control media. Different metabolites trigger various responses to tomato root exudates soaked in the bacterial suspension (Feng et al. 2021).

The research results concluded that the addition of  $MnCl_2$  and  $CuCl_2$  at concentrations of 35 ppm to 55 ppm in *Bacillus subtilis* growth media could increase the density of bacterial cells. However, the administration of micronutrient  $FeCl_2$  at concentrations of 35 ppm to 55 ppm decreased cell density. Furthermore, the administration of  $MnCl_2$  and  $CuCl_2$  increased the mortality of *M. incognita* by up to 24.25%. These micronutrients can also suppress *M. incognita* chemotaxis in tomato seedlings by up to 17%.

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