

## Secondary metabolites produced by endophytic bacteria against the Root-Knot Nematode (*Meloidogyne* sp.)

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**Abstract.** Maulidia V, Soesanto L, Syamsuddin, Khairan K, Hamaguchi T, Hasegawa K, Sriwati R. 2020. Secondary metabolites produced by endophytic bacteria against the Root-Knot Nematode (*Meloidogyne* sp.). *Biodiversitas* 21: 5270-5275. Endophytic bacteria live and colonize in plant tissues without causing disease to their plant host. Among several processes, these bacteria can produce secondary metabolites that can help in the defense of plant host against pathogens. This study aimed to identify endophytic bacteria as biocontrol agents against *Meloidogyne* sp. in tomato plants. Six endophytic bacteria candidates from the genus *Pseudomonas*, *Arthrobacter*, *Bacillus*, and *Serratia* were isolated from *Solanum Lycopersicum*, *Psidium guajava*, *Pinus merkusii*, *Dendrocalamus asper*, *Albizia chinensis*, and *Theobroma cacao* L, respectively. The average mortality of *Meloidogyne* sp. by endophytic bacteria was 70,27% to 95,46%. From these, *Bacillus thuringiensis* AK08 produced compounds of the secondary metabolites such as flavonoid, phenol, tannins, terpenoids, steroids, saponins, and alkaloids. The best result of the average incubation period, number of galls in the root, number of nematodes at the root, and the number of nematodes in the soil on tomato plant were shown by *B. thuringiensis*. The major compounds in GC-MS analysis of *B. thuringiensis* were cholest-5-en-3-ol (3.beta.)-carbonochloridate (25.35%). *Bacillus thuringiensis* not only has rules as bio-insecticide but also has nematocidal effect.

**Keywords:** *Bacillus thuringiensis*, biocontrol agent, GC-MS analysis, mortality, nematocidal effect

### INTRODUCTION

Endophytic bacteria associated with plant tissues are known to benefit their hosts through growth and for biological protection against diseases. In mutualistic associations, endophytes (i.e. bacteria that colonize plant tissues) are protected from nematode attack by their host plant, which in turn provides shelter and nutrition. These bacteria are potential producers of secondary metabolites (Abdallah et al. 2019; Verma et al. 2019). Seven endophytic bacteria, *Aerococcus viridans*, *Bacillus megaterium*, *Bacillus subtilis*, *Pseudomonas chlororaphis*, *Pseudomonas vesicularis*, *Serratia marcescens*, and *Sphingomonas paucimobilis* effectively against root-knot nematode (Muthukumar et al. 2017).

Plant-parasitic nematodes (PPN) constitute a serious threat for crops in quantity and quality, causing global crop losses annually. Lower case for biocontrol agents such as endophytic bacteria against PPN is particularly timely given the negative impact of the use of chemical nematicides. Awareness-raising for enhancing biocontrol tactics should be better communicated (Abd-Elgawad and Askary 2020; Liu and Park 2018). Plant-Parasitic

Nematodes (PPN), such as the Root-Knot Nematodes (RKN) (*Meloidogyne* sp.) secrete cell-wall degrading enzymes (e.g. cellulase, hemicellulases, pectinases) that breakdown plant cell walls, facilitating their invasion. RKN can attack those parts of plants that are below the surface soil, especially roots, tubers, and pods. The symptoms in this part of the plant are known as gall. Moreover, RKN causes a reduction in the efficiency of the root system function, due to which plants can be susceptible to other pathogens such as fungi and bacteria. The severely affected roots are shorter than healthy roots with few lateral roots and root hair, this being caused by nematodes feeding on root cells, and causing tissues disrupted; consequently, translocation of water and nutrient is compromised (Bruzos and Grayston, 2019; Hashem et al. 2011).

According to Munif et al. (2013), the utilization of four endophytic bacteria (*Pantoea agglomerans*, *Cedecea davisae*, *Enterobacter* sp. and *Pseudomonas putida*) by seed treatment, root immersion, or application of watering to the ground, can suppress the formation of galls from pathogens *Meloidogyne incognita* to 56%. Endophytic bacteria such as *Bacillus* sp., *Serratia* sp., *Paenibacillus* sp., *Enterobacter* sp., and *Streptomyces* sp. isolated from

*Coffea canephora* plant showed significant nematicidal activities (85.8%) against *Meloidogyne incognita* (Hoang et al. 2020). Two endophytic bacteria isolated from *Piper nigrum* L. showed high inhibition (53.6% to 65.8%) against root-knot nematode in vitro, which can be applied as bio-control agents to suppress soil-borne pathogens (Wiratno et al. 2019).

According to Malfanova et al. (2011), *Bacillus subtilis* was isolated from the hogweed plant *Heracleum sosnowskyi*, can significantly stimulate the growth of tomato plants and protect the plant against *Meloidogyne* sp. Endophytic bacteria isolated from the roots of *C. rotundus* are capable of reducing the number of *M. incognita* in the roots as well as reducing the number of galls on the roots of tomato plants infected with *M. incognita* (Pradana et al. 2017). So, it is necessary to identify the secondary metabolites produced by endophytic bacteria against *Meloidogyne* sp. in tomato plant as biocontrol agent.

## MATERIALS AND METHODS

### Procedures

#### Culture of the endophytic bacteria

The endophytic bacterial isolates were: *Arthrobacter* sp. (AM08; MT598017) isolated from *Solanum lycopersicum* L., plant root, *Pseudomonas aeruginosa* (AJ14; MT598020), isolated from *Psidium guajava* L. plant root, *Pseudomonas mosselii* (AB06; MT598025) isolated from *Dendrocalamus asper* (Schult with f.) Backer ex Heyne., *Bacillus cereus* (AP12; MT598023) isolated from *Pinus merkusii* L. plant root, *Bacillus thuringiensis* (AK08; MT598028) isolated from *Theobroma cacao* L. plant root, and *Serratia marcescens* (AS09; MT598027) isolated from *Albizia chinensis* L. plant root. Bacterial isolates were grown in nutrient agar (NA, branch) media, and incubated at room temperature for 48 hours (Hallman et al. 1997).

#### Extraction of RKN from *Apium graveolens* L. roots

Nematodes extracted from samples of celery roots (*Apium graveolens* L.) were infected with root purge nematodes using the Baermann funnel method. First roots were cleaned and cut into pieces (1 cm approximately) and weighed as much as 1 gram. The pieces of roots were soaked in sterile water for 6 h on the Baermann funnel (Dababat et al. 2007).

#### In vitro assays of endophytic bacteria against RKN

RKN mortality was determined by the inhibitory test. A cell pellet of endophytic bacteria, previously grown for 48 hours, was dissolved in sterile water. Then 5 mL of bacterial cell pellet with the density -  $10^3$  CFU mL<sup>-1</sup> was added to 5 mL of nematode extract (120 second-stage juveniles (J2)), meanwhile 5 mL sterile water and 5 mL of nematode extract for control treatment, followed by 24h incubation at 26°C. Nematode mortality was determined by microscopic observation using Nikon 102 microscope (Deen et al. 2014). Nematodes were considered dead if no movement was observed after mechanical stimulation. The observation of nematode using formula mortality nematodes by (Faria et al. 2013).

$$\text{Mortality\%} = \frac{[(\text{mortality\% in treatment} - \text{mortality\% in control}) / (100 - \text{mortality\% in control})] \times 100.}$$

#### Extraction of secondary metabolites from endophytic bacteria

In order to extract the secondary metabolites, each endophytic bacteria were firstly inoculated in liquid NB media and incubated for 2 days at 120 rpm. Later, the bacterial suspension was centrifuged at 3000 rpm. Cell pellet was dried in oven at 50°C temperature, and suspended with 50 mL methanol MeOH) was air distilled, then the extracted endophytic bacteria can be used for secondary metabolites testing (Shekhawat and Shah, 2013). Extracts were tested for the presence of secondary metabolites such as flavonoids, phenols, tannins, terpenoids, steroids, saponins, and alkaloids (Harborne, 1987).

#### Qualitative analysis of bacterial secondary metabolites

The qualitative phytochemical analysis was performed by the methods of Harborne (1987), this analysis includes flavonoids, phenols, tannins, terpenoids, steroids, saponins and alkaloids.

#### Evaluation of nematicidal effect of endophytic bacteria against RKN

The evaluation of the nematicidal effect of endophytic bacteria against *Meloidogyne* sp. was carried out under greenhouse conditions, susceptible Karina variety of tomatoes was used for the experiment. Tomato seeds were surface sterilized with sodium hypochlorite (5,25% v/v) for 5 minutes, rinsed with sterilized water, and then dried. Seeds were immersed in each bacterial suspension (app.  $10^3$  CFU mL<sup>-1</sup>) for 3 hours. Tomato seeds were planted in a 2 kg polybag containing soil medium and manure (2:1). *Meloidogyne* sp. (J2) was inoculated. After 3 weeks, tomato plants were collected and the number of galls, number of nematodes at the root, and the number of nematodes in the soil were determined according to (Munif et al. 2013).

#### GC-MS analysis

Extract of endophytic bacterium *B. thuringiensis* AK08 was sent for Gas Chromatography-Mass Spectroscopy analysis. The GC-MS was carried out on TRACE 1300 GC, TSQ 8000 TRIPLE QUADRUPOLE MS fitted with TG 5MS (30m X 0.25mm, 0.25µm) column and S/SL Injector. The injector temperature was kept at 250°C and MS transfer line temperature had kept at 250°C along with ion source temperature also 250°C. The column temperature was programmed between 60°-280°C at 10°C/min using helium as carrier gas at a carrier flow rate of 1ml min<sup>-1</sup>. Injection volume had 1.0 µl prepared in DMSO having Split flow 1ml min<sup>-1</sup>. The mass spectra had taken at 75 eV with mass scan range from m/z 40-500 amu. The individual constituents were identified by comparing their mass spectra with those of standard using NIST (National Institute of Standards and Technology, U.S. Department of Commerce) compounds (Sparkman et al. 2011).

### Data analysis

This study used a complete randomized non-factorial design. Data were subjected to perform analysis of variance (ANOVA) and means were separated ( $p < 0.05$ ) by Duncan's Multiple Range Test (DMRT). All analyses were conducted using SPSS version 25.

## RESULTS AND DISCUSSION

Based on the results of nematode mortality percentage, the statistical test showed very significant difference between endophytic bacteria isolates and control (Figure 1) The number followed by different letters is not significant at the level  $\alpha$  0.05 (DMRT Test). The treatment of *B.thuringiensis* exhibited the highest (95.46%). While 82.93%, 81.05%, 78.85%, 73.36% and 70.27% nematode mortality was recorded in *P. aeruginosa*, *B. cereus*, *P. mosselii*, *S. marcescens* and *Arthobacter* sp. respectively.

Results of qualitative test revealed that all the six species of endophytic bacteria contain phenols, alkaloids, and tannins (Table 1) Saponins were observed in all five endophytic bacterial species except *Arthobacter* sp. Only three bacterial species i.e. *Arthobacter* sp., *P. mosselii*, and *B. thuringiensis* contain flavonoids. The presence of terpenoids and steroids was observed only in *B. thuringiensis*. Alkaloids were found in all six species of endophytic bacteria by the Dragendorff method. However, in the Meyer method, it was found only in *P. aeruginosa* and *B. thuringiensis*. In the Wagner method, alkaloids were only found in *B. cereus*. Species *B. thuringiensis* produced all secondary metabolites such as flavonoids, phenols, tannins, terpenoids, steroids, saponins, and alkaloids, compared to other endophytic bacterial species, which indicates the most positive secondary metabolite content. *B. thuringiensis* AK08 showed the highest mortality rate in in vitro test (95,46%). This result could be due to highest content in secondary metabolites.

Results of nematicidal effect of endophytic bacteria against RKN revealed that the incubation period (day) control treatment showed the fastest emergence of nematode symptoms at 42 days after planting, while *B. thuringiensis* AK08 treatment had the longest incubation period of 62 days after planting compared with control, with no statistical difference from other treatments (Table 2). The number of galls in root, number of nematodes in root, and in the soil were found lower in all six bacterial species than in control. Of the six endophytic bacterial

species *B. thuringiensis* AK08 showed the most nematicidal effect against RKN. In the observation of the number of galls in the root, the control treatment has the highest (97) number of galls, while in treatment with *B. thuringiensis* AK08 only 36 galls were counted. *B. thuringiensis* AK08 showed the lowest (27) number of nematodes in root whereas in control it was 171. The lowest number of nematodes in the soil was 81 in *B. thuringiensis* AK08 while highest 240 was found in control treatment, which significantly differs from all bacterial treatments.

In this study, methanol (MeOH) was used as extraction solvent since it is the most efficient method for obtainment of secondary metabolites. The methanol (MeOH) extraction was characterized and identified by GC-MS analysis. The interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) with more than 62,000 patterns. The spectrum of each unknown component was compared with the spectrum of the known components stored in the NIST library. The active principles along with their retention time (Rt), molecular formula, molecular weight, and concentration percentage (area %) are shown in Tables 3. The GC-MS analysis of bacteria extraction revealed that the steroids were the major compounds in methanol (MeOH) extraction of *B.thuringiensis* including 5-.alpha.-Androst-2-en-17-.beta.-ol, 17-methyl- (13.08%), cholest-5-en-3-ol (3.beta.)-, carbonochloridate (25.35%), and cholest-5-en-3-ol (3.beta.)-, carbonochloridate (17.78%) (Table 3).

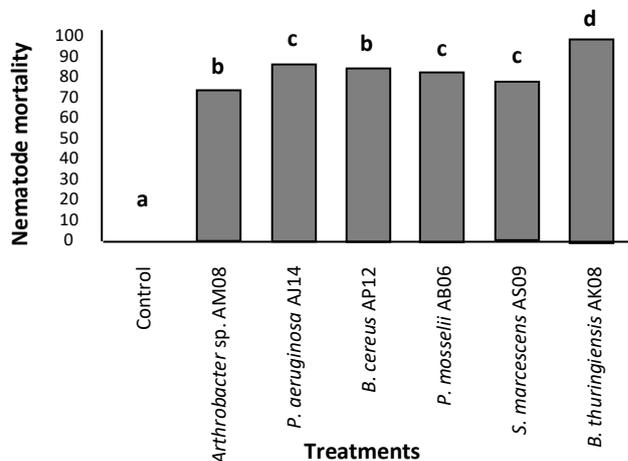


Figure 1. Mortality percentage of *Meloidogyne* sp. after treatment with endophytes bacteria

Table 1. Qualitative analysis of bacterial secondary metabolites

Endophytes bacteria species	Secondary metabolites								
	Flavonoids	Phenols	Tannins	Terpenoids	Steroids	Saponins	Alkaloids		
							D	M	W
<i>P. aeruginosa</i> (AJ14)	-	+	+	-	-	+	+	+	-
<i>Arthobacter</i> sp. (AM08)	+	+	+	-	-	-	+	-	-
<i>B. cereus</i> (AP12)	-	+	+	-	-	+	+	-	+
<i>P. mosselii</i> (AB06)	+	+	+	-	-	+	+	-	-
<i>S. marcescens</i> (AS09)	-	+	+	-	-	+	+	-	-
<i>B. thuringiensis</i> (AK08)	+	+	+	+	+	+	+	+	-

Note: D: Dragendorff; M: Meyer; W: Wagner

**Table 2.** Effect of endophytes bacteria against RKN in tomato plant

Treatment	Incubation period (day)	Number of galls in the root	Number of nematodes in the root	Number of nematodes in the soil
Control	42 a	97 d	171 e	240 b
<i>P. aeruginosa</i> (AJ14)	54 b	64 c	45 b	92 a
<i>Arthrobacter</i> sp. (AM08)	56 b	55 b	55 c	99 a
<i>B. cereus</i> (AP12)	57 b	66 c	55 c	107 a
<i>P. mosselii</i> (AB06)	57 b	70 c	85 d	102 a
<i>S. marcescens</i> (AS09)	54 b	69 c	76 d	96 a
<i>B. thuringiensis</i> (AK08)	62 b	36 a	27 a	81 a

Note: The number followed by different letters are not significant at the level  $\alpha$  0.05 (DMRT Test)

**Table 3.** Compounds identified in the crude methanol extract of *B. thuringiensis* AK08 by GC-MS analysis

No. peak	Retention time (min.)	Area (%)	Molecular weight (m/z)	Chemical name
1	3.541	0.33	87.05	2,2-Dimethoxybutane
2	41.490	0.22	83.00	n-Pentadecanol
3	41.761	0.26	55.00	E-14-Hexadecenal
4	42.061	0.22	55.00	n-Tridecan-1-ol
5	43.338	0.20	149.00	1,2-Benzenedicarboxylic acid, monobutyl ester
6	43.489	0.19	57.00	3-Eicosene, (E)-
7	44.453	0.69	74.00	Hexadecanoic acid, methyl ester
8	47.326	0.32	55.00	trans-2-Decen-1-ol, trifluoroacetate
9	47.639	1.14	83.05	Behenic alcohol
10	47.690	1.09	57.00	Octadecane, 1-chloro-
11	47.880	0.27	67.00	1,8,11-Heptadecatriene, (Z,Z)-
12	47.986	0.90	55.00	6-Octadecenoic acid, methyl ester, (Z)-
13	48.461	0.24	74.00	Hexacosanoic acid, methyl ester
14	48.495	0.39	143.00	2-(4-Fluorophenoxy)-N'-(1-styrylethylidene)acetylhydrazide
15	48.607	0.30	85.00	Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one, 3-hydroxy-, (3.beta.,17.beta.)-
16	50.882	13.08	105.05	5- $\alpha$ -Androst-2-en-17- $\beta$ -ol, 17-methyl-
17	51.465	1.47	91.05	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde
18	51.541	1.97	215.10	Androstane-3,17-diol, 17-methyl-, (3.alpha.,5.alpha.,17.beta.)-
19	51.826	2.18	81.00	Retinol, acetate
20	52.168	2.55	105.05	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate
21	52.475	0.64	74.00	7,10,13-Eicosatrienoic acid, methyl ester
22	52.765	3.86	57.00	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate
23	53.554	2.83	185.00	185.00 2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol
24	53.646	3.00	95.00	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde
25	53.938	25.35	147.10	Cholest-5-en-3-ol (3.beta.)-carbonochloridate
26	54.750	3.32	83.00	Retinol, acetate
27	55.293	0.58	69.00	Retinoic acid
28	56.531	17.78	81.00	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate
29	57.035	1.57	105.05	Isopimara-9(11),15-diene
30	57.263	3.02	349.15	2-Naphthalenol, 2,3,4,4a,5,6,7-octahydro-1,4a-dimethyl-7-(2-hydroxy-1-methylethyl)
31	57.615	0.48	143.10	Androsta-1,4,6-triene-3,17-dione
32	57.957	4.54	145.05	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate
33	58.406	1.23	105.00	10-12-Pentacosadiynoic acid
34	59.055	0.46	278.90	Terephthalic acid, isobutyl 2,2,2-trichloroethyl ester
35	59.392	3.34	81.00	Longifolenaldehyde

## Discussion

*Bacillus thuringiensis* AK08 showed promising results in the control of RKN both in vitro and in planta experiments. The mortality rate against RKN was 95,46% in in vitro trials. Hu et al. (2017) showed similar result with three endophytic bacteria from genus *Bacillus*, namely *B.*

*cereus*, *B. cereus*, and *B. altitudinis* has the highest mortality activity above 90%. According to Bui et al. (2020), *Bacillus* sp. and *Paenibacillus* sp. had more than 95% of mortality activity against *M. graminicola*. *Bacillus* sp. and *Pseudomonas* sp. effectively inhibited *Meloidogyne* sp. in vitro by 59.7% and 64.1%, respectively.

Our isolate *B. thuringiensis* AK08 showed the presence of 7 secondary metabolites while the other isolates were less diverse in metabolite content. The endophytic bacteria play a significant role in the production of bioactive compounds such as alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, and phenols (Singh et al. 2017). These compounds also have important roles in therapeutic applications such as anti-cancer, antioxidant, antimicrobial, anti-inflammatory, and immunosuppressive agents. These metabolites can also act as biofilm, toxins, virulence factors (Singh et al. 2019). Secondary metabolites were categorized into alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, saponins, tannins, terpenoids, tetralones, xanthenes, and many others. The production of bioactive substances by endophytic bacteria, has been directly associated with the host microorganisms, which may include genetic information from higher plants. This allowed them to better adapt to the host plant and perform certain functions, such as protection from various types of pathogens (Gouda et al. 2016).

*Bacillus thuringiensis* AK08 has effectively controlled the *Melydogyne* sp. It reduces galls in root and number nematodes in root and in soil and also increases the incubation period on the tomato plant. Hu et al. (2017) used three species of endophytic bacteria i.e. *B. cereus* BCM2, *B. cereus* SZ5, and *B. altitudinis* CCM7 in pot experiment showed similar results that *B. cereus* BCM2 could significantly reduce the number of galls and egg mass from 81.2 % and 75.6% on tomato roots. Munif et al. (2015), reported that two endophytic bacterial isolates are MSJ1H and AGS1F, were able to suppress the number of root gall caused by *Meloidogyne* sp. by 54% to 67%. Tran et al. (2019), reported that *B. megaterium* significantly inhibited nematodes in the soil and pepper plant from 81.86% and 73.11%. Various bacterial endophytes reportedly by produce antibiotics (that inhibit pathogen proliferation) (Glick, 2020). According to Vertrivelkalai (2018), endophytic bacteria from genus *Bacillus* sp. significantly reduced nematode population in soil and root of tomato plants. The mechanisms by which reduction in nematode population may be due to competition for space and nutrients; and mortality of juveniles induced by secondary metabolites such as 2,4 Diacetylphloroglucinol, lytic enzymes, antibiotics, hydrogen cyanide, and toxic metabolites like bacillopeptidase, subtilin E and  $\beta$  lactamase that produced by *Bacillus* sp. (Dunne et al. 1998).

The GC-MS analysis revealed that the steroid compounds were predominant constituents in the methanolic *B. thuringiensis* extract. The major compounds reported in methanolic extract of *B. thuringiensis* were androstane and cholestane. Androstane is a steroid compound with a gonane core and contain 19 of carbon (C-19), and can exist as either of two isomers, known as  $5\alpha$ -androstane and  $5\beta$ -androstane. An orally bioavailable adrenal steroid analog with potential antineoplastic activity. Cholestane, is a saturated tetracyclic triterpene contain 27 of carbon (C-27), and available in low abundance in Rhodopyhtes organism, this compound, in general, has

antineoplastic activity. Cholestane mostly fragmented at m/z 217 (from molecular ion 372). This specific fragment, first looks for the 372 molecular ions of cholestane, and then fragments that molecular ion further to its m/z 217 fragments in order to improve identification of specific isomers. Gas chromatography-mass spectrometry (GC-MS) analysis of 6-chlorohydroxyquinol; 2,3,4,6-tetrachlorophenol and tetrachloro-hydroquinone using mixed culture of *B. cereus* and *S. marcescens* (Singh et al. 2009). [1,2- $\alpha$ ] pyrazine-1,4-dione, 3-Keto-1-aza-2,3-dihydrobenzopyran, 3-(4-pyridyl) acrylic acid, 9-octadecenoic acid (Z)-methyl ester, and dioctyl hexanedioate. Abdallah et al. (2016) identified key compounds using GC-MS analysis in bioactive chloroform extracts of *B. cereus*, which belonged to the family of phytic acid. The other compounds identified are phenol 3,5-dimethoxy, benzoic acid 3,5-dihydroxy,2-hydroxy-1- isoindolinone, 3-isobutylhexahydroppyrolo. Beta phenylethyl butyrate, Benzene acetic acid, Mefenoxam antimicrobial compounds that produce by *Arthobacter* sp. (Munaganti et al. 2016). *B. thuringiensis* produces crystal proteins which have nematicidal effect against plant-parasitic nematodes such as *Bursaphelenchus xylophilus*, *M. hapla*, *Pratylenchus scribneri*, *Tylenchorhynchus* sp., and *Ditylenchus destructor* (Huang et al. 2018; Quan et al. 2008). In general, *B. thuringiensis* performed best and seen as a biocontrol agent for *Meloidogyne* sp. on tomato plants. Although *B. thuringiensis* is described as bio-insecticide, our results showed that is also capable of nematode control, being its nematicidal effect due to their secondary metabolites content such as cholest-5-en-3-ol (3.beta.), carbonochloridate which inhibits the nematodes growth.

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