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

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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

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

50

51 **1. Introduction**

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

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

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

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

82 PGPR rhizobacteria may exhibit their biocontrol capacity through ISR by inhibiting the
83 growth of plant pathogens, including nematodes, in addition to other direct and/or indirect
84 effects on plant growth (Majeed et al. 2018; Singh 2018). Many PGPR rhizobacteria related to
85 the *Bacillus* group are promising biocontrol tools against pathogens (Choudhary and Johri
86 2009; Kloepper et al. 2004; Thakur and Singh 2018; Fira et al., 2018). Pineapple plants
87 primed with methyl jasmonate (ISR) showed strong down-regulation of the multiplication of
88 nematode populations (Soler et al. 2013). Based on the results of previous studies, we
89 concluded that pineapple nematodes could be managed efficiently without using pesticides
90 combining two agricultural practices. First, an eco-friendly cropping system based on
91 pineapple / sunn hemp (*C. juncea*) rotation to reduce the initial biotic stress on pineapple
92 plants (inoculum of nematodes). Then, the induction of systemic resistance by applying a
93 chemical elicitor, or using pineapple endophytic bacteria to help maintain low nematode
94 populations under pineapple. In their recent review article, Abd-Elgawad and Askary (2020)
95 suggested developing similar approaches after reviewing the factors that determine the
96 success of biological agents in controlling plant-parasitic nematodes.

97

98 **2. Material and Methods**

99 2.1. Planting material and Design of the eco-friendly cropping system for pineapple.

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

102 The eco-friendly cropping system was established in a field representative of the area
103 pineapple production with young volcanic andosol with ~34% of thin soil and gravels, mainly
104 pumice. The design included two blocks each containing four plots. As preliminary soil
105 cleaning, each plot was first planted with *Crotalaria retusa*, then *Crotalaria juncea* for one
106 year. Then a rotation system without pesticide was established in each block for two cycles.
107 Each of the four plots in the 2 blocks was planted successively by sunn hemp as a controlled
108 fallow, then pineapple followed by a spontaneous grass fallow, followed by eggplants
109 (rotation system). A block showed the four crops grown simultaneously, one per plot. Each
110 plot received the complete sequence of the rotation (four crops) after four cycles. Every year,
111 all the crops were destroyed and the residues incorporated in the soil as a preparation for the
112 next step of the rotation. For every new cycle, each plot was planted with a different crop
113 according to the sequence as indicated before. Pre-planting fertilizer was applied under the
114 plastic mulch at the dose as recommended in Martinique (30 g.plant⁻¹ of dolomite and 25
115 g.plant⁻¹ of a compound 12N-4P₂O₅-24K₂O-6MgO). Subsequently, during vegetative growth,
116 the plants received foliar fertilizer applications (0.5 g.plant⁻¹ of urea and 0.5 g.plant⁻¹ sulfate
117 of potash during the first four months, then 1 g.plant⁻¹ up to eight months). Eggplants planted
118 on plastic mulch received the same pre-planting fertilizer as pineapple.
119 Symphyliids were not a problem in this particular field, which is located at sea level, because
120 in Martinique their populations grow mainly at higher altitudes where temperatures are cooler
121 and soil humidity higher (personal observation). This allowed us to target only one “soil-
122 borne pathogen” (nematode).

123

124 2.2. Isolation and identification of endophytic bacteria from pineapple root system.

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

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

136 amplification. PCR amplification were performed using the primers FGPS1509 (5'-
137 AAGGAGGGGATCCAGCCGCA-3') and FGPS6 (5'-GGAGAGTTAGATCTTGGCTCAG-
138 3') as follows (25 µl reaction): 1X Green GoTaq® Reaction Buffer (Promega, Charbonnieres,
139 France), 0.6 units of GoTaq® DNA Polymerase (Promega), 0.4 µM of each primer, 1 µl crude
140 DNA extract, 160 µM of each dNTP, with the following cycling conditions: 94°C for 3min;
141 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
142 min. PCR products were then sequenced using the primer 16S-1080r (5'-
143 GGGACTTAACCCAACATCT-3') (Genoscreen, Lille, France).

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

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

155

156 2.3. Stimulating defenses in greenhouse and field conditions

157 2.3.1. Applications of methyl jasmonate or *Bacillus* sp. GVS2 on pineapple.

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

163 2.3.2. ISR and bacterial experiment in the greenhouse.

164 While still on their parental plants, suckers from three different groups of pineapple growing
165 in a field nursery of MD-2 received three stimulations at four day intervals with the three
166 treatments according to Soler et al. (2013). Then, samples of 20 suckers per treatment
167 harvested one week after the last stimulation, were planted in 500 ml pots filled with andosol
168 that had previously been sterilized in a Systec VE-150 sterilizer at 120 °C for 20 min in a
169 greenhouse, and received one application of osmocote 5 g per plant (10N - 11P₂O₅ -18K +

170 2MgO), and one application of foliar fertilizer Mairol® (1g.L⁻¹). After one month of root
171 growth and with no further stimulation treatment other than those originally applied in the
172 field, the plants were inoculated with 4,000 individuals.plant⁻¹of reared populations of *R.*
173 *reniformis*. The increase in these populations was evaluated 45 days later to assess the impact
174 of defenses induced by the elicitor treatment or bacterial applications.

175 2.3.3. ISR and bacterial experiment in the field.

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

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

195 2.4. Evaluation of *R. reniformis* populations.

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

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

206 2.5. Evaluation of pineapple growth in the ISR and bacterial experiment

207 Twenty-five 'D leaves' per plot were weighed to assess plant growth according to Py et al.
208 (1987). Root length densities (RLD) were estimated according to Chopart et al. (2015) to
209 assess soil exploration by the pineapple roots at eight months of age using Racine2 software
210 (CIRAD). RLD were evaluated for each individual plot with three replicates corresponding to
211 3 soil profiles.

212 2.6. Statistical analysis:

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

219

220 3. Results

221 3.1. Reduction of *R. reniformis* inoculum in the eco-friendly cropping system

222 After eight months in the second year, the populations of nematodes (*R. reniformis*) were low
223 regardless of the crop considered. Under pineapple and sunn hemp the number of individuals
224 $.100g^{-1}$ of thin soil were respectively 497 and 608, but the populations in the eggplants
225 increased from 259 to 3,456 individuals $.100g^{-1}$ of thin soil, showing the highest populations
226 of nematodes of the different plots. After the destruction of pineapple, the soil under
227 traditional spontaneous grass fallow contained intermediate populations (1,742
228 individuals $.100g^{-1}$ of thin soil) between the populations under sunn hemp or pineapple, and
229 eggplants, (Fig.1).

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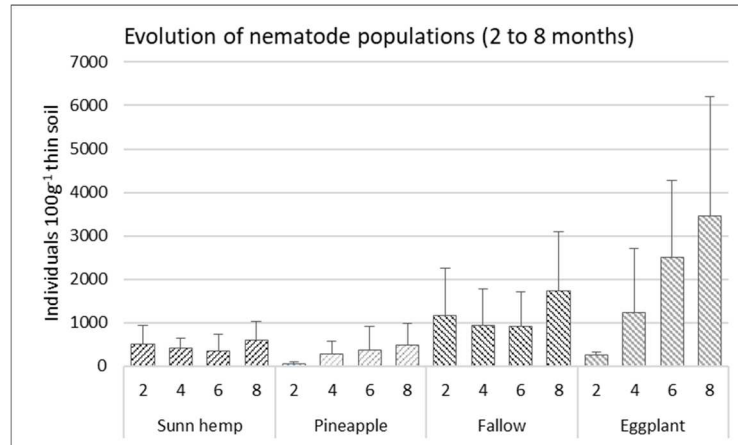


Fig.1. Evaluation of *R. reniformis* populations in soil samples of the 2nd year of the rotation system.

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.

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237 3.2. Isolation and identification of endophytic bacteria from pineapple root system.

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

246

247 3.3. Impact of induced systemic resistance on *R. reniformis* populations

248 3.3.1. ISR elicitation or *Bacillus* sp. GVS2 applications on MD-2 in controlled
 249 conditions

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

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

257 The populations were significantly lower following the two treatments compared with the
258 control (4,852, 5,777 and 9,442 individuals ($p_{0.05} = 0.014$) respectively for the *Bacillus* sp.
259 GVS2 and the methyl jasmonate treatments, and for the control, (Fig.2). The detailed statistics
260 concerning the vermiforms, the larvae (juveniles) and eggs also revealed similar differences
261 with respectively 4,256, 4,969 and 7,862 individual vermiforms ($p_{0.05} = 0.014$), and 3,376,
262 3,935 and 6,310 larvae ($p_{0.05} = 0.026$), finally 596, 807 and 1,579 eggs ($p_{0.05} = 0.026$).

263 3.3.2. Control of *R. reniformis* populations in the field.

264 In plots in which eggplant was grown as the previous crop, the increases in the populations
265 of nematodes were higher in pineapple controls than on pineapples elicited with methyl
266 jasmonate or treated with the *Bacillus* sp. GVS2. Calculated with non-parametric tests of
267 Kruskal & Wallis, the p-values_(0.05) indicated significant differences for MD2, $p_{unilateral} =$
268 0.037, $p_{GVS2\ vs\ Control} = 0.019$ and $p_{Mejasm\ vs\ Control} = 0.039$, using a multiple comparison Dunn's
269 test, (Fig.3). For Victoria (Queen), the same tests indicated non-significant differences
270 between treatments and the control ($p_{unilateral} = 0.087$, and with the multiple pair comparison
271 $p_{GVS2\ vs\ Control} = 0.05$, $p_{Mejasm\ vs\ Control} = 0.062$. The number of nematodes in samples taken on
272 pineapple after sunn hemp tended to be lower than those taken on pineapple after eggplant
273 (Control_{sunn hemp}, with 3,352 individuals for MD2 and 5,521 individuals for QV versus
274 Control_{eggplants}, with 10,322 individuals for MD2 and 11,223 individuals for QV). The
275 differences between treatments for both varieties were not significant (Table 1).

276 The average of thin soil in the samples was 34% of the soil samples (ranging from 21% to
277 49%) thus increasing the heterogeneity of the population evaluations.

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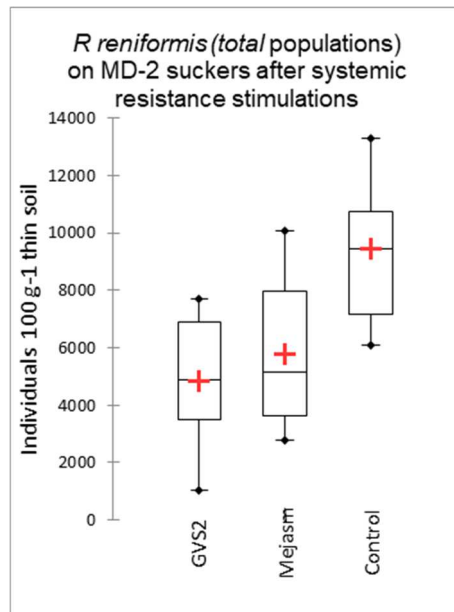


Fig 2. *R. reniformis* populations 45 days after inoculation of nematodes on MD-2 and Victoria (Queen) Pineapple with the ISR stimulation or *Bacillus* sp. GVS2. Suckers were treated while still on the parental plants in a MD-2 field nursery, harvested and transferred to the greenhouse, then after rooting,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

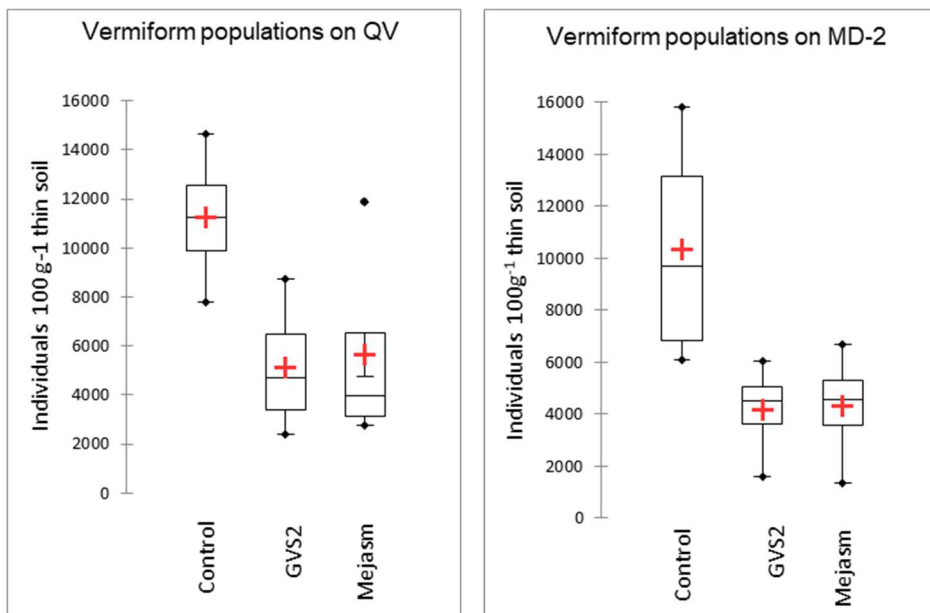


Fig. 3. *R. reniformis* populations at 8 months on MD-2 and Victoria (Queen) pineapple with the ISR stimulation or *Bacillus* sp. GVS2 (previous crop: eggplants). Pineapple were planted following eggplants to obtain a higher nematode inoculum. Methyl jasmonate ('Mejasm' 10^{-4} M; 10ml.plant^{-1}), *Bacillus* sp. GVS2 (*Bacteria* suspension, 'GVS2', 10^6 CFU ml^{-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, $p_{0.05}$)

282 3.4. Pineapple growth: D leaves (FD) and root length density (RLD).

283 3.4.1. D leaf weights

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

287 3.4.2. Root length density, RLD

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

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

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

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Table 1: *R. reniformis* populations at 8 months on MD-2 and Victoria (Queen) Pineapple

303

with ISR stimulation or bacterial treatment (previous crop: *C. juncea*).

Pineapple variety	Control	<i>Bacillus</i> 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

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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
<i>C. juncea</i>	MD-2	63.0 ± 16.1
	Victoria (Queen)	41.6 ± 11.5

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

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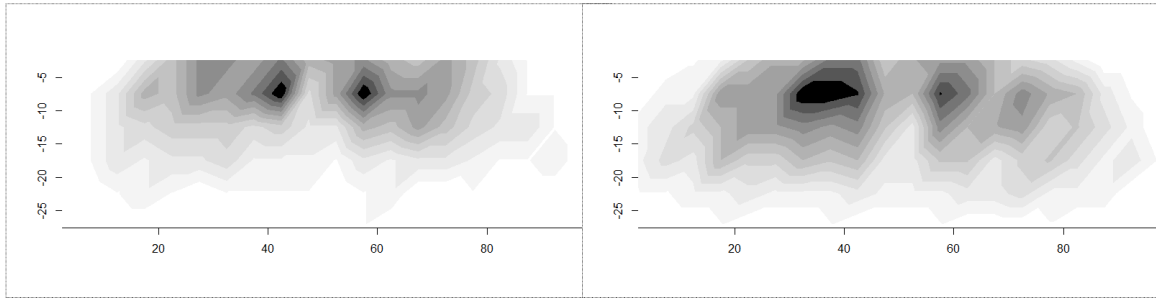


Fig. 4. Maps of root length density (RLD) for the 2 pineapple varieties MD-2 (left) and Victoria (Queen) (right).

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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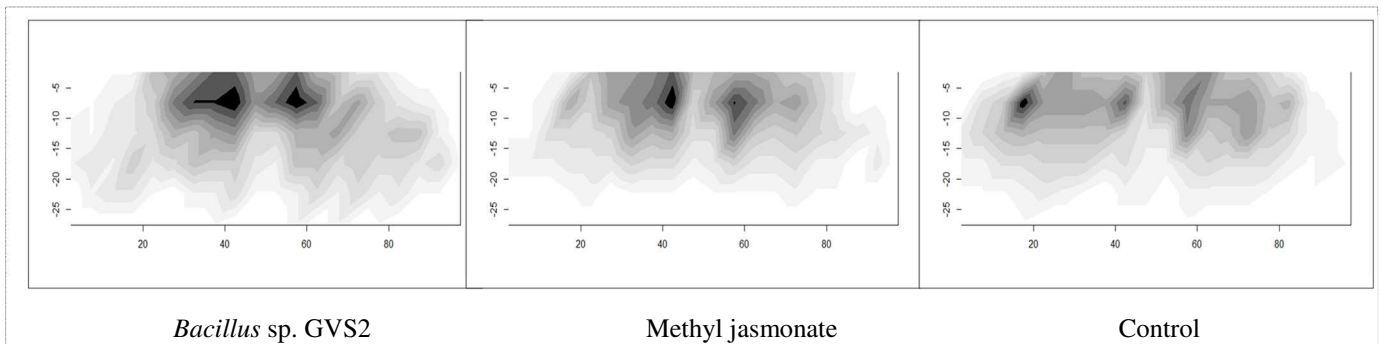


Fig. 5. Impact of stimulation treatments on root length density (RLD): *Bacillus* sp. GVS2, Methyl jasmonate and Control RLD averaging data of the two pineapple varieties and for the three replicates each, meaning 6 values for each point observed on the profiles. Each map shows darker areas of higher 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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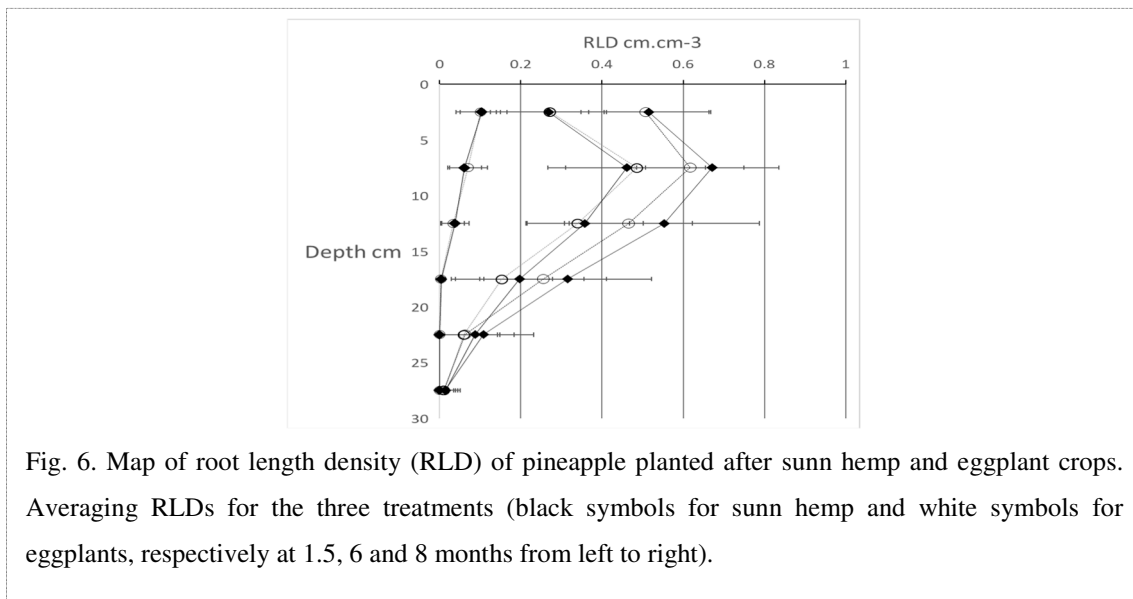


Fig. 6. Map of root length density (RLD) of pineapple planted after sunn hemp and eggplant crops. Averaging RLDs for the three treatments (black symbols for sunn hemp and white symbols for eggplants, respectively at 1.5, 6 and 8 months from left to right).

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356 **4. Discussion**

357 4.1. Management of *R. reniformis* inoculum with the eco-friendly cropping system

358 The eco-friendly cropping system proposed for pineapple included sunn hemp, *C. juncea*, as
359 controlled fallow. Like several other crotalaria species, *C. juncea* does not allow
360 multiplication of the two main "soil-borne pathogens" for pineapple, *R. reniformis* and
361 symphyllids (Marie-Alphonsine et al. 2017; Wang et al. 2003). In our conditions in
362 Martinique, French West Indies, this cover plant was able to limit the increase in *R.*
363 *reniformis* populations of the initial inoculum during the eight months of the field experiment.
364 This confirmed similar results obtained with *C. retusa* that was even more effective in the
365 same experimental site (Soler et al. 2016). *C. retusa* is much more toxic for nematodes and
366 symphyllids due to higher concentrations of pyrrolizidines than sunn hemp, respectively 12.2
367 $\mu\text{g}\cdot\text{g}^{-1}$ seed dw in *C. retusa*, and $< 0.25 \mu\text{g}\cdot\text{g}^{-1}$ in *C. juncea* (Fletcher et al. 2009; Ji et al.
368 2005; Thoden and Boppré 2010). *Crotalaria* spp. are legumes and root nodulation is also a
369 precondition for them to produce the pyrrolizidines responsible for their non-host status for
370 nematodes (Irmer et al. 2015).

371 According to Rabie and Tustin (2009) sunn hemp did not control *Pratylenchus brachyurus*
372 species as efficiently as it did with *R. reniformis*. Fortunately, the *P. brachyurus* present in
373 Martinique is not as aggressive towards pineapple as it is in the acidic soils in West or South
374 Africa (Sarah et al. 1991). In our experimental site under sunn hemp, the populations of *R.*
375 *reniformis* were replaced by increased populations of ectoparasites that are less harmful for
376 pineapple, mainly *Helycotylenchus multincinctus* as well as *Criconemoides* spp. (Berimey
377 2012).

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

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

389 In addition to its soil sanitizing capability in the eco-friendly cropping system, sunn hemp can
390 produce considerable biomass, up to 10 to 15 tons.ha⁻¹ of dry matter at the flowering stage
391 depending on growth conditions (Soler and Dorey, 2017). Given they are legumes, *Crotalaria*
392 spp. may also supply nitrogen thanks to their N-fixing ability when *Bradyrhizobium* spp are in
393 symbiosis within their root nodules (300 to 450 kg N.ha⁻¹). The nitrogen content of these
394 cover plants ranges from 3% to 5%, and helps raise the nitrogen level in the soil.
395 Controlled fallow with sunn hemp thus helped reduce nematode inocula and further re-
396 infestation of pineapple, and the sunn hemp produced a high volume of nitrogen rich biomass.
397 Biomass is one of the characteristics that provide a better environment for the following
398 crops, in our case pineapple and eggplants, in the eco-friendly cropping system (Dobbelaere
399 et al. 2003; Soler et al. 2016).

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401 4.2. Methyl jasmonate and *Bacillus* sp. GVS2 treatments.

402 4.2.1. Greenhouse experiment after field stimulation

403 Stimulation of systemic resistance of suckers still growing on the parental plants of the MD-
404 2 variety in a field nursery was effective despite the stresses resulting from the transfer of
405 the suckers to greenhouse and the delay due to their rooting before being inoculated with the
406 nematodes, three months after inoculation/stimulation in the field nursery. This experiment
407 reveals an interesting feature of the priming of defenses in pineapple through chemical
408 induced ISR or by endophytic bacteria application, resilience. This leads us to consider
409 reducing monthly applications of the elicitor methyl jasmonate or of the *Bacillus* sp. GVS2.
410 The production of disease-free plants in the nursery, that integrates both practices, can help
411 control nematodes during the establishment of new pineapple plots through systemic
412 resistances induced by elicitors as methyl jasmonate, or through the application of the
413 endophytic bacteria *Bacillus* sp. GVS2 (whatever the mechanism of defense is in this case).

414

415 4.2.2. Field experiment

416 The ISR triggered via the jasmonic acid pathway, or the application of *Bacillus* sp. GVS2
417 significantly reduced the growth of the nematode populations on MD-2 pineapple but not on
418 Victoria (Queen). Nevertheless, the growth of nematode populations and differences
419 between treatments and controls were very similar in the two pineapple varieties. The data
420 for Victoria (Queen) were actually very close to being significant in the pairwise comparison
421 giving $p_{GVS2 \text{ vs Control}} = 0.05$, $p_{Mejasm \text{ vs Control}} = 0.062$ when the limit for the statistical test was
422 $\alpha=0.05$. The enumerations of nematodes in the soil samples were expressed as the number of

423 individuals.100g⁻¹of thin soil. This thin soil only represented an average of 34% (range 21-
424 49%) of the total soil in the samples. This heterogeneity could have impacted statistical
425 analysis of the nematode enumerations shown in figure 2. In addition, we had only four
426 replicates per individual plot, which could have been a limiting factor for more accurate
427 evaluations of nematode populations. Nematodes grow in aggregated populations which
428 means infestations are heterogeneous at field level, particularly in the case of the low
429 infestations observed in this experiment, that could also result from the rotation system used
430 to reduce parasitic pressure before planting pineapple.

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

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

446 4.2.3. RLD and D leaves.

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

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

463 4.2.4. Comments on the endophytic bacteria, *Bacillus* sp. GVS2

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

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

484

485 5. Conclusion

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

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

505

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610

611

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).