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RESEARCH PAPER

Ensilaged biostimulants promoting root health and control of *Radopholus similis* in banana (*Musa* AAA) cv. Grande Naine

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Abstract This study evaluates plant growth promotion and the suppressive effect of the burrowing nematode (*Radopholus similis*) in banana *Musa* AAA cv Grande Naine, by two ensilaged biostimulants (EBSs) in two greenhouse trials and in two different commercial farms. Conductive (CS) and suppressive (SS) soils to plant parasitic nematodes were used for EBSs production. The EBSs were incorporated in the growth substrate at 10% w/w before planting the in vitro banana plants and before *R. similis* inoculation in the greenhouse trials. In commercial banana plantations, the treatments were applied every four months by incorporating 500 g of the EBS into the soil in front of the successional sucker of each banana plant. The results showed that both EBSs were effective, stimulating the root growth and reducing *R. similis*. The EBS with CS reduced *R. similis* consistently in greenhouse and field evaluations. The data suggests the potential of EBSs to promote unfavorable conditions for the burrowing nematode reproduction and more favorable conditions to the development and production of the crop, which could contribute to promote more sustainable banana production.

Keywords Fermentation · Plant parasitic nematodes · Root health · Soil suppressiveness

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Introduction

According to the Food and Agriculture Organization of the United Nations [FAO] (2020), bananas are the fruits with the biggest international consumption. During 2020, the global banana market generated more than US$22.8 billion. However, this crop faces serious phytosanitary problems that place its global production at risk. Such is the case of the new variant of "fusariosis" whose causal agent is the phytoparasitic fungus *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Dita et al., 2018). Banana is also affected by plant parasitic nematodes, such as *Radopholus similis*, *Helicotylenchus multicinctus*, *Meloidogyne* spp. and *Pratylenchus coffeae* (Araya, 2004). These pathogens could destroy the banana root system with their stylet, causing plant collapse or severely reducing bunch weight (Araya & Vargas, 2018; Bechem et al., 2018; Jaramillo et al., 2019; Thammaiah et al., 2019). Chemical nematicides are the most common tools to control nematodes in this crop (Vargas et al., 2006; Aguirre et al., 2016). Currently, the international banana market demands fruits with less chemical residues, grown more environmentally friendly and with safer conditions for labor workers. In the medium long term, these commercial and environmental requirements could partially or totally restrict the use of nematicides (Das and Borgoain, 2018).

Most of the banana-producing areas in the world have favorable or conductive soil conditions for the reproduction and establishment of *R. similis* (Aguirre et al., 2016). On the other hand, in some banana-producing regions, farms have also been found where throughout the year the populations of *R. similis* remain well below the economic threshold of 8000 individuals per 100 g of root (Zum Felde et al., 2005; Vargas et al., 2012; Montenegro, 2013). The type of soil in these areas is called "suppressive", since plant parasitic nematodes do not survive in them despite the existence of favorable environmental conditions and the presence of both a susceptible host and a virulent inoculum (Cook & Baker, 1983). Such suppressiveness can be attributed to biotic or abiotic factors associated with the rhizosphere (Akhtar et al., 2015, Steinberg et al., 2019). An important characteristic of these soils is that, when sterilized, they lose their suppressiveness (Mendes et al., 2011; Vargas et al., 2012). In some cases, suppressiveness could be transferred to conductive zones by removal and transport of soils between areas (Westphal, 2005); however, for banana producers, this would not be profitable. On the other hand, detecting and extracting biological control agents (BCA) from suppressive soils is limited, because the majority of soil microbiota (99%) cannot be cultivated in artificial media (Paul, 2015).

Currently, the main integrated nematode management strategy is based on increasing local biological diversity to stimulate the natural suppressive potential present in most agricultural soils (Stirling 2014., Akhtar et al., 2015, Steinberg et al., 2019). It is assumed that greater biological diversity leads to greater natural suppressiveness, since pathogens face greater antagonism, predation, and competition with other local biotic communities seeking nutrients and energy (Westphal, 2005). The stimulation of suppressiveness could be obtained through the addition of different organic amendments and carbon sources enriched with BCA (Stirling, 2014). In this sense, various cultural practices, including the use of cover and rotation crops, organic and mineral fertilizers, dolomitic lime, as well as reduced tillage systems, could improve the quality and health of the soil and its potential capacity to suppress root pathogens (Abawi & Widmer, 2000; Segura et al., 2021; Segura-Mena et al., 2021). The use of amendments with higher concentrations of beneficial microbes produced by fermentation during a silage or composting process, could help control soil-borne diseases including fusarium wilt disease in banana (Shen et al., 2013; Zhang et al., 2014). During the silage process, organic acids are synthesized by fermentation of the sugar present in the molasses, which reduces the pH. This low acidity preserves the biostimulant, stimulates beneficial microbiota and reduces pathogens, especially phytopathogenic bacteria. Besides the potential multiplication of biocontrol agents, this type of fermentation induces the multiplication of lactic bacteria, recently detected as helpful in the control of plant-endoparasitic sedentary nematodes such as *Meloidogyne* spp. (Seo et al., 2019). However, many of these ecological-friendly measures have not been studied in detail to control species with mobile endoparasitic ecology such as *R. similis*.

The incorporation of EBSs near the rhizosphere in conductive soils, could increase microbiological diversity and suppression of *R. similis* in the rhizoplane, while increases the organic matter content closer to the roots. These organic fermentation techniques are currently used in banana organic farming in Costa Rica with successful results in the reduction of diseases and an increase in growth of banana plants. These favorable

results in organically cultivating farms, prompted us to study this technique in two identical experiments in greenhouse and two identical experiments in commercial plantations, in order to determine if the application of suppressive or conductive soils from the ensilage processes, reduce *R. similis* populations and improve plant growth in banana crops.

Materials and methods

Ensilaged bio-stimulant preparation for greenhouse and field experiments

Both EBSs were developed through an adaptation of the methodology of Torres Pérez et al. (2022). Said adaptation consisted of replacing the forest mulch that the researchers used as a biological additive for silage fermentation, with filtered aqueous suspensions 1/7 v/v of native soil (conductive or suppressant) and water. The remaining procedure was followed according to the indicated methodology.

Nematode inoculum preparation for greenhouse experiments

The population of *R. similis* were isolated in a commercial banana farm from the Siquirres Canton, Costa Rica. The roots were liquefied and sifted according to Taylor and Loegering (1953). A sample of the liquefied material (200 g) was taken to extract *R. similis* using the modified Baermann funnel technique (Hallmann & Subbotin, 2018). Slides with nematodes were checked using a binocular microscope and the morphological description of Sikora et al., (2018) was used for *R. similis* identification. The collected nematodes were reproduced in carrot disks according to the protocol of Speijer & De Waele (1997). To preserve active *R. similis* specimens, individuals were transferred to new carrot discs every 3 months and kept at 25 °C for reproduction.

Greenhouse experiments

The experiments were established in a greenhouse of the Research Center at the National Banana Corporation (CORBANA), Costa Rica. (10° 15′ 54″N and 83° 46′ 26″W). The first experiment was established between January and April 2020, with potting soil from Guácimo Canton, Limón Province (Soil 1). The second was carried out between April and July 2020 with soil from Siquirres Canton, Limón Province (Soil 2). Soil 1 consisted in 78% sand, 4% silt and 18% clay. Each 100 g of Soil 1 contained 250 *R. similis*, 1250 *H. multicinctus*, 500 *Megalaima incognita* and 250 *P. coffeae* individuals. The Soil 2 had 38% sand, 24% silt, and 38% clay. Each 100 g of this soil contained 125 *R. similis*, 125 *H. multicinctus*, 125 *M. incognita*, and no *P. coffeae* was found. The texture of the substrates was measured by an adaptation of the Bouyoucos methodology (Beretta et al., 2014). The Baermann funnel was used for soil nematodes extraction (Cesarz et al., 2019).

The treatments were as follows: T1: Untreated control, T2: Nematicide Oxamyl 24% of a.i. in liquid suspension (LS) (100 ppm a.i. /plant, applied 15 days after transplant), T3: EBS with SC (128 g of DM/plant) and T4: EBS with SS (128 g of DM/plant). The experimental design was completely randomized with 15 replications per treatment. To apply EBSs treatments, the unsterilized soil substrate was mixed with EBS CS or EBS SS at 10% w/w, respectively. Seven days after, the plastic pots of 1.8 L were filled with the prepared substrates and a phase IV in vitro banana plant (plant 10 cm high and three developed leaves) was planted in each pot (Fig. 1A). Each pot was inoculated 15 days after transplanting with approximately 500 nematodes (adults and juveniles) of *R. similis*.

Twenty days after planting, as preventive control of black sigatoka (*Pseudocercospora fijiensis Morelet*) Mancozeb at 60% of a.i. in encapsulated suspension (ES) (30 g a.i./L) was applied every 15 days. Fertilization was done once a week with Hoagland solution (100 ml/pot).

The experiments ended 90 days after sowing. For data collection, fresh weight (g) of root, pseudostem and foliage was measured using an electronic balance as well as the height (cm) from the base to the point of intersection between the sheaths of the last two leaves and the diameter of the pseudostem (cm) at the base of each plant. Also, the number of *R. similis*, *H. multicinctus*, *M. incognita*, *P. coffeae* were counted. Thereafter, total nematodes (sum of the four genera of nematodes) in 100 g per roots was calculated. Nematodes were extracted and quantified following Taylor and Loegering (1953).

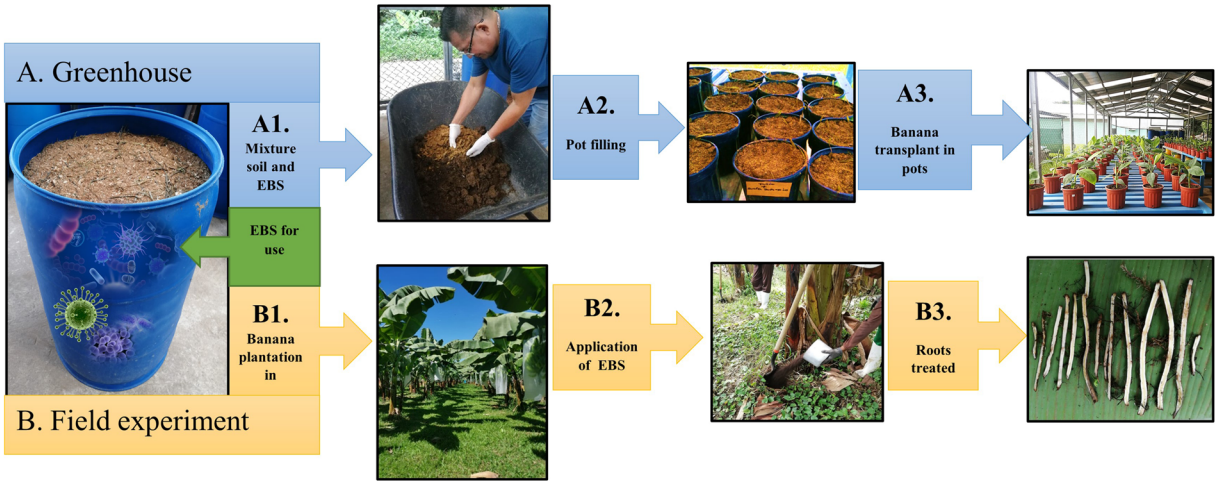

Fig. 1 General outline of the methodology for use of ensilaged biostimulant (EBS) in experiments of A) greenhouse and B) commercial banana plantation

Field experiment

Two identical experiments were set up in two commercials bananas fields. The first experiment was established between August 2020 and January 2021 in Guácimo Canton (10° 15′ 52″ N and 83° 38′ 15″ W) in soil with 78% sand, 4% silt and 18% clay of a 6-years-old banana crop. During the experimental period, this area registered a monthly average precipitation of 279.2 mm, 25.5 °C and a relative humidity of 87.4%. The second experiment was carried out between March and August 2021 in Siquirres Canton (10° 6′ 56″ N and 83° 29′ 25″ W) in soil with 51% sand, 42% silt and 7% clay of a 10-year-old banana crop. During the experimental period, this zone registered a monthly average precipitation of 316.4 mm, 25.8 °C of temperature and 90.5% of relative humidity. The texture of the soils was measured by an adaptation of the Bouyoucos methodology (Beretta et al., 2014). Both experimental areas: a) were planted with the Grande Naine cultivar (*Musa* AAA Cavendish subgroup) at a density of 1700 plants/ha, b) were divided in rectangular sections of land of approximately 25 × 50 m separated by drainage canals, where banana was planted.

The treatments consisted of T1: Untreated control, T2: EBS with SC (250 g of DM/plant) and T3: EBS with SS (250 g of DM/plant). The experiment was established under a randomized complete block design with 6 replicates. The experimental unit was formed in a plot with approximately 80 plants. One month before starting the experiment, all plants were injected with 1.2 g a.i. of Oxamyl at 24% in LS to standardize nematode populations throughout the experimental area. During the experimental period of 6 months, a total of two applications were made, one at the beginning of the experiment and one four months later. EBSs were incorporated into the soil near the root system of the youngest plant (sucker) (Fig. 1B). The total area of each experiment was 1.2 ha with 1440 plants. No nematicide molecules were included in either of the two field evaluations.

For data collection, repeated measurements were made each month for a total of 6 samplings for the monitoring of nematode populations and biometric variables of the root. The fresh weight of the total and functional root (g), percentage of functional root (%), which consists of the proportion of the weight in grams of the healthy root tissue with respect to the weight of the necrotic root, were measured. Root sampling was performed as follows: 4 recently flowered plants were selected from each plot. Roots of 6750 mL of soil collected in front of the sucker of each productive unit were subtracted. The production unit is made up of the pseudostem of three plants united in successional phenological phases. The roots collected from the 4 selected plants were pooled to form a composite sample per plot. The number of *R. similis*, *H. multicinctus*, *M. incognita* and total nematodes (summatory of the individuals of *R. similis*, *H. multicinctus* and *M. incognita*) in 100 g of root were also evaluated. In order to quantify the weight variables and the number of root nematodes, the method of Taylor & Loegering (1953) was used.

Statistical analysis

For the data from the greenhouse experiments, the biometric variables were analysed using analysis of variance (ANOVA) after normality evaluation. The number of each particular nematode genera was studied by analysis of deviance (McCullagh & Nelder, 1989) with $log(x + 1)$ as transformation function, where x is the dependent variable, and errors are assumed to be negative binomially distributed. For the analysis of the field experiments, average per plot across the monthly evaluations were obtained for every measured variable (including the nematode variables) and compared with ANOVA after normality evaluation. Treatments means were compared with the Tukey test for all the variables of all experiments. All the analyses were performed in *R* version 4.0.2 (R Core Team, 2014) with its "lm" function to perform the LM analyses, "glm.nb" function of the "MASS" package (Venables & Ripley, 2002) to perform the GLM analysis, and "emmeans" package (Rusell, 2021) to compare treatment means.

Results

Greenhouse experiment

In the first experiment, both EBSs showed a growth-promoting effect ($P \leq 0.0014$, Table 1) in plants inoculated with *R. similis*, which was observed with 32 and 36% more fresh foliar weight, 12 and 13% more height and 12 and 15% more pseudo stem diameter in the plants applied with CS and SS EBS, respectively, when compared to the control ($P \leq 0.05$, Table 1). The EBSs promoted greater growth in fresh foliar weight and height when compared to the nematicide ($P \leq 0.0500$, Table 1). In the second experiment, only EBS with CS promoted growth in all its variables with respect to the control, nematicide and EBS with SS ($P \leq 0.0075$, Table 1).

EBS with CS showed a suppressive effect on all nematodes reducing levels of *R. similis* between 76 and 57%, *H. multicinctus* between 71 and 69% and total nematodes between 73 and 64% in both repeated experiments, in comparison to the control ($P \leq 0.0500$, Table 2). While EBS with SS reduced *H. multicinctus* by 79%, and total nematodes by 61% in the first experiment, only *R. similis* was reduced by 60% in the second experiment when compared to the control ($P \leq 0.0500$, Table 2).

Field experiment

None of the EBSs promoted root growth expressed as fresh weigh or increased the percentage of functional roots in both experiments ($P \leq 0.7439$, Table 3). However, both EBSs showed nematode suppression of *R. similis* under field conditions. This is supported by the reduction of 48 to 53% and 31 to 43% of *R. similis* in the first and second experiment in comparison to the control ($P \leq 0.0500$, Table 4). The responses to *H. multicinctus* and total nematodes were variable depending on the site and the EBSs used. In Guácimo, the density of *H. multicinctus* were only reduced

Table 1 Growth promotion effect of plants infested with *R. similis* treated with ensiled biostimulants (EBS) prepared from either a conductive (CS) or a suppressive (SS) under greenhouse conditions

Treatment	TRIAL I: Guácimo soil				TRIAL II: Siquirres soil			
	Fresh roots weight (g)	Fresh foliar weight (g)	Height (cm)	Pseudo stem diameter (mm)	Fresh roots weight (g)	Fresh foliar weight (g)	Height (cm)	Pseudo stem diameter (mm)
Control[a]	31.33 a	126.88 a	30.64 a	18.76 a	9.76 a	31.21 a	13.86 ab	9.86 a
Nematicide[b]	32.65 a	135.04 a	30.13 a	19.75 ab	10.32 ab	35.53 a	14.53 b	10.29 a
EBS with CS	32.25 a	168.06 b	34.47 b	21.08 bc	12.24 b	52.17 b	17.53 c	12.13 b
EBS with SS	34.66 a	173.06 b	34.67 b	21.73 c	8.69 a	30.00 a	12.66 a	9.87 a
Standard error	1.53	5.00	0.99	0.40	0.61	1.84	0.58	0,31
Pr>F	0.4998	≤0.0001	0.0014	≤0.0001	0.0014	≤0.0001	≤0.0001	≤0.0001

Means in the same column sharing a common letter do not differ significantly according to Tukey test ($P > 0.05$). [a] Control: untreated plants, [b] Nematicide: 100 ppm of oxamyl (commercial formulation 24% liquid solution) by plant, EBS: applied to 10% (w/w)

Table 2 Effect of ensiled biostimulants (EBS) prepared from either a conductive (CS) or a suppressive (SS) soil on nematodes population on in vitro banana plant infested with *R. similis* under greenhouse conditions

Treatment	TRIAL I: Guácimo soil			TRIAL II: Siquirres soil		
	R. similis[b]	*Helicotylenchus multicinctus*[b]	Total nematodes[c]	*R. similis*[b]	*H. multicinctus*[b]	Total nematodes[c]
Control[a]	21,739±8748 b	4366±1407 b	29,533±7115 b	25,440±5325 b	23,970±4729 b	50,287±9372 b
Nematicide	6303±2248 ab	756±218 a	8355±1784 a	6750±3387ab	21,614±4119 b	38,878±7000 b
EBS with CS	5194±1790 a	1232±355 a	7735±1596 a	10,883±2364 a	7310±1393 a	17,882±3458 a
EBS with SS	9708±3345 ab	917±264 a	11,505±2374 a	10,188±2213 a	22,563±4300 b	32,443±6274ab
Pr>F	0.0256	≤0.0001	≤0.0001	0.0075	≤0.0001	0.0023

Means and standard error in the same column sharing a common letter do not differ significantly according to Tukey test ($P > 0.05$). [a] Control: Untreated plants, Nematicide: 100 ppm of oxamyl (commercial formulation 24% liquid solution) by plant, EBS: applied to 10% (w/w). [b] Number of nematodes (number/100 g of root) was transformed to $log(x + 1)$ prior to statistical analysis. [c] Total nematodes is the sum of the numbers of *R. similis*, *H. multicinctus*, *M. incognita* and *P. coffeae*

significantly under SS inoculated fields, but both EBSs reduced total nematodes. While in Siquirres, both EBSs were not effective to reduce density of *H. multicinctus* while EBSs with SS was able to reduce total nematodes.

Discussion

R. similis is one of the plant parasitic pathogens most difficult to control because its migratory endoparasitic behavior, which provides it with a type of armor within the root tissue. On the other hand, the semi perennial behavior of its host (banana), favors its constant reproduction and dissemination (Volcy, 2011; Cobon et al., 2019). This study showed that EBSs with CS had a suppressive effect on *R. similis* both at the two greenhouse experiments and at the two field evaluations. On the other hand, EBSs with SS were effective in reducing the endoparasite only at one of the two greenhouse evaluations, but they were effective in both field evaluations. In this sense, the native soil in EBSs (CS or SS) and/or the remaining microbiota after the silage process, showed an important interaction with the microbiota present at the soil manipulated for pot experiments (as it is the case for Guácimo soil for *R. similis* or Siquirres soil for *H. multicinctus*). Additionally, the inoculation levels of EBSs in all pot was 10% v/v, while at the field, soil levels are far lower and only for the sucker under each production unit. From the side of plant growth stimulation, the EBSs are made up of a diverse group of soil microbiota in which native microorganisms with biostimulant, biocontrol or biofertilizing qualities could be present. Such argument was evidenced on growth variables of the greenhouse evaluation, probably by the quantity of EBSs proportion with respect to the total soil (10% v/v). However, already under field conditions

Table 3 Growth promotion effect of plants treated with ensiled biostimulants (EBS) prepared from either a conductive (CS) or a suppressive (SS) under field conditions

Treatment	TRIAL I: Guácimo soil			TRIAL II: Siquirres soil		
	Total root (g)	Functional root (g)	Functional root (%)	Total root (g)	Functional root (g)	Functional root (%)
Control[a]	27.7 a	23.3 a	83.6 a	28.0 a	22.8 a	80.9 a
EBS with CS	24.3 a	21.1 a	86.2 a	29.9 a	24.7 a	83.1 a
EBS with SS	26.6 a	21.8 a	82.8 a	29.7 a	24.6 a	82.9 a
Standard error	1.86	1.42	2.17	1.91	1.49	1.79
Pr>F	0.4326	0.5371	0.5245	0.7439	0.5902	0.4065

Means in the same column sharing a common letter do not differ significantly according to Tukey test ($P > 0.05$). [a] Control: untreated plants, EBS with CS or SS: 250 g dry material was applied per production unit (mother plant and sucker together).

Table 4 Effect of ensiled biostimulants (EBS) prepared from either a conductive (CS) or a suppressive (SS) soil on nematodes population in banana under field conditions

Treatment	TRIAL I: Guácimo soil			TRIAL II: Siquirres soil		
	R. similis[b]	*Helicotylenchus multicinctus*[b]	Total nematodes[b]	*R. similis*[b]	*H. multicinctus*[b]	Total nematodes[c]
Control[a]	33,221 a	2529 a	36,119 a	23,122 a	5281 a	28,436 a
EBS with CS	17,357 b	2148 ab	19,918 b	15,927 b	5700 a	21,638 ab
EBS with SS	15,702 b	1187 b	17,062 b	13,122 b	4406 a	17,706 b
Standard error	4019	278	5645	1763	852	2381
Pr>F	0.0147	0.0173	0.0094	0.0039	0.5616	0.0205

Means in the same column sharing a common letter do not differ significantly according to Tukey test ($P > 0.05$). [a] Control: Untreated plants, EBS: applied to 10% (w/w). [b] Nematodes (number/100 g of root) was transformed to $log(x + 1)$ prior to statistical analysis; [c] Total nematodes is the sum of the numbers of *R. similis*, *H. multicinctus*, *M. incognita* and *P. coffeae*

where there are multiple factors of production, the beneficial effects of EBSs on banana growth were probably dispersed.

To elucidate the mechanism that explains the EBSs nematode suppressive effect, it is necessary to consider the changes experienced by the native microbiota of SC and SS during their exposure time to three consecutive stages: a) during the silage process, b) in the rhizosphere after the incorporation of EBSs into the soil, and c) during their subsequent colonization of the rhizoplane, where the highest concentration of potentially nematode-suppressing and radical-stimulating microbiota is presumed to be present. Likewise, once the EBSs were incorporated into the soil, near the banana rhizosphere, a rapid biochemical interaction between the silage microbiota and its metabolites, with the biotic and abiotic factors of the soil, possibly began.

Since *R. similis* remains sheltered within the root environment, it seems likely that the suppression of the endoparasite is due to the activation of defensive mechanisms of the plant due to the effect of EBSs, or to some biological control agent present in the EBS that could act as an endophyte limiting the access or reproduction of the nematode within the intraradical environment (Poveda et al., 2020). The EBSs raw materials included melina sawdust (*Gmelina arborea*), carpet grass (*Axonopus compressus*), semoline, and molasses, which, once fermented, were converted into sugars, amino acids, enzymes, cellulose, lignin, and organic acids. The elicitors of the activation of these defense mechanisms could come from prebiotic molecules synthesized from the biological decomposition of raw materials formed at the EBSs process in the soil. Selby et al., (2016) presented evidence, that aqueous extracts of four ray grass (*Lolium perenne*) cultivars showed elicitor qualities in annual crops, demonstrating that such metabolites may affect soil-friendly environment for nematodes.

On the other hand, during the biological decomposition of EBSs, constituting materials near the root, native microbiota could have synthesized a series of by-products, metabolites, elicitors-effectors with prebiotic qualities near the rhizoplane, which could have promoted the suppression of *R. similis* (Bonanomi et al., 2020; Trabelsi & Mhamdi, 2013; Xue et al., 2015). Likewise, during the decomposition of these EBSs at the soil, different fungal growth was also observed at the applied area, which seems to be the interaction between the microbiota of the EBSs and the native microbiota of the soil. Besides, EBSs probably promoted phytohormone-producing microbiota, phosphorus and potassium solubilizers, as well as free-living nitrogen fixers with biofertilizing or biostimulant qualities that promoted healthier root tissue in the plant (Bonanomi et al., 2010). Even thought, EBSs with SC reduced *H. multicinctus* at the two greenhouse evaluations, the same were not effective at the field level. In contrast, EBSs with SS reduced *H. multicintus* in both greenhouse and field experiments. This effect on different plant-parasitic nematode species gave us the point that different mechanisms could be involved in the control of these nematodes with different ecological strategies (*R. similis* as migratory endoparasite and *H. multicintus* as migratory ectoparasite).

To understand in greater depth the mechanisms associated with the suppression and bio-stimulation of EBSs, new research phases are proposed. For example, include metabarcoding studies of the constitutive taxa of the biostimulants and their effect in the rhizoplane

microbiome at the field, during a defined period and in the presence of *R. similis*. Likewise, the study of different control mechanisms against the endoparasite as direct antimicrobial effect or the action of metabolites of the soil microbiota would be important. The search for alternatives to chemical control of the burrowing nematode in banana cultivation is urgently needed and must continue in near future in order to implement a sustainable crop production system.

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