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Project: Microbial uptakes for sustainable management of major banana pests and diseases (H2020 MUSA - 727624)

Deliverable D3.1 (WP3)

Data on biology and effectiveness of selected EBCAs from different regions and climates, including risk analysis data

Project Acronym MUSA

Document Type Deliverable

Document Number D4

Primary Author(s) UA

Document Version / Status Draft (ver. 1)

Distribution Level PU (public)

Topic H2020-SFS-2016-2017 (H2020-SFS-2016-2)

Date May 31st, 2019

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 727624

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1. Introduction

In the previous activities within the MUSA Project, the Consortium isolated and selected endophytic biocontrol agents (EBCAs strains) effective *in vitro* and *in planta* against the main pathogen associated with Banana Plants (*Fusarium oxysporum* f. sp. *cubense* (*Foc,* Panama Disease; PD). Besides, our Consortium has focused efforts to isolate native entomopathogenic fungi (EF) and nematophagous fungi (NF) from banana fields to develop antagonists for *Cosmopolites sordidus* (BW) and plant parasitic nematodes. Besides, in this Consortium we aim to isolate and identify soil-borne microorganisms (EBCAs) able to colonise plant tissue as endophytes with capabilities to promote plant growth and induce plant defence. Experiments to generate these resources have been performed under laboratory, greenhouse and field conditions in the main locations of Banana plant production in Europe, Central America and Africa. Our results indicate that banana fields are good niches for antagonistic microorganisms to the main banana pest and diseases. This deliverable provides data on parasitism/antagonism of EBCAs to BW and PD. Interactions among EBCAs and with banana pest and diseases agents are also reported. Data available on Risk Analysis of EBCAs are also provided. Potential volatile organic compounds (VOCs) repellent on BW and preliminary field test data of this VOCs on BW are also reported. Finally, we provide data on genomic resources on EBCAs and Banana pests.

2. Prevalence and Colonisation of Endophytic Biocontrol Agents (EBCAs)

2.1 Parasitism of Banana Weevil (BW) by Entomopathogenic Fungi (EF)

- **African Isolates**

Most (19) of the fungal isolates tested from ICIPE and RealIPM exhibited pathogenicity against the BW, with mortality ranging from 3 – 54 %. The *Beauveria* isolates exhibited the highest degree of pathogenicity, with isolates ICIPE 648, ICIPE 273 and ICIPE 660 causing mortality levels of 54%, 46% and 45% respectively (Table 1). Furthermore, these three isolates exhibited a high biological ability to sporulate on weevil cadavers (Table 2), an indication of their potential to self-disseminate in the field following the first inoculation. Three isolates: ICIPE 700, ICIPE 69 and ICIPE 281 showed no pathogenicity against the weevils (Table 1).

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Table 1. Mortality of adult BW after exposure to fungal antagonists.

No.	Isolate code	Description, Source	% BW mortality (mean ± se)
1	ICIPE 648	*Beauveria*, ICIPE	53.5 ± 11.99
2	ICIPE 273	*Beauveria*, ICIPE	46.25 ± 14.75
3	ICIPE 660	*Beauveria*, ICIPE	45 ± 14.52
4	ICIPE 644	*Beauveria*, ICIPE	35.63 ± 11.15
5	Bb 01	*Beauveria*, Real IPM	35.28 ± 10.8
6	Bb 02	*Beauveria*, Real IPM	33.5 ± 8.7
7	ICIPE 284	*Beauveria*, ICIPE	32.5 ± 13.73
8	ICIPE 622	*Beauveria*, ICIPE	26.88 ± 8.91
9	ICIPE 609	*Beauveria*, ICIPE	21.25 ± 9.34
10	ICIPE 662	*Beauveria*, ICIPE	18.88 ± 3.94
11	ICIPE 631	*Beauveria*, ICIPE	17.5 ± 7.5
12	ICIPE 647	*Beauveria*, ICIPE	8.33 ± 4.77
13	ICIPE 281	*Beauveria*, ICIPE	0 ± 0
14	TRC 900	*Trichoderma*, Real IPM	7.78 ± 2.61
15	TRC 901	*Trichoderma*, Real IPM	2.5 ± 2.5
16	ICIPE 700	*Trichoderma*, Real IPM	0 ± 0
17	ICIPE 62	*Metarhizium*, ICIPE	7.5 ± 7.5
18	ICIPE 78	*Metarhizium*, ICIPE	7.5 ± 4.79
19	ICIPE 69	*Metarhizium*, ICIPE	0 ± 0
20	ICIPE 682	*Iseria*, ICIPE	2.5 ± 2.5
21	ICIPE 712	*Fusarium*, ICIPE	2.5 ± 2.5

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Table 2. Spore production (mean ± SE) on BW cadavers, 21 days after death.

No	Isolate code	Spore production per dead BW (× 10^7)
3	ICIPE 660	6.3 ± 0.91
2	ICIPE 273	3.7 ± 0.69
8	ICIPE 622	3.3 ± 1.26
1	ICIPE 648	2.8 ± 0.83
7	ICIPE 284	2.2 ± 0.75
10	ICIPE 662	2 ± 1.69
4	ICIPE 644	0.8 ± 0.31
5	Bb 01	0.3 ± 18
6	Bb 02	0.2 ± 3

A total of ten bacterial and/or fungal isolates were obtained after sampling three sites in Kenya highlands (Kapcherop Forest**,** Suam orchards, Trans nzoia), Mau Forest, Kericho County. Out of the ten bacterial and fungal isolates that were identified, three isolates, together with existing RealIPM isolates, were screened for their efficacy against BW *in vitro*. Tested isolates included: *Beauveria bassiana* (Bb 01, 02 and 03) *Metarhizium* (Met78) and *Trichoderma* (TRC900, TRC901) species. For each of the isolates, ten weevils were treated through spraying with 10 ml of a spore suspension (1.0×10^8 spore ml^{-1}) using a hand sprayer with fine mist. Treated weevils were maintained in 250 ml plastic containers and provided with banana corms (~200 g) as food. Each isolate assay was replicated four times. Dead weevils were checked every five days for 40 days and checked for mycosis. Seven of the most pathogenic fungal isolates were tested for their potential to sporulate on BW cadavers. As a result, all tested isolates exhibited pathogenicity against BW, with mortality ranging from 14 - 47%. Tested *B. bassiana* isolates showed the highest degree of pathogenicity (*Beauveria* 03, 01 and 02) which did not differ significantly in mortality levels (47%, 45% and 40%, respectively). Isolates *Metarhizium* 78*, Trichoderma* 900 and TR01 exhibited pathogenicity towards BW. However, the degree of BW mortality was low (17%, 14% and 16%) respectively. A second trial was conducted to evaluate the efficacy *Bacillus subtilis*

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Bb04, *B. subtilis* REAL IPM isolate, TR 01, *Trichoderma* TR900, as biofertilizers under greenhouse condition. These strains promoted growth in banana plantlets (Table 3).

Table 3. Microbial isolates collected from Kenya Highlands and their activities related to Plant-Growth Promotion (PGP) and biocontrol of banana weevils (BW).

Collected isolates id. No.	Molecular ID	Origin	*In vitro*- against BW	PGP - endophytes: greenhouse condition
E0000188001	*Beauveria bassiana*	Kenya Highland, Suam Orchards (Trans Nzoia Orchard 2)	YES	-
E0000188002	*Penicillium parvum* clade	Kenya Highland (Kapcherop Forest, Phase 1)	-	-
E0000188003	*Fusarium* sp.	Kenya Highland, Mau Forest; Phase 2	-	-
E0000188004	*Bacillus* sp. (*B. amyloliquefaciens*)	Kenya Highland Suam Orchards (Trans Nzoia, Orchard 1)	-	YES
E0000188005	*Fusarium* sp. (*F. oxysporum*)	Kenya Highland, Mau Forest, Phase 2	-	-
E0000188006	*Trichoderma* sp. (*T. viridae*)	Kenya Highland, Kapcherop Forest, Phase 2	YES	YES
E0000188007	*Thielaviopsis* sp.	Kenya Highland, Kapcherop Forest, Phase 2	-	-
E0000188008	*Talaromyces amestolkiae*	Kenya Highland, Mau Forest, Phase 2	-	-
E0000188009	*Penicillium citrinum*	Kenya Highland, Mau Forest, Phase 1	-	-
E0000188010	*Penicillium chermesinum*	Kenya Highland, Kapcherop Forest, Phase 1	-	-

Canary Islands isolates

Pathogenicity bioassays of EF

Pathogenicity bioassays were performed by placing in contact *G. mellonella* larvae with a 15-day-old colony of EF grown in CMA. We also tested pathogenicity of EF isolates by dipping larvae on EF spore suspensions in either SDW or 0.05% Tween 20 in SDW (10E+6 spores/ml). We finally discarded this method because larvae were still alive after 20 days incubation (just like mock-inoculated controls). This could be because larvae cuticle

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presents waxes that repel aqueous spore suspensions. For this reason, we finally performed pathogenicity bioassays by placing larvae in contact with an EF colony sporulated on solid medium.

In the pathogenicity tests EF isolated from banana crop soils were compared with similar EF strains from the Plant Pathology Laboratory Collection (University of Alicante). We observed that the most virulent strain was *Beauveria bassiana* 1TS11 isolated from Tenerife, followed by *B. bassiana* 19TS04 and *Metarhizium anisopliae* 4TS04, also from Tenerife, and three fungi from the UA collection (*B. bassiana* 203, *B. bassiana* 53 and *M. anisopliae* 46). These fungi generated *G. mellonella* 100% mortality after two days (Fig. 1). In the second replicate of this test the same virulence was obtained. In the experiments at environmental humidity, the results are similar to the moist chamber ones, with a delay to achieve 100% mortality but with the same pathogenic characteristics of the strains (Fig. 2 and 3). This suggests that EF strains from banana fields are active under environmental stress (low humidity).

Figure 1. Moist chambers of *G. mellonella* larvae after exposure with entomopathogenic fungi isolated from Tenerife soils, *B. bassiana* 19TS04 (left) and *M. anisopliae* (right).

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Table 4. Number of spores adhered to *G. mellonella* larvae used in the virulence tests in moist chambers and under environmental humidity, and estimated survival mean time (time in days when 50% of the larvae are dead) in each incubation condition (with/without humidity).

Isolate	Environmental Humidity		Moist chamber	
	Spores (mean ± SE)	Estimated survival mean time (days)	Spores (mean ± SE)	Estimated survival mean time (days)
Lecanicillium lecanii 131	4.02E+05 ± 4.04E+05	15.3	3.27E+05 ± 5.75E+04	13.033
Lecanicillium psaliotae	7.12E+05 ± 5.55E+05	15.133	1.62E+05 ± 9.70E+04	5.4
Beauveria bassiana 203	9.08E+06 ± 5.46E+06	4.5	2.18E+05 ± 4.07E+04	1.767
Beauveria bassiana 53	1.51E+06 ± 1.73E+06	6.133	2.55E+05 ± 1.63E+05	2.167
Beauveria bassiana 119	4.23E+04 ± 1.10E+04	6.533	1.00E+04	2.393
Metarhizium anisopliae 46	2.22E+06 ± 1.06E+06	5.467	1.73E+05 ± 1.42E+05	1.733
Beauveria sp. 19TS04	6.96E+06 ± 4.62E+06	4.714	1.69E+06 ± 5.59E+05	1.821
Beauveria sp. 1TS11	1.10E+07 ± 1.02E+07	4.75	1.23E+06 ± 4.45E+05	1.367
Metarhizium sp. 4TS04	8.08E+05 ± 6.97E+05	4.714	2.13E+05 ± 1.96E+05	2
Lecanicillium sp. 5TS08	1.52E+07 ± 9.03E+06	11.607	1.17E+06 ± 2.44E+05	11.467
Lecanicillium sp. 2TS05	4.54E+06 ± 8.65E+05	10.714	1.94E+06 ± 9.61E+05	9.967
Lecanicillium sp. 6TS01	2.70E+06 ± 1.71E+06	7.893	1.53E+06 ± 1.12E+06	8.6
Control	-	15.864	-	14.267

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Regarding the moist chambers tests, *B. bassiana* 19TS04 despite having more inoculum per larva (1.69E+06 ± 5.59E+05) than *B. bassiana* 1TS11 (1.23E+06 ± 4.45E+05) is significantly less pathogenic, with 1.821 and 1.367 estimated survival mean time, respectively, indicating that 50% of individuals are dead at these times. In the case of *M. anisopliae* 4TS04, having less inoculum (2.13E+05 ± 1.96E+05) than *B. bassiana* 19TS04, no effect was found in the cumulative survival rate (no significant difference). Lastly, *L. lecanii* 2TS05 and *L. lecanii* 5TS08 treated larvae did not differ from uninoculated ones. This indicates low pathogenicity of both *L. lecanii* isolates to *G. mellonella* test (Table 4).

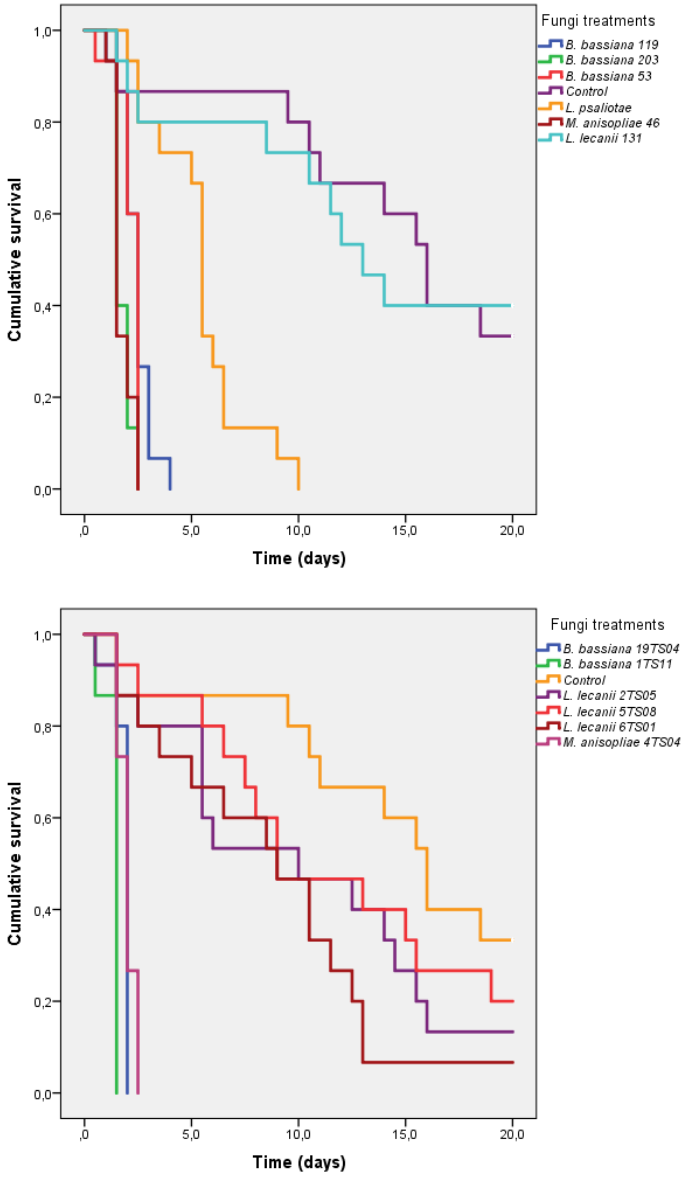

Figure 2. Pathogenicity bioassay of EF on *Galleria mellonella* under incubation on moist chamber conditions. Top: UA isolates. Bottom: MUSA isolates.

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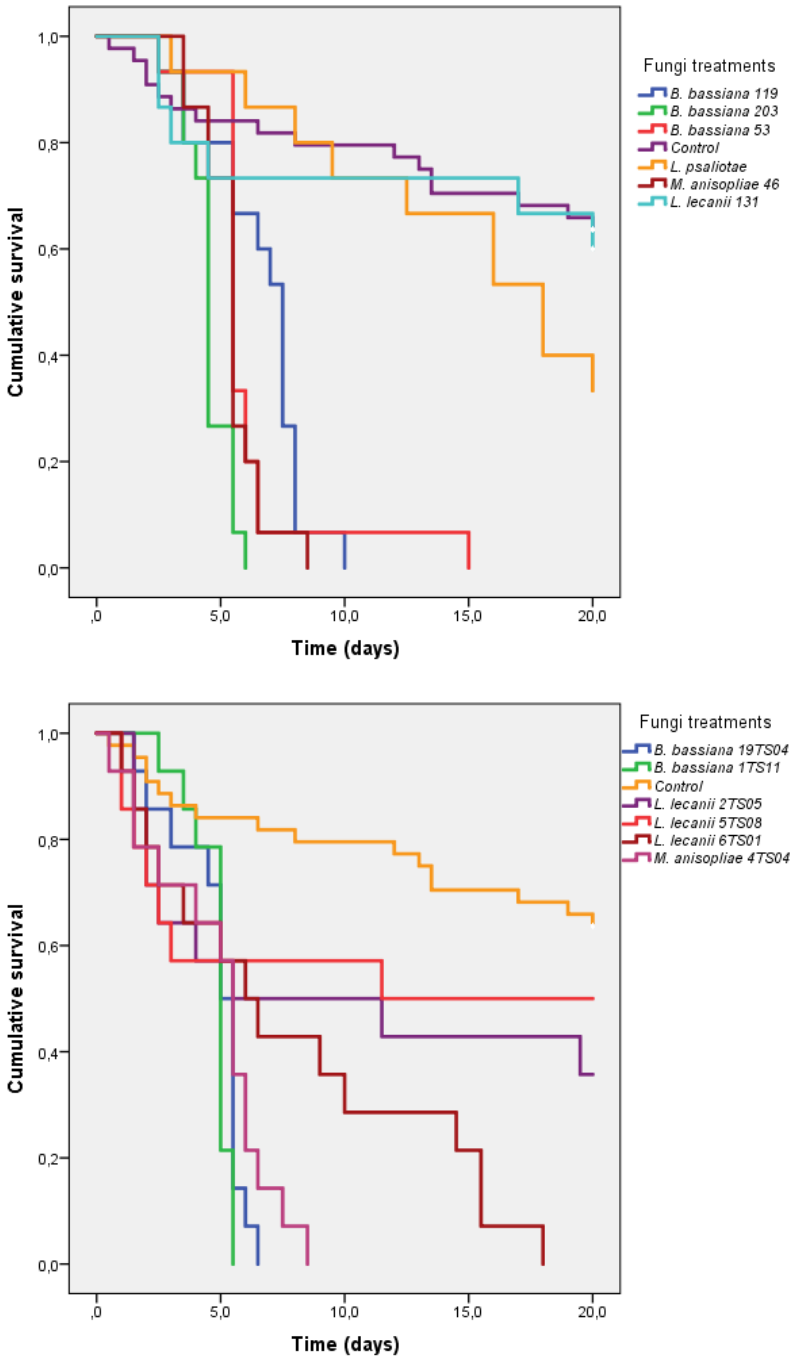

Figure 3. Pathogenicity bioassay of EF on *G. mellonella* under incubation without extra humidity (environmental moisture). Top: UA isolates. Bottom: MUSA isolates.

We have also performed a pathogenicity test on *Cosmopolites sordidus* larvae (Fig. 4-6). Larvae used in this trial comprised sizes between 0.5 and 2 cm (included several larval stages), were very sensitive to handling and dryness. Larvae were placed in Petri dishes with moist filter paper. This test was carried out with only two EF isolates from banana soils, due to the small number of larvae available from laboratory rearing (see before). The mortality of the larvae was very high in the first hours, even the control larvae (exposed to

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uninoculated CMA). Even so, *Metarhizium* sp. 4TS04 was slightly more virulent than *Beauveria* sp. 19TS04 with 87% and 69% mortality after 1.5 days, respectively. However, the differences were not significant. In the control, the mortality at 1.5 days is 60%. Both EF achieved full mortality (100%) at 2.5 days while controls did so at 6 days.

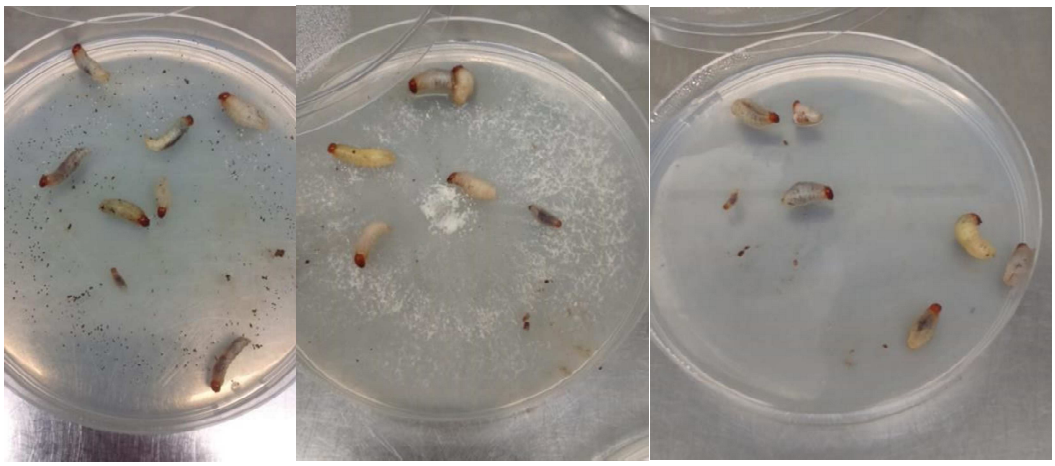

Figure 4. *C. sordidus* larvae exposed to entomopathogenic fungi isolated from Tenerife soil samples. Left: *M. anisopliae*. Middle: *B. bassiana.* Right: control (uninoculated).

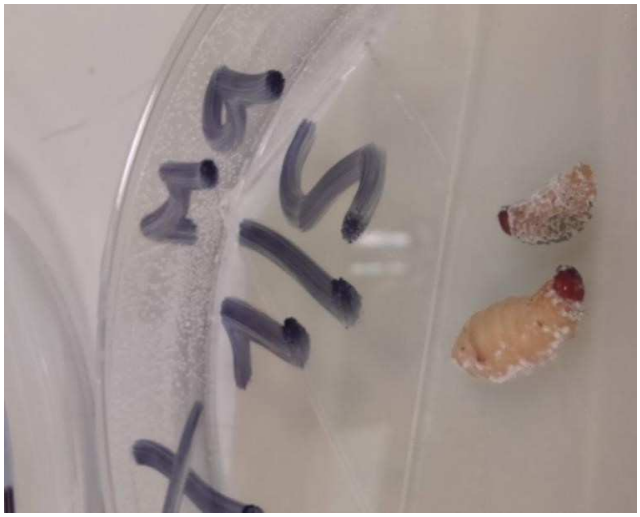

Figure 5. *C. sordidus* larvae inoculated with *M. anisopliae* 4TS04 from Tenerife. Fungal sporulation on the insects.

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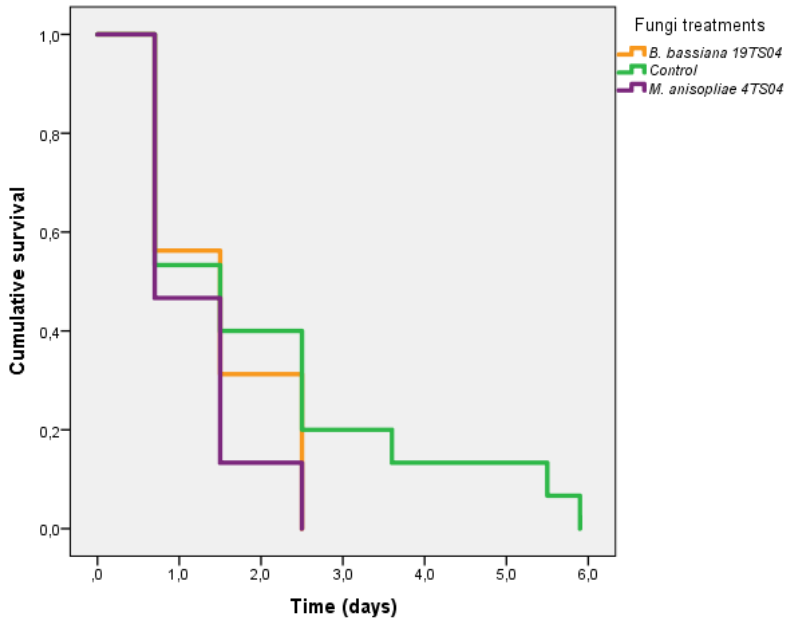

Figure 6. Pathogenicity test of isolated EF on *Cosmopolites sordidus* larvae under moist chamber conditions.

2.2 Parasitism of BW by Entomopathogenic nematodes

Biological data on BW antagonists. Numerical results of parasitism assays carried out to evaluated BW antagonisms by microorganisms of EPN.

Cosmopolites sordidus (BW) was observed in Cuba first time in 1944. It is the main insect pest in banana/plantain crops and is present in all the country. BW populations build up slowly and their damage becomes increasingly important in successive crop cycles. Yield losses are estimated between 19 to 34 %.

Banana and plantain productive systems in Cuba are monoculture (medium and large areas, mainly in flat lands) and intercropping (flat and mountain areas). For BW management, in Cuba an Integrated Pest Management (IPM) program was developed using mainly cultural and biological tactics. (Figure 7)

The behaviour of several cultivars of banana and plantain inoculated with BW were evaluated by Castellón et al. in 2018. The authors found differences between cultivars, that would be useful for farmers in the selection of material for BW infested fields.

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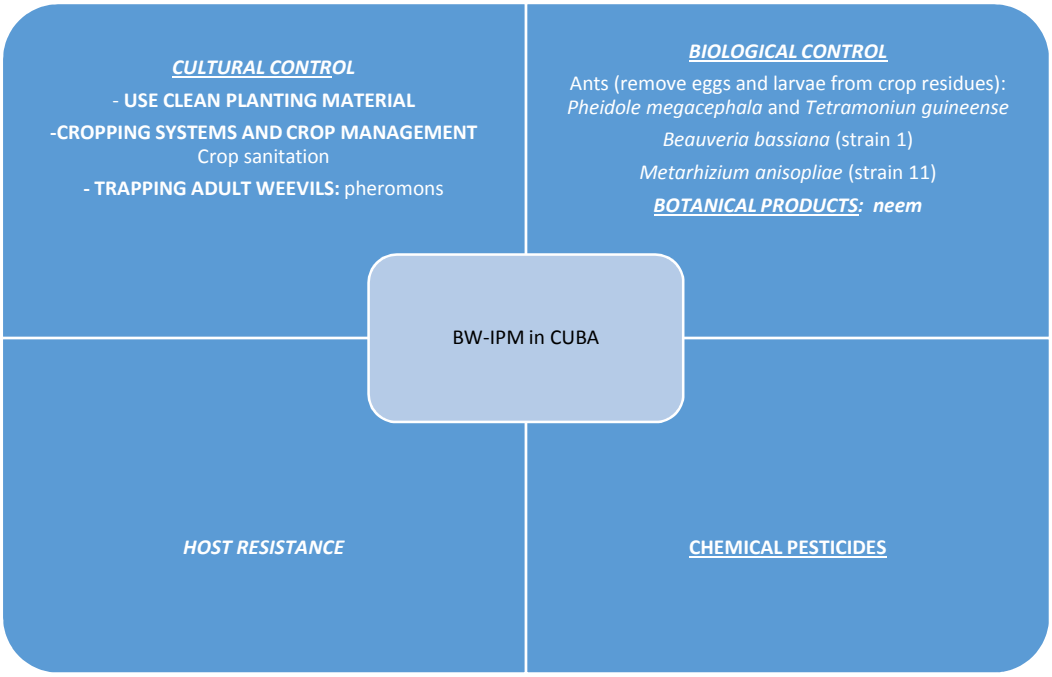

Figure 7. IPM suggested by Martínez *et al.* (2006) for Banana Weevil in Cuba. (Note: The chemical control is suggested but actually it is not useed by farmers).

Cultivars with > 40% BW infestation: Manzano criollo, Hembra ¾, Macho rojo, Macho indio.

Cultivars with 20% BW infestation: FHIA 18, Tamburu, Burro ¾, Burro criollo, Burro enano, Burro amarillo and CEMSA ¾.

Tolerant cultivars: FHIA-01, FHIA-23, Burro CEMSA, Burro Pelipita, INIVIT PB-2003, FHIA-03, FHIA-20, FHIA-20, FHIA- 21, Dwarf yawa, INIVIT PV-0630, INIVIT PV-2011, INIVIT PB-2012, Hua mua, Maia maoli, Boungfu.

Biological control of pest in Cuba has been successful using isolates of *B. bassiana* and *M. anisopliae*. However, there are few studies under laboratory and field conditions on the efficacy of EPNs in BW management.

EPNs have been evaluated for BW management in several Latin-American countries in laboratory and field assays. New isolates of EPNs have been obtained in Cuba and their effect has been evaluated in laboratory, using *Heterorhabditis amazonensis* strain HC1 as control strain. This strain is used in Cuba for pests control. Two experiments were carried out using at the CENSA Nematology Lab. facilities.

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BW colonies. *C. sordidus* adults were collected in *Musa* spp. fields, using two types of traps: sandwich and disc (modified, Figure 8) located in fields without biological or chemical treatments, in Mayabeque Province (western region of Cuba).

Figure 8. Sandwich trap (left) and modified disc trap (right)

BW colonies were established in laboratory conditions in plastic trays using banana pseudostem fragments as diet (Figure 9).

Figure 9. Quarantine cabinet for banana weevil adults for laboratory studies.

Effect of EPN *Heterorhabditis amazonensis* strain HC1 on BW adults. BW adults for this trial proceeded from San José de las Lajas Municipality (Mayabeque Province). Adults with similar weight and size from the quarantine cabinets were used in EPNs trial in the Nematology Lab.

Five BW adults were placed per Petri dish and were sprayed with five different concentrations of EPNs (125, 250, 500, 2500, 5000 infective juveniles) per adult and control (distilled water) (*Heterorhabditis amazonensis* strain HC1), using three plates per each treatments. Nematodes were obtained from *G.mellonella* infected larvae.

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In each plate, small disinfected pieces of banana pseudostem were placed for BW feeding. Plates were sealed with parafilm and kept at 27°C. BW mortality was evaluated every 12 hours, from the inoculation day til day 21. Mortality data were used in Probit Analysis to calculate the LT_{50}, LT_{90}, LC_{50} and LC_{90}. Dead BW were placed (individually) in Petri dishes. Four-five days afterwads, dead bodies were dissected and EPNs were observed. Symptoms of infected BW were described.

H. amazonensis strain HC1caused BW adults mortality (Figures 10 and 11). This strain must be used as a reference in studies relative to characterization of new EPNs isolates from banana/plantain fields in western Cuba (WP2).

Figure 10. Dead BW adults showed, as symptomatology of EPN effects, reduction of mobility, hard cadavers and maintained their dark brown color, odorless. The weevil shrinks its legs when it dies

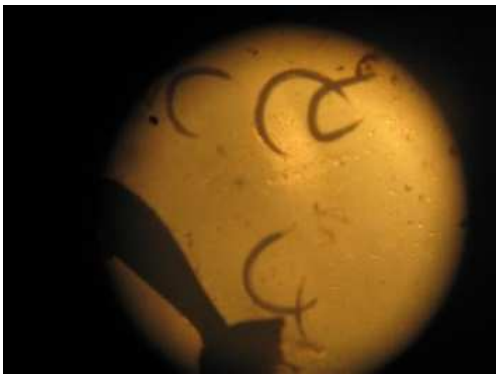

Figure 11. Hermaphrodite females emerging form dead body of BW, indicative of EPNs pathogenicity in adults

The LC_{50} and LC_{90} of *H. amazonensis* strain HC1 were estimated for BW. The LC_{50} was estimated in > 1800 infective juvenile (IJ) per BW adult; LC_{90} was estimated in > 6000 IJ/adult. These results must be used to analyse the efficacy of new EPN isolates in trials (Table 5 and 6). Around 50 % of BW adults died at 417 hours (~17 days) and 90 % at 693 hours (~29 days).

The HC1 strain caused significant mortality on BW adults (Figure 12), with highest mortality in the treatment with highest concentration (5000 infective juveniles per BW adult).

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Table 5.

LETHAL CONCENTRATION (LC)			
	Confidence limit: 95 % for concentrations		
Probability	Estimation	Inferior limit	Superior limit
.500	1872.366	816.781	3265.612
.550	2283.130	1292.318	3984.803
.600	2700.511	1709.664	4781.433
.650	3131.908	2093.658	5652.179
.700	3586.535	2464.735	6603.409
.750	4077.150	2840.635	7654.486
.800	4623.472	3240.232	8843.895
.850	5260.277	3690.098	10246.211
.900	6061.523	4241.096	12025.680

Table 6.

LETHAL TIME (LT)			
	Confidence limit: 95 % for time		
Probability	estimation	Inferior limit	Superior limit
.500	417.653	362.552	519,350
.550	444.629	383.693	558.651
.600	472.040	405.009	598.751
.650	500.371	426.912	640.326
.700	530.228	449.889	684.244
.750	562.448	474.594	731.731
.800	598.327	502.019	784.694
.850	640.148	533.902	846.514
.900	692.768	573.925	924.391

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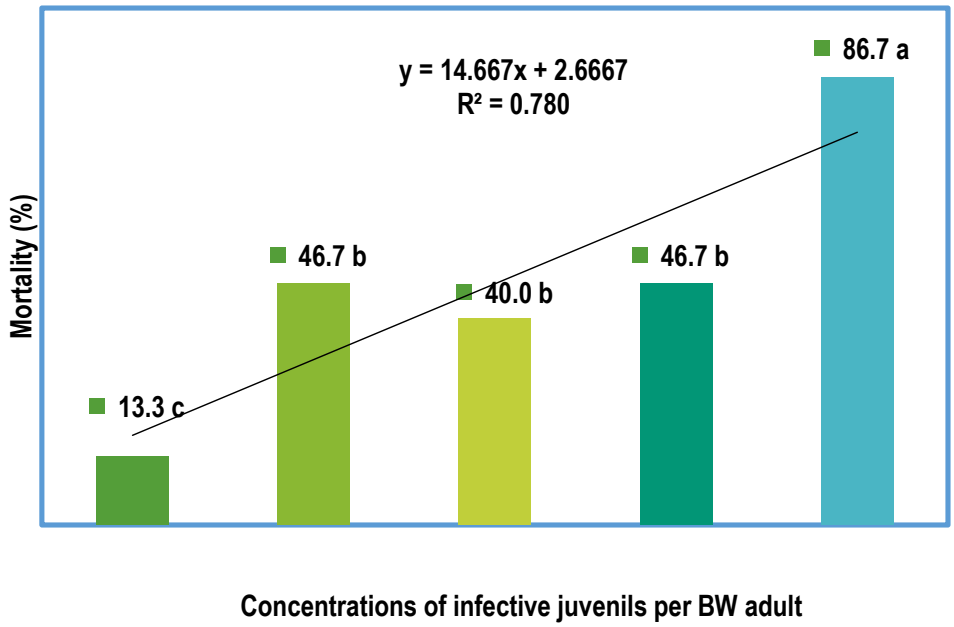

Figure 12. Mortality (%) of *C. sordidus* adults caused by different concentrations of infective juveniles of *Heterorhabditis amazonensis* strain HC1.

BW mortality with three EPN doses ranged from 40 to 47 % (Figure 12). The highest concentration (5000 IJ/ BW adult) caused > 85 % mortality in adults. These results are really high compared with mortality from literature data and show the potential of HC1 strain for BW management.

Some authors noted that the EPN do not cause mortality in adults in trials *in vitro*: meanwhile, other noted that some particular strain of EPN affected BW adults. According to results obtained in this experiment, the dose of 5000 IJ per BW adult was selected for future experiments, and each trial must be evaluated, at least, during 21 days.

Efficacy of different EPNs isolates against BW. Five isolates (Table 7) were screened for their *in vitro* efficacy against BW under laboratory conditions at CENSA, using strain HC1 of *H. amazonensis* as reference. All isolates proceeded from banana/plantain soils located in Pinar del Río Province (western region of Cuba) and were obtained previously during WP2. They are deposited in the Nematology Laboratory at CENSA.

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Table 7. Details about Cuban isolates using in trials of efficacy of EPN against BW adults (more details in Del. D2.1 from WP2).

Taxonomic name	Code n.	Collection location	No. in graphic with results
Heterorhabditis sp.	PR-C1	NL-CENSA	Isolate 1
Heterorhabditis sp.	PR-C2	NL-CENSA	Isolate 2
Heterorhabditis sp.	PR-CSJ	NL-CENSA	Isolate San José
Heterorhabditis sp.	PR-C4	NL-CENSA	Isolate 4
Heterorhabditis sp.	PR-C5	NL-CENSA	Isolate 5

Symbiotic bacteria associated to the five EPN strains were characterized using API and several growth media. All bacteria belonged to *Photorhabdus* spp. They are shrt bacilli, gram negative, form red colonies on McConkey medium and give positive reaction in egg and milk media. BW adults for this trial were collected Nueva Paz Municipality (Mayabeque Province). They were placed in quarantine cabinetes, until used for experiments.

Figure 13. BW adults with similar weight and size, proceeded from the quarantine cabinets maintained in laboratory conditions were used for experiments in the Nematology Lab

In Petri dish with filter paper (Fig. 13), ten BW adults per plate were sprayed with EPNs (5000 infective juveniles per BW adult). Two controls were performed: a negative one using distilled water and a positive one with *H. amazonensis* reference strain HC1. Three plates were used per treatment. Nematodes were obtained from *G. mellonella* infected larvae.

In each plate, disinfected small pieces of banana pseudostem were placed for feeding BW. Plates were sealed with parafilm and kept at 27°C. BW mortality was evaluated every 12 hours, from the inoculation day til day 25. Mortality data were analyzed by ANOVA.

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EPN caused significant mortality on BW vs. negative control applied with distilled water (Fig. 14). The highest mortality (30 %) was caused by *H. amazonensis* HC1 strain. *Heterorhabditis* sp. isolates from Cuban banana/plantain also caused BW mortality. The maximum mortality was over 15 % .

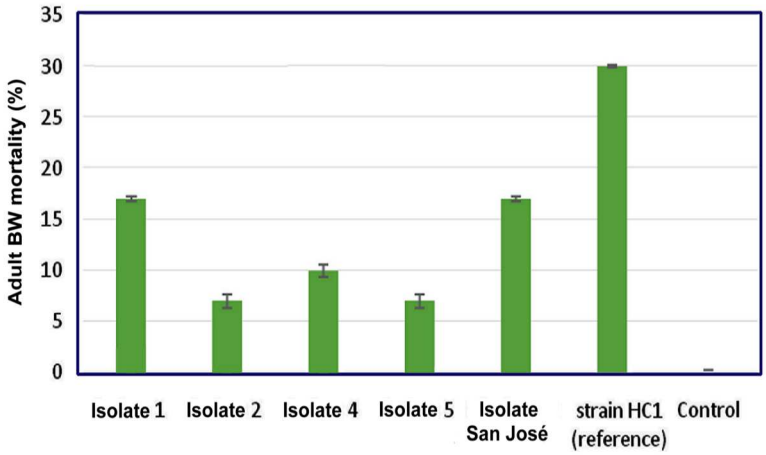

Figure 14. Mortality of BW by EPNS from Cuban banana/plantain fields.

2.3 Colonisation of Banana Plants by EBCAs

A total of 1097 single/pure bacterial and/or fungal isolates were obtained after two samplings rounds in the Canary Islands (Tenerife, La Palma and La Gomera). Bacterial and fungal isolates were tested against representative isolates of *Fusarium oxysporum* f. sp. *cubense* (Foc) races. As a result, 150 strains showed antagonism against one or more Foc tested races. These strains have been characterized as for the presence of common traits related to biocontrol (BC) and plant-growth promotion (PGP) abilities, and have been identified at the molecular level by sequencing of several housekeeping genes. A preliminary risk analysis study of the EBCAs selected was carried out. Table 8 shows: bacteria/fungi codes at IAS collection, molecular identification, origin, *in vitro* antagonism results against different Foc races, number of positive activities related to biocontrol (BC) and plant growth promotion (PGP), and potential risks of selected EBCAs. Table 9 shows detailed information about activities related to BC and PGP for each strain. 29 most promising strains were analysed, based on BC and PGP activities and their ability to antagonize against Foc. Strains marked in blue colour have been selected for future *in planta* biocontrol assays against Foc together with two bacterial strains isolated from olive.

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Table 8. Most promising banana root endophytes from Canary Islands based on activities related to Biocontrol (BC) and Plant-Growth Promotion (PGP), and their ability to in vitro antagonize *Fusarium oxysporum* f. sp. *cubense* races.

ISOLATES	Molecular ID*	Origen	*In vitro* antagonism against *Foc*			PGP and BC activities	Risk
Bacteria			TR4	STR4	R1		
IAS-B-102	*Pseudomonas chlororaphis*	Tenerife Farm 00 (Escuela Capataces)	Yes	No	Yes	7	No
IAS-B-103	*Serratia marcescens*	Tenerife Farm 02 (Temaso)	+/-	Yes	+/-	7/8	Yes (1)
IAS-B-197	*P. chlororaphis*	Tenerife Farm 01 (Siverio)	Yes	Yes	Yes	6	No
IAS-B-301	*P. chlororaphis*	Tenerife Farm 01 (Siverio)	Yes	Yes	Yes	5	No
IAS-B-364	*P. chlororaphis*	Tenerife Farm 00 (Escuela Capataces)	Yes	Yes	No	7	No
IAS-B-444	*P. chlororaphis*	Tenerife Farm 02 (Temaso)	Yes	Yes	Yes	6	No
IAS-B-471	*P. chlororaphis*	Tenerife Farm 02 (Temaso)	Yes	Yes	Yes	5	No
IAS-B-478	*P. chlororaphis*	Tenerife Farm 02 (Temaso)	Yes	Yes	Yes	6	No
IAS-B-481	*P. chlororaphis*	Tenerife Farm 02 (Temaso)	Yes	Yes	Yes	7	No
IAS-B-483	*P. chlororaphis*	Tenerife Farm 02 (Temaso)	Yes	Yes	+/-	5	No
IAS-B-504	*P. chlororaphis piscium*	Tenerife Farm 02 (Temaso)	Yes	Yes	Yes	7	No
IAS-B-793	*P. protegens*	Tenerife Farm 04 (Malpaís - Colpon Agrícola)	Yes	Yes	Yes	6	No
IAS-B-931	*P. chlororaphis aurantiaca*	La Palma Farm 07 (Siso, Fuencaliente)	Yes	Yes	ND	6	No
IAS-B-944	*P. chlororaphis aureofaciens*	La Palma Farm 07 (Siso, Fuencaliente)	Yes	Yes	ND	5	No
IAS-B-962	*P. chlororaphis piscium*	La Palma Farm 08 (Ortiz, Tijarafe)	Yes	Yes	ND	5	No
IAS-B-966	*P. chlororaphis piscium*	La Palma Farm 08 (Ortiz, Tijarafe)	Yes	Yes	ND	5	No
IAS-B-1013	*P. chlororaphis*	Tenerife Farm 09 (Escuela Capataces)	Yes	Yes	ND	5	No
IAS-B-1040	*Serratia marcescens*	La Gomera Farm 11 (David, San Sebastián)	Yes	Yes	ND	7/9	Yes (1)
IAS-B-1054	*P. chlororaphis aureofaciens*	Tenerife Farm 09 (Escuela Capataces)	Yes	Yes	ND	5	No
IAS-B-1075	*P. chlororaphis aureofaciens*	La Gomera Farm 10 (Hermigua)	Yes	Yes	ND	5	No
IAS-B-1090	*Serratia marcescens*	La Gomera Farm 11 (David, San Sebastián)	Yes	Yes	ND	8	Yes (1)
Fungi							
IAS-B-54	*Fusarium* sp.	Tenerife Farm 00 (Escuela Capataces)	Yes	Yes	Yes	2	Yes (2)
IAS-B-65	*Fusarium oxysporum*	Tenerife Farm 00 (Escuela Capataces)	Yes	Yes	Yes	1	Yes (2)
IAS-B-67	*Fusarium oxysporum*	Tenerife Farm 00 (Escuela Capataces)	Yes	Yes	Yes	1	Yes (2)
IAS-B-69	*Fusarium proliferatum*	Tenerife Farm 00 (Escuela Capataces)	Yes	Yes	Yes	1	Yes (2)
IAS-B-505	*Fusarium solani*	Tenerife Farm 02 (Temaso)	Yes	Yes	Yes	4/5	Yes (2)
IAS-B-918	ND	Tenerife Farm 05 (Fco Pacheco, Arico)	Yes	Yes	ND	2	ND
IAS-B-968	ND	Tenerife Farm 06 (La Caldera, Adeje)	Yes	Yes	ND	2	ND
IAS-B-1057	ND	Tenerife Farm 09 (Escuela Capataces)	Yes	Yes	ND	1	ND

*Based on DNA *gyrB* and *16S* RNA gene sequences for bacteria and ITS and elongation factor for fungi. ND, not determined. (1) *Serratia marcescens* is often associated to nosocomial infections. It is considered a harmful human pathogen which has been known to cause urinary tract infections, wound infections and pneumonia. However, these strains showed many enzymatic activities related to BC and PGP. It would be interesting to evaluate the potential of metabolites secreted to culture media against *Foc*. (2) These fungal strains could be potential phytopathogens.

Table 9. Phenotypes traditionally associated with biocontrol/plant growth promotion.

ISOLATES	Butanediol	Catalase	Phytase	HCN	Protease	Siderophores	β-Glucosidase	Phosphatase	Xylanase	Amylase
Bacteria										
IAS-B-102	-	+	+	+	+	+	-	+	-	-
IAS-B-103	+	+	+	-	+	+	+	+	-	+?
IAS-B-197	-	+	+	+	+	+	-	+	-	-
IAS-B-301	-	+	+	+	+	+	-	-	-	-
IAS-B-364	-	+	+	+	+	+	-	+	-	-
IAS-B-444	-	+	+	-	+	+	-	+	-	-
IAS-B-471	-	+	+	+	+	+	-	-	-	-
IAS-B-478	-	+	+	+	+	+	-	+	-	-
IAS-B-481	-	+	+	+	+	+	-	+	-	-
IAS-B-483	-	+	+	+	+	+	-	-	-	-
IAS-B-504	-	+	+	+	+	+	-	+	-	-
IAS-B-793	-	+	+	+	+	+	-	+	-	-
IAS-B-931	-	+	+	+	+	+	-	+	-	-
IAS-B-944	-	+	+	+	+	+	-	-	-	-
IAS-B-962	-	+	+	+	+	+	-	-	-	-
IAS-B-966	-	+	+	+	+	+	-	-	-	-
IAS-B-1013	-	+	+	+	+	+	-	-	-	-
IAS-B-1040	+	+	+	-	+	+	+	+	+?	+?
IAS-B-1054	-	+	+	+	+	+	-	-	-	-
IAS-B-1075	-	+	+	+	+	+	-	-	-	-
IAS-B-1090	+	+	+	-	+	+	+	+	-	+
Fungi										
IAS-B-54	-	+	-	-	-	-	+	-	-	-
IAS-B-65	-	+	-	-	-	-	+	-	-	-
IAS-B-67	-	+	-	-	-	-	+	-	-	-
IAS-B-69	-	+	-	-	-	-	+	-	-	-
IAS-B-505	-	+	+	-	-	+	+	-	-	+?
IAS-B-918	-	+	-	-	-	-	+	-	-	-
IAS-B-968	-	-	-	-	-	-	+	-	+	-
IAS-B-1057	-	-	-	-	-	-	+	-	-	-

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One hundred and thirty (130) fungal isolates were recovered from internal tissues of healthy banana roots from three different banana production systems in Costa Rica. These were: organic (52 isolates), fallow (42 isolates) and conventional (36 isolates, Fig. 15). All fungal isolates are deposited in the endophyte collection at EARTH University. The most frequent fungus recovered belonged to genus *Trichoderma*. The plant growth promotion of nine *Trichoderma* isolates (3 from organic, 3 from fallow and 3 from conventional renovation) were tested on tissue-culture plants of Grande Naine (Musa AAA) cultivar. Plants were challenged with a spore suspension of each isolate of *Trichoderma* spp. under greenhouse conditions at EARTH University. Isolates Endo 1, Endo 2 and Endo3 from organic systems as well as Endo 4 from fallow increased banana shoot weight respect to that of untreated control (Fig. 16). In addition, preliminary results on biocontrol activity towards the burrowing nematode *Radopholus similis* were conducted under greenhouse conditions. The endophytes reduced nematodes penetration in the root systems in comparison to control (Fig. 17).

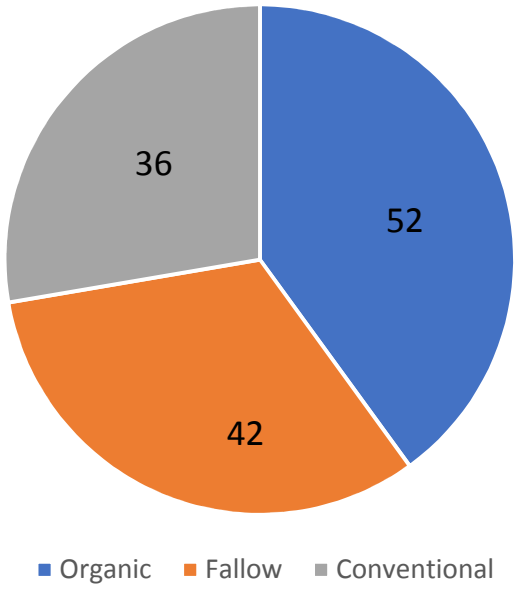

Figure 15. Isolation of 130 endophytic fungi from three different banana production system: organic (52), fallow (42) and conventional (36) at EARTH University.

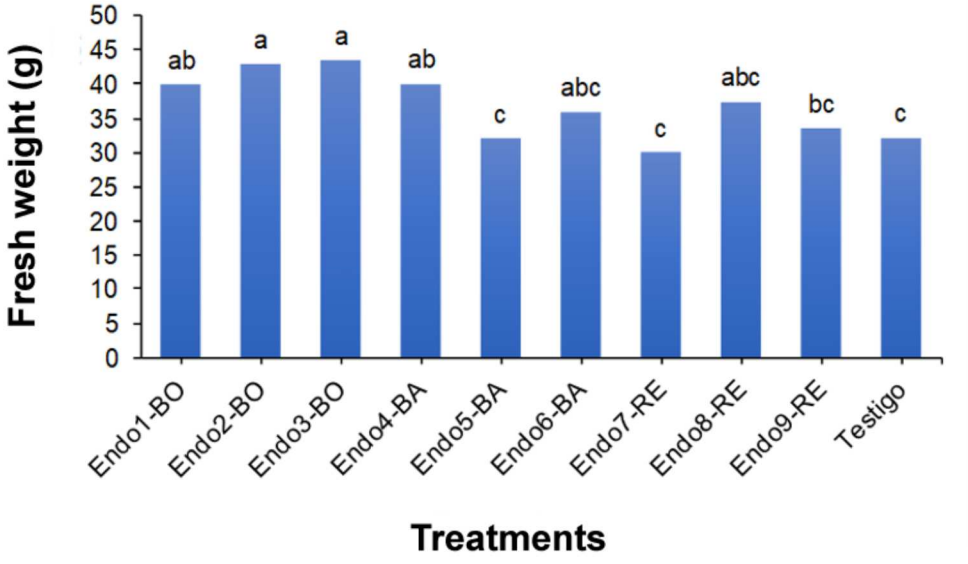

Figure 16. Effect of Trichoderma isolates on the plant growth promotion on tissue cultured plants of Grande Naine.

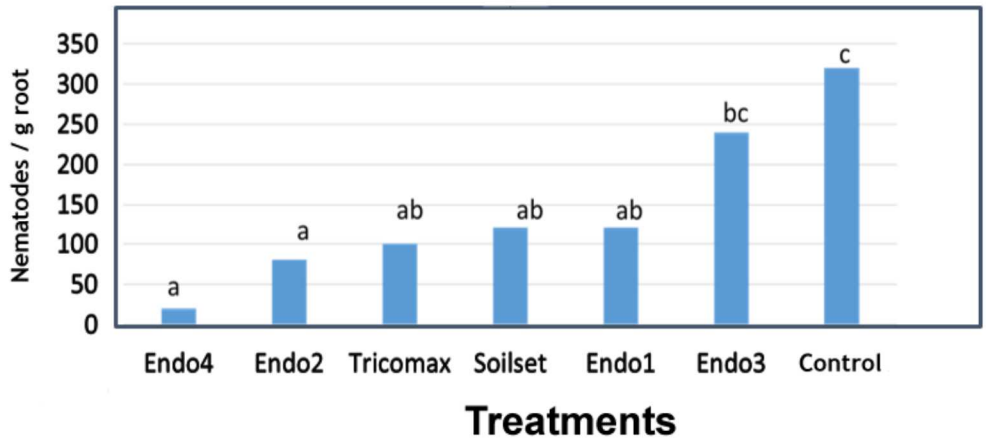

Figure 17. Effect of the endophytes on the penetration of *R. similis* on the root under greenhouse conditions at EARTH University.

There are currently two bioassays in progress for evaluating the biocontrol activity towards burrowing nematode *R. similis* and Panama disease. Both bioassays will be assessed at the end of July 2019.

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***In vivo* test of *Pochonia chlamydosporia* VC21 endofitic activity.** *P. chlamydosporia* INEM-VC-21 was isolated from kiwi roots attacked by *Meloidogyne* spp. coming from Metaponto (Matera, Italy) by IPSP. The strain has been deposited on 2013.03.07 at the International Depositary Authority DSMZ (Germany) with the deposit number: DSM26985.

The following experimental test aimed at evaluating the endophytic activity of *P. chlamydosporia* strain VC21 on tomato and to evaluate the efficiency of two different inoculation modalities: foliar and soil. Four treatments were prepared: 1) Leaf treatment (F); 2) Leaf treatment control (FC); 3) Soil treatment (T); 4) Soil treatment control (TC).

After 24 days from seeding the plants were inoculated with a conidial suspension of *Pochonia chlamydosporia* VC21 at the concentration of 10^8 CFU / ml. The modalities of inoculation in the various thesis were the following: in treatment 1 (F) the conidial suspension was applied by spraying until the leaf surface was saturated; in treatment 2 (FC) the nebulization of sterile distilled water was performed; in treatment 3 (T) soil was inoculated with 10 ml of conidial suspension per plant; in treatment 4 (TC) soil was inoculated with sterile distilled water (Fig. 18).

Figure 18. Inoculation of isolate Vc21 conidial suspensions

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Two weeks after the inoculum two plants were harvested per treatment and from each plant we sampled two leaves, two pieces of root and two of stem. The surface of the samples was sterilized as described for the seeds and then plated on Petri dishes containing PDA supplemented with tetracycline, streptomycin and penicillin (2 mg / l each). The plates were incubated in the dark at 25 ° C in order to allow growth of the fungus and its re-isolation. For identification of *P. chlamydosporia* VC21 a Real-Time PCR was set up using the specific POSP F1 primers (5'GCGCCACATGGTTGAAGAGC 3 'and POSP R1 (5'TGTACCGCCTTAATCCAAG 3'), designed based on the GenBank sequence AJ427460 corresponding to complete VCP1 gene, which codes for an alkaline serinprotease (Rosso & Ciancio, 2005).

From portions of the stem of the treatment T (soil inoculum) it was possible to re-isolate the fungus with morphological characteristics that referred to the inoculated strain (Fig. 19). Real Time PCR showed an amplified product for the VCP1 gene portion, confirming the VC21 isolate identity (Fig. 20 – 21; Table 10).

Figure 19. Re-isolation of *P. chlamydosporia* from portions of stem

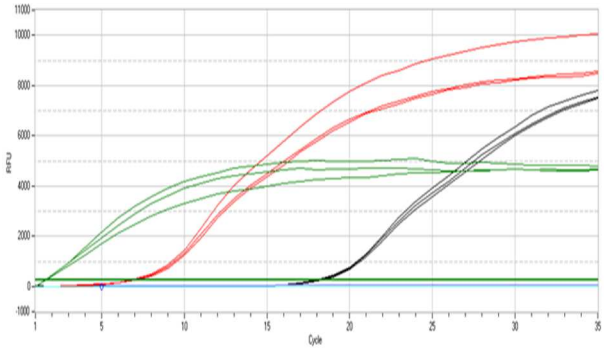

Figure 20. RtPCR amplified VCP1 products from fungi re-isolated from soil.

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Table 10. qPCR results for identification of *P. chlamydosporia*.

Fluorophore	Isolated fungi	Sample	Cq	Melting Temp.
SYBR	Unknown	*Pochonia* like	18,15	86,3
SYBR	Unknown	*Pochonia* like	18,23	86,2
SYBR	Unknown	*Pochonia* like	18,36	86,2
SYBR	Positive Control	Vc21	7,15	86,2
SYBR	Positive Control	Vc21	7,02	86,2
SYBR	Positive Control	Vc21	6,86	86,3
SYBR	Positive Control	F1	1,58	86,7
SYBR	Positive Control	F1	1,62	86,4
SYBR	Positive Control	F1	1,66	86,1

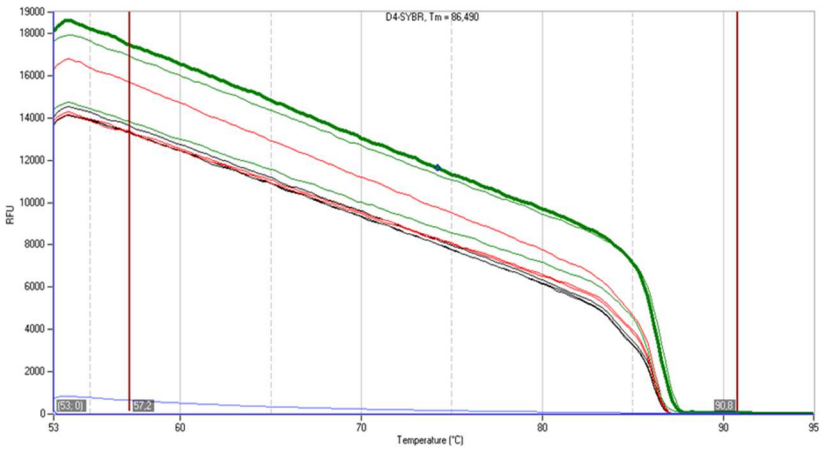

Figure 21. Melting Curve Results

The results show that the amplification took place for positive controls but also for the treated samples. Given the specificity of the primers used, the re-isolated fungi were identified as *P. chlamydosporia* VC21. The analysis of the melting curve further confirms this, since the Tm of the controls is similar to that of the sample. In conclusion, the test confirmed the *in vivo* endophytic activity of VC21 strain of *P. chlamydosporia*. The method of inoculation allowed the fungus to be re-isolated from plant portions after its introduction as a conidial suspension into soil.

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Tricalcic phosphate solubilization by *Pochonia clamydosporia*

Pochonia clamydosporia solubilizes a proportion of tricalcium phosphate (insoluble P) present in soil. The mechanism by which P solubilization takes place appears to be related to the production of a phytase. *Pochonia clamydosporia* was grown on selective agar medium until complete sporulation.

The specific medium used to detect the phosphorus-solubilizing microorganisms was the Pikovskaya broth, containing tricalcic phosphate. Four agar pieces (1 × 1 cm) of *P. chlamydosporia* culture were placed in the flasks containing 100 ml of Pikovskaya broth medium with addition of insoluble phosphate in the form of tricalcium phosphate. Three repetitions of the same thesis were prepared. The flasks were then incubated in a shaker at 24 ° C for 10 days. After incubation, the culture broth was centrifuged at 5000 rpm for 5 min to remove microbial cells and the insoluble material present. The supernatant was removed, decanted and filtered. Soluble P was determined by ICP readings (Rashid et al. 2004). Soluble P was periodically quantified up to 10 days, to verify the progress of solubilization of tricalcium phosphate during the fungus growth (Table 12).

Table 12. Phosphate solubilization *by Pochonia chlamydosporia Vc21.*

time (days)	**ppm (ICP)**	**P Solubilization (%)**
3	226,5	22,1
5	196,8	19,2
7	392,5	38,3
10	230,4	22,5

2.4 Interactions among EBCAs

After isolation and mass screening for the antagonistic activity against *Foc*, 96 EBCAs isolates were obtained. Forty-four of them were used to construct a synthetic microbial community, namely SynCom1.0, which was tested *in planta* against *Foc* TR4. Since the biocontrol obtained was only moderate, SynCom1.0 was modified by reducing the number of members to the seven most effective as resulted *in vitro*. Therefore, SynCom1.1 was defined (Table 13). SynCom1.1 was assayed *in planta* and, again, moderate control of Fusarium wilt was obtained.

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Table 13. Composition of SynCom1.1.

Putative genus	Isolate
Pseudomonas sp.	P1C1
	PS5
	P1A1
Bacillus sp.	BT1
	BN8.2
Streptomyces sp.	St2AOB1
Trichoderma sp.	T2C1.4

Cellophane-agar method (metabolites released in the agar). Almost all SynCom1.1 members were inhibited partially or completely each other (Figure 23). Interestingly, *Streptomyces* sp. St2AOB1 did not inhibit other SynCom1.1 members. *Pseudomonas* sp. PS5 and *Bacillus* sp. BT1 inhibited only *Bacillus* sp. BT1, *Streptomyces* sp. St2AOB1, respectively, and (partially) *Trichoderma* sp. T2C1.4, but not the other SynCom1.1 members. Strains such as P1A1, P1C1 (*Pseudomonas* spp.), and BN8.2 (*Bacillus* spp.) inhibited most SynCom1.1 members. On the other hand, *Trichoderma* sp. T2C1.4 and the *Pseudomonas* spp. strains, especially PS5, were the most resistant against other SynCom1.1 members, with *Bacillus* sp. BT1 and *Streptomyces* sp. St2AOB1 as most sensitive. *Trichoderma* sp. T2C1.4 was inhibited by PS5, partially, and BN8.2, completely.

Overlapping-plates method (volatile organic compounds, or VOCs). Only *Trichoderma* sp. T2C1.4 was inhibited by VOCs produced by *Pseudomonas* spp. strains P1A1, P1C1 (partial inhibition) and *Bacillus* sp. BT1 (complete inhibition), and only when these strains were grown on LB agar (Fig. 22). T2C1.4 was also inhibited by *Pseudomonas* sp. PS5 and *Bacillus* sp. BN8.2, but just in the sporulation and not in the mycelium growth. SynCom1.1's members grown on PDA did not produce toxic VOCs (Figure 23).

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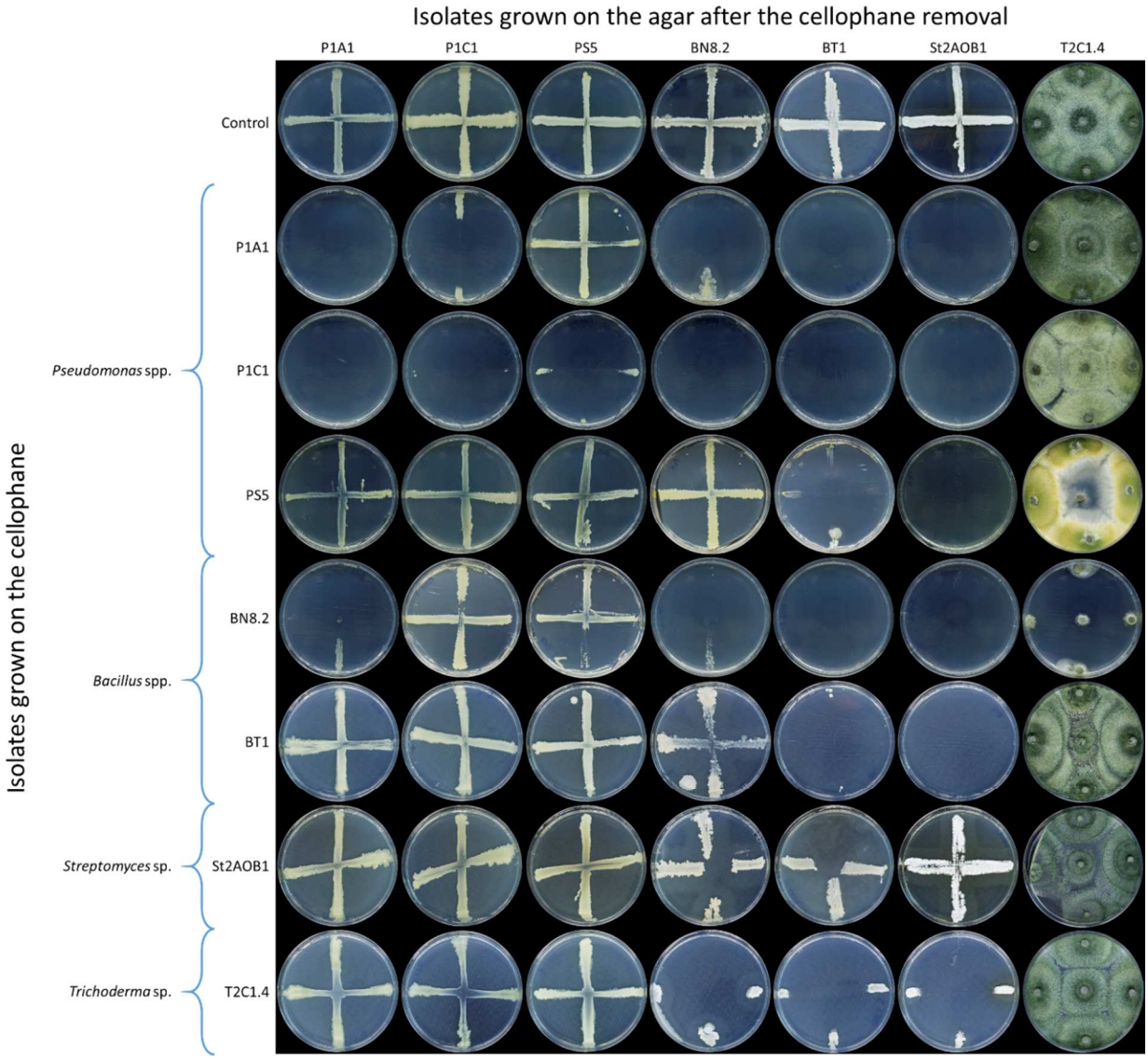

Figure 22. Cross antagonism among SynCom1.1 members due to metabolites released in the agar using a cellophane-agar assay.

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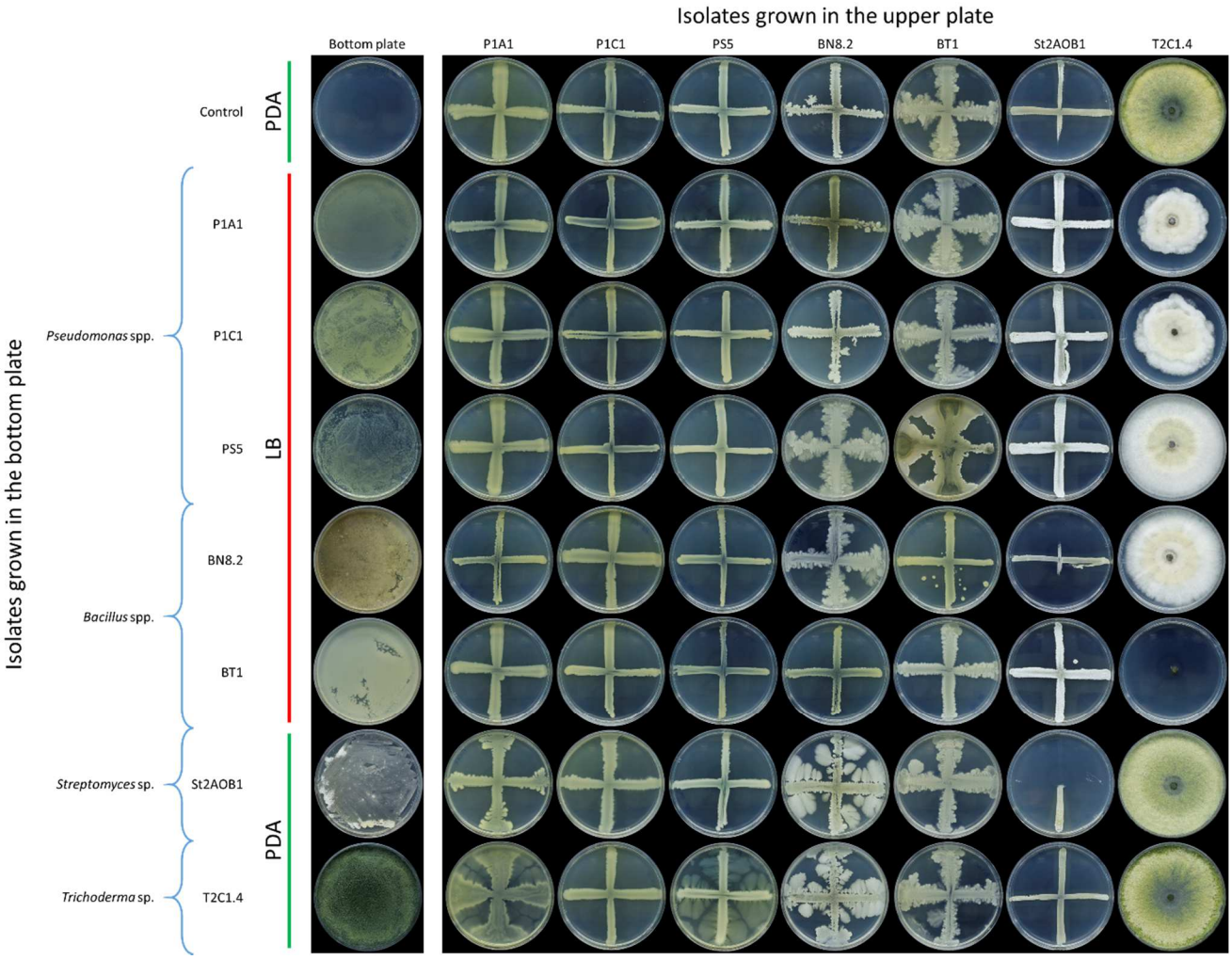

Figure 23. Cross-antagonism among SynCom1.1's members due to volatile organic compounds (VOCs) in an overlapping-plate assay. In the bottom plates, bacteria were grown on LB agar and fungi on PDA.

2.5 Interactions of EBCAs with *Fusarium oxysporum* f. sp. cubense

Cellophane-agar method (metabolites released in the agar). All SynCom1.1 members inhibited partially *Foc* R1 and *Foc* TR4, except St2AOB1 against *Foc* TR4 (Fig. 24). The highest inhibition of *Foc* was observed for PS5 and BN8.2.

Overlapping-plates method (volatile organic compounds, VOCs). The effect of VOCs produced by SynCom1.1, growing either on LB agar or PDA, was moderate or null (Fig. 25). Notably, when grown on LB agar, BT1 strongly inhibited *Foc* R1.

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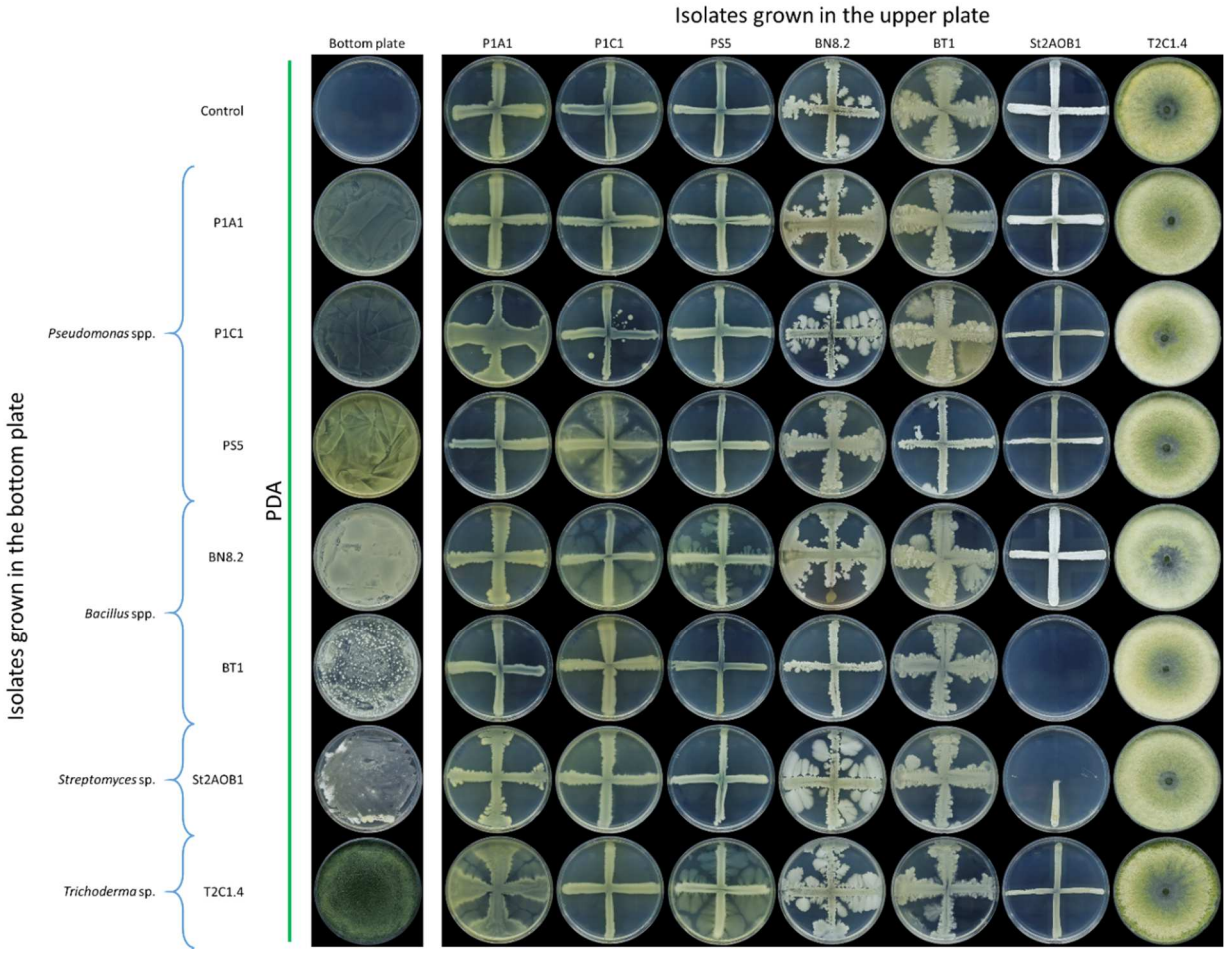

Figure 24. Cross-antagonism among SynCom1.1's members due to volatile organic compounds (VOCs) in an overlapping-plates assay. In the bottom plates, both bacteria and fungi were grown on PDA.

2.6 Interactions of *Fusarium oxysporum* f. sp. cubense with EBCAs

Cellophane-agar method (metabolites released in the agar). Both *Foc* R1 and TR4 were able to inhibit partially *Pseudomonas* spp. strains and completely (or almost completely) *Bacillus* spp. strains and *Streptomyces* sp. St2AOB1 (Fig. 27). Interestingly, *Trichoderma* sp. T2C1.4 was resistant against metabolites produced by *Foc*.

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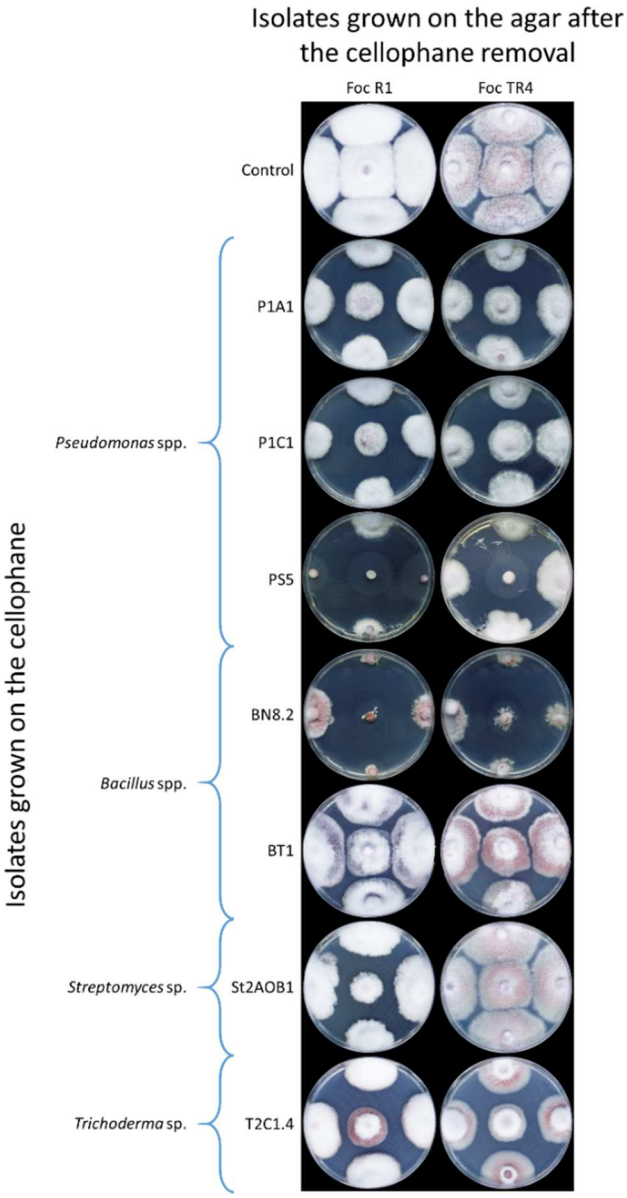

Figure 25. Effects of metabolites released in the agar by SynCom1.1 members against *Fusarium oxysporum* f. sp. *cubense* (*Foc*) in a cellophane-agar assay.

Overlapping-plates method (volatile organic compounds, or VOCs). Both *Foc* R1 and TR4 did not produce VOCs toxic against SynCom1.1's members (Fig. 28). Among the SynCom1.1's members, *Bacillus* sp. BN8.2 and *Pseudomonas* spp strains, especially PS5, were the most effective against *Foc* because of their diffusible metabolites. Also, *Bacillus* sp. BT1 and *Pseudomonas* sp. P1A1 produced VOCs effective against *Foc* R1, but not TR4.

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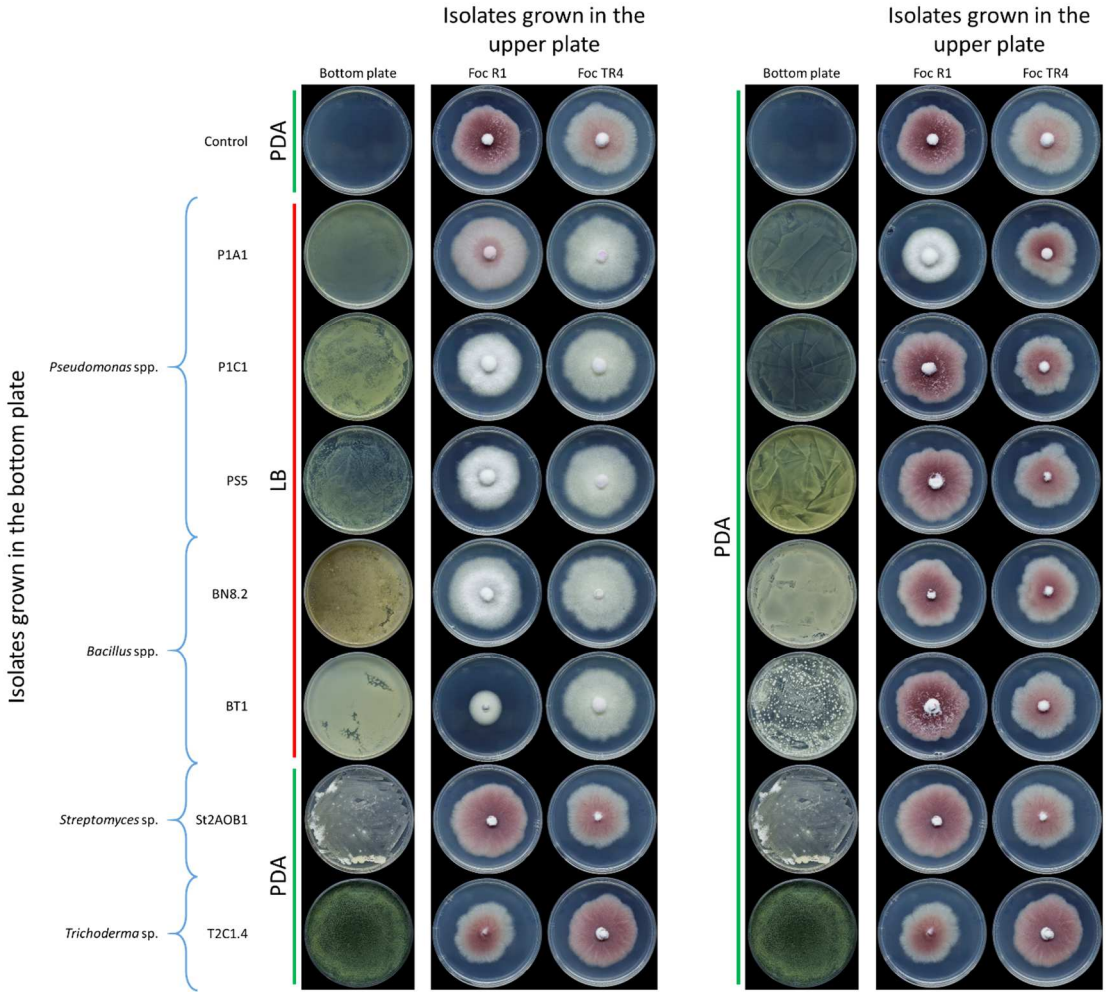

Figure 26. Effects of volatile organic compounds (VOCs) produced by SynCom1.1 members against *Fusarium oxysporum* f. sp. *cubense* (*Foc*) in an overlapping-plate assay.

We point out that EBCAs may exert antagonism not only against a pathogen like *Foc* but also against other beneficial microorganisms, a trait that would hamper the mixture of different EBCAs. In fact, in our experiments, some of SynCom1.1 members were able to inhibit each other. In particular, overall, cross-antagonism occurred mostly between strains belonging to different genera, but poorly between those of the same genus. Interestingly, *Trichoderma* sp. T2C1.4 and the *Pseudomonas* spp. strains, especially PS5, were the most resistant against diffusible metabolites produced by other SynCom1.1 members, though T2C1.4 was sensitive to VOCs. In contrast, strains such as *Streptomyces* sp. St2AOB1 and *Bacillus* sp. BT1 were sensitive to most SynCom1.1 members, and therefore they should be discarded. *Pseudomonas* spp. P1A1 and P1C1 inhibited most bacterial strains but not T2C1.4.

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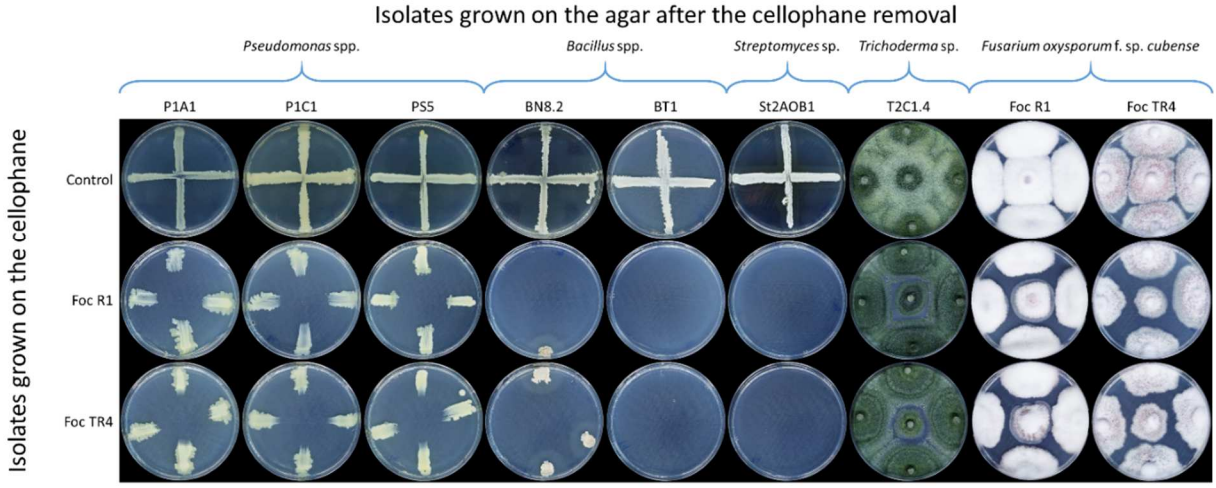

Figure 27. Effects of metabolites released in the agar by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) against SynCom1.1 members in a cellophane-agar assay.

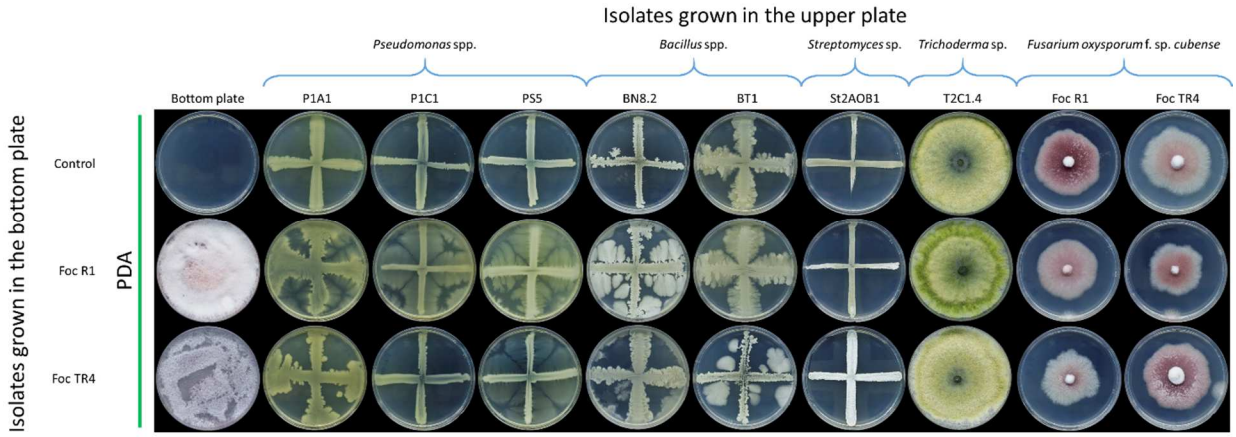

Figure 28. Effects of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) against SynCom1.1 members due to volatile organic compounds (VOCs) in an overlapping-plates assay.

We bring attention to an aspect poorly investigated in the selection of EBCAs: may a pathogen, such as *Foc*, antagonize beneficial microorganisms? In our experiments we observed that both *Foc* R1 and TR4 were able to release in the agar metabolites inhibiting some of SynCom1.1 members. Interestingly, *Trichoderma* sp. T2C1.4 was resistant against metabolites produced by *Foc*. Also, *Pseudomonas* spp. strains were partially resistant against *Foc* metabolites. Both *Foc* strains did not produce VOCs toxic to our EBCAs. Therefore, our SynCom1.1 must be further modified by discarding some members, keeping only the following strains:

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- *Pseudomonas* sp. PS5, partially resistant to *Foc*, reciprocally compatible with either P1A1 or BN8.2
- *Pseudomonas* sp. P1A1, partially resistant to *Foc*, but sensitive to BN8.2, reciprocally compatible with PS5
- *Trichoderma* sp. T2C1.4, resistant to *Foc* but sensitive to other strains
- *Bacillus* sp. BN8.2, sensitive to *Foc*, P1A1, and T2C1.4, reciprocally compatible with PS5

However, based on the *in vitro* reciprocal compatibility, it is difficult to define a single community with more than two members, *e.g.*, PS5+P1A1 or PS5+BN8.2. On the other hand, since *in vivo* results might not fully correlate with the *in vitro* assays, *in vivo* tests with single strains and different combinations of them would definitely delineate the best microbial community in terms of composition and biocontrol efficacy.

Antagonism activity of *Bacillus subtilis* SGB0013 and *Bacillus mycoides* SGB603

Bacillus subtilis SGB0013, *Bacillus mycoides* SGB603 have been isolated from soil (MS Biotech, Larino, Italy). *Fusarium oxysporum* was isolated from an infected soybean plant (MS Biotech). Both EBCAs showed in vitro antagonism versus *Fusarium oxysporum* (Fig. 29).

B. subtilis SGB0013 *B. mycoides* SGB603

Figure 29. Dual culture with *Fusarium oxysporum*

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2.7 Risk analysis of EBCAs

Potential EBCAs for applications to *Trichoderma* spp. and *Pochonia* spp. for banana pest biomanagement have been reported by partners in D2.1 and D2.2. We herein report efforts for Risk Analysis Assessment.

- *Trichoderma* spp.:

1. Evaluation of the toxicity / pathogenicity of *Trichoderma asperellum* in freshwater fish *Poecilia reticulata* (Guppy). Result: Not toxic or pathogenic to fish.

2. Evaluation of the toxicity / pathogenicity of *Trichoderma asperellum* in earthworm. Result: Not toxic or pathogenic in earthworms.

3. Evaluation of the acute oral toxicity of *Trichoderma asperellum* in rats. Result: administered orally in rats, in high concentration and at a single dose, does not produce mortality, does not induce the appearance of signs or toxic alterations, nor causes infectivity or pathogenicity in the conditions of the assay.

4. Assessment of the acute toxicity / dermal pathogenicity of *Trichoderma asperellum* in rabbits. Result: does not produce mortality in rabbits administered by dermal route with a limit concentration, does not induce the appearance of signs or toxic alterations in rabbits administered by dermal route with a limit concentration. It does not cause pathogenicity in rabbits administered by dermal route with a limit concentration.

5. Evaluation of the ophthalmic irritability of the product *Trichoderma asperellum* in rabbits. Result: the test carried out under the experimental conditions evaluated indicate that the bioproduct whose active biological agent is *Trichoderma asperellum*, does not present an irritant or ophthalmic corrosive potential.

6. Evaluation of the dermal irritability of the product *Trichoderma asperellum* in rabbits Results: The test carried out under the experimental conditions evaluated indicated that the active biological agent *Trichoderma asperellum*, does not present corrosive potential or primary dermal irritant.

Tests were carried out by Laboratorio de Toxicología. Centro de Bioactivos Químicos. Carretera a Camajuaní, km 3 ½, No. 284, El Gigante, Santa Clara, Villa Clara (Cuba). Authorised for Risk Analysis.

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- *Pochonia* spp.

Similar analysis are available for Klamic, a bioproduct based on *P. chalamydosporia* var. *catenulata*. Besides *P. chlamydosporia* has been isolated as a natural endophyte from banana plant roots in Canary Island (by CSIC). *P. chlamydosporia* has also been detected from metagenomic studies in rhizosphere soils from banana plantations (CNR).

– EF

Entomopathogenic fungi are virulent on BW (Coleoptera) but also on *Galleria mellonella* (Lepidoptera) (UA). See section 3.1.

- EPN

Elements for EPN Risk analysis available in Cuba: the registration process of biocontrol agents in Cuba have distinctive elements, such as, the active participation of different ministries for the analysis of each product. All biopesticides must be evaluated by the National Director of Environmental Health in Public Health Ministry and for General Director on Plant Heath Center (in the case of biopesticides for agricultural purposes). The process is described in "Procedimiento para el registro de plaguicidas biológicos (Procedure for biological pesticides register)". This process produced the publication of biopesticide in the Official List of Authorized Pesticides. In Cuba as in the UE, EPN do not require registration, but their importation and export are regulated by Resolution 180/2007, Annex 12 and 13 (Science and Technology Minister-CITMA, 2007). Therefore EPNs are considered no risk organisms.

– Technical Report about toxicity on NEMATECH product from MS Biotech Spa, Larino Italy.
Inoculated microorganism in the product: spores of *Pochonia chlamydosporia* and *Glomus* spp.
Equipment and Methods of Analysis: analytical method used as UNICHIM 1651: 2003.
The seeds of plant species (vascular mono- and dicotyledons), such as sorghum (*Sorghum saccharatum*), or cress (*Lepidium sativum*) or cucumber (*Cucumis sativus*), were used to perform the phytotoxicity tests lasting 24 hours, in the dark at a temperature of 25 ± 2 ° C with detection at the end of the test, counting the number of seeds germinated and the length of their radical system.

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The toxicity of an environmental sample is evaluated according to the change of the answer given by the body when exposed to the test matrix, compared to the control. The test in liquid phase was made from purified water, quality of 2 or better, according to UNI EN ISO 3696. The aqueous extract of the test sample (obtained after a preliminary treatment) was diluted to a concentration of 75 and 50%. Five ml aliquots of each of the two samples obtained (most the same number of controls with water) are placed in Petri dishes containing tissue paper. In each capsule 10 seeds of *Lepidum sativum* and other test plants were added, allowed to swell for one hour in distilled water. The capsules were incubated at 27 ° C for 72 hours under appropriate RH conditions.

After this period, the seeds germinated were counted and the root length was measured with a ruler. Length measurements were also carried out after extension of root and shoots, with the help of tweezers. The Index of germination (Ig) was calculated as follows:
Ig% = (Lc Gc / Gt Lt) · 100 Where:

Gc = number of germinated seeds in the middle of the sample
Gt = number of germinated seeds in the middle of control
Lk = average root length in sample
Lt = average root length in control

In the official method the reference value below which a result is considered unsatisfactory is a percentage of phytotoxicity of 60%. The analysis showed that the samples tested gave a value of 75%. In conclusion, the tested product was considered free of phytotoxic effects, indeed its use favors and makes up optimal plant growth.

3. EBCAs selected compounds/volatiles for BW field biomanagement

3.1 Evaluation of BW behaviour

Previous work of UA on behaviour modification of Red Palm Weevil (*Rynchophorus ferrugineus*) was applied to BW. Preliminary work showed low response of BW in laboratory bioassays. Therefore effect of light and starvation were tested on BW behaviour. Our results indicate that starvation and darkness drive BW mobility (Figures 30 and 31).

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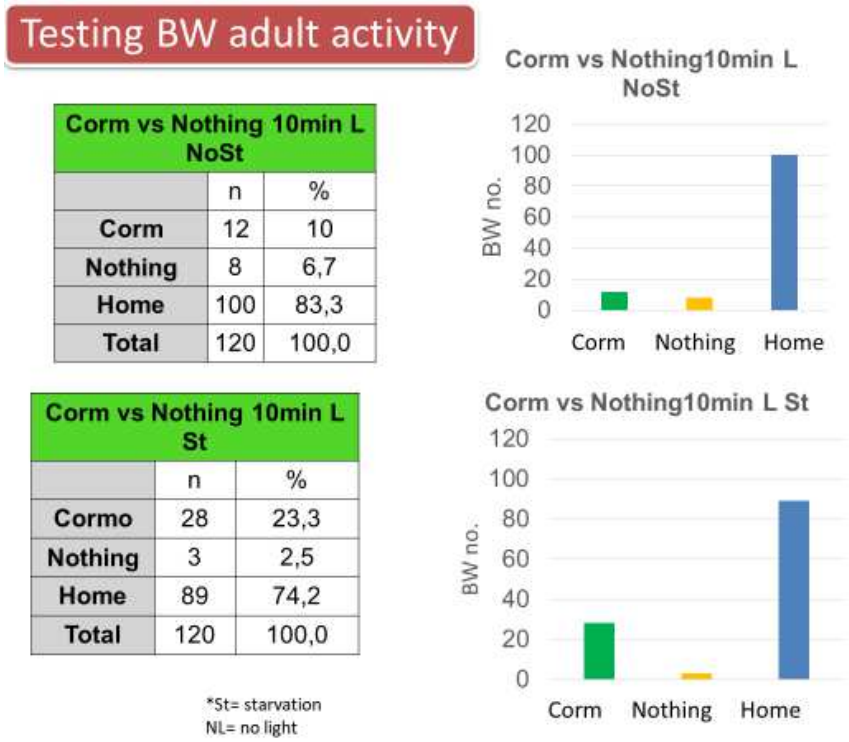

Figure 30. Effect of light and starvation on BW mobility.

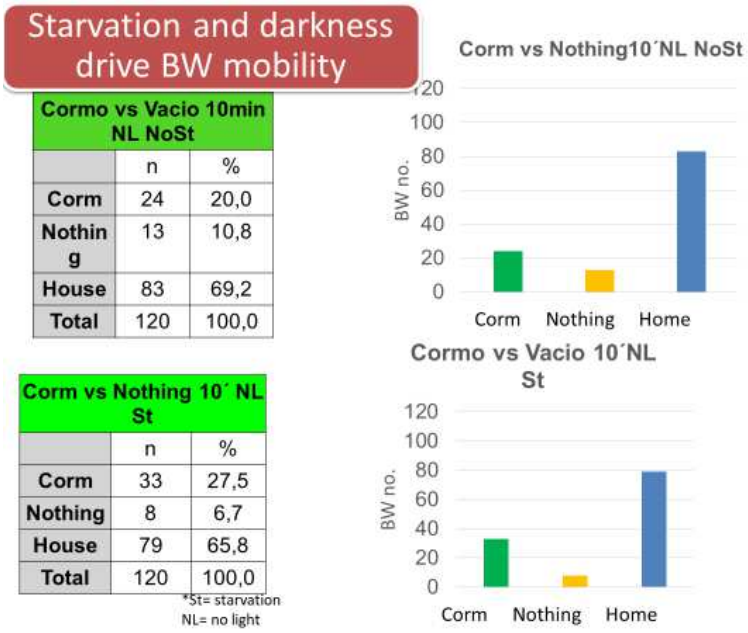

Figure 31. Effect of darkness and starvation on BW movility

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3.2 BW repellent bioassays

Previous conditions (see 3.1) were tested on BW to evaluate the effect of two volatile organic compounds (VOCs; C1 and C2) on insect behaviour. These VOCs repel BW under laboratory conditions (Figure 32).

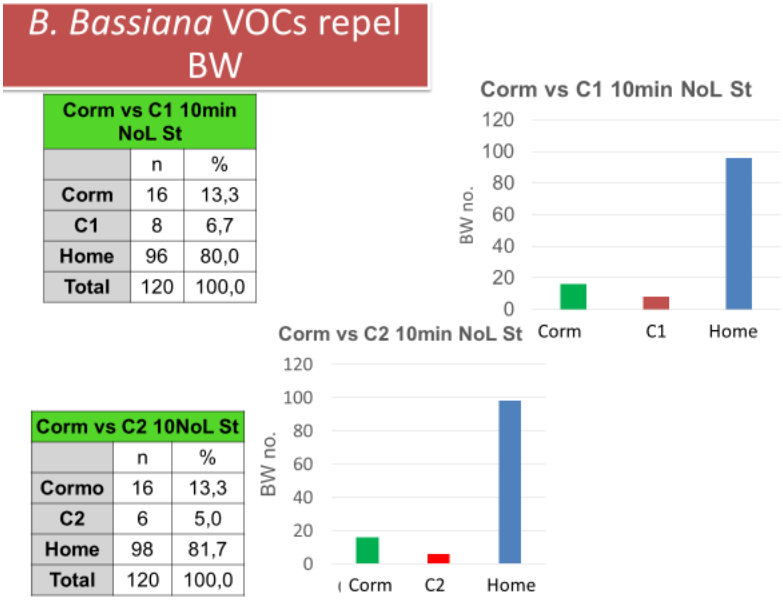

Figure 32. Effect of VOCs on BW behaviour

3.3 Detection of new volatiles from Banana Soil EBCAs isolates

Gas Chromatography (GC-MS) was used to identify VOCs in EF (*B. bassiana* and *M. anisopliae*) strains from banana soils. Several VOCs (Figs. 33) are being tested for their potential to manipulate BW behaviour.

3.4 BW repellents under field conditions

Volatile organic compounds (C1 and C2) found repellent to BW under laboratory conditions were tested, in collaboration with Partner 4 (Coplaca), under natural conditions. Banana weevil traps were placed in fields natural infested with BW in the Canary Islands including BW pheromone and the combination of either C1 or C2 and the pheromone (PR1 and PR2, respectively, Fig. 34).

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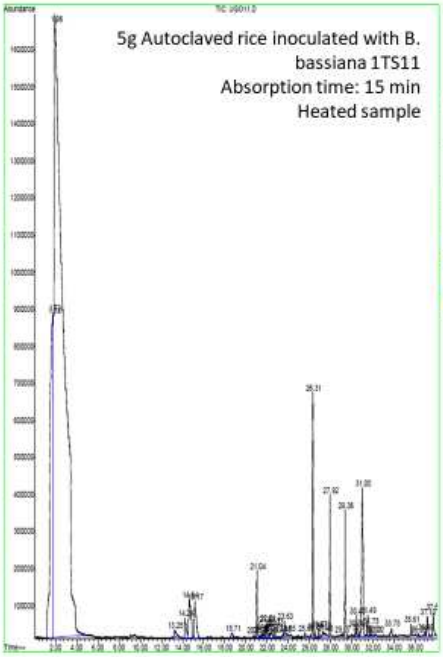
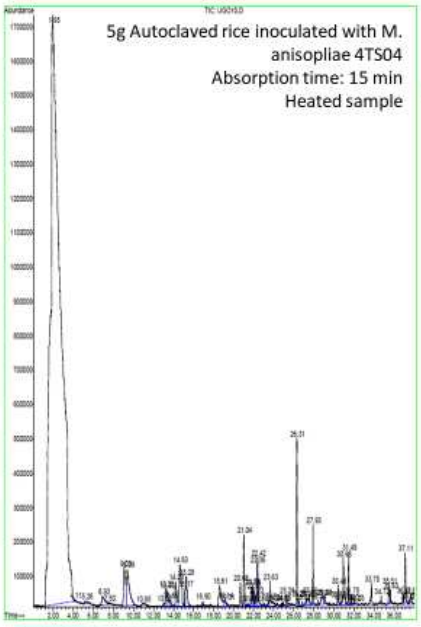


Main Bb1TS11 (B soils) VOCs

VOC	Rt (min)	Match (%)	Área
1	14,63	90	1,18
2	21,04	91	0,66
3	26,31	90	1,88
4	27,93	38	1,08
5	29,36	53	1,08
6	31	95	0,16



Main Ma4TS04 (B soils) VOCs

VOC	Rt (min)	Match (%)	Área
1	9,09	97	1,06
2	9,39	91	1,44
3	14,63	86	1,27
4	21,04	78	0,71
5	22,36	90	0,3
6	22,42	64	0,56
7	26,31	90	1,37
8	27,93	35	0,69
9	30,95	83	0,81
10	31,49	78	0,4
11	37,11	46	0,77

Figure 33. VOCs of EF from banana soils.

Preliminary results show a moderate BW repulsion by the VOCs, especially C2, under field conditions (Fig. 35).

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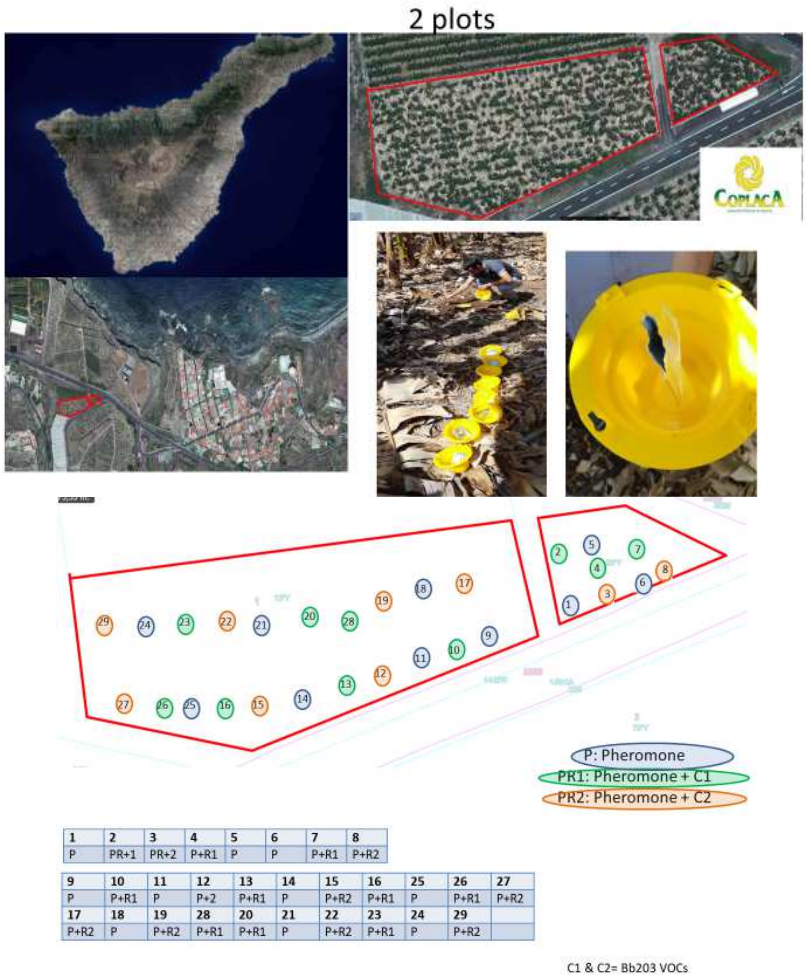

1	2	3	4	5	6	7	8
P	PR+1	PR+2	P+R1	P	P	P+R1	P+R2

9	10	11	12	13	14	15	16	25	26	27
P	P+R1	P	P+2	P+R1	P	P+R2	P+R1	P	P+R1	P+R2
17	18	19	28	20	21	22	23	24	29	
P+R2	P	P+R2	P+R1	P+R1	P	P+R2	P+R1	P	P+R2	

Figure 34. Field testing of VOCs on BW.

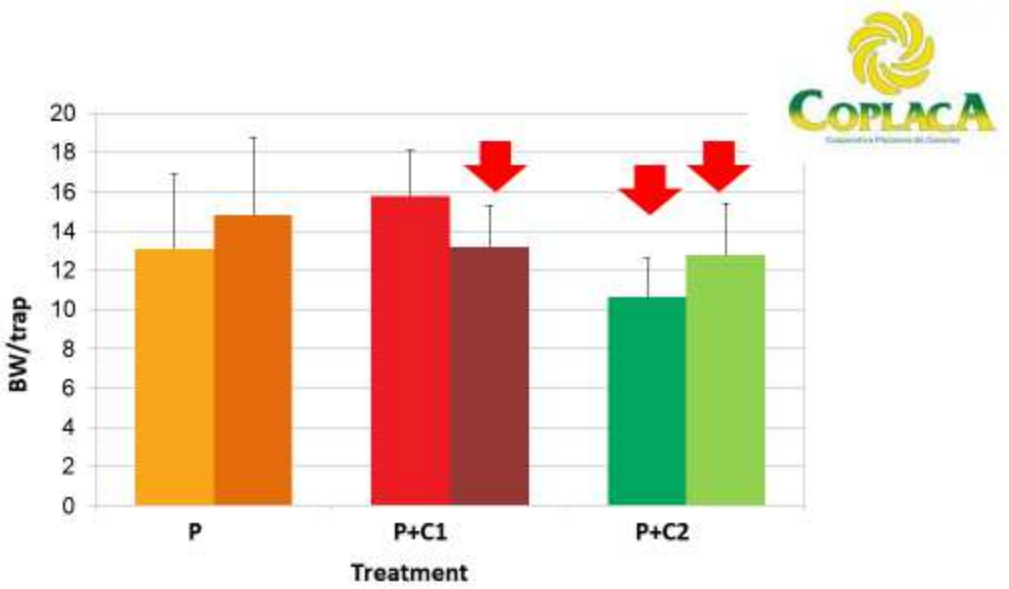

Figure 35. VOCs effect on BW field capture.

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4. Genomic resources from selected EBCAs

4.1. Molecular Identification of isolated EF

After ITS PCR, an electrophoresis of the PCR products showed a single band of ca. 500 base pairs (Fig. 36). These bands were excised and the DNA was purified for Sanger sequencing. After obtaining ITS sequences of EF isolates from Tenerife soils they have been molecular identified using comparisons with ITS sequences from NCBI. We chose for identifications matches with NCBI sequences with coverage and similarity of 100% (Table 14).

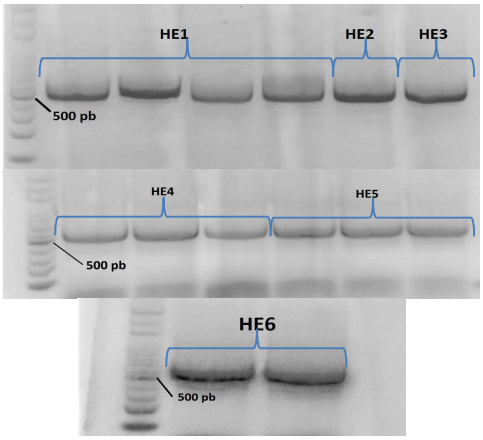

Figure 36. Electrophoretic analysis of the ITS PCR amplification (ITS1 and ITS4F primers) of EF strains isolated from Tenerife soil samples. HE1 = *B. bassiana* 1TS11, HE2 = *B. bassiana* 19TS04, HE3 = *M. anisopliae* 4TS04, HE4 = *L. lecanii* 5TS08, HE5 = *L. lecanii* 2TS05 and HE6 = *L. lecanii* 6TS01.

Table 14. Analysis of ITS sequence homology of entomopathogenic fungal strains obtained from banana soils compared with the NCBI database.

Sample code	BLASTn	Coverage (%)	Identity (%)	Acc. No.	Identification
1 TS11	*Beauveria bassiana*	100	100	KT378236.1	*Beauveria bassiana 1TS11*
2 TS05	*Simplicillium lamellicola*	100	100	KT004573.1	*Lecanicillium lecanii 2TS05*
4 TS04	*Metarhizium anisopliae*	100	100	MH483917.1	*Metarhizium anisopliae 4TS04*
5 TS08	*Simplicillium lamellicola*	100	100	KT004573.1	*Lecanicillium lecanii 5TS08*
6 TS01	*Simplicillium sp.*	100	100	KY305078.1	*Lecanicillium lecanii 6TS01*
19 TS04	*Beauveria bassiana*	100	100	MH483769.1	*Beauveria bassiana 19TS04*

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4.2. EF Phylogenetic Analysis

For the phylogenetic analysis Neighbour-Joining method has been used (Saitou and Nei, 1987). After the analysis the isolates were placed near different strains of species and identified by comparison with the NCBI database. ITS sequences from other strains used in the phylogenetic analysis were extracted from the NCBI database. The tree was drawn to scale and the length of the branches represents the evolutionary distance of strains analysed, calculated with the Maximum Composite Likelihood method (Tamura et al., 2004) (Fig. 37).

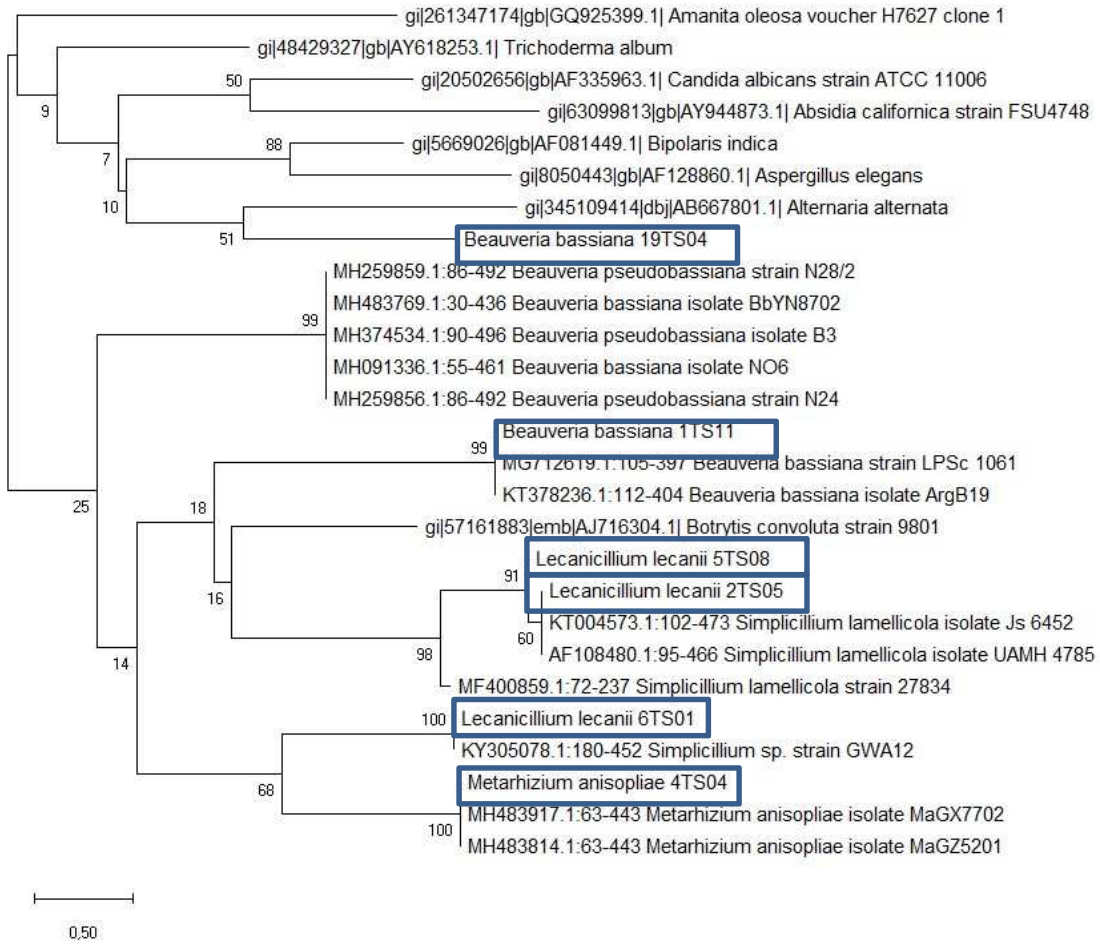

Figure 37. Phylogenetic tree using ITS sequences obtained from the EF isolated from banana crop soils (blue boxes) compared with other fungal ITS sequences from the NCBI database. Bootstrap values are indicated in the nodes (1000 replicates) (Felsenstein, 1985).

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4.3. Updating *Pochonia chlamydosporia* genome annotation and public release via GenBank

Background and relevance to MUSA. The fungal species *Pochonia chlamydosporia* (Pc) is a soil fungus that infects and destroys the eggs and kills females of plant-parasitic nematodes. It is one of the EBCAs specifically mentioned in the Description of WP3. UA are using Pc strain 123 in their experimental system in WP3. However, despite a genome sequence being publicly available (Larriba et al. 2014), no annotation was publicly available in the public databases, though partial annotation was published in the supplementary data of UA research papers (Larriba et al. 2014; Aranda-Martinez et al. 2016). Annotated genome sequences are required for UA and other partners transcriptomics experiments and it is essential that a standardised version of the annotation is publicly available. Therefore, UNEXE provided bioinformatics support to UA to generate a genome annotation that satisfies the quality-control criteria for submission to GenBank and is as consistent as possible with the partial annotation used in previous studies (Larriba et al. 2014; Aranda-Martinez et al. 2016).

Methods. Specifically, we have used the MAKER pipeline (Campbell et al. 2014) to re-annotate the previously published genome sequence, after updating the sequence based on evidence from expressed sequence tags and targeted Sanger sequencing (see Fig. 38). The output of the MAKER pipeline was then cleaned using Genome Annotation Generator tools (Geib et al. 2018) and converted into the NCBI's Sequin format, which is required for submission to the public repository, using tbl2asn (https://www.ncbi.nlm.nih.gov/Sequin/). Generating this final annotation was an iterative process that required trialling various options and parameter values at UNEXE and responding to feedback on the resulting annotation from the UA biological expertise.

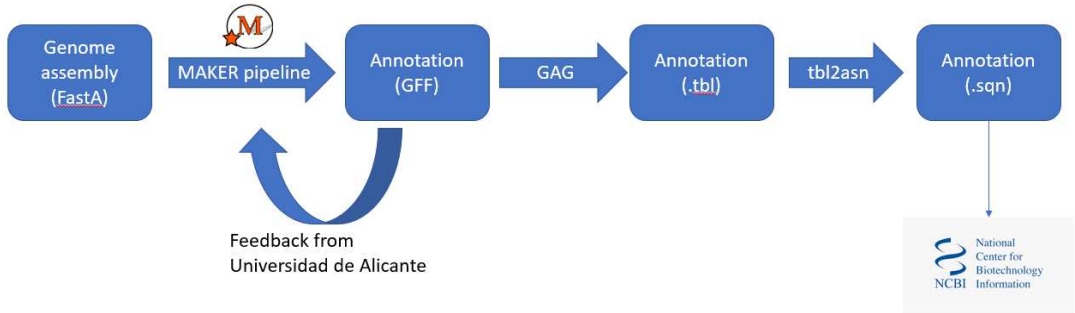

Figure 38. Overview of the workflow for annotation and submission to GenBank.

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Result and conclusion. The final annotation has now been agreed by all relevant partners and has been submitted to GenBank (PRJNA68669, GCA_000411695.2).

Bioinformatics pipeline for *Pochonia chlamydosporia* transcriptomics. Having received the first batch of RNAseq data from UA, UNEXE created and executed a bioinformatics pipeline for quality control and identification of differentially expressed genes. The pipeline has been tested on previously published data Pc 123 RNA-seq data (Larriba et al. 2014) and confirmed to give consistent results. Our pipeline is based on tried-and-tested open-source tools including: FastQC (Andrews n.d.), Trimmomatic (Bolger, Lohse, and Usadel 2014), HISAT2 (Kim, Langmead, and Salzberg 2015), StringTie (Kim et al. 2016), Trinity (Grabherr et al. 2011) and DESeq2 (Love et al. 2014). Deployment of our pipeline on the first batch of MUSA project RNA-seq data from UA revealed a technical issue with the data (contamination with rRNA) and will be updated after arrival of the new data from UA.

4.4. Genome sequence and annotation of *Brevibacillus laterosporus* strains that produce broad spectrum antimicrobials.

Background /relevance. David Studholme's MUSA presentation at the Belgrade BioInformatics Conference in June 2018, led to a collaboration on strains of *Brevibacillus laterosporus* (BGSP7, BGSP9 and BGSP11) isolated from silage in Serbia that produce broad-spectrum antimicrobials and thus augment the collection of potential EBCAs.

Results/conclusions. UNEXE contributed to bioinformatics analysis of these three genome sequences: *https://www.ncbi.nlm.nih.gov/bioproject/PRJNA432906/.*

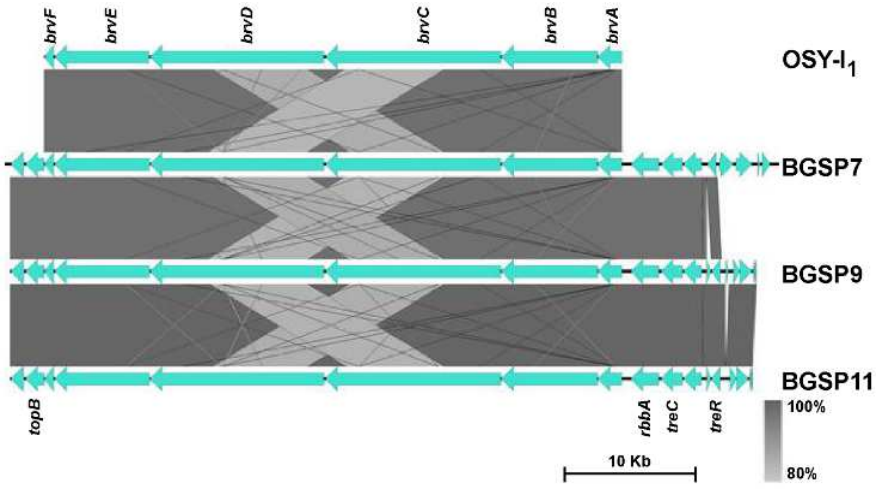

Figure 39. Comparative analysis of *brevibacillin* gene cluster among *B. laterosporus* strains.

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This revealed the presence of gene clusters associated with the production of non-ribosomally synthesized peptides (brevibacillin [see Fig. 39], bogorol, gramicidin S, plipastatin and tyrocin) and bacteriocins (laterosporulin, a lactococcin 972-like bacteriocin, as well as putative linocin M18, sactipeptide, UviB and lantipeptide-like molecules). Purification of a number of antimicrobial molecules from each isolate suggests that they can be considered as potent biocontrol strains that produce an arsenal of antimicrobial molecules active against bacteria, fungi and insects.

4.5. Genome sequencing of *Fusarium* TR4 from outbreak in UK

The fungal pathogen Foc TR4 has been detected in the UK, on banana plants at The Eden Project rainforest biome: *http://www.promusa.org/blogpost580-TR4-present-in-the-UK*. UNEXE have extracted genomic DNA from this pathogen, isolated at The Eden Project and have submitted them for sequencing at UNEXE. Data will be delivered and analysed by the end of June 2019. This will enable identification of the likely geographic origin of the pathogen, by comparing its genome against those of previously sequenced TR4 from various geographical locations.

Survey / meta-analysis of EBCAs

UNEXE has initiated a meta-analysis of published results, which aims to summarize current evidence for biocontrol of banana pests and diseases. We searched literature for "biocontrol" and "banana" to 2000 and included only microbial biocontrols (not plant extracts or exudates). The results were divided into fungal pathogens, and insect and nematode pests. The top five biocontrol microorganisms were *Pseudomonas fluorescens*, *Trichoderma viride*, *Trichoderma harzianum*, *Bacillus amyloliquefaciens* and *Fusarium oxysporum*. The top five for biocontrol of *Fusarium oxysporum* f. sp. *cubense* (Foc) were *F. oxysporum, P. fluorescens*, *T. harzianum*, *T. asperellum* and *B. amyloliquifaciens*. Nematode field research lags behind.

This meta-analysis quantified two metrics: disease effect and yield effect for each study. It also identified some limitations in currently available publications; e.g. diverse protocols make comparisons difficult and standard errors rarely reported. Standard protocols and clear reporting are urgently required. It also revealed that some important diseases (e.g. banana *Xanthomonas* wilt) have not yet been addressed by biocontrol studies.

Of some concern is the finding that much of the reported studies are laboratory-based and field trials (most of which are from India) under-represented. Effect sizes in the field were

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approximately half those observed in culture. For control of Foc, non-pathogenic Foc, *P. fluorescens* and bacterial endophytes show most promise.

The meta-analysis is in-progress and before completion needs to be expanded to encompass the Spanish language literature. Discussions have been initiated with Dr Heyker Lellani Baños Díaz (CENSA, a MUSA partner in Cuba) on abstracting more data from the Spanish-language literature, and with PROMUSA for hosting the data on their website.

4.6. Metagenomics of Banana Rhizosphere Soils

In order to study the ecology of microbial antagonists associated to banana crops in Tenerife, (Canary Islands, Spain), metagenomic data were produced from rhizosphere soil of cv Pequeña Enana and control soils collected from adjacent sites, without banana roots. Microbial communities were characterized using the variable V3 and V4 regions of the 16S rRNA ribosomal gene for bacteria and the ITS region fungal communities. Sequences produced were deposited on NCBI SRA database with accession project number PRJNA540248: "A metagenomic study of banana nematode antagonists in Canary Islands" (Table 15). Banana root-associated microorganisms were isolated from soil samples collected in Tenerife by serial dilution technique and identified by ribosomal ITS sequences (Table 16). Endophytism of *P. chlamydosporia* (MUT 6232) and *Acrostalagmus luteoalbus* (J2_3B1) was demonstrated in nematode tolerant and susceptible banana roots. The endophytic fungus was tested on *in vitro* banana germoplasm: cv. Gran Enano (ITC1256) and Gros-Michel (ITC1122), obtained from Biodiversity (*www.crop-diversity.org*). Assays were carried out *in vitro* with 30-days-old plantlets on Murashige and Skoog -agar medium or with *in vitro* derived plants, grown in planting trays in growth chamber.

The banana-endophytes interaction was performed in axenic bioassays in plastic boxes during 20 days. Endophytism was tested by incubation of surface sterilized root fragments on petri dishes with water agar, for 15 days. After incubation, roots were observed under a steroscope. *P. chlamydosporia* (MUT 6232) and *Acrostalagmus luteoalbus* (G2_3B1) were re-isolated from banana roots confirming the endophytic behaviour for both fungi.

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Table 15: Reference data for soil samples associated with cv Pequeña Enana rhizosphere in Tenerife used for metagenomic studies.

Sample	Location	Accession NCBI
M1	Tenerife	PRJNA540248
M2	Tenerife	PRJNA540248
Nord 1	28º22'03,53" N/16º48'10,93" O - Z = 77 m	PRJNA540248
Nord 2	28º22'03,53" N/16º48'10,93" O - Z = 77 m	PRJNA540248
Nord 3	28º22'03,53" N/16º48'10,93" O - Z = 77 m	PRJNA540248
Nord C1	28º22'03,53" N/16º48'10,93" O - Z = 77 m	PRJNA540248
Nord C2	28º22'03,53" N/16º48'10,93" O - Z = 77 m	PRJNA540248
Nord C3	28º22'03,53" N/16º48'10,93" O - Z = 77 m	PRJNA540248
Nord 4	28º22'08,55" N/16º48'06,20" O - Z = 64 m	PRJNA540248
Nord 5	28º22'08,55" N/16º48'06,20" O - Z = 64 m	PRJNA540248
Nord 6	28º22'08,55" N/16º48'06,20" O - Z = 64 m	PRJNA540248
Nord C4	28º22'08,55" N/16º48'06,20" O - Z = 64 m	PRJNA540248
Nord C5	28º22'08,55" N/16º48'06,20" O - Z = 64 m	PRJNA540248
Nord C6	28º22'08,55" N/16º48'06,20" O - Z = 64 m	PRJNA540248
Nord 7	28º22'36,19" N/16º44'07,56" O - Z = 22 m	PRJNA540248
Nord 8	28º22'36,19" N/16º44'07,56" O - Z = 22 m	PRJNA540248
Nord 9	28º22'36,19" N/16º44'07,56" O - Z = 22 m	PRJNA540248
Nord C7	28º22'36,19" N/16º44'07,56" O - Z = 22 m	PRJNA540248
Nord C8	28º22'36,19" N/16º44'07,56" O - Z = 22 m	PRJNA540248
Nord C9	28º22'36,19" N/16º44'07,56" O - Z = 22 m	PRJNA540248
Sud 1	28°10'07.4"N/16°26'14.6"W	PRJNA540248
Sud 2	28°10'07.4"N/16°26'14.6"W	PRJNA540248
Sud 3	28°10'07.4"N/16°26'14.6"W	PRJNA540248
Sud C1	28°10'07.4"N/16°26'14.6"W	PRJNA540248
Sud C2	28°10'07.4"N/16°26'14.6"W	PRJNA540248
sud C3	28°10'07.4"N/16°26'14.6"W	PRJNA540248
Sud 4	28°09'20.3"N/16°48'00.6"W	PRJNA540248
Sud 5	28°09'20.3"N/16°48'00.6"W	PRJNA540248
Sud 6	28°09'20.3"N/16°48'00.6"W	PRJNA540248
Sud C4	28°09'20.3"N/16°48'00.6"W	PRJNA540248
Sud C5	28°09'20.3"N/16°48'00.6"W	PRJNA540248
sud C6	28°09'20.3"N/16°48'00.6"W	PRJNA540248
Sud 7	28°12'25.5"N/16°49'37.2"W	PRJNA540248
Sud 8	28°12'25.5"N/16°49'37.2"W	PRJNA540248
Sud 9	28°12'25.5"N/16°49'37.2"W	PRJNA540248
Sud C7	28°12'25.5"N/16°49'37.2"W	PRJNA540248
Sud C8	28°12'25.5"N/16°49'37.2"W	PRJNA540248
sud C9	28°12'25.5"N/16°49'37.2"W	PRJNA540248

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Table 16. Banana root-associated microorganisms isolated from samples soil collected in Tenerife.

Isolates	Organism	Location	Gene* sequenced
J2-3B1	*Acrostalagmus luteoalbus*	Tenerife	ITS1-4 ribosomal gene
J2-4D1	*Acremonium antarcticum*	Tenerife	ITS1-4 ribosomal gene
J5-1D1	*Sarocladium kiliense*	Tenerife	ITS1-4 ribosomal gene
J7-2E1	*Acremonium sp*	Tenerife	ITS1-4 ribosomal gene
J8-1D1	*Sarocladium kiliense*	Tenerife	ITS1-4 ribosomal gene
J8-2E1	*Acremonium sp*	Tenerife	ITS1-4 ribosomal gene
J9-2E1	*Phialemonium inflatum*	Tenerife	ITS1-4 ribosomal gene
J12-2E1	*Phialemonium inflatum*	Tenerife	ITS1-4 ribosomal gene
J16-1D1	*Acremonium nepalense*	Tenerife	ITS1-4 ribosomal gene
T1-2	*Lecanicillium saksenae*	Tenerife	ITS1-4 ribosomal gene
T1-3	*Lecanicillium psalliotae*	Tenerife	ITS1-4 ribosomal gene

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