Field management of *Rotylenchulus reniformis* on pineapple combining crop rotation, chemical-mediated induced resistance and endophytic bacterial inoculation

Authors: A. Soler[1ψ], P-A. Marie-Alphonsine[1], P. Quénéhervé[2], Y. Prin[3], H. Sanguin[4], P. Tisseyre[5], R. Daumur[1], C. Pochat[1], E. Dorey[1], R. Gonzalez Rodriguez[6], N. Portal[7], J. Smith-Ravin[8]

[1] CIRAD, UPR GECO, F-97455 Saint-Pierre, Réunion, France.
GECO, Univ Montpellier, CIRAD, Montpellier, France.

[2] IRD, UMR IPME, F-97285 Le Lamentin, Martinique, France

[3] CIRAD, UMR LSTM, F-34398 Montpellier, France.

[4] CIRAD, UMR BGPI, F-34398 Montpellier, France

[5] LSTM, Univ Montpellier, CIRAD, IRD, Institut Agro, Montpellier, France.

[6] Université Ciego de Avila, Centro Bioplantas, Cuba.

[7] Université Ciego de Avila, Cuba.

[8] Université des Antilles, Martinique

[ψ] corresponding author alain.soler@cirad.fr

Highlights:

- Eco-friendly pineapple cropping systems are potential alternative to pesticides
- Pineapple - Crotalaria rotations reduces *R. reniformis* infestation
- Induced systemic resistances were shown to be efficient on pineapple nematodes in field
- An endophytic bacteria *Bacillus* sp. GVS2 reduced *R. reniformis* multiplication .

Keywords: Induced systemic resistances, biocontrol, cover plant, crop rotation, integrated ecological management, plant parasitic nematodes.

Abstract:

In Martinique or La Réunion, French authorities recently banned the use of pesticides for the management of "soil-borne pathogens" on pineapple after several decades of intensive monoculture where the natural balance between beneficial and harmful communities of soil organisms has disappeared. Today, increasing infestation of pineapple by the nematode *R. reniformis* and other "soil-borne pathogens" causes severe damage to the crop. New cropping systems with innovative ecological nematode management are needed. An eco-friendly cropping system, which comprised rotation of sunn hemp (*C. juncea*), pineapple, a natural grass fallow and another cash crop, eggplant, consistently reduced the inoculum of

nematodes. Nematode populations were reduced by 86.4%, 82% and 46.5% respectively under pineapple, sunn hemp and grass fallow compared to infestations of eggplant (3,456 nematodes per 100g of soil) after several rotations. Integrating a chemical induction of systemic resistance or application of an endophytic bacteria recovered from pineapple roots, *Bacillus* sp. GVS2, helped maintain low populations of nematodes during the vegetative cycle. On two pineapple varieties, MD2 and Victoria (Queen), two different treatments were applied monthly in the field, methyl jasmonate (10^{-4} M, 10 ml per plant), or a suspension of *Bacillus* sp. GVS2 (10^{8} CFU.ml^{-1}, 10 ml per plant) isolated from pineapple roots. After eight months, the nematode populations were reduced respectively on MD2 and Victoria (Queen) by 58.3% and 50.3% with the methyl jasmonate and by 59.6% and 54.3% with the *Bacillus* sp. GVS2 compared to controls. Because of the efficiency of sunn hemp in reducing the inoculum of nematodes, no significant differences in vegetative growth were observed using the D leaf weight and the estimated root length densities (RLD). The potential of pest management through eco-friendly cropping systems for pineapple with a biocontrol for nematodes with no pesticide is discussed.

1. Introduction

In most production areas, pineapple is grown in intensive monocultural cropping systems based on the systematic application of pesticides. Among them, nematicides represent the main cost for the farmer and a strong threat to population health (Py et al., 1987; Sanewsky et al., 2018). These intensive systems no longer provide ecosystem services as regulatory functions that allow a natural balance between beneficial and harmful organisms. In response to the strong societal demand to reduce environmental pollution and health risks, local authorities are limiting the use of nematicide. As a result increasing nematode populations, mainly *R. reniformis*, were observed in French overseas territories (Massé et al, 2020; Soler et al., 2019). The challenge for the pineapple research is to design innovative cropping systems that respect the agroecology concept, i.e. that promote agriculture that preserves the environment.

Improving soil conditions using cover plants before planting pineapple is not a new concept. Wang et al. (2002) proposed several crotalaria species to be grown before pineapple. In Martinique, a rotation system for pineapple that includes *Crotalaria* species as *C. retusa* was proposed (Soler et al. 2016). However, *C. retusa* produces less biomass than other crotalaria species, along with high levels of pyrolizidins that resulted in severe toxicity for cattle and leading to select other less toxic species such as *C. juncea.*

Plants recognize the presence of pathogens and then trigger defense responses at cellular level using efficient systemic signaling mechanisms, induced systemic resistance (ISR) or systemic acquired resistance (SAR). This is now referred to as the immune system of plants (Villena et al. 2018) that allows plants to develop a strategy of ´primed defenses'. Primed plants are in a state of enhanced alert that will allow a faster and stronger reaction to pathogen attacks than in unprimed plants (Li et al., 2020; Conrath, 2011; Conrath et al., 2006).

The ISR signaling pathway is involved in interactions between the plant and necrotrophic pathogens but also in mutualistic interactions between the plant and PGPR (De Vleesschauwer and Höfte 2009). The host-associated microbiome plays a major role in maintaining plants in good health by adjusting the jasmonic acid and ethylene signaling pathways. ISR induction may protect aboveground tissues of both dicots and monocots from necrotrophic pathogens, but the original infestation by the pathogen must not be too challenging for the plant (Rodriguez et al. 2019; Walters et al. 2013; Wasternack 2014).

PGPR rhizobacteria may exhibit their biocontrol capacity through ISR by inhibiting the growth of plant pathogens, including nematodes, in addition to other direct and/or indirect effects on plant growth (Majeed et al. 2018; Singh 2018). Many PGPR rhizobacteria related to the *Bacillus* group are promising biocontrol tools against pathogens (Choudhary and Johri 2009; Kloepper et al. 2004; Thakur and Singh 2018; Fira et al., 2018). Pineapple plants primed with methyl jasmonate (ISR) showed strong down-regulation of the multiplication of nematode populations (Soler et al. 2013). Based on the results of previous studies, we concluded that pineapple nematodes could be managed efficiently without using pesticides combining two agricultural practices. First, an eco-friendly cropping system based on pineapple / sunn hemp (*C. juncea*) rotation to reduce the initial biotic stress on pineapple plants (inoculum of nematodes). Then, the induction of systemic resistance by applying a chemical elicitor, or using pineapple endophytic bacteria to help maintain low nematode populations under pineapple. In their recent review article, Abd-Elgawad and Askary (2020) suggested developing similar approaches after reviewing the factors that determine the success of biological agents in controlling plant-parasitic nematodes.

2. Material and Methods

2.1. Planting material and Design of the eco-friendly cropping system for pineapple.

Young suckers of two pineapple varieties, MD-2 and Victoria (Queen), were used as planting material (average weight 250 g for MD-2 and 180 g for Queen).

The eco-friendly cropping system was established in a field representative of the area pineapple production with young volcanic andosol with ~34% of thin soil and gravels, mainly pumice. The design included two blocks each containing four plots. As preliminary soil cleaning, each plot was first planted with *Crotalaria retusa,* then *Crotalaria juncea* for one year. Then a rotation system without pesticide was established in each block for two cycles. Each of the four plots in the 2 blocks was planted successively by sunn hemp as a controlled fallow, then pineapple followed by a spontaneous grass fallow, followed by eggplants (rotation system). A block showed the four crops grown simultaneously, one per plot. Each plot received the complete sequence of the rotation (four crops) after four cycles. Every year, all the crops were destroyed and the residues incorporated in the soil as a preparation for the next step of the rotation. For every new cycle, each plot was planted with a different crop according to the sequence as indicated before. Pre-planting fertilizer was applied under the plastic mulch at the dose as recommended in Martinique (30 g.plant^{-1} of dolomite and 25 g.plant^{-1} of a compound 12N-4P_2O_5-24K_2O-6MgO). Subsequently, during vegetative growth, the plants received foliar fertilizer applications (0.5 g.plant^{-1} of urea and 0.5 g.plant^{-1} sulfate of potash during the first four months, then 1 g.plant^{-1} up to eight months). Eggplants planted on plastic mulch received the same pre-planting fertilizer as pineapple.

Symphylids were not a problem in this particular field, which is located at sea level, because in Martinique their populations grow mainly at higher altitudes where temperatures are cooler and soil humidity higher (personal observation). This allowed us to target only one "soil-borne pathogen" (nematode).

2.2. Isolation and identification of endophytic bacteria from pineapple root system.

A collection of 51 endophytic bacteria strains were isolated from the three pineapple cultivars, Victoria (Queen), MD-2 and Smooth Cayenne, in Martinique. After careful washing in water, 2 cm root fragments were surface-sterilized with 0.5 % NaOCl for 5 min. After rinsing in sterilized water, they were manually ground in sterilized water, and serially diluted up to 10^{-5} in water. The 10^{-3} to 10^{-5} bacterial suspensions were plated on N-free medium (Dimargon, Debereiner et al., 1976) at 28°C for 7 days to select for diazotrophic strains. The isolates were identified by phylogenetic analysis of partial 16S rRNA gene sequences according the following protocol.

Lysate cell suspension containing genomic DNA from individual isolates was obtained by suspending a colony in 100 µl sterilized water heating at 95 °C for 5 min and chilled on ice. Crude DNA extracts were stored at -20° C prior to polymerase chain reaction (PCR)

amplification. PCR amplification were performed using the primers FGPS1509 (5'-AAGGAGGGGATCCAGCCGCA-3') and FGPS6 (5'-GGAGAGTTAGATCTTGGCTCAG-3') as follows (25 µl reaction): 1X Green GoTaq® Reaction Buffer (Promega, Charbonnieres, France), 0.6 units of GoTaq® DNA Polymerase (Promega), 0.4 µM of each primer, 1 µl crude DNA extract, 160 µM of each dNTP, with the following cycling conditions: 94°C for 3min; 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 90 s; a final elongation step at 72°C for 10 min. PCR products were then sequenced using the primer 16S-1080r (5'-GGGACTTAACCCAACATCT-3') (Genoscreen, Lille, France).

Phylogenetic analysis of 16S rRNA sequences obtained from the isolates was performed in the MEGA X software package (Kumar et al., 2018) using the multiple alignment software MUSCLE, the maximum likelihood method, and bayesian estimation of the best-fitting model of molecular evolution. Nodal robustness of the tree was assessed by bootstrapping (500 replicates). 16S rRNA relatives from NCBI database corresponding to type material were used for the phylogenetic affiliation of isolates.

A *Bacillus* sp. GVS2 was pre-selected for its ISR capacities against *Rotylenchulus reniformis* on pineapple vitroplants in controlled conditions (data not shown), and was used for greenhouse and field experiments of ISR induction on pineapple cv MD-2 and Victoria (Queen). The sequences are available under the bioproject PRJEB38137 (https://www.ebi.ac.uk/ena/data/view/PRJEB38137).

2.3. Stimulating defenses in greenhouse and field conditions

2.3.1. Applications of methyl jasmonate or *Bacillus* sp. GVS2 on pineapple.

Pineapple defenses against the nematode were stimulated either through an application of methyl jasmonate (Mejasm) as ISR elicitor (input of 20 ml per plant 10^{-4} M on the soil), or by inoculating each plant with 10 ml of *Bacillus* sp. GVS2 (7 ml on the soil and 3 ml on the plant) at a concentration of 10^8 CFU ml^{-1}). An application of water (10 ml per plant) was used as control.

2.3.2. ISR and bacterial experiment in the greenhouse.

While still on their parental plants, suckers from three different groups of pineapple growing in a field nursery of MD-2 received three stimulations at four day intervals with the three treatments according to Soler et al. (2013). Then, samples of 20 suckers per treatment harvested one week after the last stimulation, were planted in 500 ml pots filled with andosol that had previously been sterilized in a Systec VE-150 sterilizer at 120 °C for 20 min in a greenhouse, and received one application of osmocote 5 g per plant (10N - $11P_2O_5$ -18K +

2MgO), and one application of foliar fertilizer Mairol® (1g.L^{-1}). After one month of root growth and with no further stimulation treatment other than those originally applied in the field, the plants were inoculated with 4,000 individuals.$plant^{-1}$of reared populations of *R. reniformis*. The increase in these populations was evaluated 45 days later to assess the impact of defenses induced by the elicitor treatment or bacterial applications.

2.3.3. ISR and bacterial experiment in the field.

The field was prepared for this experiment as follows: After two rotations of the eco-friendly cropping system, the plots previously planted with sunn hemp and eggplants in each block were used for the subsequent ISR experiments in the field. The pineapple suckers were first grown in greenhouse using the techniques described above. The pineapple suckers received the stimulating treatments only once after the first rooting (one month). Then, after one additional month of growth, the plants were transferred to the field where the treatments were again applied once a month (i.e. seven times before the 8^{th} month after planting).

The pineapple plots received 1,000 pre-treated plants in plot, half being MD-2 and the other half Victoria (Queen), planted in double rows. Each treatment modality was replicated twice in each block of the rotation system, giving a total of four replications per block for both pineapple cultivars. Additional rows of non-treated MD-2 were planted as borders around the plots. Within each block, the two plots not used for the ISR experiment were planted with sunn hemp to maintain a spatial arrangement corresponding to the rotation system, but the crotalaria-controlled fallow replaced the spontaneous grass fallow to provide the same 'environment' for the ISR plots in the two blocks while respecting the spatial arrangement of the eco-friendly cropping system. No pesticide treatment had been applied in the different plots since the original crotalaria plantation. The nematode populations were evaluated eight months after transfer to the field (meaning on nine-month old plants evaluated as the correct forcing time).

2.4. Evaluation of *R. reniformis* populations.

All *R. reniformis* populations were evaluated by counting the individuals (vermiforms) using a sieving-centrifugation method (Jenkins 1964). For soil sampling, 500 g samples were prepared with sub-samples of soil (~100 g) collected at in the root system the base of each plant. The sub-samples were then mixed and a 250 ml aliquot of soil formed the final sample from each plot for enumeration of nematodes. The nematode populations under each crop of the rotation were evaluated every 2 months during 2 rotations. The last count of the 2^{nd} rotation made in the experimental system was used as indicators of the initial inoculum for pineapple in the following ISR experiments, respectively high inoculum or low inoculum for

eggplants or sunn hemp. The nematode populations under pineapple in the ISR experiment were counted after eight months of growth.

2.5. Evaluation of pineapple growth in the ISR and bacterial experiment

Twenty-five 'D leaves' per plot were weighed to assess plant growth according to Py et al. (1987). Root length densities (RLD) were estimated according to Chopart et al. (2015) to assess soil exploration by the pineapple roots at eight months of age using Racine2 software (CIRAD). RLD were evaluated for each individual plot with three replicates corresponding to 3 soil profiles.

2.6. Statistical analysis:

As nematodes live in aggregated populations, statistics on nematode populations were made with non-parametric tests (Kruskal-Wallis non-parametric bilateral tests followed by a multiple comparison Dunn's test). Other measurements were analyzed with classical parametric tests. Maps of density for RLD were designed using R software and results were combined by variety and by treatment. Symptoms of root damage were rare or absent, consequently these observations were not included in the study.

3. Results

3.1. Reduction of *R. reniformis* inoculum in the eco-friendly cropping system

After eight months in the second year, the populations of nematodes (*R. reniformis*) were low regardless of the crop considered. Under pineapple and sunn hemp the number of individuals $.100g^{-1}$ of thin soil were respectively 497 and 608, but the populations in the eggplants increased from 259 to 3,456 individuals $.100g^{-1}$ of thin soil, showing the highest populations of nematodes of the different plots. After the destruction of pineapple, the soil under traditional spontaneous grass fallow contained intermediate populations (1,742 individuals.$100g^{-1}$ of thin soil) between the populations under sunn hemp or pineapple, and eggplants, (Fig.1).

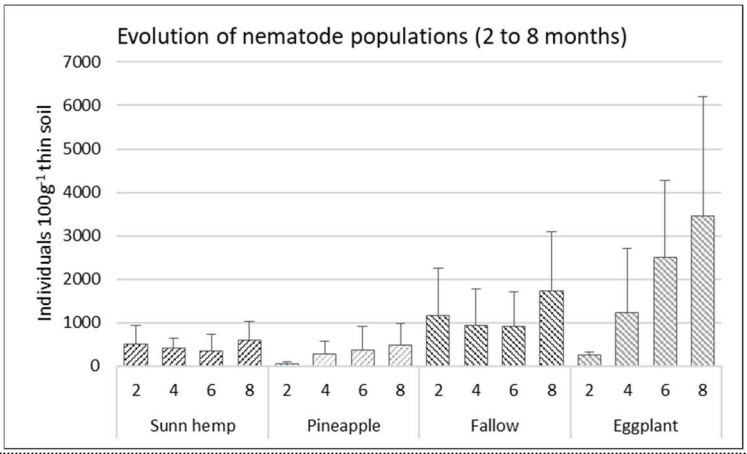

Fig.1. Evaluation of *R. reniformis* populations in soil samples of the 2nd year of the rotation system.
The rotation included successively a sunn hemp planted fallow, then pineapple followed by a spontaneous grass fallow, then a second cash crop 'eggplant'. Nematodes were counted according to Jenkins et al., 1964 every 2 months from 2nd to 8th month.

3.2. Isolation and identification of endophytic bacteria from pineapple root system.

Fifty one isolates able to grow on N-free medium were identified from the three pineapple varieties MD-2, Victoria (Queen) and Smooth Cayenne, in different areas of pineapple production in Martinique. The isolates belonged to Proteobacteria (57%), Firmicutes (41%) and Actinobacteria (2%) phyla, and were affiliated to 10 genera, mostly *Burkholderia* (35%) and *Bacillus* (39%) (Phylogenetic tree as supplementary material).The *Bacillus cereus* group was equally identified in the three pineapple varieties. A *Bacillus* sp. GVS2 was selected for the field experiments based on its ability to reduce the *R. reniformis* populations on MD-2 pineapple in controlled conditions (see 3.3.1.).

3.3. Impact of induced systemic resistance on *R. reniformis* populations

3.3.1. ISR elicitation or *Bacillus* sp. GVS2 applications on MD-2 in controlled conditions

The MD-2 suckers, treated directly on the parental plants in a field nursery, were grown using the same eco-friendly cropping system as the one used in the field experiment (rotation system including *Crotalaria juncea*). The suckers were harvested one week after the last applications in the nursery and transferred to the greenhouse, and then the roots were allowed to develop for a month before inoculation with the nematodes. The nematode populations

were evaluated 45 days later, i.e. a total of three months after field stimulation of ISR or inoculation with the bacteria.

The populations were significantly lower following the two treatments compared with the control (4,852, 5,777 and 9,442 individuals ($p_{0.05} = 0.014$) respectively for the *Bacillus* sp. GVS2 and the methyl jasmonate treatments, and for the control, (Fig.2). The detailed statistics concerning the vermiforms, the larvae (juveniles) and eggs also revealed similar differences with respectively 4,256, 4,969 and 7,862 individual vermiforms ($p_{0.05} = 0.014$), and 3,376, 3,935 and 6,310 larvae ($p_{0.05} = 0.026$), finally 596, 807 and 1,579 eggs ($p_{0.05} = 0.026$).

3.3.2. Control of *R. reniformis* populations in the field.

In plots in which eggplant was grown as the previous crop, the increases in the populations of nematodes were higher in pineapple controls than on pineapples elicited with methyl jasmonate or treated with the *Bacillus* sp. GVS2. Calculated with non-parametric tests of Kruskal & Wallis, the p-values$_{(0.05)}$ indicated significant differences for MD2, $p_{unilateral}$ = 0.037, $p_{GVS2\ vs\ Control}$ = 0.019 and $p_{Mejasm\ vs\ Control}$ = 0.039, using a multiple comparison Dunn's test, (Fig.3). For Victoria (Queen), the same tests indicated non-significant differences between treatments and the control ($p_{unilateral}$ = 0.087, and with the multiple pair comparison $p_{GVS2\ vs\ Control}$ = 0.05, $p_{Mejasm\ vs\ Control}$ = 0.062. The number of nematodes in samples taken on pineapple after sunn hemp tended to be lower than those taken on pineapple after eggplant (Control$_{sunn\ hemp}$, with 3,352 individuals for MD2 and 5,521 individuals for QV versus Control$_{eggplants}$, with 10,322 individuals for MD2 and 11,223 individuals for QV). The differences between treatments for both varieties were not significant (Table 1).

The average of thin soil in the samples was 34% of the soil samples (ranging from 21% to 49%) thus increasing the heterogeneity of the population evaluations.

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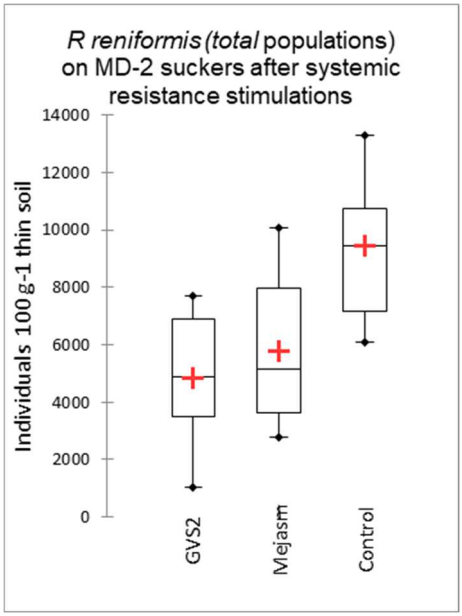

Fig 2. *R. reniformis* populations 45 days after inoculation of nematodes on MD-2 and Victoria (Queen) Pineapple with the ISR stimulation or *Bacillus* sp. GVS2.
Suckers were treated while still on the parental plants in a MD-2 field nursery, harvested and transferred to the greenhouse, then after rooting, inoculated with 4,000 reared individuals populations. (GVS2 and Mejasm significantly lower than Control, $p_{0.05}$).

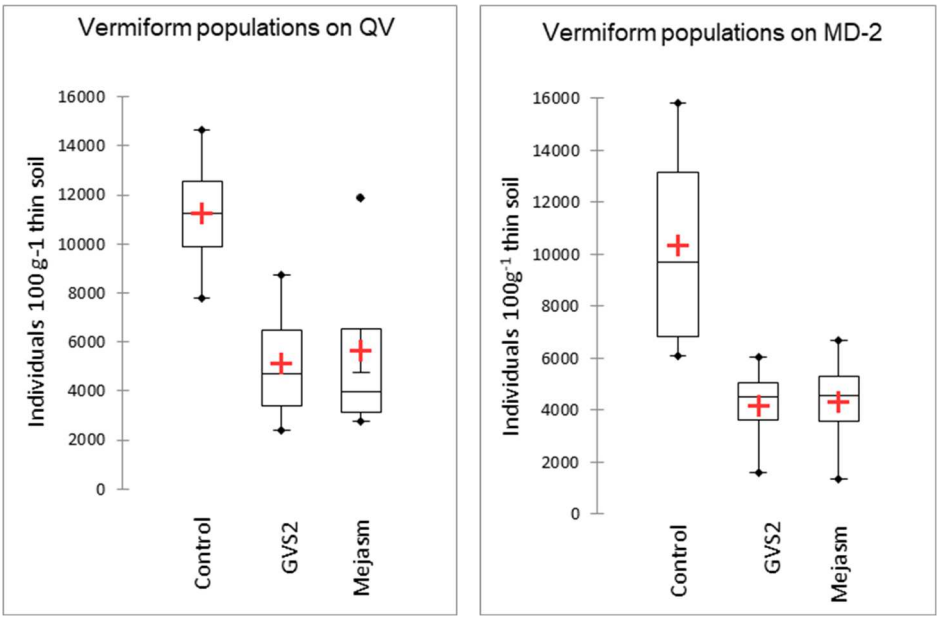

Fig. 3. *R. reniformis* populations at 8 months on MD-2 and Victoria (Queen) pineapple with the ISR stimulation or *Bacillus* sp. GVS2 (previous crop: eggplants).
Pineapple were planted following eggplants to obtain a higher nematode inoculum. Methyl jasmonate ('Mejasm' 10^{-4} M; 10ml.plant^{-1}), Bacillus sp. GVS2 *(Bacteria suspension, 'GVS2', 10^{6} CFUml^{-1}; 10ml.plant^{-1}), and applications with water (control) were the treatments applied every month up to the 7th month. (For the 2 varieties, GVS2 and Mejasm significantly lower than Control, $p_{0.05}$)*

3.4. Pineapple growth: D leaves (FD) and root length density (RLD).

3.4.1. D leaf weights

The average D leaf weights of the Victoria (Queen) were lower than those of MD-2 but no effect of the previous crop was observed, and there were no significant differences between D leaf weights after the different treatments in the two varieties (Table 2).

3.4.2. Root length density, RLD

The root system in the Victoria (Queen) plants was more developed than in MD-2 plants as shown by the RLD profiles across the ridges in the maps of RLD (Fig.4).

The RLD showed that the length of the roots was slightly reduced by the methyl jasmonate treatment (Fig.5) but the roots were still longer directly under the treated plants than under the control plants. The maps also showed that the RLD of plants inoculated with the *Bacillus* sp. GVS2 were higher not only around the plants but also in depth.

The effect of the crop grown before pineapple was highlighted by the calculations of RLD. The RLDs were slightly reduced when eggplants were the previous crop. During the RLD development from 1.5 months to 6 and 8 months (Fig. 6), RLD was higher in pineapple planted after sunn hemp than in pineapple planted after eggplants at almost all stages and depths. Pineapple roots were rarely observed below a depth of 30 cm.

Table 1: *R. reniformis* populations at 8 months on MD-2 and Victoria (Queen) Pineapple with ISR stimulation or bacterial treatment (previous crop: *C. juncea*).

Pineapple variety	Control	*Bacillus* sp. GVS2	Mejasm
MD-2	3,552 ±1,053	3,474 ±2,017	2,408 ±1,356
Victoria (Queen)	5,521 ±2,337	6,069 ±1,324	7,839 ±3,005

Table 2: Average pineapple D leaf weight for MD-2 and Victoria (Queen) at 8 months

Previous crop	Pineapple variety	D leaf Weight (g)
Eggplant	MD-2	60.4 ± 16.5
	Victoria (Queen)	44.7 ± 10.2
C. juncea	MD-2	63.0 ± 16.1
	Victoria (Queen)	41.6 ± 11.5

The average D leaf weights in Table 1 include all treatments.

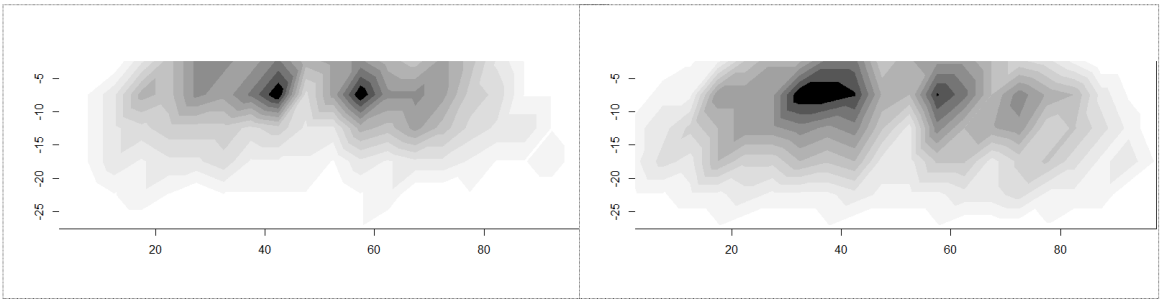

Fig. 4. Maps of root length density (RLD) for the 2 pineapple varieties MD-2 (left) and Victoria (Queen) (right).
Profiles were made transversally across the ridges at a depth of 30 cm and included the roots of 2 plants. One plant was just on top of the profile, the second was 12 cm behind because the suckers are planted at 24 cm intervals along the ridges in 2 parallel lines offset by 12 cm. Measurement and evaluation of RLD were made according to Chopard et al., (2015) and RLD maps were designed with R software. Figure 4 shows RLD per variety averaging data of the three treatments (stimulations and control) for both varieties.

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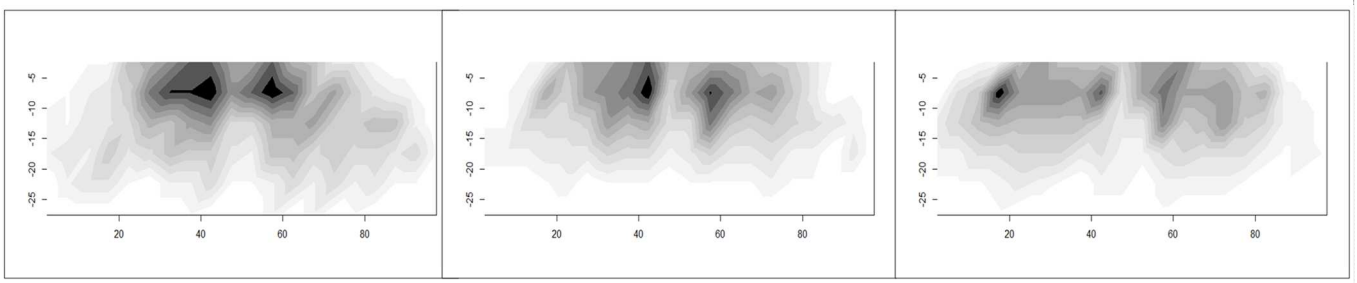

Bacillus sp. GVS2 Methyl jasmonate Control

Fig. 5. Impact of stimulation treatments on root length density (RLD): *Bacillus* sp. GVS2, Methyl jasmonate and Control
RLD averaging data of the two pineapple varieties and for the three replicates each, meaning 6 values for each point observed on the profiles. Each map shows darker areas of higher density of roots around 40 and 60 cm, which correspond to the location of 2 pineapple plants planted in double rows.

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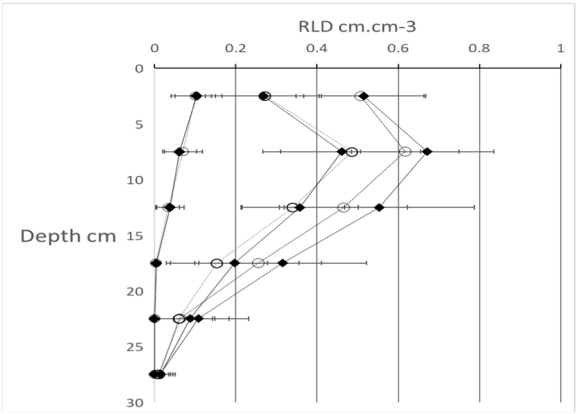

Fig. 6. Map of root length density (RLD) of pineapple planted after sunn hemp and eggplant crops. Averaging RLDs for the three treatments (black symbols for sunn hemp and white symbols for eggplants, respectively at 1.5, 6 and 8 months from left to right).

4. Discussion

4.1. Management of *R. reniformis* inoculum with the eco-friendly cropping system

The eco-friendly cropping system proposed for pineapple included sunn hemp, *C. juncea,* as controlled fallow. Like several other crotalaria species, *C. juncea* does not allow multiplication of the two main "soil-borne pathogens" for pineapple, *R. reniformis* and symphylids (Marie-Alphonsine et al. 2017; Wang et al. 2003). In our conditions in Martinique, French West Indies, this cover plant was able to limit the increase in *R. reniformis* populations of the initial inoculum during the eight months of the field experiment. This confirmed similar results obtained with *C. retusa* that was even more effective in the same experimental site (Soler et al. 2016). *C. retusa* is much more toxic for nematodes and symphylids due to higher concentrations of pyrrolizidines than sunn hemp, respectively 12.2 $\mu g.g^{-1}$ seed dw in *C. retusa*, and $< 0.25\ \mu g.g^{-1}$ in *C. juncea* (Fletcher et al. 2009; Ji et al. 2005; Thoden and Boppré 2010). *Crotalaria* spp. are legumes and root nodulation is also a precondition for them to produce the pyrrolizidines responsible for their non-host status for nematodes (Irmer et al. 2015).

According to Rabie and Tustin (2009) sunn hemp did not control *Pratylenchus brachyurus* species as efficiently as it did with *R. reniformis*. Fortunately, the *P. brachyurus* present in Martinique is not as aggressive towards pineapple as it is in the acidic soils in West or South Africa (Sarah et al. 1991). In our experimental site under sunn hemp, the populations of *R. reniformis* were replaced by increased populations of ectoparasites that are less harmful for pineapple, mainly *Helycotylenchus multicinctus* as well as *Criconemoides* spp. (Berimey 2012).

Our results also showed that a conventional spontaneous grass fallow left a higher inoculum of *R. reniformis* in the soil than sunn hemp as a controlled fallow. A floristic analysis of weeds found in the spontaneous grass fallow in this experimental site showed that about half of the weed species were good or potential hosts for *R. reniformis* (Soler et al., 2016). Under the weeds, the survival of the "soil-borne pathogens" allowed faster re-infestation of the following crop, pineapple or eggplant, which allow the multiplication of this nematode.

In this experimental site, the nematodes were the main target of our management. Due to the unfavorable pedoclimatic conditions for symphylids, in the present study they did not represent a strong additional biotic stress for pineapple. Nevertheless, this eco-friendly cropping system based on a rotation with a crotalaria that is not a symphylid host plant would also have helped control them (Marie-Alphonsine et al. 2017; Soler et al. 2016).

In addition to its soil sanitizing capability in the eco-friendly cropping system, sunn hemp can produce considerable biomass, up to 10 to 15 tons.ha^{-1} of dry matter at the flowering stage depending on growth conditions (Soler and Dorey, 2017). Given they are legumes, *Crotalaria* spp. may also supply nitrogen thanks to their N-fixing ability when *Bradyrhizobium* spp are in symbiosis within their root nodules (300 to 450 kg N.ha^{-1}). The nitrogen content of these cover plants ranges from 3% to 5%, and helps raise the nitrogen level in the soil.

Controlled fallow with sunn hemp thus helped reduce nematode inocula and further re-infestation of pineapple, and the sunn hemp produced a high volume of nitrogen rich biomass. Biomass is one of the characteristics that provide a better environment for the following crops, in our case pineapple and eggplants, in the eco-friendly cropping system (Dobbelaere et al. 2003; Soler et al. 2016).

4.2. Methyl jasmonate and *Bacillus* sp. GVS2 treatments.

4.2.1. Greenhouse experiment after field stimulation

Stimulation of systemic resistance of suckers still growing on the parental plants of the MD-2 variety in a field nursery was effective despite the stresses resulting from the transfer of the suckers to greenhouse and the delay due to their rooting before being inoculated with the nematodes, three months after inoculation/stimulation in the field nursery. This experiment reveals an interesting feature of the priming of defenses in pineapple through chemical induced ISR or by endophytic bacteria application, resilience. This leads us to consider reducing monthly applications of the elicitor methyl jasmonate or of the *Bacillus* sp. GVS2. The production of disease-free plants in the nursery , that integrates both practices, can help control nematodes during the establishment of new pineapple plots through systemic resistances induced by elicitors as methyl jasmonate, or through the application of the endophytic bacteria *Bacillus* sp. GVS2 (whatever the mechanism of defense is in this case).

4.2.2. Field experiment

The ISR triggered via the jasmonic acid pathway, or the application of *Bacillus* sp. GVS2 significantly reduced the growth of the nematode populations on MD-2 pineapple but not on Victoria (Queen). Nevertheless, the growth of nematode populations and differences between treatments and controls were very similar in the two pineapple varieties. The data for Victoria (Queen) were actually very close to being significant in the pairwise comparison giving $p_{GVS2\ vs\ Control} = 0.05$, $p_{Mejasm\ vs\ Control} = 0.062$ when the limit for the statistical test was $\alpha=0.05$. The enumerations of nematodes in the soil samples were expressed as the number of

individuals.100g^{-1}of thin soil. This thin soil only represented an average of 34% (range 21-49%) of the total soil in the samples. This heterogeneity could have impacted statistical analysis of the nematode enumerations shown in figure 2. In addition, we had only four replicates per individual plot, which could have been a limiting factor for more accurate evaluations of nematode populations. Nematodes grow in aggregated populations which means infestations are heterogeneous at field level, particularly in the case of the low infestations observed in this experiment, that could also result from the rotation system used to reduce parasitic pressure before planting pineapple.

The priming of the plants followed by monthly applications of methyl jasmonate, or by the application of endophytic bacteria *Bacillus* sp GVS2 succeeded decrease the growth of the *R. reniformis* inocula left by the previous crop (eggplant). These observations could not be confirmed when the previous crop was *C. juncea* because the eco-friendly cropping system was very efficient in reducing nematode inocula before the ISR experiment began. After *C. juncea*, the differences between populations after ISR stimulations or *Bacillus* sp GVS2 applications, were not significant. The results also confirm our initial hypothesis that including a rotation with sunn hemp may considerably reduce the risk of biotic stresses, giving pineapple an efficient protection against nematodes.

Several biotic stresses (symphylids, nematodes, pathogenic fungi, insects) or additional abiotic stresses would have led to more energy resources being used by the plant for its defenses. The energy costs induced by the accumulation of biotic and abiotic stresses or high level infestations of one pathogen, would have made it more difficult for the pineapple to establish efficient ISR or reduced the efficiency of the *Bacillus* sp. GVS2 against a specific target like *R. reniformis* (Choudhary 2012; Suzuki et al. 2012).

4.2.3. RLD and D leaves.

Methyl jasmonate is an inhibitor of root growth (Staswick et al. 1992), so either the methyl jasmonate applications, or the jasmonic acid produced by the plant after the stimulation could have been responsible for the slight inhibition of root growth observed in treated plants. Nevertheless, under methyl jasmonate treatment, these roots were expected to bear smaller nematode populations than the roots of the controls as shown by the nematode counting. The endophytic *Bacillus* sp. GVS2 established a beneficial interaction with pineapple roots, as in our experiment root growth tended to be enhanced by the bacterial treatment. These bacteria were able to help control nematodes but they could have several other beneficial impacts as PGPR effects on the plant they colonized, besides a direct biocide effect on nematodes (Rodriguez et al. 2019; Shafi et al. 2017; Singh 2018). In fact,

based on D leaf weights, the plant growth of both varieties was not affected by the levels of nematode populations. The larger RLDs in Victoria (Queen) than on MD-2 are the result of a varietal effect as the Victoria (Queen) is known to have a strong root system when planted in fertile soils (Py et al. 1987). The RLD results could not show significant differences but they were reported as agronomic observations that enlighten the positive effect of the rotation, and ISR or bacterial treatments on pineapple as shown in the RLD maps in figure 5.

4.2.4. Comments on the endophytic bacteria, *Bacillus* sp. GVS2

The mechanism of reduction of the multiplication of nematodes by non-phytopathogenic rhizobacteria has been shown to be linked to ISR defenses or antibiosis . The *Bacillus* sp. GVS2 was part of several related bacteria species isolated from pineapple that have still not been tested for systemic resistance. As mentioned by Kloepper et al. (2004), mixing several endophytic bacteria can enhance the PGPR and ISR effects compared with a single bacterial strain. To select the endophytic bacteria, the decision was taken to focus on diazotrophic endophytic bacteria and on preliminary results obtained in greenhouse experiments. The *Bacillus* sp. GVS2 grew without any problem on the N-free medium, but the actual diazotrophic status of the isolated bacteria should now be determined by molecular techniques using a primer set specific to the *nif* gene. In the present experiments, it was not possible to determine if an ISR was actually elicited by the *Bacillus* sp. GVS2, or if the decrease of multiplication was a biocide effect, or both. Molecular and enzymatic experiments to determine which defense genes were activated would provide the necessary information to conclude about the mechanism involve with the bacteria.

Some of the species in the *Bacillus* group have a direct biocide effect that is not necessarily linked to systemic resistances (Hashem et al. 2019; Shafi et al. 2017). However, both methyl jasmonate and *Bacillus* sp. GVS2 slowed down equally the development of the nematode populations on pineapple all experiments. The positive impact against *R. reniformis* observed for the two treatments was found also the same resilience when applied on suckers growing on their parental plants.

5. Conclusion

The decreases of the nematode multiplication obtained at field level in these different experiments showed that managing *R reniformis* with ISR induced by methyl jasmonate compared to a defense provided by the endophytic bacteria *Bacillus* sp. GVS2 were very similar in a context of eco-friendly cropping system, and showed the same resilience. After several rotations of this system including *Crotalaria* sp., the populations of nematodes were

reduced even on plants which host status for *R. reniformis* such as pineapple or eggplants are supposed to allow its multiplication. In addition, the reduction of biotic stress made it possible to stimulate effective systemic resistances because these multi-pathogenic defenses were mobilized by plants for fewer targets (smaller inocula and control of other "soil-borne pathogens" such as symphylids). It is also the case for the endophytic bacteria *Bacillus* sp. GVS2 which provided a protection against nematodes similar to the one provided by the methyl jasmonate (ISR). Previous results suggested that in pineapple, systemic resistances are variety-dependent physiological processes. However, the two varieties MD-2 and Queen Victoria (Queen) responded equally and effectively to the two modes of protection (ISR by methyl jasmonate or biological protection with *Bacillus* sp. GVS2). Finally, both greenhouse and field experiments supported the hypothesis that endophytic bacteria from pineapple root system, like the *Bacillus* sp. GVS2, may be a promising tool for a biocontrol against *R. reniformis* as well as ISR elicitors against pineapple pathogens, when combined with a cropping system based on rotation with non-host cover crops.

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612 Phylogenetic relationship of bacterial endophytes isolated from roots of pineapple varieties
613 MD-2 (red), Victoria (Queen) (green) and Smooth Cayenne (blue). The phylogenetic tree was
614 constructed using the maximum likelihood method and the Tamura-Nei model with a discrete
615 gamma distribution with invariant sites for distance correction. Levels of bootstrap value (500
616 resamplings) are indicated by black circles (if >80 %) or open circles (if between 50 and 80
617 %). The scale bar shows the number of base changes per sequence position. The 16S
618 reference sequences correspond to the closest sequences from material types (NCBI
619 database).