Metabolites of Bacterial-Feeding Nematodes Stimulate Bacterial Indole-3-Acetic Acid (IAA) Synthesis

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Abstract

Interorganismal metabolites play significant roles in regulating behaviors and communications between organisms. Nematodes are the most abundant animals on earth, and function well in soil ecosystem due to their interactions with microbes. Bacterial-feeding nematodes stimulate the activity of indole-3-acetic acid (IAA)-producing bacteria and increase the content of IAA in soil. However, we do not fully understand how bacterial-feeding nematodes interact with bacteria and affect IAA synthesis. In this study, the model nematode Caenorhabditis elegans and three species of soil-dwelling IAA-producing bacteria (Bacillus amyloliquefaciens JX1, Arthrobacter pascens ZZ21 and A. chlorphenolicus L4) were employed to determine the effect of nematodes on the IAA biosynthesis of bacteria. Then the metabolites and extracts of C. elegans were tested the effect on three bacterial IAA synthesis (but only A. pascens ZZ21 for the extracts). Lastly, two soil-dwelling bacterial-feeding nematodes (Mesorhabditis sp. and Acrobeloides sp.) and two IAA-producing bacteria (B. amyloliquefaciens JX1 and A. pascens ZZ21) were subsequently used to explore the universality of this interaction. Our results showed that the metabolites or extracts of nematodes could promote the IAA biosynthesis of IAA-producing bacteria, and implied this stimulatory effect maybe widely spread in metabolites of bacterial-feeding nematodes and IAA-producing bacteria, but vary with nematodes and bacteria species. Our findings indicate that bacterial-feeding nematodes could mediate the interaction between nematodes and bacteria by their metabolites, except for their feeding behavior, and offer insights into the ecological function of the metabolites of nematodes.

Introduction

Interorganismal metabolites functioned as a kind of special language to regulate the behaviors and physiological process of many organisms, especially in soil ecosystem, which harbor the most diverse populations of bacteria, fungi, nematodes, protists and plants (Demain and Fang 2000; Bardgett and van der Putten 2014; Haichar et al. 2014; Zhalnina et al. 2018; Schmidt et al. 2019). Metabolites released by soil organism play important roles in maintaining the soil conditions and other physical parameters to facilitate them to face environmental stress. For example, plants could produce specialized metabolites that are able to modulate root microbiota, attracting, inhibiting or even killing soil microorganisms, so as to promote plant growth and defense pathogens (Lebeis et al. 2015; Sasse et al. 2018; Stringlis et al. 2018; Zhalnina et al. 2018; Huang et al. 2019). Correspondingly, bacterial exudates are capable of promoting plant infection by pathogens (Kong and Hong 2016) or acting as antibiotics that are antagonistic to pathogens (Junaid et al. 2013; Molina-Santiago et al. 2015; Tyc et al. 2017). Hence, understanding the interaction between soil organisms will have considerable bearing on maintaining a stable soil system and further increasing crop yields.

Nematodes, as one of the major functional groups in soil, play different roles in the ecosystem due to the diversity of feeding habits (Clarholm 1985; Yeates et al. 1993). Bacterial-feeding nematodes are the most abundant component of soil nematodes. They influence the quantities, activities and communities of soil microbes through metabolic activities and feeding behaviors, thereby affecting nutrient transformation and circulation, as well as regulating plant growth and development (Bouwman et al. 1994; Ferris et al.
In previous studies, we found that bacterial-feeding nematodes improved the activities of bacteria and increased the content of soil indole-3-acetic acid (IAA) (Mao et al. 2006; Mao et al. 2007; Cheng et al. 2011; Jiang et al. 2012). IAA is the most common naturally phytohormone with the capacity to regulate plant growth and development (Woodward and Bartel 2005; Teale et al. 2006; Patten et al. 2013). It also serves as a signal in the communications and interactions among soil organisms (Spaepen et al. 2007; Kochar et al. 2013; Lee et al. 2017). To our knowledge, bacterial-feeding nematodes do not have the ability to secrete IAA, so it is reasonable to attribute the increase of IAA to the interaction between nematodes and IAA-producing bacteria. However, it is still not fully understood how bacterial-feeding nematodes interacts with IAA-producing bacteria, and how nematodes modulate the bacterial IAA synthesis. Several researches have shown that the metabolites of plants are capable of promoting bacteria to produce IAA. For example, Prinsen et al. (1991) and Theunis et al. (2004) demonstrated that the flavonoids, produced by host plants and accumulated in the rhizosphere, are able to stimulate the root-symbiotic bacterium *Rhizobium* spp. to synthesize IAA. Jasim et al. (2014) reported that the metabolites (piperine and other compounds) extracted from *Piper nigrum* could stimulate the endophytic bacteria to produce IAA. Besides, the nematodes could secrete lot of metabolites, including amino acids, sugars and organic acids (Kaplan et al. 2009). Recent studies demonstrated that the metabolites of nematodes play a significant ecological role in maintaining the balance of soil ecosystem, like regulating the behavior of nematodes, eliciting plant defenses and pathogen resistance (Edison 2009; Manosalva et al. 2015; Reilly and Srinivasan 2019; Manohar et al. 2020). Therefore, we hypothesized that the bacterial-feeding nematodes could regulate the IAA synthesis of bacteria by their secretory metabolites.

The objective of this study was to determine if the metabolites of bacterial-feeding nematodes could influence the IAA biosynthesis of bacteria. The experiment was carried out in the liquid mineral medium. First, we used the model nematode *Caenorhabditis elegans* and three species of soil-dwelling IAA-producing bacteria (*Bacillus amyloliquefaciens* JX1, *Arthrobacter pascens* ZZ21 and *A. chlorphenolicus* L4) to test the phenomenon that nematodes could increase the IAA biosynthesis of bacteria. Second, we explored if metabolites and extracts of *C. elegans* would influence the IAA biosynthesis of three soil-dwelling bacteria (but only *A. pascens* ZZ21 for the extracts). Finally, the metabolites of another two soil-dwelling bacterial-feeding nematodes (*Mesorhabditis* sp. and *Acrobeloides* sp.) and two soil-dwelling bacteria (*B. amyloliquefaciens* JX1 and *A. pascens* ZZ21) were employed to explore the universality of this interaction. We speculated that the metabolites of nematodes play a major role in inducing the IAA biosynthesis of these bacteria, and this maybe a universal interplay between bacterial feeding nematodes and IAA-producing bacteria, at least in our study system.

**Results**

Same with the results of previous study, all the three tested bacteria showed an increased ability to synthesize IAA in the presence of *C. elegans*, especially in the middle and late stages of cultivation (5th and 7th days) (Fig. 1). As we can see from Fig. 1, *A. pascens* ZZ21 was more sensitive to *C. elegans* than
B. amyloliquefaciens JX1 and A. chlorophenolicus L4. For A. pascens ZZ21, the maximum amount of increased IAA appeared on day5, but for another two bacteria species, it appeared on day7. Besides, the maximum amount of IAA of A. pascens ZZ21 was more than other two species.

In another way, our results demonstrated that the metabolites of C. elegans could promote the IAA synthesis of three IAA-producing bacteria. While, it showed different pattern for different bacteria species, but same as the direct action of C. elegans (Fig. 2). B. amyloliquefaciens JX1 and A. pascens ZZ21 began to increase IAA synthesis on day2, and continued until the third day. While, for A. chlorophenolicus L4, there was no uniform pattern for their IAA synthesis over time. What's more, the amount of IAA synthesized by A. pascens ZZ21 increased with incubation time, but not for B. amyloliquefaciens JX1 and A. chlorophenolicus L4. Based on this, we selected A.pascens ZZ21 and C. elegans extracts to further validate the reactive patterns of metabolites on bacterial IAA synthesis. It turned out that the extracts of C. elegans also accelerate the IAA synthesis of A. pascens ZZ21, and increase with incubation time (Fig. 3).

The two soil-dwelling bacteria (B. amyloliquefaciens JX1 and A. pascens ZZ21) showed a similar promotion of IAA synthesis by metabolites of Mesorhabditis sp. and Acrobeloides sp. with that of C. elegans secreted metabolites. The only difference was the reactive time of these two bacteria. For C. elegans, both bacteria began to react on day2 and continued to day3 (Fig. 2), while for Mesorhabditis sp., they showed a strong reaction at the beginning, and the difference did not disappear until the third day (Fig. 4). For Acrobeloides sp., both bacteria began to response until the 3th day (Fig. 4). Moreover, the same bacteria reacted differently to the metabolites of different nematodes. For example, A. pascens ZZ21 began to increase the IAA synthesis on the first day when incubated with metabolites of Mesorhabditis sp., but did not begin until the third day when incubated with metabolites of Acrobeloides sp.

Besides, the levels of bacterial IAA production increased with no significant effect on the growth of bacteria after the metabolites of bacterial-feeding nematodes were supplied to the medium, expect A. chlorophenolicus L4 on day1 (Fig. 5). These results suggested that the increase of IAA content in the medium was not caused by the increase of the quantities of bacteria.

**Discussion**

**Bacterial-feeding nematodes metabolites promote bacterial IAA synthesis**

Understanding the extremely complex interactions between soil fauna and microorganisms is important to explore how soil ecosystems function and maintain stability (Huang et al. 2019; Ludewig et al. 2019; Akduman et al. 2020). Although predation directly mediate the interaction between organisms, metabolites also play important roles, like mediating their communication, protecting themselves from toxic substances (Karlovsky 2008; Liebeke et al. 2015). Our study demonstrated that the metabolites of bacterivorous nematodes accelerated the IAA synthesis of IAA-producing bacteria without significantly altering the quantities of bacteria (Fig. 2, Fig. 4 and Fig. 5). This result was similar with our previous work
(Cheng et al. 2016) and those of Vandeputte et al. (2005), who reported that the leafy galls extracts significantly increased the level of IAA production of *R. fascians* strain D188-5 with no major effect on the growth of the strain. Our result means that in addition to feeding behavior, bacterial-feeding nematodes could stimulate the IAA synthesis of bacteria by non-feeding behavior, and this provides new evidence to support the important role of nematode metabolites. However, we did not identify the active components in metabolites, which requires further exploration.

The metabolites of *C. elegans* mainly contain organic acids, amino acids, sugars and pheromones, and some of these metabolites have been confirmed to regulate the development and social behaviors of nematodes (Kaplan et al. 2009; Reilly and Srinivasan 2019). For example, nacq#1, which is an N-acylated glutamine, could promote onset of sexual maturity of *C. elegans* hermaphrodites (Ludewig et al. 2019). Ascarosides, the pheromone secreted by plant-parasitic nematodes, can be recognized by plants to modify their immune system to reduce infection (Manohar et al. 2020). Nevertheless, we do not know whether these metabolites could affect the metabolism of microorganism that interact with nematodes. Our results proved that the metabolites of bacterial-feeding nematodes could stimulate the metabolism (synthesis of IAA) of IAA producing bacteria. Moreover, for IAA-producing bacteria, the synthesis of IAA are affected by numerous factors, like environmental and genetic conditions (Spaepen et al. 2007; Patten et al. 2013; Molina et al. 2018). In this study, we controlled the conditions except for the treatments, so we speculated that the IAA-producing bacteria recognize the active ingredients of metabolites, and elicit a series of gene expression that enhance the synthesis of IAA. Of course, many further rigorous experiments are needed to confirm this speculation.

**Stimulatory effects of bacterial-feeding nematodes metabolites on bacterial IAA synthesis vary with nematode and bacteria species**

In our study, we found the stimulatory effects of bacterial-feeding nematodes metabolites on bacterial IAA synthesis maybe a universal interplay between bacterial-feeding nematodes and IAA-producing bacteria, at least in our study system. However, the stimulatory effects of metabolites varied with the species of nematodes and bacteria. Our findings are similar to a previous study, which different species of bacterial-feeding nematodes and IAA-producing bacteria led to the difference of IAA content in soil (Cheng et al. 2011). The difference in the diversity, property, action concentration and activity of metabolites prompt a complex communication network of soil system, which maintain the dynamic balance of the system (Karlovsky 2008; Reilly and Srinivasan 2019; Schmidt et al. 2019). The bacteria responded much faster to metabolites of *C. elegans* and *Mesorhabditis* sp. than *Acrobeloides* sp. This may due to *C. elegans* and *Mesorhabditis* sp. belong to c-p1 ‘enrichment opportunists’, while the *Acrobeloides* sp. are c-p2 ‘general opportunists’, which give rise to higher rates of metabolism of *C. elegans* and *Mesorhabditis* sp. (Bongers and Ferris 1999). It is reasonable to speculate that the difference in metabolic rate of nematodes leads to the difference in metabolite concentration within same time, which leads to the difference in react time of IAA-producing bacteria. Even so, we cannot rule out other factors that affect the activity of IAA-producing bacteria, like the diversity and activity of metabolites in different nematodes.
In addition to the differences in the characteristics of nematodes, the quality of bacteria and their sensitivity to external stimulus may also induce the different reactive pattern to metabolites. For example, *Yersinia pestis* can form biofilms on the body and head of *C. elegans* to inhibit the feeding of nematodes (Darby et al. 2002). While, in *Pseudomonas* spp., they can also resist predation by *C. elegans* with secreting toxic metabolites that kill nematodes (Neidig et al. 2011; Nandi et al. 2015). The IAA synthesis of bacteria is a complicated metabolic network, which modulated by many transcription factors, enzymes and intermediates (Spaepen et al. 2007; Duca et al. 2014; Garcia et al. 2019; Liu et al. 2019). Furthermore, the location of IAA biosynthesis genes in the genome, the expression mode of IAA synthesis genes and different transcriptional regulators are diverse in different bacteria (Spaepen et al. 2007; Patten et al. 2013; Duca et al. 2014). Therefore, the differences in reaction patterns and time of synthesis of IAA may be due to the fact that metabolites of nematode stimulate different synthesis pathways in three different soil-dwelling bacteria, although we do not know the exact mechanism.

**Theoretical implications for soil ecological functions governed by nematodes metabolites**

The metabolites of soil nematodes contribute to modulate surrounding environment, conduct intraspecific and interspecific communications, regulate their behaviors, growth and development as well as affect other organisms (Braendle 2012; Kaplan et al. 2012; Srinivasan et al. 2012). However, most of these studies focused on the effects of nematode metabolites on the nematodes themselves or on the communication between nematodes and plants, and little about the effects on bacterial IAA synthesis (Manosalva et al. 2015; Ludewig et al. 2019; Rwilly et al. 2019). To some extent, our research makes up for this gap, albeit in laboratory conditions, which are much simpler than the natural soil environment.

In the natural soil system, the factors that influence the IAA synthesis are much more complicated, such as the content of organic matter, the distribution of nematode metabolites. We do not rule out the possibility that the effect of nematode metabolites on the bacterial IAA synthesis differ in the mineral liquid medium and in a natural soil environment. Nevertheless, the mineral liquid medium method avoids many confounding factors such as uneven distribution of bacteria and nematode metabolites, and the influence of other compounds on the IAA synthesis of bacteria, focusing on the effects of nematodes metabolites on bacterial IAA synthesis. The IAA productions of these tested bacteria increased in the presence of bacterial-feeding nematodes, the metabolites or the extracts of nematodes. Theoretically, these results imply that the metabolites of bacterial-feeding nematodes may increase the IAA content in soil and stimulate the plant growth, similar with the results of previous studies (Mao et al. 2007; Cheng et al. 2011; Xu et al. 2015; Jiang et al. 2020). This possibility merits further studies in more realistic soil environments.

**Conclusions**

Our results showed that the metabolites of nematodes could promote the IAA biosynthesis of bacteria, which means that in addition to feeding behavior, bacterial-feeding nematodes also function in the nematodes-bacteria interaction system by non-feeding behavior. These results offer insights into the
ecological function of nematode metabolites. We propose that further studies should be performed to explore the regulatory mechanism of nematodes metabolites on IAA biosynthesis of bacteria, including the response of genes or pathways related to IAA synthesis in bacteria to nematode metabolites and identifying the active ingredients derived from the metabolites of nematodes.

**Methods And Materials**

**Preparation of nematodes, their secreted metabolites and extracts**

*C. elegans* N2 was obtained from the CGC (Caenorhabditis Genetics Center), and two soil-dwelling bacterial-feeding nematodes *Mesorhabditis* sp. and *Acrobeloides* sp. were obtained from the alluvial soil from Banqiao Town, Nanjing City, Jiangsu Province, China (Liu et al. 2018).

The nematodes were cultivated at 20 °C on freshly prepared nematode growth medium (NGM) (3 g NaCl, 2.5 g peptone, 17 g agar and 975 mL H₂O were autoclaved; when cooled to 55 °C, supplied with 25 mL 1 M KPO₄ buffer (pH 6.0), 1 mL 1 M CaCl₂, 1 mL 1 M MgSO₄ and 1 mL 5 mg/mL cholesterol in ethanol, which were filtered through 0.22-μm filter). The worms were usually fed with *Escherichia coli* OP50, a standard food for bacterial-feeding nematodes in laboratory, or other three IAA-producing bacteria when required. After reached the desired stage, the worms were harvested from the petri plates with M9 buffer (5 g NaCl, 3 g KH₂PO₄, 6 g Na₂HPO₄, 1 mL 1 M MgSO₄, H₂O to 1 L autoclaved) and collected by the modified Baermann funnel method (Liu et al. 2008). To remove the medium and bacteria from the nematode cuticle, the worms were washed and centrifuged three times with M9 buffer. They were then placed in M9 buffer for 30 min at 22 °C at 250 rpm to digest the bacteria in their guts. After washing three times by sterile water, the worms were collected for further experiments. All the operations were ensured sterility.

For collecting nematodes-secreted metabolites, the worms need to be incubated in sterile water for 1 hour at 22 °C at 250 rpm with a density of ~15000 worms/mL. Then, the metabolites were collected by gentle centrifugation, filtered through 0.22-μm filter, lyophilized and stored at -80 °C. The metabolites secreted by one worm in 1 hour is defined as 1 worm equivalent (WE) (Srinivasan et al. 2008). For collecting nematodes extracts, the worms were incubated in sterile water for 1 hour at 22 °C at 250 rpm and then broken up by Ultrasonic Cell Crusher (XO-1000D) with 60 % duty ratio (working 3s with rest 3s) for 45 min. Then, the extracts were collected by centrifugation, filtered through 0.22-μm filter, lyophilized and stored at -80 °C.

**Bacteria and culture conditions**

Three IAA-producing bacteria used in this study were *Bacillus amyloliquefaciens* JX1, *Arthrobacter pascens* ZZ21 and *Arthrobacter chlorophenolicus* L4 from our laboratory (Table 1). *B. amyloliquefaciens* JX1 was isolated from the same alluvial soil with the two soil-dwelling nematodes (Yu et al. 2015). *A. pascens* ZZ21 was isolated from the forest soil from Zijin Mountain in Nanjing City, Jiangsu Province, China (Li et al. 2018). *A. chlorophenolicus* L4 was isolated from the red soil from the Red Soil Ecological...
Station of Chinese Academy of Sciences. \textit{E. coli} OP50 was obtained from the CGC. These three IAA-producing bacteria were inoculated into mineral liquid medium (5 g glucose, 2 g (NH$_4$)$_2$SO$_4$, 0.5 g NaH$_2$PO$_4$, 0.5 g K$_2$HPO$_4$, 0.2 g MgSO$_4$·7H$_2$O and 0.1 g CaCl$_2$·2H$_2$O with 1 L H$_2$O at pH 7.0) at 1% (V/V) for 3 days. 200 mg/L tryptophan was added to the medium to stimulate bacterial IAA synthesis.

Table 1 Descriptions of the IAA-producing bacteria used in the study

| Bacteria                     | Gram strain | Accession number |
|------------------------------|-------------|------------------|
| \textit{B. amyloliquefaciens} JX1 | G+          | JX424611         |
| \textit{A. pascens} ZZ21       | G+          | KF515608         |
| \textit{A. chlorophenolicus} L4 | G+          | JQ277449         |

Detection of the effect of bacterial-feeding nematodes and their metabolites on bacterial IAA synthesis

Bacterial IAA production affected by \textit{C. elegans}

The nematode \textit{C. elegans} and three IAA-producing bacteria (\textit{B. amyloliquefaciens} JX1, \textit{A. pascens} ZZ21 and \textit{A. chlorophenolicus} L4) were employed to investigate the effect of nematodes on bacterial IAA synthesis. \textit{C. elegans}, fed with the corresponding IAA-producing bacteria, were added to medium at 80 worms/mL (the amount of nematodes added was based on the experimental results of Jiang et al. (2016)). After that, the medium were moved to the incubator set at 22 °C for 7 days (the suitable incubating temperature and time for the growth of nematodes). The groups without nematodes were controls. The concentration of IAA in the medium was detected on day1, 2, 3, 5 and 7.

Bacterial IAA production affected by \textit{C. elegans}-secreted metabolites and extracts

The nematodes \textit{C. elegans} and three IAA-producing bacteria mentioned above were employed to investigate the effect of nematodes-secreted metabolites on bacterial IAA synthesis. \textit{C. elegans}-secreted metabolites were added to medium at 2000 WE/mL (equivalent to the metabolites that secreted by 80 worms for 1 day). Then, the medium were moved to the incubator set at 30 °C for 3 days. The groups without \textit{C. elegans}-secreted metabolites were controls. The concentration of IAA in the medium was detected every day.

In order to facilitate the enrichment of nematode metabolites, another ultrasonic fragmentation method was used to collect the metabolites (called extracts). Based on the results of above experiments, \textit{C. elegans} and \textit{A. pascens} ZZ21 were selected to investigate the effect of nematodes extracts on bacterial IAA synthesis (see results). The extracts of \textit{C. elegans} were added to medium 2000 WE/mL. After that, the medium were moved to the incubator set at 30 °C for 3 days. The groups without \textit{C. elegans} extracts were controls. The concentration of IAA in the medium was detected every day.

Bacterial IAA production affected by soil-dwelling bacterial-feeding nematodes-secreted metabolites
Besides, we selected two soil-dwelling bacterial-feeding nematodes (*Mesorhabditis* sp. and *Acrobeloides* sp.) and two IAA-producing bacteria (*B. amyloliquefaciens* JX1 and *A. pascens* ZZ21) to investigate the effect of soil-dwelling nematodes-secreted metabolites on bacterial IAA synthesis. Soil-dwelling bacterial-feeding nematodes-secreted metabolites were added to medium at 2000 WE/mL. Subsequently, the medium were moved to the incubator set at 30 °C for 3 days. The groups without nematodes-secreted metabolites were controls. The concentration of IAA in the medium was measured every day.

**Quantification of IAA Levels**

IAA levels in the supernatants of samples were measured spectrophotometrically (Gordon and Weber 1951). The supernatants were equal-ratio mixed with Salkowski reagent (50 mL 35% HClO$_4$ combined with 1 mL 0.5 M FeCl$_3$). After incubating in darkness for 30 min at room temperature for color development, the absorbance was measured at 530 nm by spectrophotometer. The concentration of IAA was calculated by comparing the absorbance with a standard curve constructed with the known concentrations of IAA. Meanwhile, the OD$_{600}$ was determined at each test time.

**Statistical analysis**

Data were analyzed by SPSS 22.0 statistical software. One-way analysis of variance (ANOVA) was performed to analyze the abilities of bacteria to synthesize IAA under different treatments. Duncan’ test was used to assess the significant differences among the means (*P*<0.05). All figures were performed using Origin.

**Declarations**

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Figures

Figure 1

Effects of C. elegans on the abilities of different bacteria to synthesize IAA. The species of IAA-producing bacteria are labeled on the upper left corner of the box. The asterisk indicates a statistically significant difference (* P < 0.05, ** P < 0.01, *** P < 0.001). Data are expressed mean ± SE.
Figure 2

Effects of C. elegans metabolites on the abilities of different bacteria to synthesize IAA. The species of IAA-producing bacteria are labeled on the upper left corner of the box. The asterisk indicates a statistically significant difference (*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\)). Data are expressed mean ± SE.

Figure 3

Effects of C. elegans extracts on the ability of A. pascens ZZ21 to synthesize IAA. The asterisk indicates a statistical significant difference (**\(P < 0.001\)). Data are expressed mean ± SE.
Figure 4

Effects of soil-dwelling bacterial-feeding nematodes secreted metabolites on the abilities of different bacteria to synthesize IAA. The species of IAA-producing bacteria are labeled on the right hand axis, and the nematodes species were labeled on the top box. The asterisk indicates a statistical significant difference (* P < 0.05, ** P < 0.01, *** P < 0.001). Data are expressed mean ± SE.
Figure 5

Effects of bacterial-feeding nematodes secreted metabolites on the growth of bacteria. The species of IAA-producing bacteria are labeled on the right hand axis, and the nematodes species were labeled on the top box. The asterisk indicates a statistical significant difference (* P < 0.05). Data are expressed mean ± SE.