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1. Explanation of the work carried out by beneficiaries and overview of progress

MUSA activities started in June 2017. During the first 18 months the beneficiaries planned and developed the collection, identification and study of endophytes and biocontrol agents (EBCAs) suitable for management of banana (*Musa acuminata*) and enset (*Ensete ventricosum*) pests and diseases. The threats considered are: plant parasitic nematodes (PPN), Panama Disease (PD, *Fusarium oxysporum* f. sp. *cubense*) and banana weevil (BW, *Cosmopolites sordidus*). The Consortium carried out several prospecting campaigns for EBCAs collection. The isolates obtained are the main component of the work developed in WP2 and WP3. Studies also concerned screening for resistance of banana and enset germplasm (WP4).

The field work has been developed in the three world regions interested by the Action: Canary Islands, sub Sahara Africa and Central America. This work has been finalized to the build up, expand and integrate national/regional microbial collections, available at participating institutions and SMEs. The beneficial organisms isolated have been subsequently investigated through laboratory and greenhouse assays for selection and testing their efficacy against the threats considered (WP3), in view of exploitation for IPM or bio-management.

The experimental work carried out, including cooperative activities of WP2, provided data informative about the response of banana germplasm lines as hosts of most promising isolates. This work has been carried out through *in vitro*, greenhouse or field studies. Testing of the EBCAs isolated and/or made available by the beneficiaries carried out included applications of *Trichoderma* spp. tested growth promotion and against *Radopholus similis* and other PPN, bacteria and endophytes used against PD, and EPN or entomopathogenic fungi applied against BW. At the same time *in vitro* and laboratory assays have been carried out on the biology and diversity of the isolates obtained, including DNA sequencing for taxonomic identifications. A number of isolates and procedures have been shared with participating stakeholders in the view of new bio-management, mass production and exploitation.

Samplings for studies of banana corm and rhizosphere metagenomics have been carried out in Tenerife (ES) and in the Uganda IITA germplasm conservation fields. These studies, still in progress, aim at identifying the links of the germplasm sampled with soil and corm microbial species having a particular interest for bio-management. The data gathered will be actually analysed in conjunction to other climatic and environmental factors.

The diversity of genes suitable for exploitation in breeding has been studied for banana and enset lines. A number of transcriptomic studies are in progress on root gene expression during parasitism by *Pratylenchus goodeyi* and *F. oxysporum* f. sp. *cubense*, including the most damaging race, tropical race 4 (TR4). Data on gene expression of banana roots have been produced in tri-trophic interactions with EBCAs such as *Pochonia chlamydosporia*, *Trichoderma* and *Streptomyces* spp. This aims at identifying growth promotion or plant defense effects, and related elicitors. Data have been also produced on the effects of natural products and compounds (i.e. chitosan, xanthines) on PPN, BW or on root and plant defense induction.

Overview of results

Eight collections have been produced thus far, with a total of 1072 isolates of fungi and bacteria and five lines of entomopathogenic nematodes (EPN, *Heterorhabditis* sp.) from Central America (specific objective 1). Details about their locations and composition are given in Deliverable D2.1. Procedures have been set up for large scale cultivation of microbial EBCAs, including EPN bioformulation and storage (Deliv. D5.1). Data informative of the response of banana germplasm lines as hosts for a range of EBCAs have been produced (Deliv. D2.2).

Dissemination, Communication and Project Management initiatives have been developed (WP10 and WP11), including the production of a Data Management Plan (Deliv. D10.6) and a Communication, Dissemination and Exploitation Plan (CDEP, Deliv. D10.1). A first set of eight Practice Abstracts has been produced, describing the issues afforded and the actual phytosanitary situation of banana crops (Deliv. D10.3).

Several experiments in controlled or field conditions have been carried out with applicatin of EBCAs against PD, PPN and BW, as described for the WP2, 3 and 4.

Three international meetings have been organized. As indicated in the report of the kick-off meeting, held on Aug. 29-31, 2017 at Tenerife (Del. D1.1), the Consortium also addressed in that occasion ethics and security issues (WP1, Del. D1.1). Two further Project meetings have been organized during 2018, the first by IITA at Kampala (Uganda) and the second by EARTH University in Costa Rica. All meetings were followed by a Workshop organized with local stakeholders on applied aspects of biological pest/disease management in banana productions.

Summary of deliverables and milestones

The following deliverables have been produced after evaluation of related milestones, carried out during the Consortium periodic meetings held in the first 18 months of activity:

Verified milestones	*Deliverables*	*WP*
-	D1.1 - NEC Requirement N. 1	1
MS2 - Microbial collections planning and start up. Sampling work planned to build local microbial collections. Decisions taken concerning: the field sampling areas in Canary Islands, Caribbean and SSA countries; the procedures for EBCAs isolations; the lab requirements to start the beneficiaries' collections with related equipments purchase; parameters for first host-range assessment tests.	D2.1- Microbial collections (bacteria, fungi) and other beneficial EBCAs and selected PGPM/soil microorganisms for applied IPM	2
	D2.2 - Report on EBCAs host-range assessment	2
MS5 - Culturing and production protocols. Identification of most appropriate EBCAs culturing and mass production methods: protocols drafts available for partners; parameters for bioformulations and storage conditions discussed and agreed on by beneficiaries.	D5.1 - Methods for large scale cultivation of microbial EBCAs and EPNs, bioformulation and storage	5
MS13 - Decisions on the CDEP.	D10.1 - Communication, Dissemination and Exploitation	10

Decisions concerning the Communication, Dissemination and Exploitation Plan agreed on by all partners through their representatives, at the issue of the MUSA kick off meeting. They consider: the measures to be implemented for dissemination; how data will be exploited and/or shared/made accessible; how to involve potential final users and stakeholders and local regional political context; the activities for education and training; the application of results and the measures to be implemented after the end of the Project; the draft of the included Data Management Plan and the most appropriate communication tools and strategies.	Plan (CDEP)	
	D 10.3 - Practice abstracts, first set	10
	D10.6 - Data Management Plan	10
MS1 - Kick off meeting organized Organization of the kick–off meeting completed: decisions about the meeting date and location in Canary Islands taken, in accordance with Coplaca; speakers identified and presentation titles agreed on; funds transferred to beneficiaries and EAB members, to allow participation to the meeting.	D11.1 - Kick-off Meeting Report	11

Summary of exploitable results

The MUSA Consortium produced eight national collections of beneficial microorganisms associated to banana plants from three different world regions. The exploitation of these microbial tools in pests/disease management and growth promotion of banana crops depends on the selection of best performing isolates. This requires testing their efficacy in conditions as close as possible to those of the local destination fields. Significant informations have been provided on the interactions of banana plant germplasm and beneficial microbial species. Data produced concern one or more of the considered pests (nematodes, banana weevil) or of the new races of *Fusarium oxysporum* f. sp. *cubense*, such as the Tropical race 4, which is actually devastating banana crops in Africa. Exploitation of best performing isolates including entomopathogenic nematodes in tropical and subtropical environments will allow the set up of sustainable IPM practices. It will also promote the development of new commercial products and bioformulation by SMEs, increasing their contribution towards a green, closed-cycle economy.

1.1 Objectives

MUSA aims at implementing practical tools for management of three severe pests and diseases of banana: Panama Disease, banana weevil and plant parasitic nematodes. The Action is developed in three world regions: Canary Islands, Sub Saharan Africa and Central America. The enset crop is also included through participating partner SARI, in collaboration with partner UNEXE. The Consortium seeks innovation in a holistic research approach combining the use of endophytes and biocontrol agents (EBCAs), testing their interactions with plant germplasm. Main objective is to set up more sustainable and environment friendly IPM methods, improving the resilience of crops, thus reducing costs and pesticide use.

The Consortium integrates stakeholders such as SMEs and producer organizations. Partners seek innovative IPM products based on novel bioformulations, selected on the basis of economic sustainability and crop resilience towards main climate drivers. Direct benefits are expected to be derived by reducing crop production costs and/or by increasing yields, in synergy with use of suitable plant germplasm and safer propagation material (*in vitro* plants, indexed plants and/or thermally treated suckers).

The focus is on products and processes improving the efficacy of the IPM strategies, as well as the profitability of new methods for local stakeholders. Partners target organic as well as conventional farmers, involved or not in commercial export, supporting thereby IPM for a broad use. In the EU region, producers aim at improving the quality and management of crops, reducing their environmental impact and sustaining the commercial promotion of organic fruits. In SSA the focus is on food security. In Central America overall goals concern food security (Cuba) and quality of exported fruit (Costa Rica).

Specific objectives

The MUSA framework globally aims at: *i)* a sustainable management of pests and diseases of banana (including plantain and enset) using EBCAs and plant resistance/tolerance-based IPM approaches, *ii)* improving EBCAs efficacy by developing bioformulations suitable for marketing, *iii)* improving yields of staple food or export-derived incomes, by inducing plant defense, *iv)* selecting effective EBCAs-germplasm combinations, under the regional conditions of the Project, *v)* defining soil/plant parameters to forecast/monitor banana and enset biotic threats, *vi)* identifying climatic/agronomic factors affecting the banana crops and pests' cycles and the economic success of the IPM practices tested and *vii)* analyzing the profitability of successful IPM strategies identified, and their social impact.

The Project has the following *specific objectives*:

1. Identify and select EBCAs for incorporation in existing IPM or bio-management of plant parasitic nematodes (PPN) Panama Disease (PD) and banana weevil (BW).
2. 2.1) Produce knowledge on the biology of pests/diseases and beneficial microorganisms, and on their life cycles on banana crops, in different regions (SSA, Canary Islands, Caribbean) with varying intensification levels. 2.2) Develop and apply locally adapted detection and preventive measures, to reduce incidence and spread of the cited pests and diseases.
3. 3.1) Identify and test suitable germplasm resources (*Musa* spp. and enset varieties) susceptible/tolerant to one or more of the pests/diseases cited, to be used alone or in combination with EBCAs, to provide a first genetic basis for IPM methodologies to reduce infestation levels and pesticide applications; 3.2) determine banana genes and molecular pathways induced by EBCAs and in the response to pests and pathogens.
4. Set up and test novel procedures for mass production, storage and application of EBCAs by industrial stakeholders and/or local producers, tailored for application in the different regions of study, depending on local agricultural and social systems.
5. Improve/sustain yields through introduction of selected EBCAs in germplasm field assays.

6. Apply microorganisms on *Musa* spp. or enset germplasm, validated through field assays to draft locally adapted bio-management strategies in farming systems from SSA, Caribbean and Canary Islands.
7. Identify most suitable field/crop indicators and ecological parameters to address monitoring issues for climatic threats, at the regional levels.
8. Overcome socio-cultural barriers affecting adoption of new farming practices and technologies, to sustain economical banana production.

Work carried out by the Consortium

A sampling survey was carried out in cooperation by CNR and Coplaca for isolation and identification of EBCAs from Canary Islands, and for collection of PPN populations (*Pratylenchus goodeyi*, *Helicotylenchus multicinctus*). An *in vitro* assay has been started for production of transcriptomic data from roots of Gran Enana, with or without inoculation of *P. goodeyi* and/or *Pochonia chlamydosporia* var. *chlamydosporia* (DSM 26985). Greenhouse assays have been produced on the effect of the fungus on *Fusarium oxysporum* f. sp. *cubense* (Foc) race TR4 using cv Gran Enana plants. As shown by different assays carried out by CSIC, UA and CNR, Gran Enana plants resulted susceptible to Foc TR4. EBCAs isolates, produced in collaboration with Coplaca and IAC-CSIC from soil samples collected by IAS-CSIC and then transferred to CNR, have been used to set up a microbial Synthetic Community (SynCom) tested for biomanagement of Foc TR4 in a quarantine conditioned greenhouse (located at University Campus, Bari, IT). A liquid-solid production method was developed by CNR and SacomLab for production of *P. chlamydosporia*. *Specific objectives: 1, 2.1.*

SacomLab isolated and developed mass production technologies for endophytes and nematode parasitic fungi. SacomLab participated in the kick-off meeting and other dissemination initiatives, and produced the MUSA logo. *Specific objectives: 1, 4.*

The UA team demonstrated the endophytism of *P. chlamydosporia* on banana roots. UA devised three systems for analysing EBCAs interactions with host plants. The nematophagous fungus *P. chlamydosporia* (Pc 123, ATCC MYA-4875; CRCT 20929) was tested on *in vitro* reared commercial plant material of cv. Pequeña Enana, using 30-day-old *in vitro* plantlets on agar medium or 6-7 week-old in *vitro* derived plants, grown in planting trays in greenhouse. The banana-EBCAs interaction schemes included axenic bioassays in magenta boxes, experiments in growth chambers and pot experiments in the greenhouse. The main objective was to test methods for inoculating *P. chlamydosporia* in banana plants, and verify if the treatments were effective in the long term. Four methods of inoculation have been developed. All treatments with *P. chlamydosporia* applied to Pequeña Enana promoted root growth. The plantlets showed growth parameters significantly higher than control (uninoculated plants), with a maximal root length achieved when treated with conidial and chlamydospore suspensions. Similar results were found for the fresh root weight. UA also isolated and tested fungal antagonists of BW. In collaboration with UNEXE, UA analysed and produced gene prediction data from the sequenced genome of *P. chlamydosporia*. *Specific objectives: 1, 2.1.*

In collaboration with Coplaca, CSIC produced a collection of EBCAs subsequently tested for their antagonistic activity *vs* Foc. Three independent pilot pathogenicity tests were carried out under non-gnotobiotic conditions to optimize banana cultivation, set

up experimental conditions and assess development of PD symptoms under controlled conditions, to tune up Foc biocontrol assays. Banana plants (*in vitro* propagated seedlings, cv. Pequeña Enana, susceptible to PD), were supplied by Partner 4 (Cultesa, a shareholder company within Coplaca cooperative). More than 100 bacterial and fungal strains showed *in vitro* antagonism of strains *vs* Foc (CAV-095) at a variable degree. These strains have been characterized as for the presence of common traits related to biocontrol and plant-growth promotion abilities. The CSIC team showed that banana roots are a rich reservoir of beneficial endophytes antagonistic against the pathogen. The three most effective isolates, that showed *in vitro* a relative inhibition index higher than 0.6, were molecularly identified as *Pseudomonas chlororaphis* and *Pseudomonas* spp. *Specific objectives: 1, 2.1.*

In order to find new effective EBCAs in a wide range of *Musa* cultivars, KU LEUVEN initiated a set of experiments to test commercially available microorganisms in different genotypes and environments/conditions: greenhouse, nursery and open field. The microorganisms provided by partner Real IPM (recently acquired by Biobest) included fungi such as *Trichoderma* or *Glomus spp.* and bacteria such as *Bacillus* spp. or *Azospirillum* sp. In a first stage, they were tested in banana as Plant Growth Promoting Microorganisms (PGPM), as they stimulated growth in other crops acting as biofertilizers and biocontrol agents. *Trichoderma* and *Bacillus* spp. were also tested for a potential effect in alleviating drought stress, one of the major abiotic factors limiting banana productions worldwide. Plants from the export cultivar Williams inoculated with *T. asperellum* under optimal irrigation significantly increased their number of leaves, leaf area and number of primary roots. Samples from different plant tissues were collected for gene expression analyses whereas the rhizosphere was sampled for microbiota analyses. In a second round of experiments in the greenhouse, *Trichoderma* was used to inoculate different banana genotypes grown either in liquid medium (Gran Enano and Yangambi km5) or in soil (Valery, Pequeña Enana and Yangambi Km5). Liquid medium plants were sampled for transcriptomic analyses to characterize genes/pathways induced after inoculation with *T. asperellum*. In parallel, two trials have been carried out in the Dominican Republic to evaluate growth promoting effect on Williams nursery plants. The nitrogen-fixing bacteria *Azospirillum* sp., the arbuscular mycorrhiza fungi *Glomus* spp., the phosphate solubilizers *Trichoderma, Bacillus* spp., and a combination of *Trichoderma* + *Bacillus* sp., + *Glomus* spp. were tested. Preliminary data indicated that the microorganisms promoted growth in banana plantlets under nursery conditions. Data from a field trial in the Dominican Republic also suggest that *Azospirillum* sp. may stimulate growth of Williams plants at early stages after inoculation. *Specific objectives: 1, 2.1, 3.1, 3.2.*

Models have been produced by UNEXE on the effects of climate changes on the susceptibility of banana crops to fungal diseases. UNEXE with SARI produced raw sequence reads and genome assemblies from 17 accessions of the Ethiopian orphan crop plant enset, using Illumina HiSeq and MiSeq platforms. A catalogue of single-nucleotide polymorphisms was inferred from the sequence data at an average density of approx. one SNP per kb of genomic DNA. *Specific objectives: 3.1, 3.2.*

Activities developed in Kenya by Real IPM aimed at the integration of tested beneficial microorganism in local banana crops. Real IPM has been engaged in:

i) Bioprospecting, by collecting microbial (bacterial and fungi) and other beneficial EBCAs for IPM from different regions in Kenya. In particular, feld surveyes were carried out in three region in Kenya: Kapcherop Forest in Marakwet County, Suam Orchards, Elgon in Trans-Nzoia, and Mau forest in Kericho County.
ii) Isolation and identification in the laboratory. A total of 10 isolates were identified by sequencing, in collaboration with CABI Microbial identification service, Egham, UK (listed in Deliverable 2.1).
iii) Screening of soil bacterial and fungal isolates for activity. Screening included use of *Bacillus amyloliquifaciens* against PD, and of *B. mycoides* vs root knot nematodes.
iv) Screening of isolates against BW were carried out through bioassays and trials in collaboration with IITA-Kenya and ICIPE, screening *Trichoderma asperellum*, *T. atroviride, Beauveria bassiana* and *Metarhizium* spp.
v) Conducting field trials with already existing isolate from Real IPM, to validate the use of the endophytes to promote plant growth of tissue culture banana plants. Source of materials were tissue culture plantlets of *Musa* spp. Grand Naine and Williams. Hybrids were obtained from Jomo Kenyatta University of Agriculture and Technology, Institute for biotechnology research, Juja, Kenya. Isolates of the following microorganism: *Bacillus* sp., *Trichoderma* sp. and *Serratia nematodiphila* were tested. They were isolated from Kenyan soils and supplied by Real IPM. Two trials were carried out under greenhouse conditions.

Eigth new staff members were recruited by Real IPM as: microbiologist (3), horticulturist (2), Coordinator, Project Assistance and Accountant. *Specific objectives 1, 2.1, 4, 5.*

Partner IITA aimed at identifying differences in the endophytic populations that contribute to maintain asymptomatic plants in PPN, BW and Fusarium wilt-infected banana crops. The following activities have been established/started:

– identification of optimal banana agroecosystems for EBCAs isolation, characterization;
– use of metagenomics, endophyte isolation and biological characterization to identify differences in microbial populations between PD-infected and asymptomatic plants;
– upon identification of diseased and healthy plants with significant difference in microbiota composition, metatranscriptomics analyses were planned to identify changes in plant and microorganisms expression profiles, between symptomatic and asymptomatic plants;
– integration of data obtained from metagenomics, metatranscriptomics and endophytes microbiological isolation analyses to identify new genera/species involved in banana tolerance to PD;
– testing commercially available EBCAs (in laboratory, screenhouse and field conditions) for potential to control PD, PPN and BW;
– isolate and culture endophytes from banana and *in vitro* assays to identify EBCAs;
– field trials to test commercially available EBCAS to manage PPN and BW.

Specific objectives 1, 2.1, 3.1, 3.2, 4, 5.

EARTH produced, evaluated and selected EBCAs, in collaboration with export-oriented producers from Costa Rica (Corbana, https://www.corbana.co.cr/). The most

effective isolates belonging to the genus *Trichoderma* were selected for evaluation in the greenhouse based on the production of inocula and fast growth *in vitro*. Nine isolates were inoculated in tissue-cultured plant of Grand Naine (AAA) for evaluation of their growth promotion effects. The best isolates selected for plant growth promotion increased the fresh biomass of plants. They are under evaluation as biological control agents of *R. similis* in greenhouse conditions. The endohytes were inoculated in test banana field for assays against PPN and performed better when compared to commercial products actually in use. *Specific objectives 1, 2.1, 3.1, 5.*

Continued mass production of referenced strains of *P. chlamydosporia* var. *catenulata* (IMI SD187) and *T. asperellum* (Ta.13) was performed at CENSA using a solid state fermentation technology, developed for two commercial products, KlamiC® y SevetriC®. They have been used for experimental assays to evaluate new applications and EBCAs. A method for *in vivo* production of EPN invasive stages (infective juveniles of *Steinernema* and *Heterorhabditis* spp. with symbiotic *Xenorhabdus* and *Photorhabdus* spp. bacteria, respectively) was modified by CENSA research team for massive use in Cuba, including quality control steps. The protocol modifications produced have been deposited in the Cuban Copyright Center (Centro Nacional de Derecho de Autor, Cuba, n. 09613-2002) as a reference for EPN production in cottage laboratories (details given in Del. D5.1). The Cuban Germplasm Bank located at INIVIT (Central region), was sampled to determine the nematofauna associated with the most important banana genotypes, to assess the resistance or susceptibility to root knot nematodes and *R. similis* of accessions, and to establish pure nematode populations. Five EPN populations have been isolated for use *vs* BW. *Specific objectives 1, 2.1, 4, 5.*

The following beneficiaries: CENSA, SARI, EARTH, IITA, ICIPE, Real IPM that have received EU funding, plan to exploit the results generated with such funding primarily in third countries not associated with Horizon 2020. The Union funding will benefit Europe's overall competitiveness (reciprocity principle), through: the increased exchange of newly produced knowledge, the development of reciprocal international collaborations both scientific and cultural, the potential for adoption by EU stakeholders of the new technologies and /or protocols produced, the potential for increased trade opportunities, the higher quality of imported fruit commodities, the reduced impact of more environment-friendly crops, and the enhanced visibility of the EU international role.

1.2 Explanation of the work carried out per WP

1.2.1 WP 1 - Ethics requirements.

During the kick-off meeting held on Aug. 29-31, 2017 at Tenerife (ES) all attending Consortium members addressed ethics and security issues and, upon agrement of all partners also reached by email by the Coordinator, produced Deliverable 1.1.

1.2.2 WP 2 - Isolation and selection of Endophytes and Biocontrol Agents (mths 1-18)

Task 2.1 EBCAs of Fusarium oxysporum *f. sp.* cubense

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A number of samplings have been performed by IAS CSIC to identify and collect EBCAs suitable for exploitation in Project tasks. Roots of cv. Pequeña Enana plants originated from five sampled farms (Fig. 1A, Table 1) representative of different orchards at Tenerife Island. Two samples per plant (5 per farm) were collected. Samples of roots and adhered (rhizosphere) soil were collected (10-15 cm depth) from the selected farms (October 2017, with guidance and collaboration of Partner 4, Coplaca, Sta. Cruz de Tenerife, Spain; Personnel involved, Dr. Ing. Javier López-Cepero, Fig. 2).

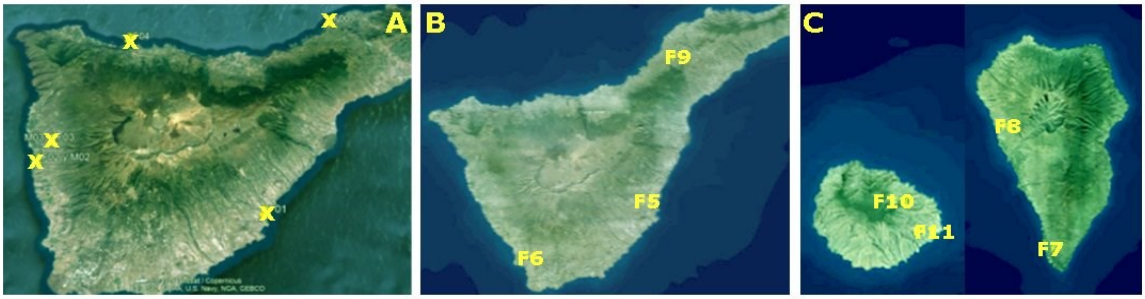

Figure 1. Maps of Tenerife showing location of surveyed farms in first (A) and second (B) samplings, and of La Gomera and La Palma islands showing location of surveyed farms (C).

Table 1. Characteristics of farms sampled in Tenerife by IAS-CSIC and Coplaca (first round).

Sampling date	Farm Nº	Farm name	Lat. (N)	Long. (W)	Altitude (msl)
09/10/2017	F00	Escuela Capataces	28°29'46"	16°25'15"	299
10/10/2017	F01	Siverio	28°10'06"	16°26'13"	37
10/10/2017	F02	Temaso	28°09'00"	16°47'36"	84
10/10/2017	M02	Temaso	28°09'00"	16°47'36"	84
10/10/2017	F03	Servicios Agrícolas Abdul	28°11'02	16°47'01"	326
10/10/2017	M03	Servicios Agrícolas Abdul	28°10'60"	16°47'02"	306
10/10/2017	F04	Malpaís - Colpon Agrícola	28°22'36"	16°44'08"	22

To increase the microbial collection generated from banana plants a second sampling (Nov. 2018, with the guidance and collaboration of Partner 4, Coplaca; Personnel involved, Dr. Ing. Javier López-Cepero) was carried out in new farms from three different islands. Root plants (cv. Pequeña Enana) were sampled from seven farms (Fig. 1 B,C and Table 2) representative of different banana orchards at Tenerife (including Farm 00 of the previous sampling), La Palma and La Gomera. Samples of roots and adhered (rhizosphere) soil were collected from healthy farms and those where incidence of PD was reduced significantly.

Figure 2. Root and soil sampling in banana farms in Tenerife.

Table 2. Characteristics of farms surveyed in Tenerife, La Palma and Gomera islands by partner 5 (IAS-CSIC), in collaboration with Partner 4.

Sampling Date	Farm	Farm name	Latitude (N)	Longitude (W)	Altitude (msl)
06/11/2018	F05	Fco Pacheco, Arico, Tenerife	28°10'67"	16°27'64"	188,64
06/11/2018	F06	La Caldera, Adeje, Tenerife	28°04'84"	16°43'32"	113,22
07/11/2018	F07	Siso, FuencalienteLa Palma	28°28'97"	17°52'58"	28,77
07/11/2018	F08	Ortiz, Tijarafe, La Palma	28°41'37"	17°57'01"	299,56
09/11/2018	F09	Escuela Capataces, Tenerife	28°29'46"	16°25'15"	299
10/11/2018	F10	Hermigua, La Gomera	28°10'82"	17°11'08"	123,15
10/11/2018	F11	David, San Sebastián, La Gomera	28°06'69"	17°08'68"	82,82

Associated (rhizosphere) soil samples were conserved at 4ºC until their transfer to Partner 1 (CNR, Bari, IT), for further isolations. Surface sterilization was carried and plates were examined for the presence or absence of contaminating microorganisms (Fig. 3B). Five different media were tested with were *ad hoc* chosen dilutions (Fig. 4).

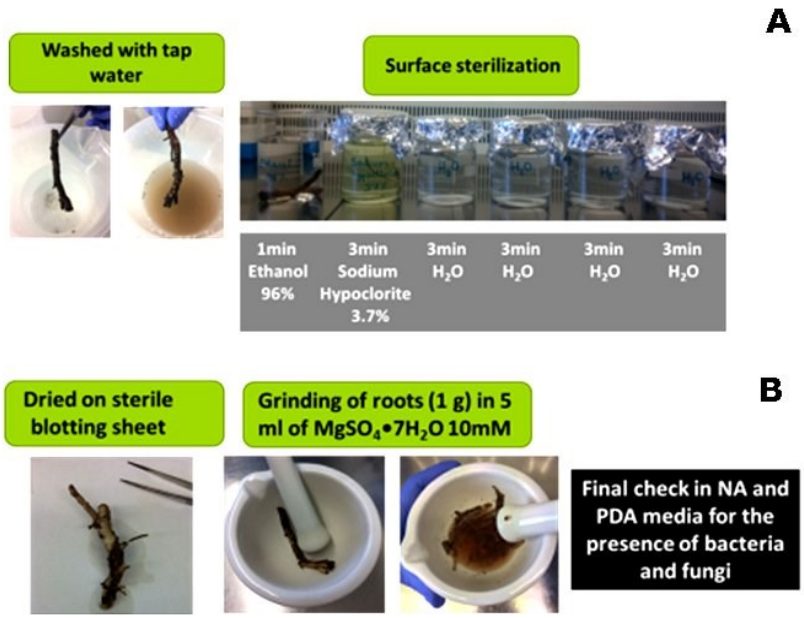

Figure 3. Banana roots surface sterilization procedure (A) and grinding protocol (B).

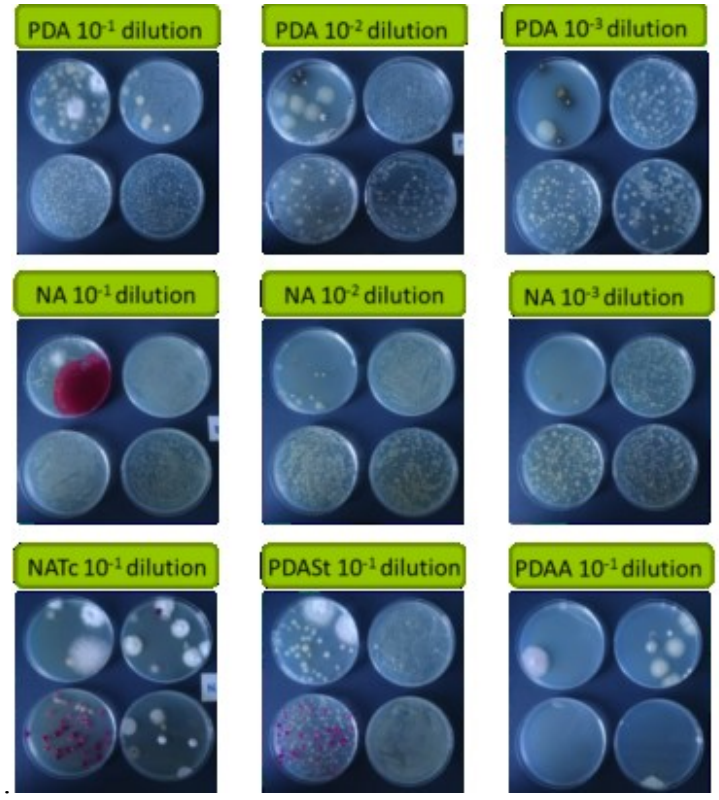

Figure 4. Examples of diversity of bacteria/fungi colony morphologies on different culturing media during the banana roots endophyte isolation procedure.

Agar plates were incubated up to 7 days at 28ºC and observed on a daily basis to check for bacterial/fungal growth. Individual colonies were transferred to PDA plate and colonies were selected according to speed of growth and morphology (colour, size, shape) (Fig. 5).

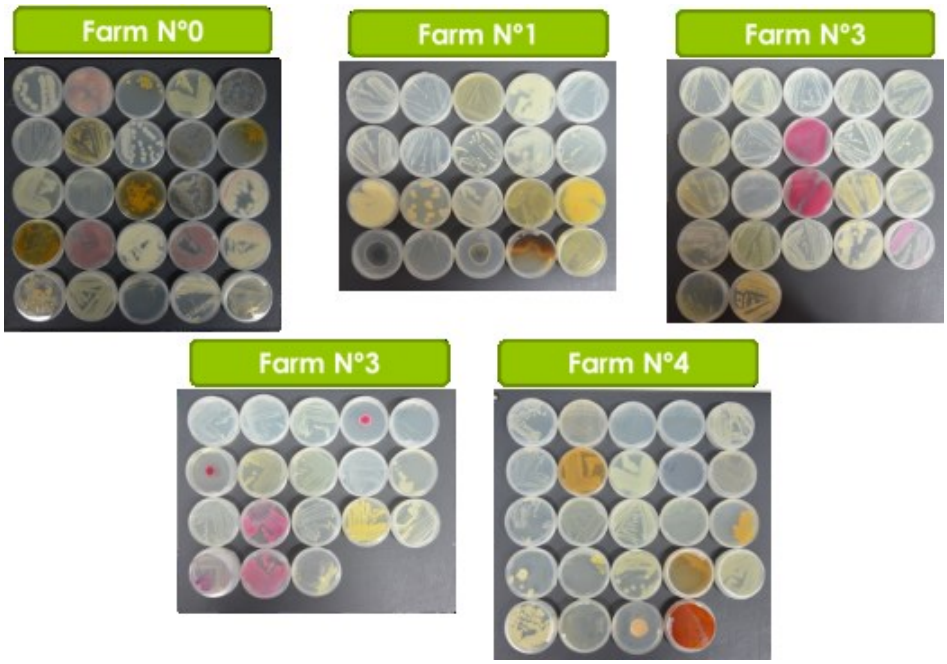

Figure 5. Examples of bacteria/fungi colony morphologies obtained from root macerates of a selected banana plant sampled at each surveyed farm

Eventually, pure cultures originating from single colonies were grown in Luria Bertani (LB, for bacteria) or PDA (for fungi) media and cryopreserved at -80ºC until use. 696 single/pure bacteria and 162 fungi isolates were obtained from the first sampling.

In vitro *antagonism assays*. Bacterial and fungal isolates were tested against a representative isolates of Foc subtropical race 4 (STR4), race 1/2 (R1/R2) (isolatesCAV-095 and CAV-2790, kindly provided Prof. Altus Viljoen, Stellenbosch Univ., South Africa) and TR4 (isolate II5, kindly Prof. Antoni Di Pietro, Córdoba University, Spain), causal agent of PD. To determine the *in vitro* antagonist activity of root endophytes, individual drops (10 µL) of CAV-095 (containing mycelium and conidia) were plated in the centre of PDA plates. Four endophytic bacteria or fungi were inoculated at four equidistant points from the pathogen spot with a sterile loop (Fig. 6). One control plate with just a suspension of the pathogen biomass was included on each trial. Currently, bacteria and fungi isolated from the second sampling are being tested against CAV-095 (STR4) and a representative isolate of Foc tropical race 4 (TR4) following the same protocol as the first sampling.

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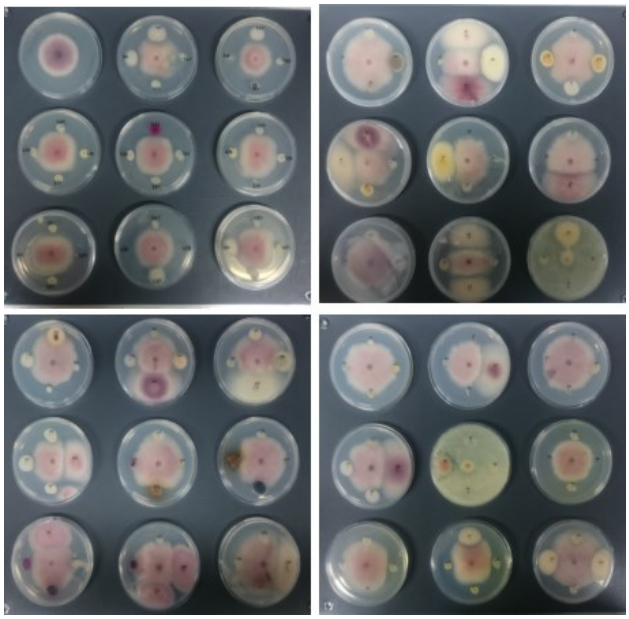

Figure 6. Results of *in vitro* antagonism assays against Foc CAV-095 at 7 days after plate inoculation (first sampling)

More than 100 strains showed *in vitro* antagonism against Foc CAV-095 at a variable degree, showing that banana roots are a rich reservoir of beneficial endophytic antagonists. The 88 strains (from the first sampling round) showing the best potential as EBCAs were selected and additional dual cultures were performed. Individual drops of Foc TR4 biomass (i.e., mycelium plus conidia) were plated in the middle of plates, and four equidistant 10-µl drops of each tested microorganism were inoculated. Control plates with just a suspension of the pathogen biomass were included on each experiment. Antagonist activity (i.e., halos or inhibition zones) was then scored. The relative inhibition index was calculated according to the equation *(Rc-Ra)/Rc*, where *Rc* is the average radius of Foc TR4 colony in the absence of antagonist microorganism and *Ra* is the average radius of Foc TR4 colony in the presence of antagonist microorganism (four equidistant points). These experiments were performed with three biological replicates per each interaction and used media. Differences were found depending on the culture medium used (PDA or NA). Inhibition of Foc growth was more frequent in NA. Twenty six and nine selected endophytes significantly inhibited growth of Foc TR4 (relative inhibition >0.30) in NA and PDA media, respectively. Isolate #793 (molecularly identified as *Pseudomonas protegens*; see below) was the most promising strain showing a relative inhibition index of 0.43 (PDA medium). However, based on the results obtained from *in vitro* tests in NA medium, the three most promising strains were #102, #248 and #301 (molecularly identified as *Pseudomonas chlororaphis* the first and *Pseudomonas* spp.). A relative inhibition index >0.60 was measured for these three *Pseudomonas* spp. isolates (Fig.7).

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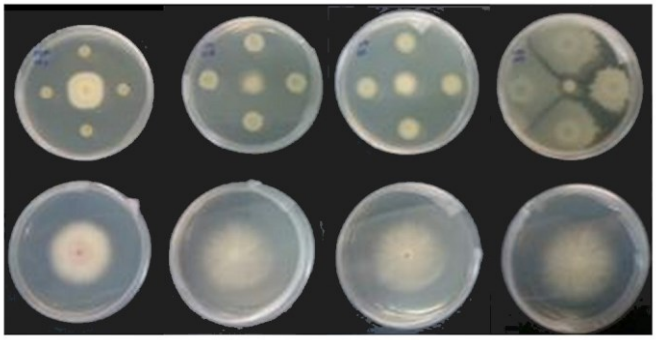

Figure 7. Endophytic strains showing the best in vitro antagonism against Foc TR4. Lower row shows control plates without antagonists.

Phenotype characterization. After the selection of 88 strains (from the first sampling round) showing the highest antagonistic activity, phenotypic characterization was performed. We performed assayas to check diverse phenotypes traditionally associated with biocontrol and/or plant growth promotion such as volatile production (e.g. 2,3 butanediol and hydrogen cyanide), enzymatic activities such as catalase, xylanase, amylase, phytase, protease and β-glucosidase, and siderophore production. Table 3 summarizes the number and frequency of isolates positive for the tested activities.

Table 3. Evaluation of phenotypes associated with biocontrol and/or plant growth promotion.

Phenotype	Number of isolates	Frequency (%)
Phytase	55	62.5
Protease	51	57.9
Xylanase	6	6.8
Catalase	84	95.4
Siderophores	67	76.1
Amylase	19	21.6
β-Glucosidase	35	39.8
2,3 Butanediol	16	18.2
HCN	30	34.1

Molecular identification. The 88 strains, selected at CSIC according to their Foc antagonistic effect, are currently being identified at the molecular level by sequencing 16S rDNA and *gyrB* genes for bacteria, and Translation Elongation Factor 1-alpha (*tef1*) and Internal Transcribed Spacer (ITS) for fungi. Data gathered so far showed a low diversity among bacteria, *Pseudomonas* being the most predominant genus. Fungal isolates showed higher diversity, with representative genera *Gloeotinia, Fusarium, Plectosphaerella, Gliocladium, Epiccocum* and *Acremonium* were found.

CNR proceeded to the isolation of *Bacillus* and *Streptomyces* spp. and other EBCAs from banana rhizosphere and subsequent selection for *in vitro* antagonistic activity against Foc, producing a collection held at IPSP CNR (Bari, IT), tested for efficacy against PD in pot-grown banana. Samples proceeded from the Oct. 2017 survey carried out by Coplaca and IAS CSIC (43 soil samples), and from a second survey, carried out on Feb. 2018, in collaboration between CNR and Coplaca, also aiming at PPN antagonists.

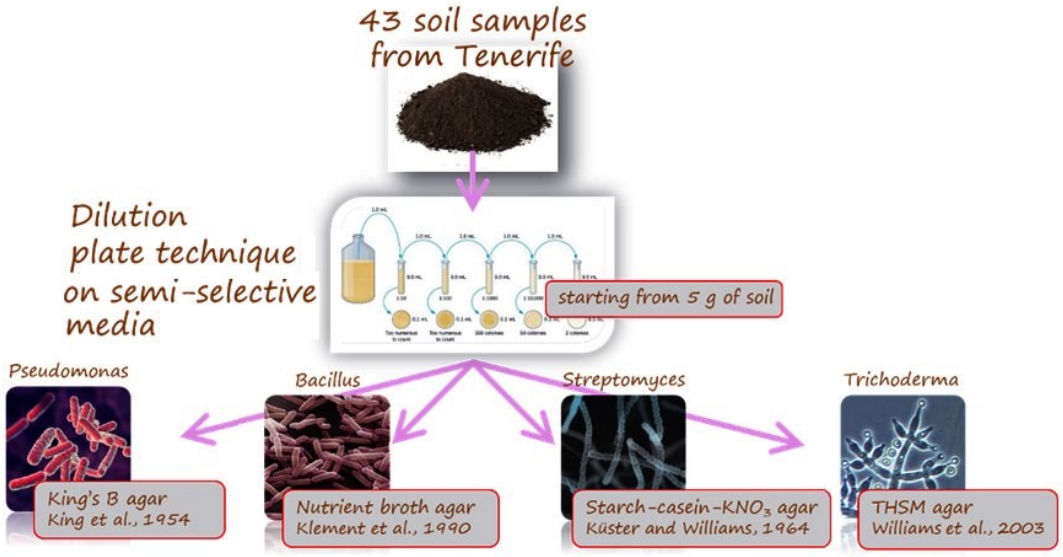

Figure 8. Procedure applied to isolate microorganisms from banana soil samples by the dilution plate technique.

Isolation of EBCAs from banana rhizosphere. EBCAs were isolated from soil onto agar media according to the dilution plate technique (Fig. 8), using 5 g of soil suspended in 50 mL physiological solution. Such suspension was used for ten-fold dilutions, and then plated on semi-selective agar media in triplicate. A total of 516 plates were used. Colonies of *Bacillus* and *Pseudomonas* spp. were visible one-two days after plating, and those of *Streptomyces* and *Trichoderma* spp. 5-7 days after plating. At this stage, isolates were identified only by the morphology of the colony (Fig. 9).

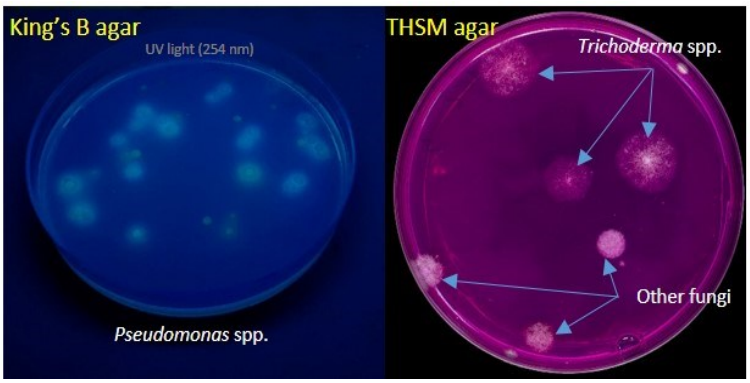

Figure 9. Isolation of microorganisms from soil by the dilution plate technique. King's B agar plate with colonies of *Pseudomonas* spp. showing fluorescence under UV light at 254 nm (left). THSM agar plate with colonies of *Trichoderma* spp. (right).

Selection of EBCAs for in vitro Foc *antagonism*. EBCAs were screened *in vitro* at CNR for their antagonistic activity against Foc race 1 (Foc R1) and Foc tropical race 4 (Foc TR4) by the dual culture method (Fig. 10). Only isolates developing an inhibition halo vs Foc were transferred to PDA for storage at -80°C with glycerol.

A total of 516 plates were used for EBCAs isolation. Since the agar media were not strongly selective, microorganism genera other than the target ones were also present in the isolation plates, especially SCPNA used for *Streptomyces* spp. However, since the antagonistic activity against Foc was a priority, promising antagonists were selected regardless of taxonomic classification. About 1000 isolates putatively belonging to *Bacillus* spp. and *Streptomyces* spp. and over 340 isolates putatively belonging to *Pseudomonas* spp. and *Trichoderma* spp. were screened for antagonism against Foc R1 and Foc TR4.

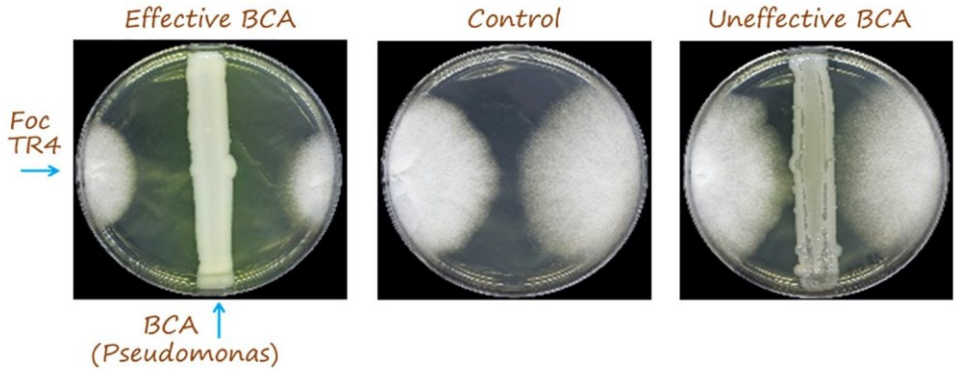

Figure 10. Dual culture method for screening beneficial microorganisms for antagonism *vs Fusarium oxysporum* f. sp. *cubense* race 1 and *tropical race* 4 (Foc).

Establishment of a microorganism collection. Finally, a total of 87 isolates were selected as Foc antagonists and stored in collection at IPSP CNR.

Evaluation of efficacy of selected EBCAs against PD in pot-grown banana. The most effective 44 isolates were used to set up a synthetic microbial community (SynCom, Table 4), tested *in planta* in two consecutive assays. Banana plants cv. Gran Enana, transplanted in pots with sterilized soil, were inoculated with known concentrations of Foc TR4 with/without added SynCom, and incubated in a conditioned quarantine greenhouse. PD symptoms were monitored periodically as well as the soil dynamics of Foc and SynCom. In a first assay, PD symptoms appeared 14 days post-inoculation (dpi) and increased afterwards. Inoculation with SynCom reduced the symptom severity of PD (Fig. 11 and 12), although not affecting the disease prevalence (percent of diseased plants).

The soil SynCom population components remained viable and active throughout the experiment and were compatible (not deleterious to) the Gran Enana roots. The pathogen impacted negatively on the chlorophyll content of leaves, an effect that the SynCom mitigated (Fig. 12). The results showed that SynCom reduced partially the PD severity, probably by indirect mechanisms, because no pathogen regulation was detected in soil. These results were confirmed in a second independent assay with a reduced SynCom (Table 5, Figs. 11-14).

Table 4. Initial composition of the SynCom 1.0 (first in planta assay, 44 isolates).

Taxon	Isolates
Pseudomonas sp.	P1C1, PS5, P1A1, PS3.1, P3E1, PS6, P4AOD1, P2D1, P4A1, P2B1, P2C1.2.1
Bacillus sp.	BT1, BS6, B2C1.2.1, BN8.1, BS7, B4C1, B2C1.2, B1A1, BN8.2, B4B1.2, B2AOB1
Streptomyces sp.	St2AOB1, St3B1.1, St4C1, St3A1, St2C1.1, St2B1, St3D1, St4AOD1, St1D1, St2A1.2, St3C1
Trichoderma sp.	T2C1.4, T2A1.1, T2A1.2, T2B1.1, T2B1.2, T2C1.1, T2C1.2, T2C1.3, T2E1, T3A1.2, TS1

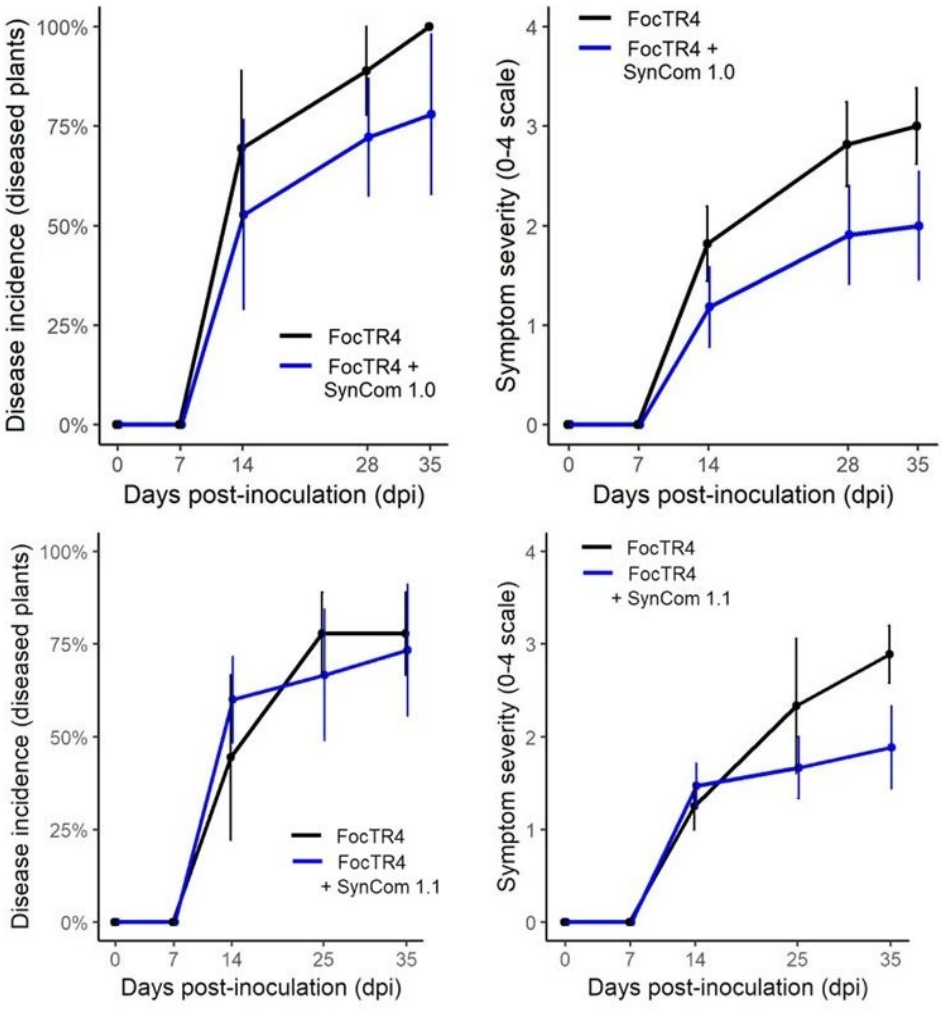

Figure 11. Experiments I (upper panel) and II (lower panel). Incidence (percent of diseased plants) and severity of Panama disease in pot-grown banana plants inoculated or not with a microbial synthetic community (SynCom). Data from four replicates (mean ± SD).

Figure 12. Symptom severity of PD in pot-grown banana plants inoculated or not with a microbial synthetic community (SynCom)..

Table 5. Composition of the reduced SynCom_1.1 (second *in planta* assay, 7 isolates).

Taxon	**Strains**
Pseudomonas sp.	P1C1, PS5, P1A1
Bacillus sp.	BT1,BN8.2
Streptomyces sp.	St2AOB1
Trichoderma sp.	T2C1.4

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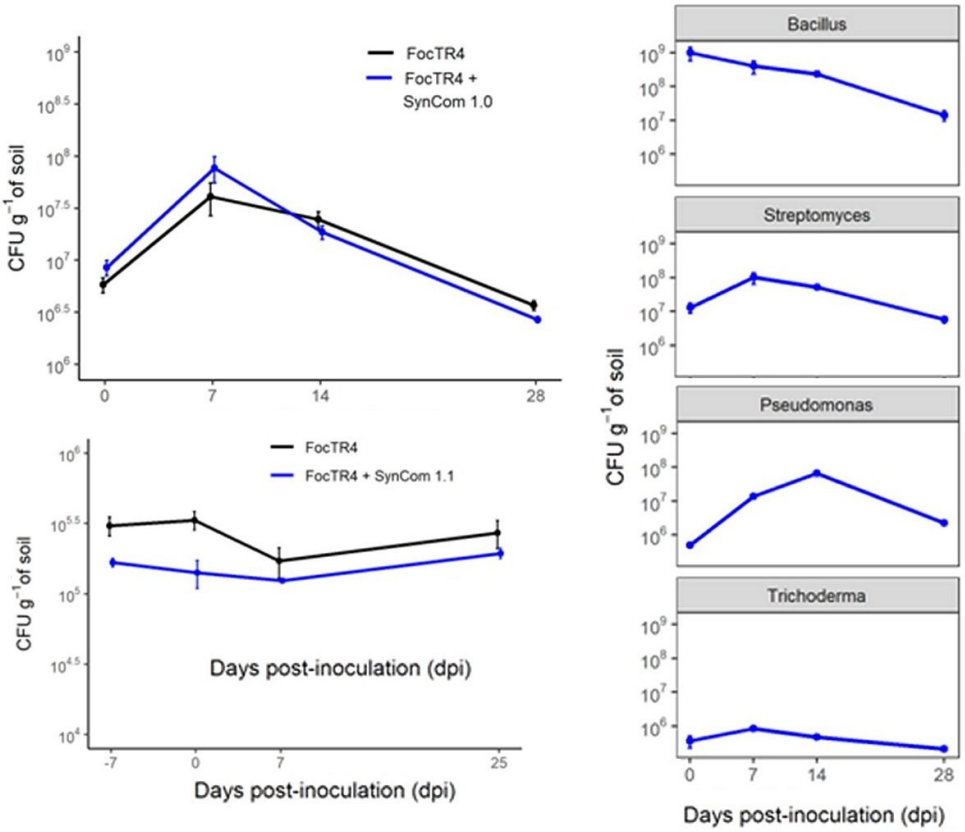

Figure 13. Population dynamics (cfu g^{-1} of soil) of *Fusarium oxysporum* f. sp. *cubense* (Foc) TR4 in experiments I (upper panel) and II (lower graph). Density changes of the microbial synthetic community (SynCom) members soil artificially inoculated (exp. I). Data from four replicates (mean ± SD).

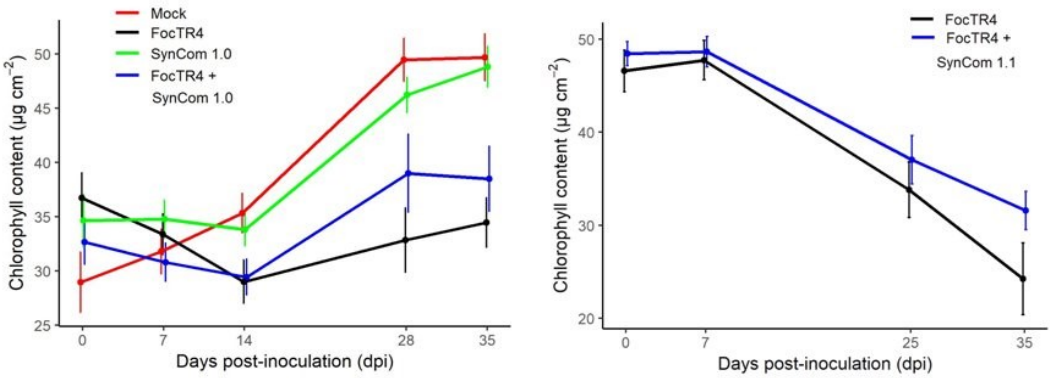

Figure 14. Experiments I (left) and II (right). Chlorophyll content of the top-three leaves of banana plants cv. Gran Enana grown in soil inoculated with of *Fusarium oxysporum* f. sp. *cubense* (Foc) TR4 and/or a microbial synthetic community (SynCom). Data from 4 replicates (mean ± SD).

A third experiment (III), was carried out to check if the SynCom could reduce the Foc TR4 inoculum in soil. Three batches of soil were prepared in triplicate with pathogen concentrations ranging from 10^3 to 10^7 cfu g^{-1} soil, and inoculated or not with the SynCom. Again, the FocTR4 population, monitored periodically, was not regulated, regardless of the SynCom concentration (Fig 15).

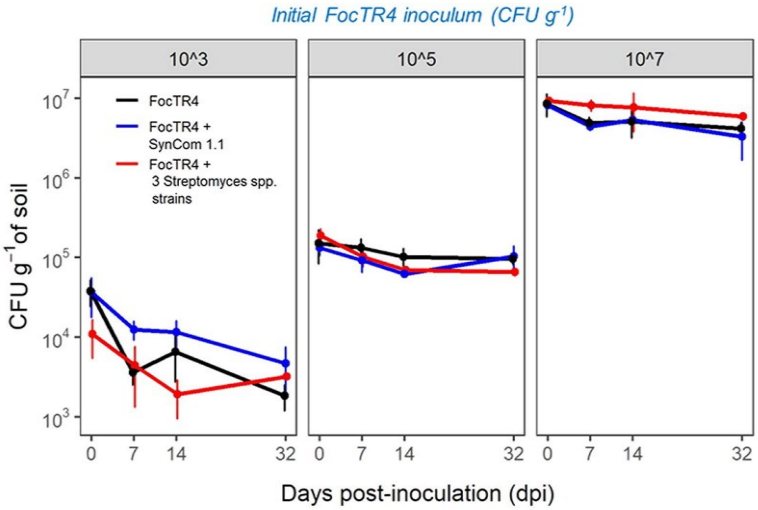

Figure 15. Experiment III. Population dynamics (cfu g^{-1} of soil) of *Fusarium oxysporum* f. sp. *cubense* (Foc) TR4 with the microbial synthetic community (SynCom 1.1) in soil artificially inoculated. Data from 4 replicates (mean ± SD).

In a forth assay, isolate of *Pochonia chlamydosporia* DSM 26985 was tested *in planta* for control of Foc TR4. Such isolate resulted effective *in vitro* against the pathogen (Fig. 16A). The *P. chlamydosporia* isolate was inoculated by soil drenching pot-grown plants with a conidial suspension with a known concentration. One week later, plants were transplanted in soil artificially inoculated with 10^4 cfu g^{-1} of Foc TR4. Plants treated with DSM 26985 showed less disease symptoms at early stages of PD development (35 dpi), compared to Foc TR4-inocualted control. However, as the disease progressed, difference between treated and untreated plants disappeared, and became undetectable at 60 dpi (Fig. 16B).

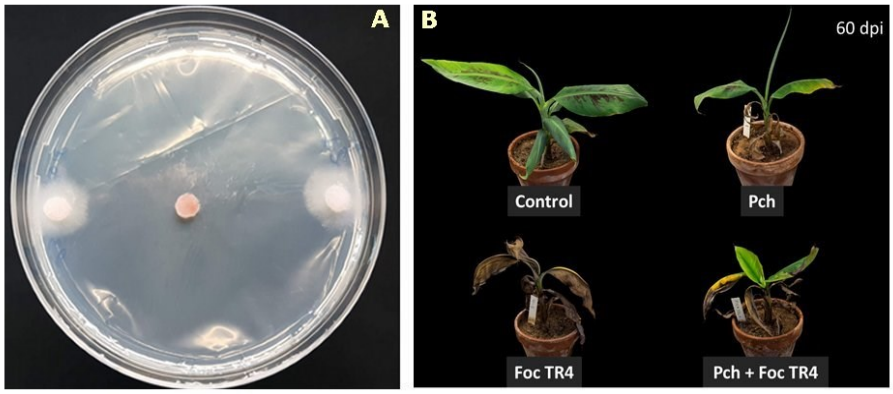

Figure 16. Colonies of Foc TR4 grown on PDA (A) in presenc of metabolites produced by *Pochonia chlamydosporia* DSM 26985 grown for 5 days on a cellophane film placed on the agar surface, allowing the fungus to spread metabolites but not hyphae, on the medium. A metabolite(s) concentration gradient was formed from the center towards the edge. After cellophane removal, the plate was inoculated with Foc TR4 as shown. The colony in the center was in the zone with highest concentration of Pc metabolites, compared with the edge. The highest concentration of Pc metabolites inhibited Foc TR4 growth, while the lowest only partially inhibited its development. Symptom severity of PD in pot-grown cv. Gran Enana plants inoculated or not with Foc TR4 and *P. chlamydosporia* DSM 26985 (B).

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Selected soil bacteria and fungi have been also isolated for PD control in Kenya by Real IPM and ICIPE. Isolation is still ongoing for screening in greenhouse and field conditions.

Isolation of beneficial EBCAs in SSA has been conducted by IITA. In Tanzania, 370 bacterial isolates were obtained from 35 corms and roots of Mchare type banana plants sampled from Arusha and Kilimanjaro. Samples were collected from 5 smallholder banana farms located approx. 10 km apart. For each farm, plants with PD symptoms and others without apparent symptoms were selected, at the same physiological maturity. A total of 4 samples (roots and corms) were collected from each farm, two from symptomatic plants and two from asymptomatic ones. Isolation was carried out according to the protocol developed by IAS CSIC and shared through the MUSA project. Pure cultures isolates are under long-term storage in glycerol at -80°C. Sampling was conducted twice, in Jan. 2018 (summer season) and July 2018 (winter - drier season). Of the 370 potential bacterial endophytes obtained, 131 proceeded from corms and 239 from roots. The isolates have been characterized using morphology (colony shape, and color) and Gram staining test. DNA has been extracted from all 370 isolates, the 16S rRNA gene amplified and sent for sequencing to a commercial provider, to complete identification.

In Uganda, a total of 534 bacterial isolates and 56 fungal isolates have been obtained by IITA and processed for long-term storage in glycerol and at -80°C. All bacterial isolates have been characterized using morphology (colony shape, and color) and Gram staining. Sampling for mycotoxin analysis was conducted twice. The first was done in March, 2018 for samples collected from cultivars Silk (AAA) and Sukali Ndizi (AAB), in Kawanda. As in Tanzania, samples were collected from plants with apparent PD symptoms and from others without apparent symptoms, at the same physiological maturity. Samples were collected from 5 symptomatic and 5 asymptomatic plants of each cultivar and field. The site was selected for the semi-controlled infection conditions that guaranteed the presence of the pathogen in both symptomatic and asymptomatic plants.

Scientists at ICIPE, IITA and Real IPM collaborated in Kenya for assessment of antagonists against BW and PPN (Table 6). They could also recover a *Beauveria bassiana* strain first isolated by NARO (Caroline Nankinga) from weevils in Uganda and used previously by both NARO and IITA, which performed efficiently in the field. As it could not be found in Uganda but it had been deposited with CABI, it was recovered from this collection and now is in use. The Foc endophyte V5W2 previously isolated by IITA demonstrated to provide good management of nematodes, as well to induce a growth stimulus, at least to plantlets. In order to gain field progress these two isolates were used to assess their efficacy in isolation as well as combined, under field conditions. Scientists from IITA, ICIPE, Real IPM and KU Leuven effectively implemented three strategies to select isolates for subsequent work. In particular by: *i)* leveraging on other/previous projects that have bioprospected microbes – such as the 1500 populations isolated under the banana Xanthomonas wilt (BXW) project (the best five populations that worked against BXW were selected for assessment in MUSA); *ii)* bioprospecting sites with Fusarium problems and isolating new popualtions for *in vitro* and *in vivo* assessment (molecular tools/identifications are applied to focus on which populations to select), and *iii)* assessing best populations from institutional collections

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(ICIPE/RealIPM) including those already marketed as products, unsing in addition a *F. oxysporum* and a *B. bassiana* populations that have been proven to work on banana and that were taken to the field to maximise assessment of previously proven populations.

Table 6. Antagonists tested in Kenya against banana weevils and nematodes.

Isolate code	Taxonomic name	Repository /Institute	Isolation Source	Isolation site	Target organism(s)
ICIPE 284	*Beauveria bassiana*	ICIPE	Soil	Mauritius	BW
ICIPE 648	*B. bassiana*	ICIPE			BW
ICIPE 647	*B. bassiana*	ICIPE	Soil	Mauritius	BW
ICIPE 662	*B. bassiana*	ICIPE			BW
ICIPE 660	*B. bassiana*	ICIPE			BW
ICIPE 609	*B. bassiana*	ICIPE			BW
ICIPE 273	*B. bassiana*	ICIPE	Soil	Mbita (Kenya)	BW
ICIPE 281	*B. bassiana*	ICIPE	Soil	Mauritius	BW
ICIPE 622	*B. bassiana*	ICIPE			BW
ICIPE 644	*B. bassiana*	ICIPE			BW
ICIPE 69	*Metarhizium anisopliae*	ICIPE	Soil	Matete (DRC)	BW
ICIPE 62	*M. anisopliae*	ICIPE	Soil	Matete (DRC)	BW
ICIPE 78	*M. anisopliae*	ICIPE	*Temnoschoita nigroplagiata*	Ungoye (Kenya)	BW
ICIPE 682	*Isaria* sp.	ICIPE			BW, PPN
ICIPE 700	*Trichoderma asperellum*	ICIPE			BW, PPN
ICIPE 710	*Trichoderma* sp.	ICIPE			BW, PPN
ICIPE 712	*Fusarium* sp.	ICIPE			BW, PPN
ICIPE 697	*Hypocrea* sp.	ICIPE			BW, PPN
ICIPE 279	*B. bassiana*	ICIPE	Coleopteran larvae	Kericho (Kenya)	BW
ICIPE 621	*B. bassiana*	ICIPE			BW
ICIPE 620	*B. bassiana*	ICIPE			BW
BW KM	*Beauveria* sp.	ICIPE	*C. sordidus*	Kalro, Mwea, Kenya	BW
SD 228	*T.asperellum*	Real IPM		Kenya	PPN
SD 298	*T.hamatum*	Real IPM		Kenya	PPN
SD 229	*B.bassiana*	Real IPM		Kenya	BW
SD 277	*B. bassiana*	Real IPM		Kenya	BW
V2W2	*Fusarium oxysporum*	IITA	Banana roots	Uganda	BW, PPN
V5W2	*F. oxysporum*	IITA	Banana roots	Uganda	BW, PPN
Bb WA	*B. bassiana*	CABI (NARO)	*C. sordidus*	Uganda	BW, PPN

In Luwero, IITA scientists selected five symptomatic and five asymptomatic banana plants of the cultivar Sukali Ndizi (AAB). This field was selected because plants were embedded in an agroforestry ecosystem with coffee and cassava plants that might

influence higher variability in the endophyte populations. In a third site, Kisoga, 5 symptomatic and 5 asymptomatic plants of cultivar Sukali Ndizi were selected. In this field banana was grown in monoculture and we could identify symptomatic and asymptomatic plants organized in distinct field locations.

Task 2.2 - EBCAs for banana and enset PPNs

Samples collected in Tenerife (Feb. 2018) by CNR showed a 77% prevalence of *Pratylenchus goodeyi*, either in northern and southern farms. *Helicotylenchus* spp. were also detected, with 81% prevalence, including *H. multicinctus* and *H. abunaami*. Nematode antagonists isolated from soil included *Drechslerella dactyloides*, *Lecanicillium* sp. (likely *L. psalliotae*, under molecular identification with ITS sequencing), and othen generalist nematode trapping fungi, under evaluation with SacomLab. The nematode populations are currently multiplied on Gran Enana plants in greenhouse conditions. A *Pasteuria* n. sp. population, available in collection at IPSP Bari and parasitic on *Helicotylenchus* spp., is under evaluation for parasitism of *H. multicinctus* and possible culturing. A delay was experienced for the isolation of PPN antagonists from enset by CNR and SARI, which is expected to start by the end of February 2019.

For isolation and selection of EBCAs from Central America, two activities were carried out at EARTH University, in three production systems: conventional renovation, fallow and organic production. A study aimed at selection of best endophytes isolated in these production system by scrrening in greenhouse conditions. A total of 150 endophytic fungi were collected in the three systems, with 53 isolates obtained from organic banana systems, 45 from fallow and 32 from conventional renovation (Fig. 17). The frequency of root colonization by endophytes was higher in organic and fallow rather than conventional renovation. The population of *Radopholus similis* was singnifically higher in the latter, in comparison to organic and fallow systems. All fungi are stored in the laboratory of plant pathology of EARTH University.

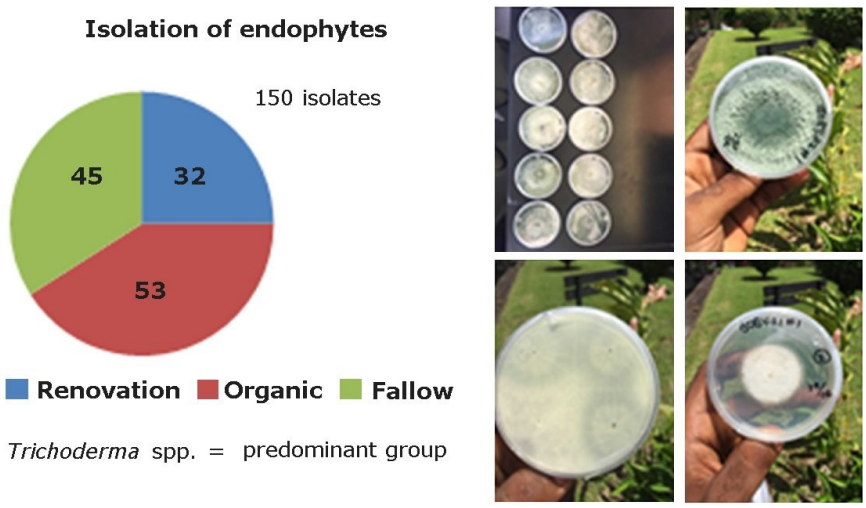

Figure 17. Isolation of endophytes from different production systems in Costa Rica: renovation, fallow and organic.

The interaction between plants and selected EBCAs was evaluated by EARTH. A total of nine isolates of *Trichoderma* spp. were selected for evaluation in the greenshouse. Three isolates of each system were selected based on the production of inocula and fast growth in petri dishes. The nine isolates were inoculated in tissue-cultured plants of Grande Naine (AAA) for evaluation of growth promotion effects. The best isolates selected for plant growth promotion were Endo 1, 2 and 3 from organic banana system and Endo 4 from fallow (Fig. 18). Currently, all the four isolates are evalauted for biological control activity against *R. similis* in greenhouse conditions. In addition, the four endohytes were inoculated in a test banana field for starting preliminary field assays against PPN.

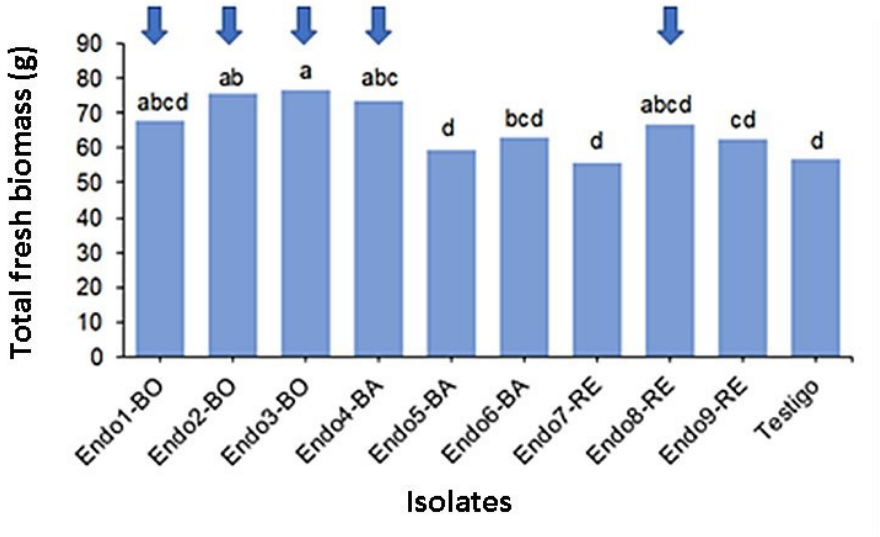

Figure 18. Effect of selected endophyte isolates on the fresh biomass production by banana cv Grand Naine in Costa Rica. Arrows show effects significantly different from control (testigo). Means with the same letters do not differ significantly (Duncan's range test, $P \leq 0.05$).

Three isolates with potential for PPN biomanagement were collected from sampling sites in Kenya by Real IPM, preliminarly identified within the biopesticides R&D laboratories, and then based on gene sequencing (performed at CABI, UK). Selected EBCAs are being tested for IPM on pests and diseases as well as known PPN. With collaboration with IITA Kenya, REAL IPM is screening isolates in the lab for PPN management.

Several surveys were conducted by CENSA for new isolates of *Pochonia*, *Trichoderma*, *Beauveria* and entomopathogenic nematodes (EPN) from banana/plantain soils, in different provinces of Cuba (Fig. 19). For the surveys, fields were selected in areas of low inputs or subsistence agriculture, when the owner farmers declared that the fields had not previously received applications of biological control agents in the history of the farm. The farm owners or managers were also asked about other details concerning the cultivars, type of agriculture, and other aspects of interest.

In each banana/plantain field, a minimum between 50 to 70 m^2 areas were sampled. The samples were distributed between the Nematology and Mycology Laboratories of CENSA for EBCAs isolation.

Figure 19. Location of fields and sampling for new isolates in Cuba.

For isolates of *Pochonia* spp. from banana and plantain, a dilution plating technique on semi-selective medium was used. After day 21, typical colonies of *Pochonia* spp. were grown on malt agar extract. From the confirmed colonies, microcultures were carried out on slides with water agar on moistened filter paper, placed in an incubator at 25ºC for 72 hours and then observed under the optical microscope to confirm the morphological characteristics of the phialides, arrangement of conidia (in heads or chains), and presence of chlamydospores. The remaining roots were used to directly obtain endophytes of *Pochonia* spp. The colonized fragments that originated colonies on petri dishes with typical characteristics of *Pochonia* were grown and the fungal

morphology was confirmed by microculturing. A total of 36 isolates were identified as *P. chlamydosporia,* 15 proceeding from roots and 3 as endophytes (Fig. 20).

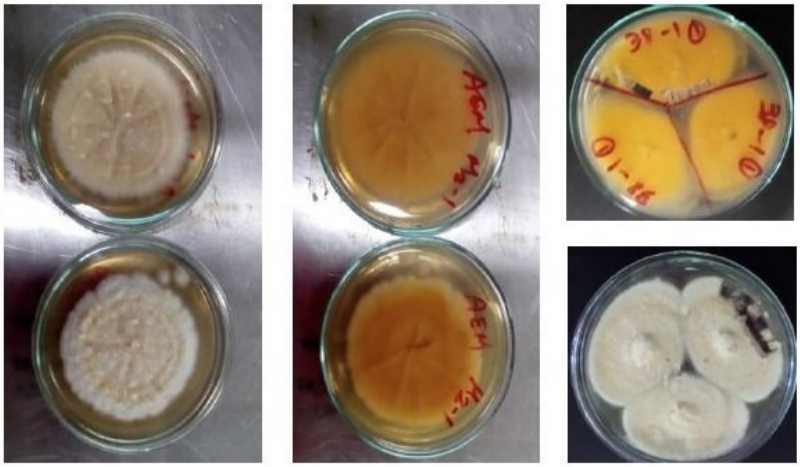

Figure 20. isolates of *Pochonia* spp. from the CENSA collection.

Out of 59 analyzed samples, proceeding from the rhizosphere of plantain and banana in the different provinces, 20 were assigned to *Trichoderma*, 5 of which as endophytes. The colonies identified as positive, isolated from the different samples, initially showed whitish coloration, with differences in texture and color shown at 72 hrs (Fig. 21).

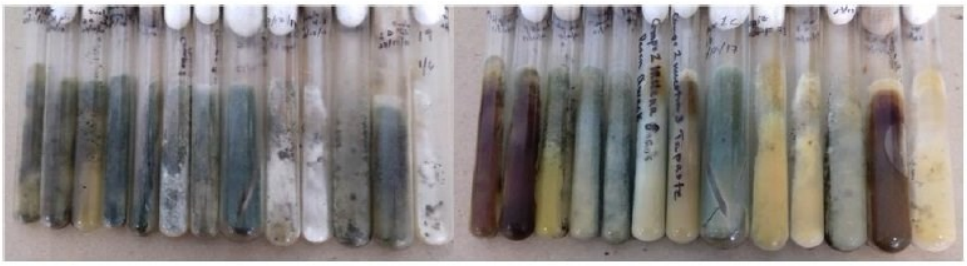

Figure 21. Cultural characteristics of the *Trichoderma* spp. isolates obtained from the rhizosphere of banana and plantain in Cuba.

UA identified fungal EBCAs from CENSA through molecular methods, by PCR with specific primers (ITS).

Task. 2.3 Antagonist and EBCAs of BW (UA task leader)

In this task UA implemented methods for isolation of entomopathogenic fungi (EF) from Canary Islands soils from banana plantations, with wide potential to be used as BW antagonists. In total 43 samples were collected in collaboration with Partner 4 Coplaca (Dr. Javier Lopez-Cepero): 3 from La Gomera, 18 from Tenerife, 10 from Gran Canaria and 12 from La Palma (Table 7) islands, sent to the Plant Pathology Lab. at the University of Alicante for EF isolation.

Figure 22. Soil sampling from banana fields.

Collection of soils from banana plantations

Soil sampling (Table 1) was performed in Canary Islands in which banana is the major crop. Number of samples collected per island was proportional to the area devoted to banana cultivation (Fig. 23). Most soil samples (18 out of 43) were taken from Tenerife, the largest island with banana as major crop. Samples covered all altitude ranges (0-100 m, 100-200 m and more than 200 m), where banana plantations are located. Soil samples were also taken from fields under sprinkler and drip irrigation (Table 7), from plantations under all crop management regimes, including ECO (plantation fields under organic production, EU Rules 834/2007 and 889/2008), with low environmental impact and fields with integrated production regimes (UNEGAP / Global Gap Rule) with a reduced use of chemicals. Finally, conventional cultivation included traditional crop management with chemical products (eg. pesticides and fertilizers). According to coordinates, soil sampling covered most of crops orientations (La Palma: eastern and western parts; La Gomera: a specific area in the northern part; Gran Canaria: along the North of the island; Tenerife: in the Northwest island slopes with prevalent banana crops, Fig. 23).

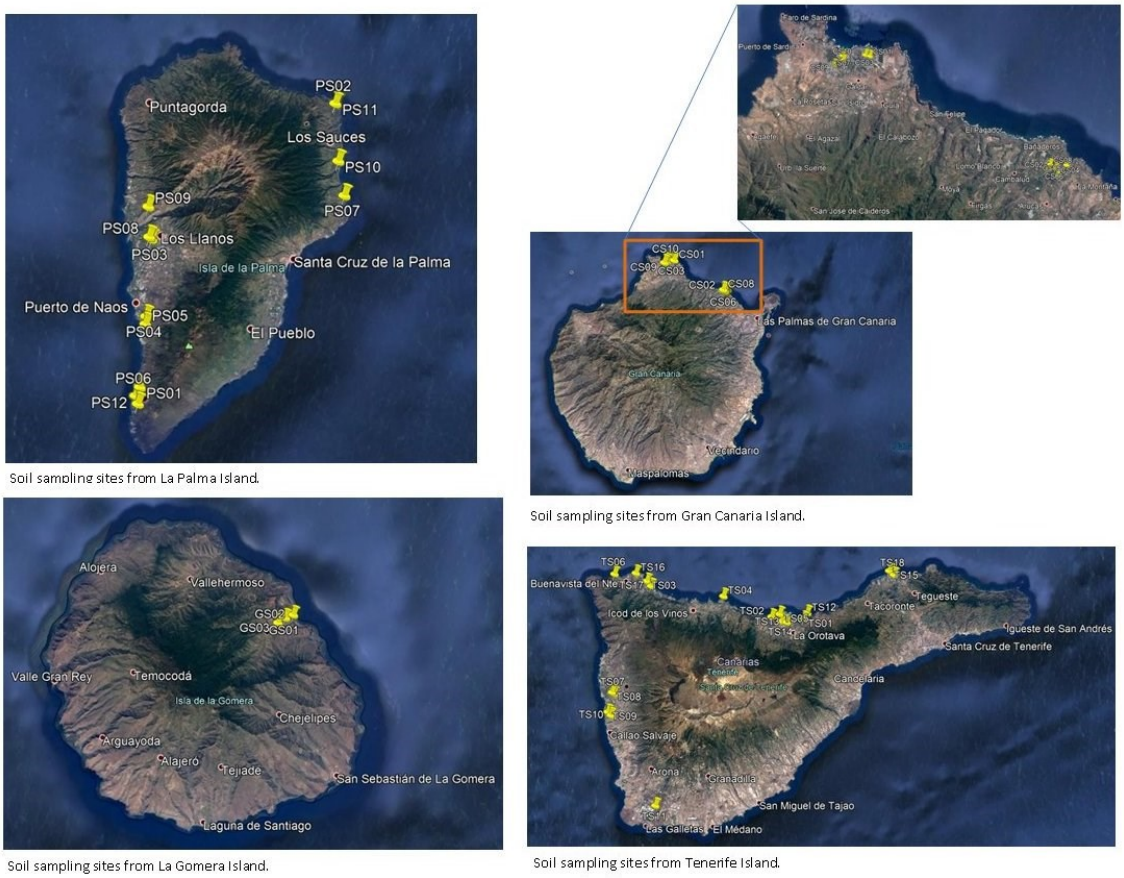

Figure 23. Soil sampling sites from banana plantation fields in Canary Islands. Bottom: percentage of the total surface of the Canary Islands sampled (A), and soil samples repartition (B, n=43).

Detection and isolation of entomopathogenic fungi (EF)

Galleria mellonella larvae were used as living baits for EF isolation. Forty g of dried soil from each screened and dried sample were placed in three replicated petri dishes, adding 10 ml of sterile distilled water (SDW) to favour EF proliferation. Eight living larvae (L3-L4) of *G. mellonella* were buried per plate, then incubated at 25 °C for 15 days in the dark and shaken periodically, to favour contact between larvae and soil (Fig. 24).

Table 7. Soil sampling data from banana fields in the Canary Islands**.**

Date	Island*	Sample	SIGPAC	Lat.	Long.	Alt. (m)	Irrigation	Crop Certification **
04/07/2017	G	GS01	38/21/16/9006/2	28°10'24"N	17°11'17"W	82	Sprinkler	U
04/07/2017	G	GS02	38/21/15/13/2	28°10'38"N	17°11'00"W	32	Sprinkler	U
04/07/2017	G	GS03	38/21/15/167/1	28°10'07"N	17°11'31"W	83	Sprinkler	U
31/10/2017	P	PS01	38/14/21/64/1	28°30'06N	17°52'16"W	56	Sprinkler	P.Int
31/10/2017	P	PS02	38/7/6/306/1	28°49'49"N	17°47'00"W	170	Sprinkler	Conv
31/10/2017	P	PS03	38/24/13/218/1	28°38'26"N	17°55'09"W	267	Sprinkler	ECO
31/10/2017	P	PS04	38/24/26/10/1	28°34'06"N	17°53'36"W	69	Sprinkler	Conv
31/10/2017	P	PS05	38/24/26/82/1	28°33'40"N	17°53'27"W	34	Sprinkler	Conv
02/01/2018	P	PS06	38/14/22/97/4	28°29'31"N	17°52'20"W	10	Sprinkler	Conv
06/02/2018	P	PS07	38/30/7/156/1	28°44'58"N	17°44'11"W	185	Sprinkler	ECO
08/02/2018	P	PS08	38/24/13/56/1	28°38'31"N	17°55'21"W	250	Sprinkler	Conv
09/02/2018	P	PS09	38/47/13/560/1	28°40'09"N	17°56'08"W	360	Sprinkler	Conv
09/02/2018	P	PS10	38/30/13/252/1	28°46'44"N	17°45'24" W	70	Sprinkler	Conv
09/02/2018	P	PS11	38/7/6/306/1	28°49'49" N	17°46'58" W	160	Sprinkler	Conv
09/02/2018	P	PS12	38/14/10/36/2	28°29'08" N	17°51'59" W	63	Sprinkler	Conv
20/07/2017	T	TS01	38/26/3/48/ 1 a 6	28°24'23"N	16°30'50"W	215	Drip	P.Int
04/08/2017	T	TS02	38/31/9/181/2	28°23'28"N	16°34'18"W	230	Drip	No
09/08/2017	T	TS03	38/42/1/32/1	28°22'23"N	16°48'51"W	50	Drip	P.Int
09/08/2017	T	TS04	38/18/3/403/2 a 5	28°23'41"N	16°40'12"W	115	Drip	P.Int
09/08/2017	T	TS05	38/28/5/9065/9	28°24'00"N	16°33'34"W	150	Drip	P.Int
10/08/2017	T	TS06	38/10/5/17/3	28°21'53"N	16°52'32"W	60	Drip	ECO
20/09/2017	T	TS07	38/19/8/9000/136	28°10'48"N	16°47'47"W	170	Drip	ECO
20/09/2017	T	TS08	38/19/7/34/8	28°10'52"N	16°48'02"W	135	Drip	P.Int
20/09/2017	T	TS09	38/1/1/82/1	28°08'54"N	16°47'43"W	70	Drip	P.Int
20/09/2017	T	TS10	38/10/51/4	28°08'42"N	16°47'16"W	80	Drip	P.Int
20/09/2017	T	TS11	38/6/4/214/6	28°01'56"N	16°38'58"W	75	Sprinkler	P.Int
09/01/2018	T	TS12	38/26/1/57/6	28°25'04"N	16°30'43"W	100	Drip	Conv
09/01/2018	T	TS13	38/26/8/43/1	28°23'29"N	16°32'30"W	91	Drip	Conv
09/01/2018	T	TS14	38/26/9/91/2	28°23'24"N	16°33'04"W	252	Drip	Conv
09/01/2018	T	TS15	38/23/1/170/1	28°31'51"N	16°23'47"W	75	Drip	Conv
09/01/2018	T	TS16	38/10/1/146/10	28°22'44"N	16°50'20"W	95	Drip	Conv
09/03/2018	T	TS17	38/42/7/6/16	28°22'04" N	16°48'13" W	72	Drip	ECO
12/03/2018	T	TS18	38/23/2/44/3	28°31'38"N	16°23'09" W	115	Drip	Conv
13/03/2018	C	CS01	35/09/2/111	28°09'28"N	15°39'01" W	75	Drip	Conv
13/03/2018	C	CS02	35/6/6/1/1	28°08'05" N	15°31'36" W	145	Drip	Conv
13/03/2018	C	CS03	35/9/2/103/2	28°09'27" N	15°39'11" W	50	Drip	Conv
13/03/2018	C	CS04	35/6/6/10/1	28°08'01" N	15°31'14" W	180	Drip	Conv
13/03/2018	C	CS05	35/6/8/272/1	28°08'24" N	15°31'03" W	135	Drip	Conv
13/03/2018	C	CS06	35/6/8/455/3	28°08'10" N	15°31'23" W	135	Drip	Conv
13/03/2018	C	CS07	35/9/1/244/1	28°09'11" N	15°39'58" W	17	Drip	Conv
13/03/2018	C	CS08	35/6/7/312/1	28°08'14" N	15°31'36" W	135	Drip	Conv
13/03/2018	C	CS09	35/9/1/260/4	28°08'49" N	15°40'07" W	32	Drip	Conv
13/03/2018	C	CS10	35/9/1/288/1	28°09'03" N	15°39'52" W	40	Drip	Conv

* G=La Gomera, P=La Palma, T=Tenerife, C=Gran Canaria. **P. Int=Integrated production, ECO=Ecological crop, Conv=Conventional crop, U=unknown.

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Figure 24. Soil samples for incubation with *G. mellonella* larvae.

After 15 days incubation, insects recovered from soil were surface sterilized with 4% sodium hypochlorite for one min, rinsed in SDW (5 minutes each time), dried on sterile filter paper and placed in moist chambers for a week, at 25°C in the dark (5 larvae per plate), to allow growth and sporulation of colonising fungi. Larvae were then plated on corn meal agar (CMA) supplemented with 50 µg/mL penicillin, 50 µg/mL streptomycin, 50 µg/mL rose Bengal and 1 mg/mL Triton X-100. Fungi present on the larvae were isolated on CMA petri dishes (Fig. 25).

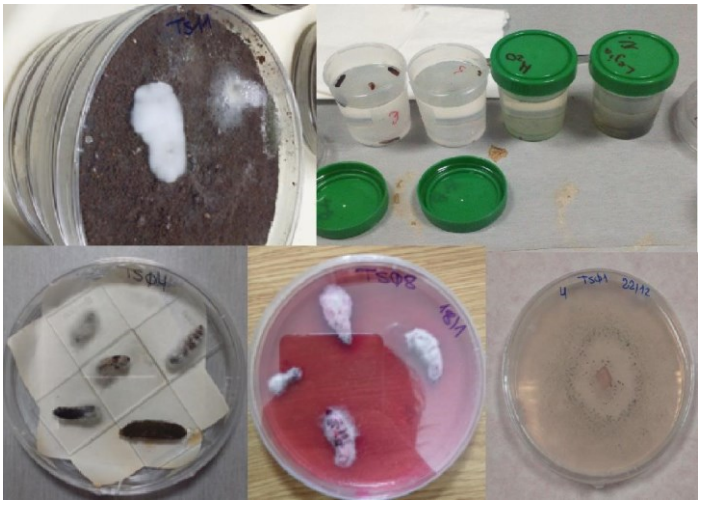

Figure 25. EF isolation from *G. mellonella* larvae.

Isolation and identification of EF

Of the 43 soil samples collected, 19 (11 from Tenerife, 3 from La Gomera and 5 from La Palma) were processed for EF isolation. EF were detected in 5 of the 19 samples processed (26.3%). All strains were isolated from soils of Tenerife (Table 8), from fields under Integrated Production certification, mostly with drip irrigation and altitude > 100 m.

UA team is currently identifying fungi isolated from the remaining banana crop soils. Recently five new samples arrived to the laboratory from Tenerife in which two new *Metarhizium* spp. strains were detected and are studied to confirm their identity. UA team is considering asking for a 2 month extension of WP2 to be able to complete this WP with a larger number of EF strains.

After cross check taxonomic data (morphologic and molecular) a species name was assigned to the EF isolates, that were submitted to the Spanish Collection of Type Cultures (CECT) for deposit, with an assigned, specific code number (Table 9).

Table 8. Entomopathogenic fungi isolated from banana crop soils in the Canary Islands.

Soil Code	Fungi	Island	Data	Altitude (m)	Irrigation	Certification
TS01	*Lecanicillium sp.*	Tenerife	20/07/2017	215	Drip	Integrated P.
TS04	*Metarhizium sp.*	Tenerife	09/08/2017	115	Drip	Integrated P.
TS04	*Beauveria sp.*	Tenerife	09/08/2017	115	Drip	Integrated P.
TS05	*Lecanicillium sp.*	Tenerife	09/08/2017	150	Drip	Integrated P.
TS08	*Lecanicillium sp.*	Tenerife	20/09/2017	135	Drip	Integrated P.
TS11	*Beauveria sp.*	Tenerife	20/09/2017	75	Sprinkler	Integrated P.

Table 9. Deposit of EF strains in CECT culture collection.

Species	*Lab code*	*CECT number*
Beauveria bassiana	B.b.1TS11	CECT 21121
Beauveria bassiana	B.b.19TS04	CECT 21122
Akanthomyces lecanii	L.l.2TS05	CECT 21123
Akanthomyces lecanii	L.l.5TS08	CECT 21124
Akanthomyces lecanii	L.l.6TS01	CECT 21125
Metarizhium anisopliae	M.a.4TS04	CECT 21126

Total strains isolated were 143 from banana soils of Canary Islands, of which 69 (48.3%) were identified, including six (8.7%) described as *bona fide* EF: *Metarhizium*, *Beauveria* and *Lecanicillium* spp. The most common fungi belonged to *Fusarium* (62.3%), followed by *Penicillium* (11.6%). The most frequent EF genus isolated was *Lecanicillium* sp. (4.3%), followed by *Beauveria* sp. (2.9%) and *Metarhizium* sp. (1.4%).

Eleven soil samples (61.1%) were processed from Tenerife, and 264 larvae of *G. mellonella* as EF baits were examined. Isolates obtained from Tenerife Island were: 29 *Fusarium* sp., 4 *Penicillium* sp., 2 *Cladosporium* sp., 2 *Mortierella* sp., 3 *Lecanicillium* sp., 1 *Metarhizium* sp. and 2 *Beauveria* sp. (Fig.26). The sample TS04 (banana plantation under integrated production) from Tenerife generated isolates of EF *Metarhizium* sp. and *Beauveria* sp. On the island of Tenerife almost 14% of the fungal isolates were EF, and it was the only island where EF were detected (Table 8). From La Gomera, La Palma and Gran Canaria soil samples no EF could be isolated.

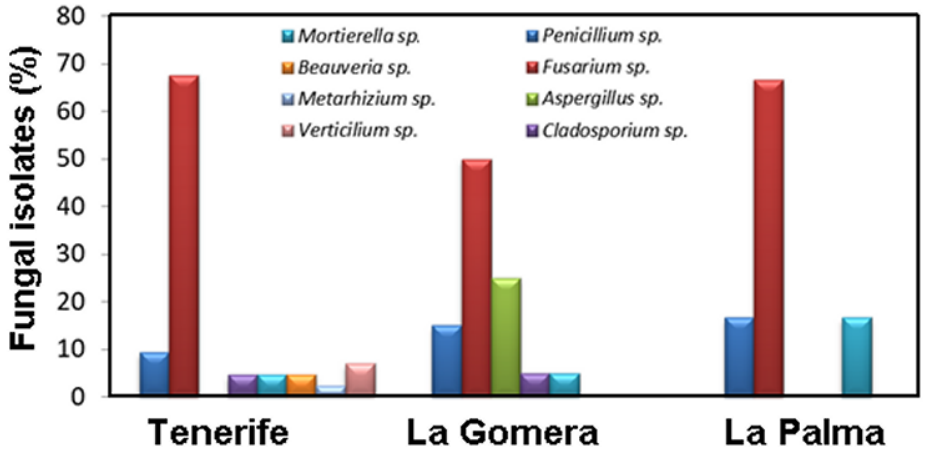

Figure 26. Fungal strains (%) isolated from La Gomera (n = 20), La Palma (n = 6) and Tenerife (n = 43).

Morphological identification of EF isolates

Fifteen-day-old colonies from fungal isolates were used to prepare microscopic slides for identification of fungal structures with light microscopy. Micro-cultures were also performed from colonies by inoculating isolates in 1×1 cm fragments of CMA on sterile slides, covered with a coverslip and kept in moist chambers to favour formation of reproductive structures easy to identify using a microscope. In this way, isolates were determined up to genus level, using general taxonomic references.

Fungal preservation

EF Isolates were preserved in filter papers following the method described below. 21-day-old colonies of EF strains on CMA were used as inoculum. A plug (5mm) of EF fresh colony was set in the middle of a CMA petri dish. Then, 5-10 pieces of sterile filter paper (5×5 mm) were placed around the initial inoculum plug. The dishes were then sealed and incubated at 25 ° C in the dark to favour fungal growth.

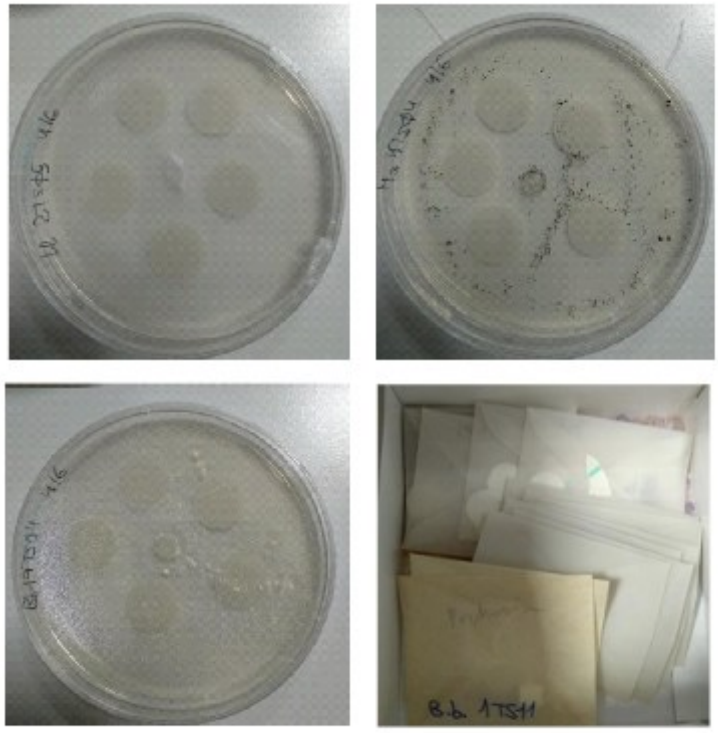

Figure 27. Preservation of EF isolates. Top and bottom left: EF strains growing on sterile filter paper. Right bottom: envelopes containing filter paper fragments after fungal growth.

The EF colonized the paper fragments for 20-25 days, that were then harvested axenically and kept in sterile Petri dishes in a desiccator with silica gel for 3-4 days. When filter papers were dry (they produced sound when shaken), they were axenically placed in sterilized envelopes, sealed and labelled with the name of the fungal strain and the code of the soil sampling location, and stored at -20 ° C with silica gel (Fig.27).

Molecular identification of EF

For EF identification, a microscopic methods (microculture) can be followed, which allows identification by studying the reproductive structures (conidiophores and conidia or chlamydospores). Besides morphological identification, DNA of the ITS region (internal transcribed spacers) of EF isolates was amplified and sequenced with standard protocols.

For PCR amplification of the ITS region and sequencing these primers were used Gel electrophoresis of PCR products showed single bands of ca. 500 bp (Fig.28). The bands were excised and the DNA was purified for sequencing, for subsequent identification by comparison with sequences available in NCBI, applying coverage and similarity rates of 100% (Table 10).

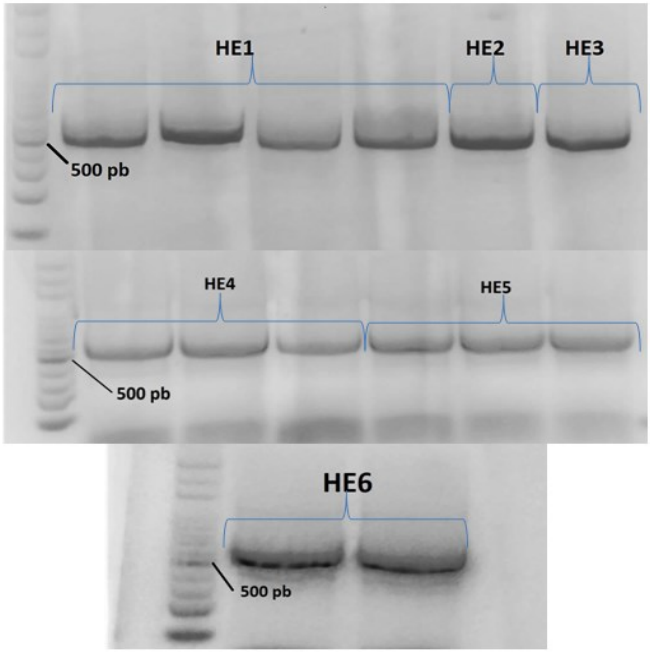

Figure 28. Electrophoretic gels of the ITS PCR amplicons of EF isolated from soil samples from Tenerife . HE1 = *B. bassiana* 1TS11, HE2 = *B. bassiana* 19TS04, HE3 = *M. anisopliae* 4TS04, HE4 = *L. lecanii* 5TS08, HE5 = *L. lecanii* 2TS05 and HE6 = *L. lecanii* 6TS01.

Sequence analysis using NCBI BLAST

Sequences obtained with the ITS primers were aligned to obtain consensus ITS regions of the fungal strains (product size 500-600 bp). Consensus sequences were compared by homology with other sequences in the NCBI database (GeneBank) using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Matching sequences with homology values higher than 95% were used to assign the associated species checked in the NCBI database. The results were crosschecked with identifications obtained microscopically.

Table 10. Analysis of ITS sequence homology of EF strains obtained from banana soils compared with NCBI closest accessions.

Sample code	BLASTn	Coverage (%)	Identity (%)	Access 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*

Phylogenetic analysis of sequences

Phylogenetical analyses were performed to confirm molecular identifications of EF strains, with retrived NCBI sequences, compared by the Neighbor join method with more than 1000 comparison using MEGA-X software. Finally a consensus phylogenetic tree was obtained including all the sequences of EF isolates (Fig. 29).

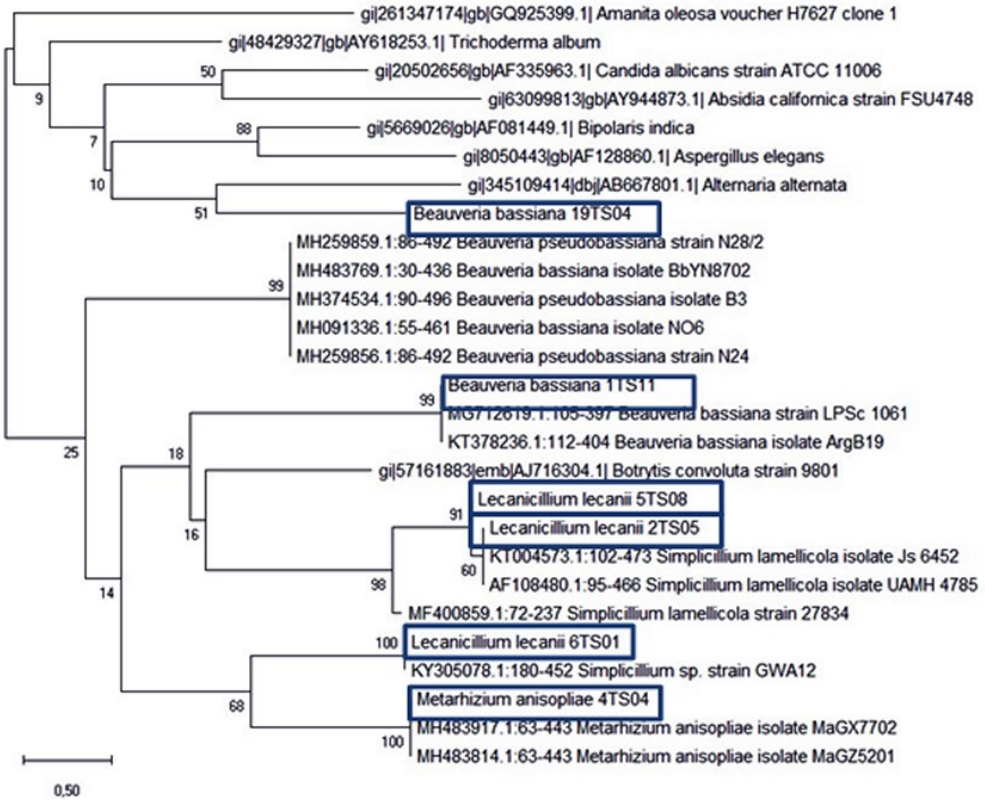

Figure 29. Phylogenetic tree of ITS sequences from the EF isolated (blue boxes) compared with other fungal ITS sequences from NCBI. Bootstrap values are shown on the nodes (1000 replicates). The tree is drawn to scale and the branch length represents the evolutionary distance calculated using the Maximum Composite Likelihood method.

Laboratory rearing of Cosmopolites sordidus

An initial population of 400 adults of banana weevil (BW) kindly given by Dr. Ana Piedra-Buena (ICIA, Tenerife, Spain), was used to rear the insects in the UA laboratory. BW were placed in 40×30 cm aerated plastic boxes at 25-30ºC in the dark, fed with fresh pieces of banana corm and pseudostem (Fig. 30), and kept humid by plates containing water. The decomposed BW diet was eliminated from the rearing boxes weekly. Water containers were also filled weekly. Banana fruits and apple slices were also tested as BW diet.

Initially, eggs of BW were set on the surface of the corm and the pseudostem of the banana tree. These were collected weekly and placed on filter paper for incubation and hatching. For this purpose, BW eggs were placed on a sterile filter paper sandwich

moistened with SDW. Paper sandwiches with BW eggs were incubated in Petri dishes at 25-30 ° C in the dark for at least 4 days. Petri dishes were then checked to detect L1 larvae of BW. Larvae were placed in holes of banana pseudostem fragments to avoid exposure to the environment. BW eggs hatching was scored weekly, using a fine brush moistened to handle the eggs and early larval stages, to avoid damage.

For insect maintenance, corm and pseudostem pieces were checked for BW adults, pupae, larvae and eggs every 20-30 days. BW larvae and pupae were placed in new breeding boxes with fresh banana corm and water to continue their development. BW Adults (ca. 50 individuals) were placed per rearing box with fresh diet and water. Before placing fresh diet, the BW rearing boxes were rinsed with 4% commercial sodium hypochlorite and then with 96% ethanol. Water containers were also cleaned and filled with fresh water.

Figure 30. BW laboratory rearing.

In the trials of BW rearing with whole banana fruits (including peel), rapid decomposition was found and no egg laying was observed. When we used apple slices BW did not use them as food source. Only banana corm allowed BW to complete its biological cycle (from egg to adult, Fig.31). Therefore, the corm and pseudostem were chosen as a diet for rearing. BW egg hatching on filter paper was carried out for 3 months (January-March, 2018). Egg hatching was 18% (19 L1 / 105 H). The L1 larvae did not survive handling when placed in fresh corm pieces and banana pseudostem.

UA team obtained new BW adults from eggs deposited in pieces of corm or pseudostem of banana tree by the original BW adult (Table 11). Viable larvae were only obtained when large pieces of corm were placed in the rearing boxes. Therefore, BW laboratory rearing is slow and requires large amounts of fresh banana material.

Table 11. Data on *Cosmopolites sordidus* laboratory rearing (January-March, 2018).

Date	Eggs	Larvae (L2-L6)	Pupae	Adults
10-jan	-	9	-	-
15-jan	-	5	-	-
23-jan	6	-	-	-
29-jan	55	-	-	-
01-feb	-	-	-	-
05-feb	14	2	1	3
07-feb	-	-	-	1
14-feb	-	-	-	-
22-feb	10	-	-	-
06-mar	-	-	-	-
12-mar	20	69	1	1
TOTAL	**105**	**85**	**2**	**5**

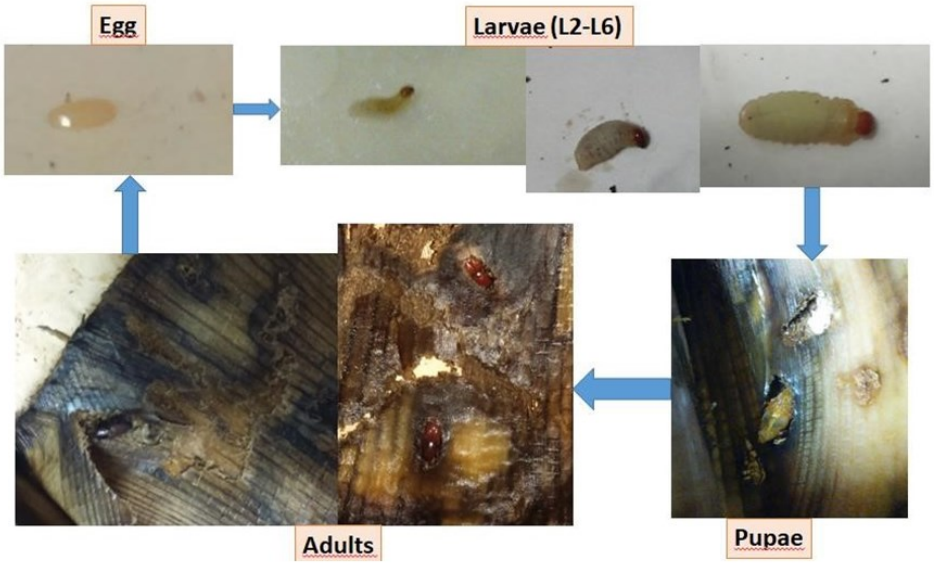

Figure 31. Stages of the banana weevil *C. sordidus*, reared under our laboratory conditions.

Pathogenicity assays

Six strains of EF isolated from Canary Islands were used for pathogenicity bioassays to check virulence: 2 *B. bassiana*, 1 *M. anisopliae* and 3 *L. lecanii.* EF pathogenicity was evaluated by testing survival of *G. mellonella* larvae inoculated with a given strain of EF under lab conditions. Further strains from the Fungal Collection of Plant pathology Laboratory at University of Alicante were also used (Table 12), to compare the virulence of isolates from Tenerife soils with that of EF from other areas.

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Table 12. EF from Plant pathology Laboratory of the University of Alicante Fungal Collection.

Isolate	Host	Origin
Beauveria bassiana 203	*Rhynchophorus ferrugineus,* red palm weevil (Coleoptera)	Daimés (Elche, SE Spain)
Beauveria bassiana 53	*Rhizotrogus chevrolati* (Coleoptera)	Setúbal (Portugal)
Beauveria bassiana 119	*Langia sp.* (Coleoptera)	Orihuela (Alicante, SE Spain)
Metarhizium anisopliae 46	*Otiorhynchus sulcatus* (Coleoptera)	-
Lecanicillium cf. psaliotae	*Phoenicococcus marlatti* (red palm scale)	Elche (Alicante, SE Spain)
Lecanicillium lecanii 131	*G. mellonella*	Alicante (SE Spain)

Living larvae of *G. mellonella* were inoculated with the above described isolates placing five larvae (L3-L4) in 9 cm dishes with a 24-day colony of each EF isolate grown in CMA. Insects were kept on the plate for 5 min, with regular shaking to favour contact with the fungus. For controls, plates with non-inoculated CMA medium were used. Pathogenicity assays were carried out in duplicate. Insects were then placed in 9 cm Petri in a moist chamber to determine the time in which the fungus kills the larvae by evaluating mortality every 12 hours (Fig. 32). A similar experiment with no added moisture (environmental humidity) was also carried out and repeated once.

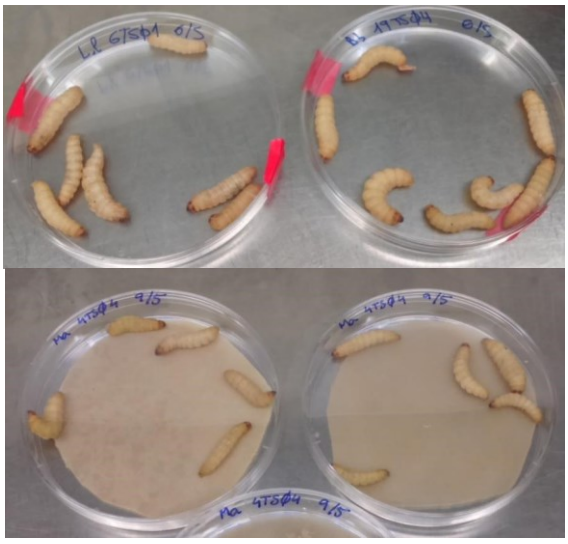

Figure 32. Larvae of *G. mellonella* inoculated with EF isolates

Pathogenicity bioassays were performed by placing in contact *G. mellonella* larvae with a 15-day-old colony of EF grown in CMA. UA team also tested pathogenicity of EF isolates by dipping larvae on spore suspensions in either SDW or 0.05% Tween 20 in SDW (10E+6 spores/ml). However, this method was discarded because larvae were still alive after 20 days incubation (just like mock-inoculated controls), likely because their cuticle waxes repel aqueous spore suspensions. For this reason a pathogenicity bioassays was finally performed by placing larvae in contact with a colony of EF sporulated on a 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 *B. bassiana* 1TS11 from Tenerife, followed by *B. bassiana* 19TS04 and *M. anisopliae* 4TS04, 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. 33 and 34A, C). In the second replicate of this test the same virulence was observed. In the experiments at environmental humidity, the results were similar to the moist chamber ones, with a delay to achieve 100% mortality but with the same pathogenic characteristics of the strains (Fig. 34B, D). This suggests that EF strains from banana fields are active under environmental stress (low humidiy).

Figure 33. Moist chambers of *G. mellonella* larvae after exposure with entomopathogenic fungi isolated from Tenerife soils, *B. bassiana* 19TS04 (left) and *M. anisopliae* (right).

Moist chambers tests data showed that *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) was significantly less pathogenic, with 1.821 and 1.367 estimated survival mean times, respectively, indicating that 50% of individuals were dead at those times. *M. anisopliae* 4TS04 having a lower inoculum (2.13E+05 ± 1.96E+05) than *B. bassiana* 19TS04, had no effect on the cumulative survival rate (no significant differences). Finally, *L. lecanii* 2TS05 and *L. lecanii* 5TS08 treated larvae did not differ from the uninoculated ones, indicating a lower pathogenicity of both isolates to *G. mellonella* (Table 13).

Table 13. Number of spores adhered to *G. mellonella* larvae used in the virulence tests in moist chambers and under environmental humidity and the estimated survival mean time (time in days when 50% of the larvae are dead) in each incubation (with and without humidity) condition.

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	10000	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

UA also performed a pathogenicity test on *Cosmopolites sordidus* larvae (Fig. 35) using larvae of sizes comprised between 0.5 and 2 cm (included several larval stages), that were very sensitive to handling and dryness. Larvae were placed in Petri dishes with moist filter paper with two EF isolates from banana soils. The larval mortality was very high in the first hours, even for control larvae (exposed to uninoculated CMA). Even so, *Metarhizium* sp. 4TS04 was slightly, although not significantly, more virulent than *Beauveria* sp. 19TS04 with 87% and 69% mortality after 1.5 days, respectively. In the control, the mortality at 1.5 days was 60% (Fig. 35D). Both EF achieved full mortality (100%) at 2.5 days while controls achieved this level at 6 days.

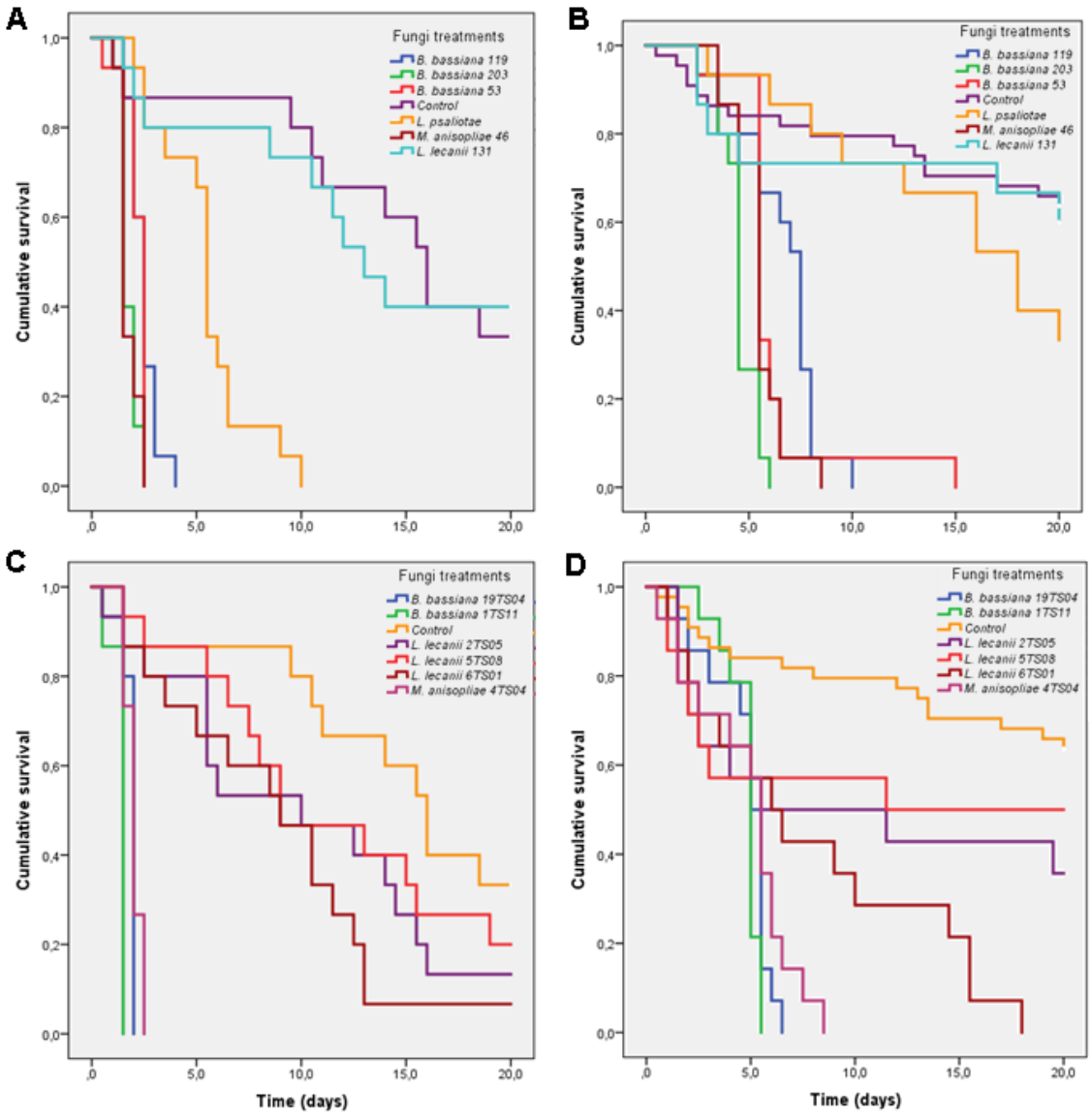

Figure 34. Pathogenicity bioassay of EF on *Galleria mellonella* under incubation on moist chamber conditions, for UA (A) and MUSA (C) isolates, and under incubation without extra humidity (environmental moisture), for UA (B) and MUSA (D) isolates.

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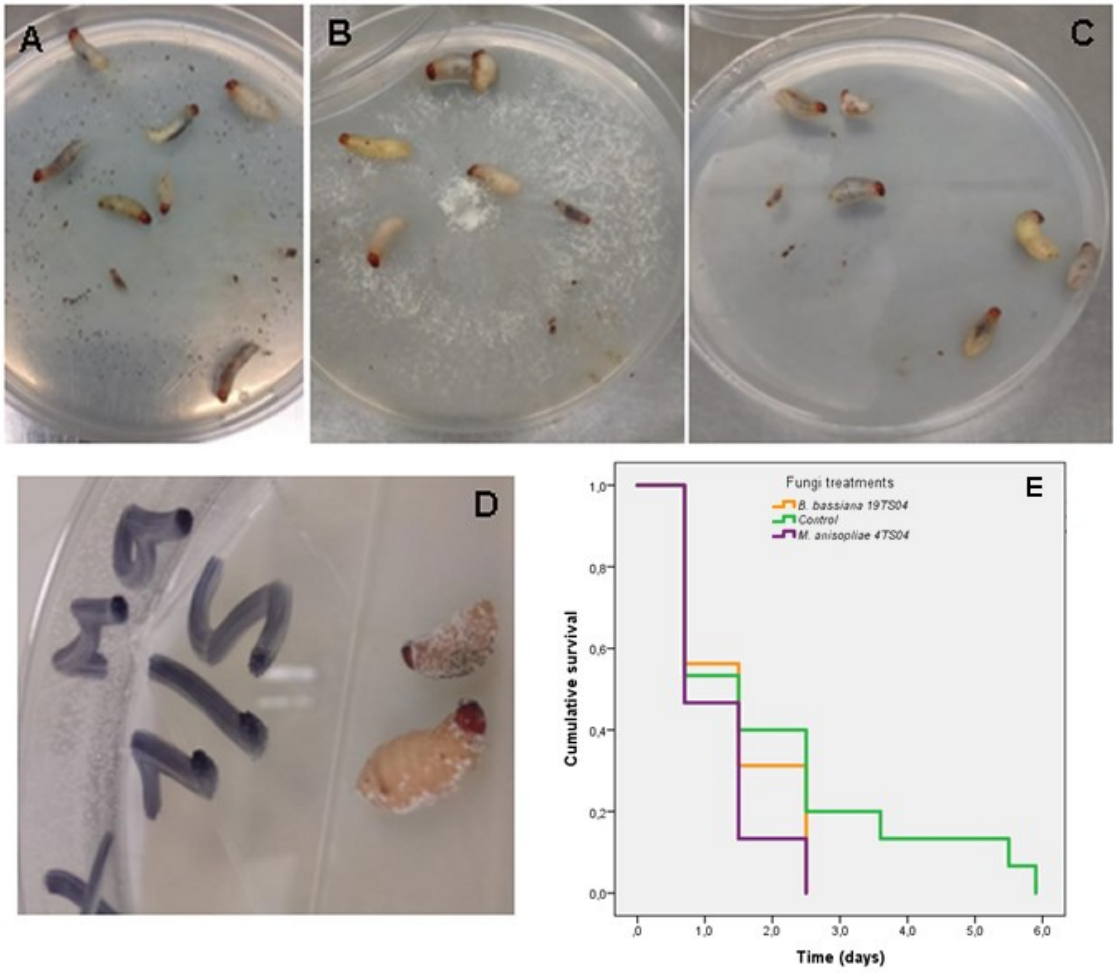

Figure 35. Larvae of *C. sordidus* exposed to EF from Tenerife: *M. anisopliae* (A), *B. bassiana* (B), control (uninoculated, C) and inoculated with *M. anisopliae* 4TS04 (D, note fungal sporulation on the insects). Pathogenicity test of isolated EF on *C. sordidus* larvae under moist chamber conditions (E).

Entomopathogenic nematodes

Lines of entomopathogenic nematodes (EPN) (Fig. 36) were obtained at **CENSA** by the baiting technique, using healthy late instar larvae of the greater wax moth *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), obtained from a laboratory culture. The EPN were maintained using cycles of infection of *G. mellonella* larvae. The juveniles were kept in clear nylon envelopes using sponges at 22ºC under laboratory conditions, and identified by a code (Fig. 37).

The *G. mellonella* cadavers from all the isolates were dark brown, suggesting the presence of the genus *Heterorhabditis*. From the second cycle of infection, some *G. mellonella* cadavers were dissected under a stereomicroscope on the fourth day and the hermaphrodite adults were observed under a microsope, corroborating their identity as *Heterorhabditis*.

Figure 36. Mass rearing process of *Galleria mellonella* using local facilities at Nematology Lab (CENSA). The late instar larva for NEP studies must be healthy and with at least 200 mg of weight.

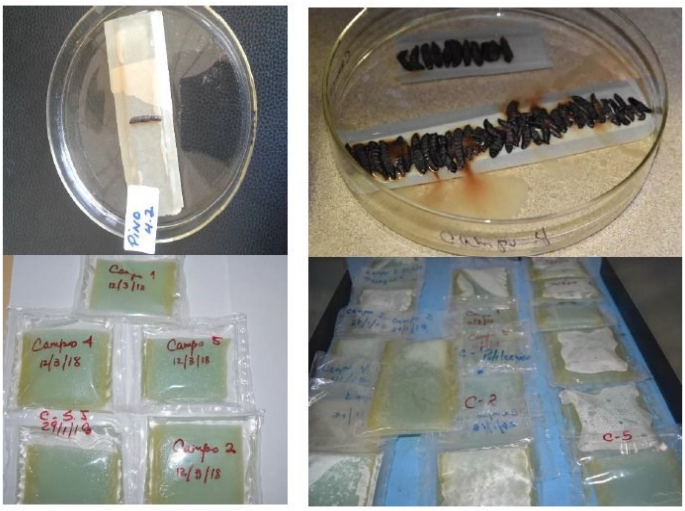

Figure 37. Infective juveniles emerging from *G. mellonella* cadavers and isolates maintained in clear polyethylene bags with sponges, under laboratory conditions at CENSA .

For *in vitro* pathogenicity assays of HC1 strain of *H. amazonensis* against BW adults (1st trial) two types of traps were evaluated to obtain BW adults. The traps used at the farm Los González were the Sandwich trap and the disc trap. The disc trap showed best results, and was recommended for BW collection (Fig. 38).

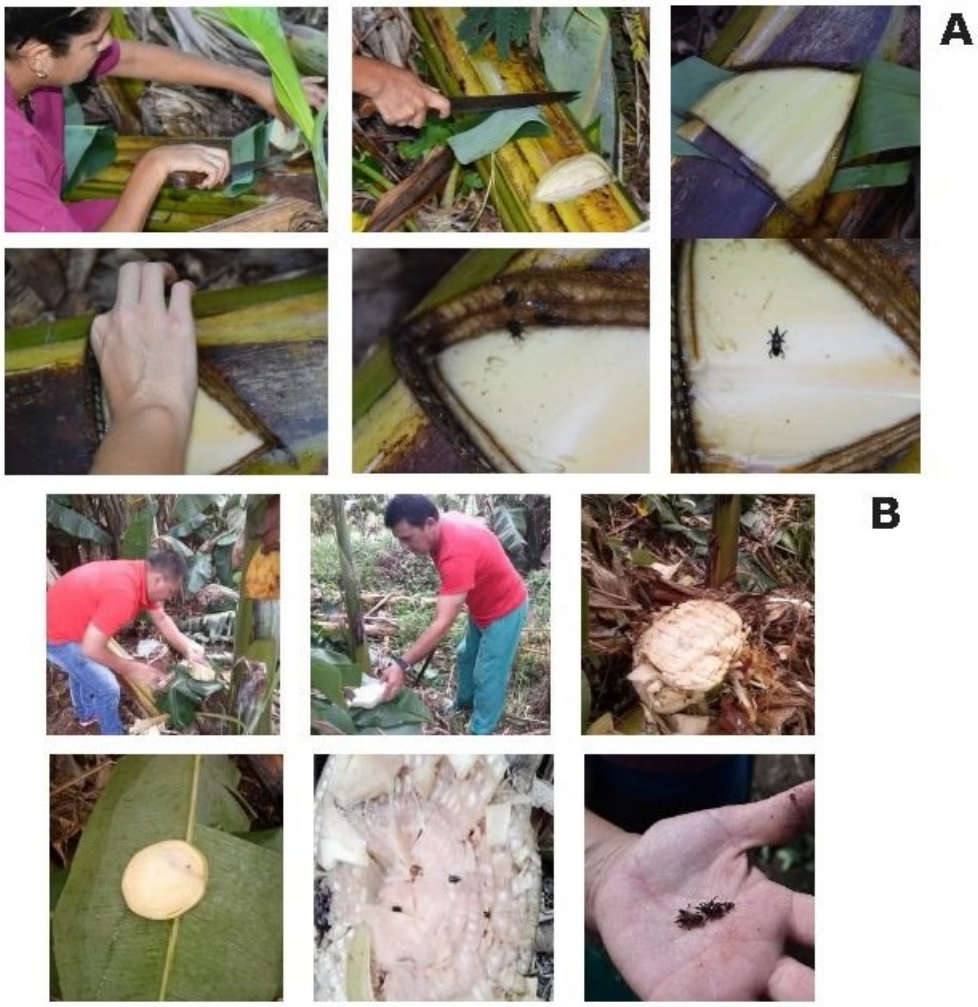

Figure 38. Sandwich trap to collect BW adults for laboratory test (A) and disc trap (according to the methodology by Lester Pupiro, Antonio de Valdivieso University, Nicaragua).

BW adults with similar weight and size, taken from a quarantine cabinet (Fig. 39), were used in EPNs trial at the Nematology Lab. In Petri dishes, five BW adults per plate were applied with five different EPNs doses (125, 250, 500, 2500, 5000 infective juveniles / ml, *H. amazonensis* strain HC1), with distilled water as a control. Three plates were used per treatment. Disinfected small pieces of banana pseudo stem were placed in each plate for insect feeding. The plates were sealed with Parafilm and kept in the incubator at 27°C. The BW mortality was daily evaluated from 12 hours to 21 days. The dead BWs were placed (individually) in a Petri dish and dissected after 4-5 days, and the hermaphrodite adults were observed. The symptoms of the affected BWs were described. A mortality around 86.7 % was obtained with the highest EPN dose, after 21 days, statistically higher ($R^2 = 0.78$) when compared to control.

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Figure 39. Cabinet produced at CENSA with local materials for BW adult quarantine assays.

IITA developed a number of pot and field assays to check the ability of a number of available isolates in management of BW and/or PPN, as follows.
Antagonistic effect of endophytic bacteria.
In vitro assays to test potential of newly isolated banana endophytes to control Foc race 1. Isolates of endophytic bacteria were tested using the dual culture method in the laboratory. Preliminary results from testing of 100 pure potential endophytic bacteria identified 11 isolates that significantly reduced growth of Foc race 1 under *in vitro* conditions. This is an ongoing activity and testing of the remaining 270 isolates from Tanzania and all isolates from Uganda is in progress.

Testing efficacy of previously isolated fungal isolates for managing nematodes and weevils.
These studies are underway in Uganda, using two fungal isolates: non-pathogenic *Fusarium oxysporum* isolate V5w2 and *Beauveria bassiana* isolate GA. The *F. oxysporum* isolate was originally isolated from roots of healthy banana plants in Uganda and preserved in sterile glycerol in Eppendorf tubes at -80°C at the Department of Plant Pathology, Stellenbosch University, Stellenbosch, South Africa. While the *B. bassiana* isolate was originally isolated from a mycosed banana weevil (*Cosmopolites sordidus*) in Uganda, freeze-dried and preserved at the culture collection unit of CABI, UK. Both fungal isolates were provided as actively growing cultures on agar plates from the respective institutes following correct procedures for importation. Cultures were then multiplied on rice grains in Uganda for experimentatal purposes.

Pot experiments. A pot experiment was set up using *in vitro* cooking banana plantlets cv. Mbwazirume (AAA-EA). The plantlets were inoculated with the fungal endophytes

Fusarium oxysporum (V5w2) and *Beauveria bassiana* (GA). The fungi were inoculated onto banana plantlets individually and in combination to assess compatibility. The plantlets were later challenged with the burrowing nematode *Radopholus similis* and banana weevil (1 : 1, F:M). Results from this preliminary experiment indicate that inoculation of banana plantlets with the fungal endophytes reduced nematode levels to 198 and 211 nematodes/5 g root weight for *F. oxysporum* and *B. bassiana* inoculated plants respectively, compared to 323 nematodes recovered per 5 g root in the control plants. Furthermore, inoculating banana plantlets with a combined inoculum of both *F. oxysporum* and *B. bassiana* greatly reduced nematode population to 33 nematodes/ 5 g root weight. Colonization of the banana rhizome by the fungal endophytes was estimated at 58% and 47% for *B. bassiana* and *F. oxysporum* respectively with no apparent penalty to plant growth. The average percentage weevil damage to the banana rhizome was estimated at 50%, 40%, 71% and 45% for plants inoculated with *B. bassiana*, a combination of *B. bassiana* + *F. oxysporum*, *F. oxysporum* and the control plants respectively. Fungal colonization of the banana roots was estimated at 37% and 43%, while colonization of the banana rhizome was estimated at 58 % and 47% for *B. bassiana* and *F. oxysporum* respectively.

Following these preliminary results, follow up pot trials were established to confirm preliminary results and further assess efficacy of these two fungal endophytes used in isolation and in combination to target 1) single nematode species, 2) mixed nematode species, 3) mixed nematode and banana weevil and 4) banana weevil infestations. The first experiment, targeting single nematode (*R. similis*) was terminated on December 18th, 2018 and analysis of samples is under way.

Task 2.4 - Host range assessment and plant receptivity assays

Evaluation of selected EBCAs vs PD in pot-grown cv Gran Enana. EBCAs isolated by CNR from banana rhizosphere soil collected in Tenerife, including fungi such as *Trichoderma* and bacteria such as *Streptomyces*, *Pseudomonas* and *Bacillus*, were used to set up a synthetic microbial community (SynCom), tested *in planta* in consecutive experiments against Foc. The SynCom (version 1.0) was constructed with 44 selected beneficial microorganisms derived from banana rhizosphere belonging to genera *Streptomyces*, *Bacillus*, *Pseudomonas* and *Trichoderma* (see Table 3). In experiments I and II, the PD symptoms appeared 14 days post-inoculation (dpi) and increased afterwards. Both versions of SynCom (1.0 and 1.1) resulted fully compatible with (not deleterious to) cv. Gran Enana plants and their inoculation partially reduced PD severity.

Interactions between endophytes and plants. In Gran Enana soil and rhizosphere, the populations of the EBCAs composing the SynCom remained viable and active throughout the experiment, although they idi not regulate the Foc TR4 population density.

A total of 9 *Trichoderma* spp. were selected in Costa Rica by EARTH for evaluation as EBCAs in a greenhouse. The best isolates were compatible with the Cavendish germplasm tested after introduction in banana fields, for preliminary assays against *R. similis* and other PPN.

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Based on results from previous work (Del. 2.2) a bioassays was implemented to compare the four best isolates with available commercial products (Soilset™ and Agromos™) commonly used as plant growth promotors. Results showed that the four endophytes selected in WP2 performed better than the commercial products (Fig. 40).

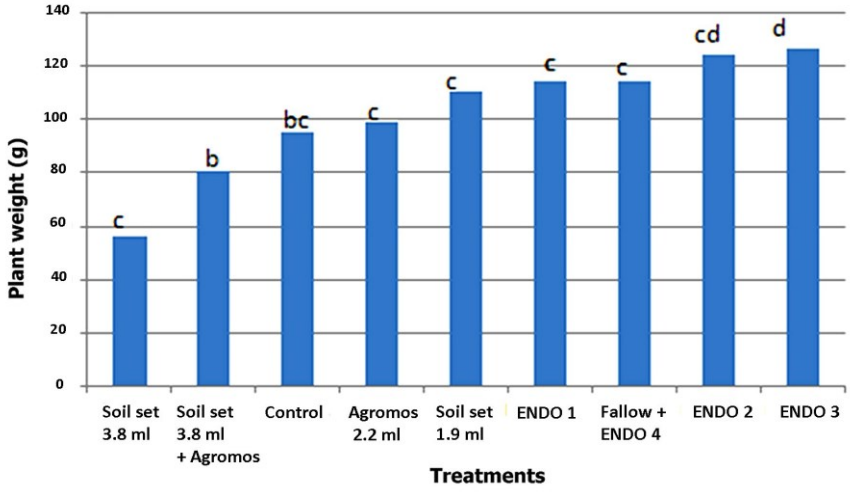

Figure 40. Effect of the endophytes on the total fresh biomass of Cavendish banana plants, in comparison with conventianl growth promoters applied on in Costa Rica.

1.2.3 WP3 - EBCAs biology in plants, pests and pathogens interactions (mths 8-18)

Task 3.1 - Molecular biology of EBCAs.
A gene expression study on Gran Enana plants artificially infected by Foc TR4, with or without additon of *P. chlamydosporia* DSM 26985 is in course at CNR, in a quarantine conditioned greenhouse. Aim is checking if root colonization by *P. chlamydosporia* elicits expression of defense genes such as *PAL 5*, *PIN II*, *PR1* and *LOX* by means of RT-PCR, at different days post-infection. The greenhouse plants have also been used for whole transcriptome analysis. A preliminary trascriptomic study has also been carried out through *in vitro* inoculation of Gran Enana roots with *Pratylenchus goodeyi* specimens extracted from Tenerife soil (50 nem per plantlet), with or without inoculation with *P. chlamydosporia*. Work is in progress.

Task 3.2 - Root biology with endophytic EBCAs in PPN and banana / enset root interactions

Subtask: 3.2.1. Microscopy analysis of the interaction of *P. fluorescens* PICF7 with PPN, and PPN / enset interactions.

Many root-associated *Pseudomonas* spp. strains have long been known to be beneficial to plants either because of their plant-growth promotion effect or their potential as biological control agents. Some of them may also establish endophytically, and have been identified and/or isolated from a wide range of plant species. *Pseudomonas fluorescens* PICF7 has been characterized by CSIC as an effective biological control agent of Verticillium wilt of olive. Moreover, this bacterium is able to colonize and persist on roots of olive (the host from which is was originally isolated), wheat, barley,

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sunflower and *Arabidopsis thaliana*. Evidence of root endophytic colonization has been obtained except for the last two species. Therefore, CSIC stated two independent pilot bioassays in order to check whether a fluorescently-tagged derivative of *P. fluorescens* PICF7 was able to colonize banana roots endophytically. For a first bioassay banana cv. Pequeña Enana plants were uprooted from the original substrate, their roots thoroughly washed in tap water without wounding and dipped for 15 min in a bacterial suspension ($1 \cdot 10^8$ cfu ml^{-1}) of *P. fluorescens* strain PICF7 (pMP4655). For the control treatment, other fifteen plants were treated similarly except that the root systems were dipped in 10 mM $MgSO_4 \cdot 7H_2O$. Plants inoculation in a second trial was performed by adding 15ml of a suspension of bacterial cells ($1 \cdot 10^8$ cfu ml^{-1} in sterile $MgSO_4 \cdot 7H_2O$ 10mM) per pot. For the control treatment, other fifteen plants were treated similarly except that the root systems were drenched with 10 mM $MgSO_4 \cdot 7H_2O$. After inoculation plants were grown in controlled chamber with 28 ± 1 °C, 75–80% RH, 14-h photoperiod of fluorescent light at 175 µmol · m^{-2} s^{-1}. Banana roots were sampled at different sampling times from bacterization. Plants were uprooted avoiding damage and the root systems were washed by dipping in tap water to remove attached sand. Roots from each plant were thoroughly analysed by confocal laser scanning microscopy. Several segments representative of the whole root system were analysed on the confocal microscope over 8 days after bacterial inoculation. Two non-bacterized, control plants were also sampled at the first and eighth days to check for plant tissue autofluorescence, possible fluorescent native bacteria or cross contamination. So far, no consistent microscopy evidence showed that strain PICF7 efficiently colonized banana roots. Further trials will be conducted in order to verify whether strain PICF7 is able to colonize banana roots and check its effects on PPN and related antagonists.

Subtask: 3.2.2 - Rhizosphere competence.

UA devised three systems for analysing interactions of inoculation with *P. chlamydosporia* on banana host plants. The isolate of *P. chlamydosporia* Pc 123 (ATCC n. MYA-4875; CRCT n. 20929) was tested on *in vitro* commercial plants of cv. Pequeña Enana, using 30-day-old *in vitro* plantlets on agar medium or 6-7 week-old in *vitro* derived plants, grown in planting trays, in the greenhouse. The banana-EBCAs interaction schemes devised were the following:

- axenic bioassays in magenta boxes for 1-7 days.
- experiments in 200 ml cups, in growth chambers for up to 30 days
- experiments in 1L/3.5 L pot, in the greenhouse for 2.5 months.

Thirty-day-old *in vitro* banana plantlets (both controls and EBCA inoculated) were planted in cups with sterilized peat moistened with 1/10 Gamborg's nutrient solution, kept in growth chambers at 24ºC and 60% RH for 10, 20 and 30 days. The effect of *P. chlamydosporia* inoculation on banana plantlets was then estimated, recording root, corm and leaf growth parameters. Three treatments to inoculate the fungus were tested (avoiding watering plants the same day after treatments, otherwise these could lose effectiveness).

Treatment 1 involved plant inoculation with *P. chlamydosporia* mycelium, using 7 mm diam. agar cores from the edge of a 21-day-old colony, placing eight cores in a planting hole dug in the cups. Treatment 2 consisted of mycelium inoculation by direct root contact using a 5 days old *P. chlamydosporia* colony. In early tests banana plantlet

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roots were placed in contact with *P. chlamydosporia* colonies on agar medium growing in petri dishes. This method appeared unsuitable as the agar dried-up after five days. As an alternative, colonies for plantlet inoculation in polycarbonate magenta boxes were used with 60 ml of poured CMA per box, autoclaved for 20 min at 121ºC. The CMA magenta were inoculated with *P. chlamydosporia* mycelial cores, and incubated for 15 days at 24ºC. The plantlets were inoculated by axenically placing their roots in contact with the *P. chlamydosporia* colony in a magenta for 5 days, and then planted in polystyrene cups with sterile peat.

Treatment 3 involved banana plantlet inoculation with 10^4 propagules (conidia and chlamydospores) per g of substrate, obtained from fungus cultures grown on pre-hydrated rice grains placed in a 250 ml Erlenmeyer flask, autoclaved 30 min at 121 °C. Autoclaved rice was inoculated by adding 10 ml sterile distilled water with 4 cores from the edge of a 21-day-old *P. chlamydosporia* colony. Flasks with inoculated rice were incubated in the dark at room temperature 3-4 weeks (Fig. 41). Twenty g of *P. chlamydosporia* inoculated rice were then shaken 1 min with 50 ml distilled water in sterile Falcon tubes. The resulting suspension was filtered and the fungus conidia and chlamydospores present in the resulting suspension were counted. Plant inoculation was performed by adding, close to the banana plantlets, a volume of suspension (ca. 500 microliters) to inoculate 10^4 propagules per g of substrate (peat). Controls include sterilized peat in cups and uninoculated plantlets.

Figure 41. Rice grains inoculated with *Pochonia chlamydosporia.*

Ten plantlets per treatment and controls were sampled 10, 20 and 30 days after planting. Root growth parameters scored were maximum root length (cm), fresh root weight (g) and the percent of total and endophytic colonization of the roots, using semi-selective media with antibiotics. Corm growth parameters were corm length (cm) and fresh and dry weight (g). The numbers of leaves per plant were counted, and maximum leaf length (cm) and fresh and dry leaf weight (g) were recorded.

Inoculations for greenhouse experiments. Experiments were carried out in a greenhouse at 24ºC and 60% relative humidity, with 6-7 week old banana plants in 1 to 3.5 L pots. First experiment was performed initially in 1 L pots. The second replicate was set in 3.5 L pots.

The main objective of this part of the MUSA project was to test methods for inoculating *P. chlamydosporia* in banana plants, and verify if the treatments were effective in the long term. Four methods of inoculation have been developed. After two and a half months, parameters of the different parts of the banana plants were recorded, such as root, corm, pseudostem and leaves.

For transplants, it was necessary to fill the 1L/3.5L pots to 80% of their capacity with peat and then moisten it. A hole was then made in the peat and a banana plant was planted in each pot. All plants were watered with an automated irrigation system, for 1 min in the morning delivering 75ml (1L pot) or 150ml (3.5 L pot) per plant, taking care for not watering plants the same day after the treatments were applied, to avoid losing effectiveness.

For 1L pots, treatment 1 involved 4 agar and mycelium cores from the edge of 21-day-old colony of *P. chlamydosporia* for each plant, arranged in the peat with roots in contact with the fungus. Treatment 2, the same as the previous one, was applied using 12 agar cores with *P. chlamydosporia* mycelium for each plant. Treatment 3 involved plant inoculation with 5000 conidia/chlamydospores (5.000 c/c) suspension per g of substrate. The conidia and chlamydospores were obtained by filtering 20 g of inoculated rice into a 50 ml tube with distilled water, then shaked for a minute. Subsequently, the suspension was filtered, counting the conidia with a microscope to ensure the 5000 c/c concentration. Treatment 4 was similar to the previous one, using 10^4 conidia/chlamydospores. Controls included peat in the 1L pot and banana plants only. For 3.5L pots experiments only treatments 3 and 4 were applied, with a single or 3 times monthly inoculations.

Growth chamber experiments. All treatments with *P. chlamydosporia* applied to Pequeña Enana promoted root growth. However, the plantlets showed growth parameters significantly higher than control (uninoculated plants), with a maximal root length achieved when treated with conidial and chlamydospore suspensions (Fig. 42). Similar results were found for the fresh root weight (Fig. 42B).

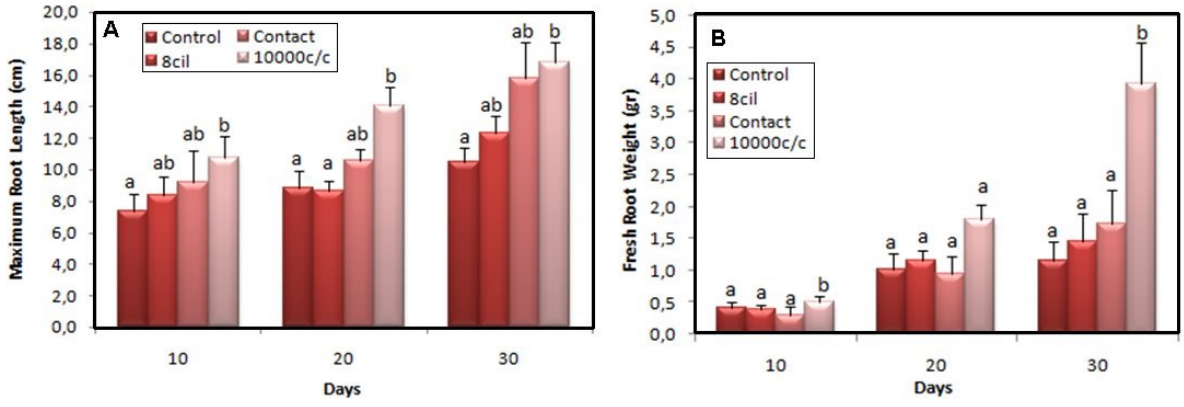

Figure 42. Effect of *P. chlamydosporia* inoculation on root growth of banana plantlets. Inoculation treatments were: 8cil (mycelium cores), fungal colony contact (Magenta box), 10000 c/c (conidia and chlamydospores).

Surface sterilization of banana plantlet roots was initially performed using 0.5% sodium hypochlorite for estimating *P. chlamydosporia* endophytic colonization. This

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concentration of was increased to 1% since 0.5% caused positive *P. chlamydosporia* growth in roots imprints (false positives for endophytic colonization). The fungus colonised endophyticaly banana plantlets roots in all treatments tested (Fig. 43).

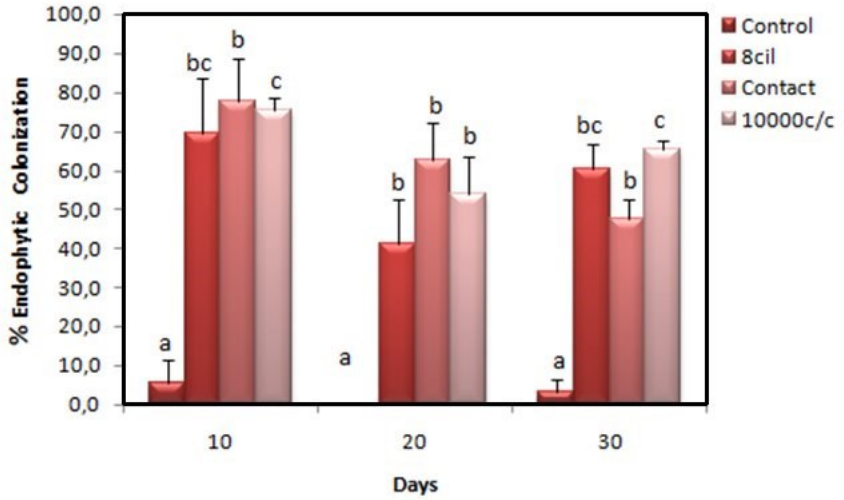

Figure 43. Endophytic root colonization of banana plantlets by *P. chlamydosporia*.

No significant difference between *P. chlamydosporia* treatments and control was found. Treatment 1 (8 agar cores) increased corm length (Fig. 44A). After 20 days (treatment 3) *P. chlamydosporia* significantly increased corm weight of banana plantlets (Fig. 44B).

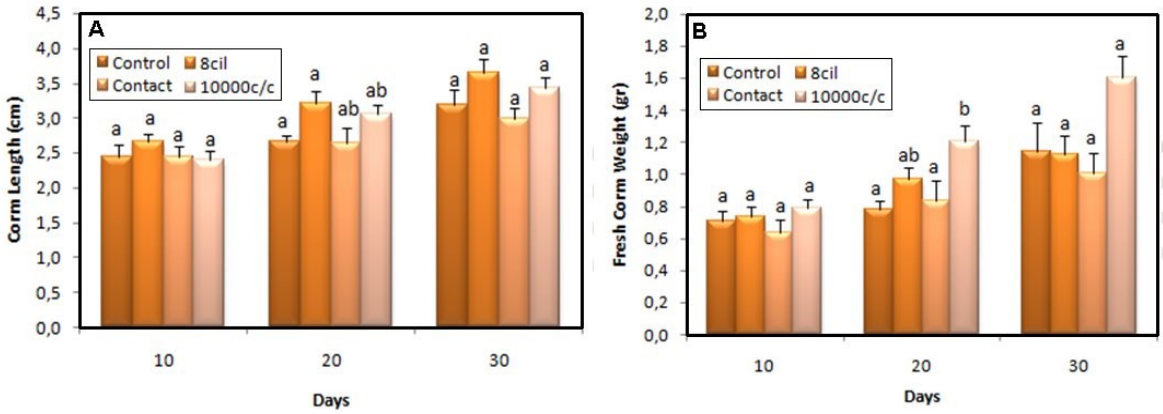

Figure 44. Effect of *P. chlamydosporia* inoculation on plantlets' corm length (A) and fresh weight (B).

Inoculation significantly increased leaf lengths at 10, 20 and 30 days after treatment 3 (inoculation with conidia and chlamydospores) (Fig. 45A) and leaf fresh weight (especially 30 days after treatment 3, Fig. 45B). Similarly, the fungus significantly increased leaf dry weight 20 days after treatment 3 (conidia and chlamydospores) (Fig. 45C). Treatments, however, did not affect significantly the number of leaves per plant, with a slight increase in plants subjected to treatment 3 only (Fig. 45D).

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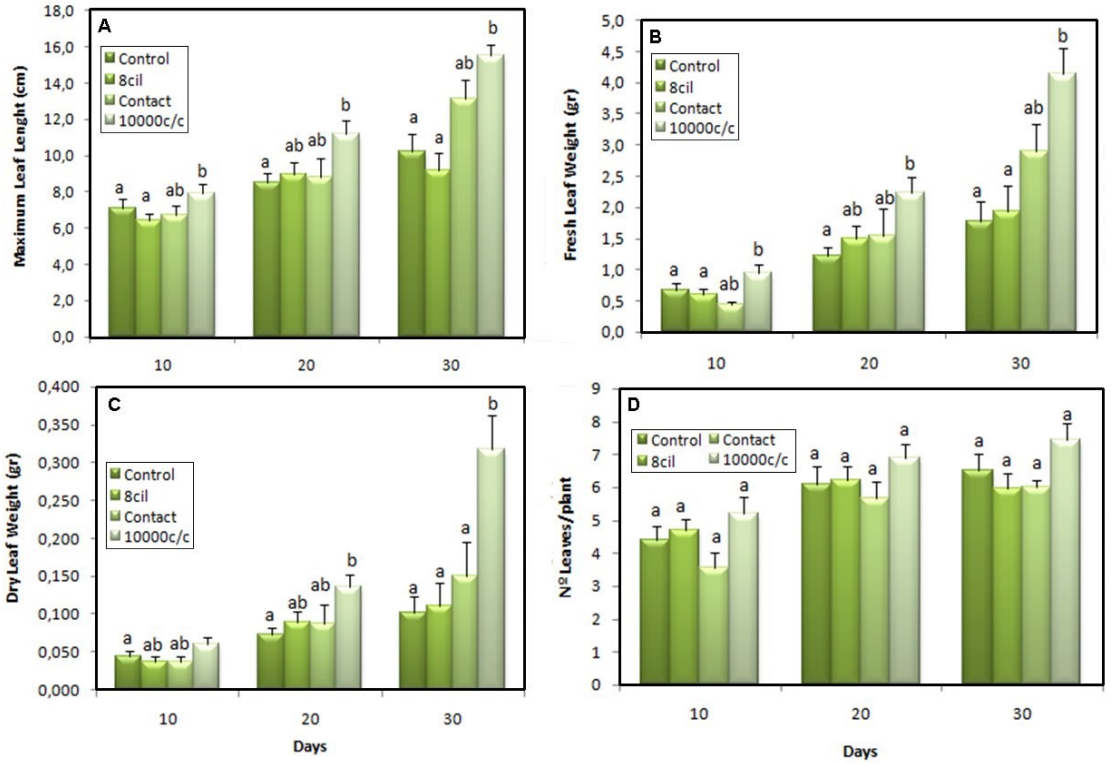

Figure 45. Effect of *P. chlamydosporia* inoculation on leaf growth on banana plantlets max. leaf length (A), leaf fresh weight (B) Leaf dry weight (C) and number of leaves per plant (D).

Greenhouse assays. Greenhouse experiments were started using 1L pots using 20 day-old plants supplied by Cultesa (Tenerife, ES). In this experiments 4 treatments were implemented (inoculation with 4 *P. chlamydosporia* plugs, inoculation with 12 *P. chlamydosporia* plugs, both from a 21-day-old colony on CMA, inoculation with 5,000 and 10,000 c/c). Plants were incubated under greenhouse conditions for 75 days (2.5 months), measuring growth parameters (root, corm, pseudostem and leaves length and weight) at the end of the experiments.

Results indicated that the fresh root weight of the banana plants was reduced when *P. chlamydosporia* mycelium was applied under this conditions. However, inoculation caused no significant differences in root length (Figure 46 A). For maximum leaves length, treatment 3 (5,000 c/c suspension) showed the best effect compared to control. However, no statistical difference was found between treatments and control, although differences were found among treatments (Fig. 46 A).

Treatments with 4 and 12 cylinders of mycelium per plant reduced fresh leaf weight respect to the control, significantly for treatment with 12 plugs (Fig. 46 B). Evaluating corm paramters, a large variability in length among samples was observed (Fig. 46 C). Therefore, no significant difference between fungal treatments and controls was found. Conidia and chlamydospores slightly increased corm weight. In addition, no significant differences between treated plants and control were found for pseudostem length and weight (Fig. 46 C, D). As this effect could be due to the smaller size of the pots used (plants could not develop rhizosphere system during the experiment) a larger pot size (3.5L) was used for subsequent experiments.

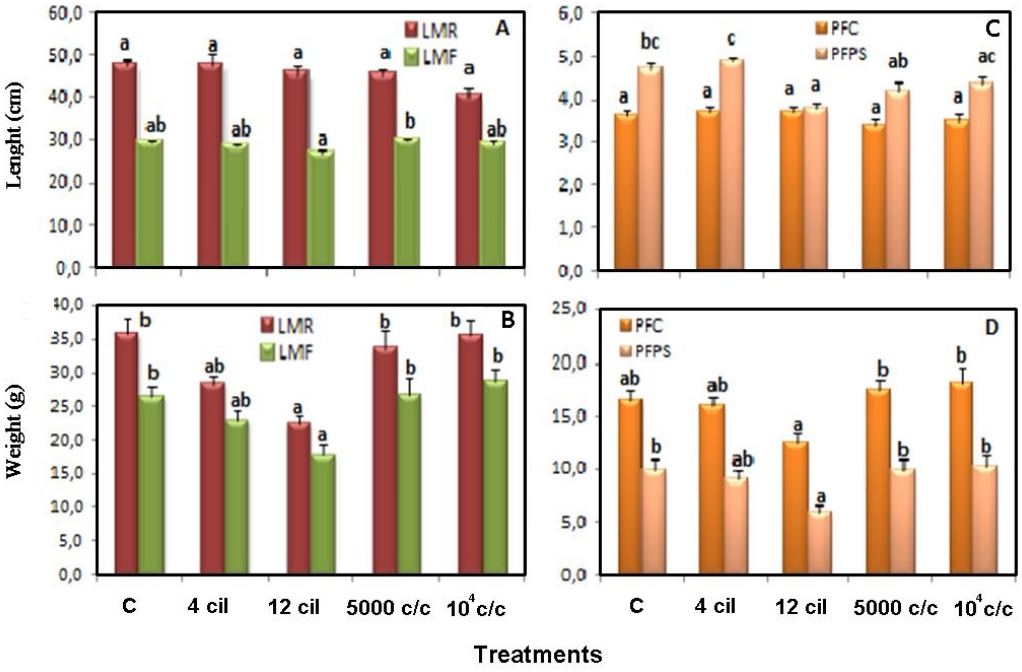

Figure 46. Effect of *P. chlamydosporia* inoculation on root growth of banana plants showing maximum values of: root and leaf length (A), root and leaf weigth (B), corm and pseudostem weigth (C) and length (D). Different letters show significant differences ($p < 0.05$).

Colonization by *P. chlamydosporia* of Pequeña Enana plants was scored under greenhouse conditions with the inoculation methods described above. For colonization, root fragments that showed occurrence of *P. chlamydosporia* were scored. Roots were surface sterilised for endophytic colonization, whereas for total colonization they were only washed with sterile distilled water.

Inoculation with conidia and chlamydospores increased endophytism and total root colonization in comparison to plants inoculated with mycelium (plugs treatments 4 cil, 12 cil; Fig. 47). *Pochonia chlamydosporia* also significanly increased ($p>0.05$) pseudostem length and fresh weight, in treated plants (Fig. 48).

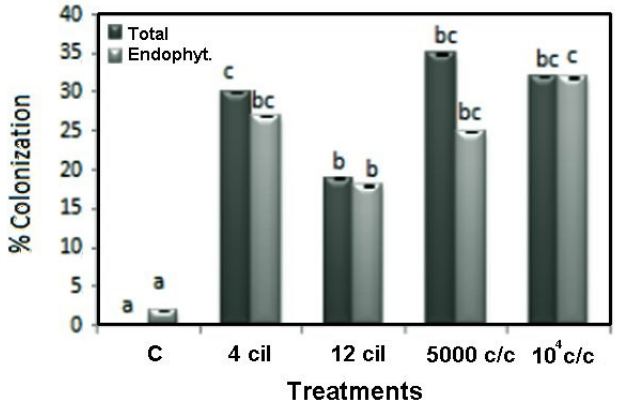

Figure 47. Colonization of Pequeña Enana roots (total and endophytic) by *P. chlamydosporia* in plants maintained in greenhouse conditions (1L pots).

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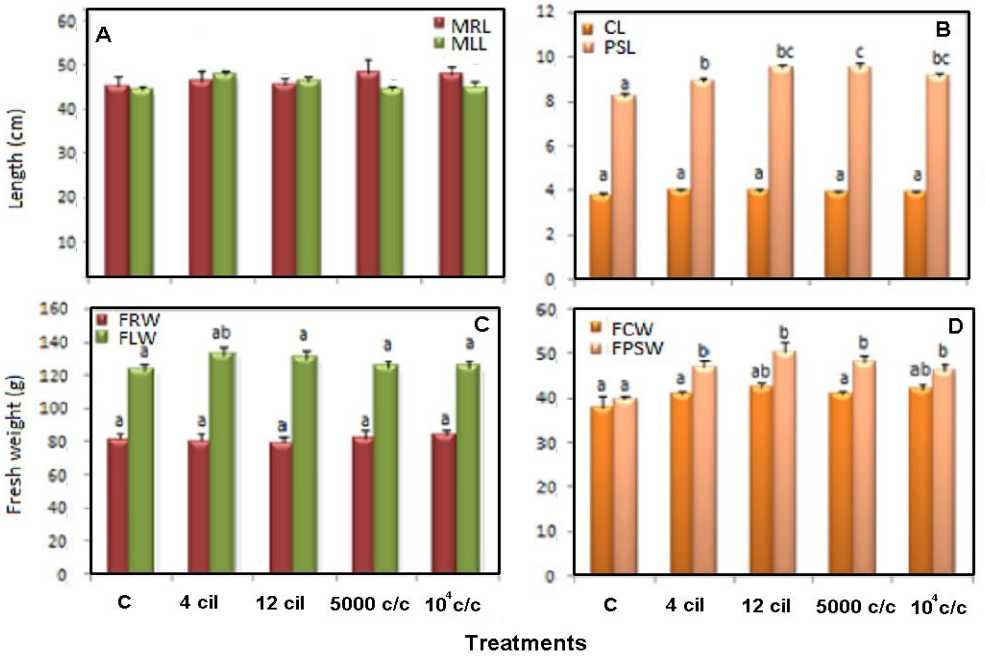

Figure 48. Effect of *P. chlamydosporia* colonization in greenhouse banana plants cv Pequeña Enana. Maximum root length (MRL) and leaves (MLL) lengths, per treatment (A). Corm (CL) and pseudostem (PSL) lengths (B). Fresh root (FRW) and leaves weights (FLW) (C). Fresh corm (FCW) and pseudostem (FPSW) weights (D). Treatments with different letters within the same parameter show significant differences from control ($p \leq 0.05$).

Effect of plant substrate on P. chlamydosporia *germination.* Use of peat as banana plant growth substrate showed (Fig. 49 and 50) that it significantly reduced ($p < 0.05$) germination of *P. chlamydosporia* conidia and chlamydospores. The effect was maintained over time (for at least 48 h). A reduction was observed in both peat extracts sterilized by filtration and not filtered, suggesting an inhibition of germination that was independent from peat-associated microbiota. The active compound(s) present in the peat, inhibitory of *P. chlamydosporia* germination, also appeared heat-resistant (withstanding steam sterilisation).

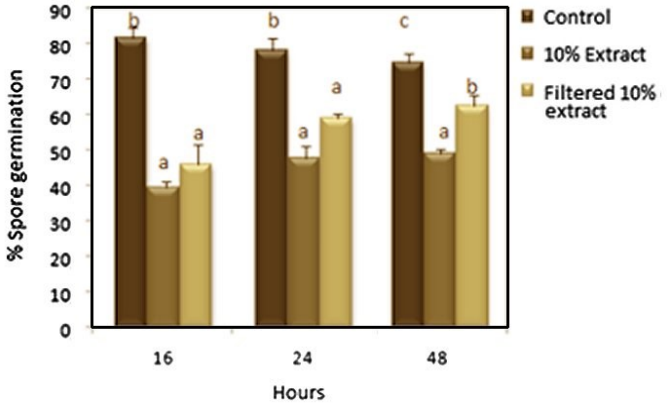

Figure 49. Banana plant growth substrate effect on germination of *P. chlamydosporia* propagules. Filtered 10% = 0.4 µm membrane filter. Treatments with different letters show significant differences from untreated control ($p < 0.05$).

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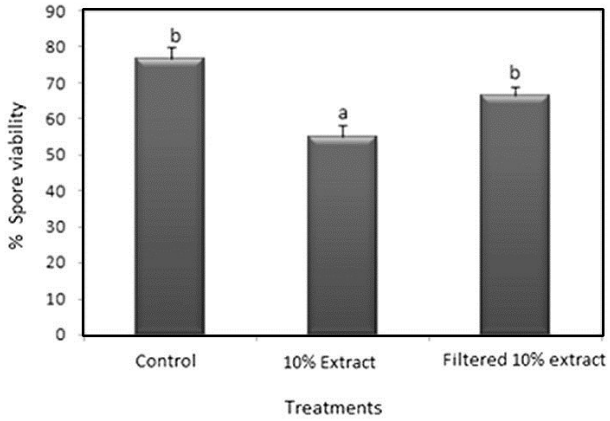

Figure 50. Banana growth substrate effect on *P. chlamydosporia* propagules viability. Filtered 10% = 0.4 µm membrane filter. Treatments with different letters show significant differences from untreated control ($p < 0.05$).

Subtask: 3.2.3 Soil receptivity

An assay is actually under evaluation at UA for the study of soil receptivity to selected *P. chlamydosporia*, based on a soil membrane technique.

Three independent pilot trials were conducted at IAS CSIC under non-gnotobiotic conditions in order to: (i) familiarize and optimize banana cultivation and ii) set up experimental conditions to assess PD symptoms development under controlled conditions. Banana plants (*in vitro* propagated seedlings, cv. Pequeña Enana, susceptible to PD), supplied by Cultesa were first acclimated under controlled growth-chamber conditions (23 ± 1 °C, 75–80% relative humidity, 14-h photoperiod of light) during fifteen days. After that, plants were transplanted to pots each containing *ad hoc* prepared substrate (peat:sand:vermiculite, 75:12.5:12.5, v:v:v) without wounding roots. Then, plants were kept in a controlled growth chamber under the same conditions mentioned above.

In the first experiment (I), 60 (five-month-old) banana plants were carefully transplanted into new pots containing the potting substrate. Then, plants were challenged with three *Fusarium oxysporum* f. sp. *cubense* (Foc) strains from (CAV-050 and CAV-095 [STR4] and CAV-183 [R1 or R2], kindly provided by Prof. Viljoen, and one from Tenerife Island (3G [undetermined race], isolated from a banana plant showing PD symptoms during the first sampling round described above), by adding 150 ml per pot of a conidia suspension of each Foc isolate ($1 \cdot 10^6$ conidial/ml). Non-inoculated plants (control) were watered just with 150 ml of water. After inoculation plants were grown in a controlled growth chamber with 28 ± 1 °C, 75–80% RH, 14-h photoperiod of fluorescent light. Pots were randomly distributed in three blocks (fifteen plants per treatment).

In the second bioassay (II), 60 (6-month-old) banana plants were challenged with three isolates from Prof. Viljoen collection (CAV-050, CAV-095 and CAV-020 [STR4]) and two from Tenerife Island (2A and 3B, undetermined race). Pots were randomly distributed in three blocks (nine plants per treatment).

Finally, a third trial (III) was conducted using 20 (one-month-old) banana plants in order to evaluate whether disease symptoms developed in a similar way to those observed in older plants. Plants were transplanted into new tray pots and before Foc inoculation they were transplanted again into larger pots to be challenged with CAV-050 (STR4).

Banana plants grew well under controlled growth-chamber conditions (Fig. 51). Plants developed well in the three experiments carried out. Forty five days after inoculation, PD symptoms were observed in Foc-inoculated plants, in contrast to non-inoculated plants (Fig. 52 and 53). In experiment I, however, plants inoculated with the R1/R2 strain did not show Fusarium wilt symptoms since 'Pequeña Enana' cultivar is not susceptible to these races (Fig. 52C). Two months after inoculation, disease progress seems to stop, and non-inoculated and inoculated plants showed a very similar aspect. Symptoms of natural senescence were observed and Foc-inoculated plants recovered from the disease. Further pathogenicity tests will be performed in order to test different inoculum densities and inoculation methods.

New pathogenicity tests are under evaluation at CSIC with a different inoculation method, increasing pot size and modifying substrate (peat:sand:vermiculite, 1/3:1/3:1/3, v:v:v), to improve disease development observed in the previous assays (Fig. 54).

Figure 51. Appearance of banana plants one day after inoculation (Experiment I; see text).

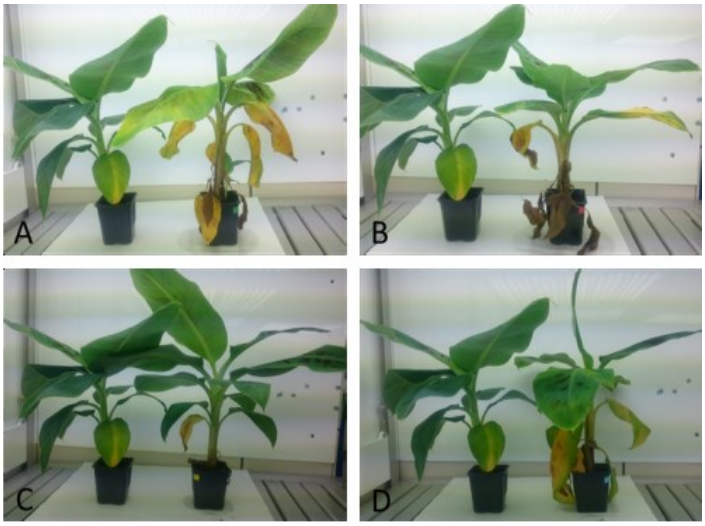

Figure 52. Symptoms observed on banana plants 51 days after Foc inoculation (experiment I). A representative control (non-inoculated) is shown on the left of each panel. (A) Foc isolate from Tenerife island, (B) CAV-095, (C) CAV-183 and (D) CAV-050.

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Figure 53. Banana plants showing first PD symptoms at 46 days after Foc inoculation (Experiment III). Control plants are shown on the left.

Figure 54. Set up of new banana plants pathogenicity tests.

Subtask: 3.2.4 Identification of *P. chlamydosporia* genes involved in plant growth promotion and PPN parasitism.

A new gene prediction of the *P. chlamydosporia* 123 genome was carried out in cooperation beteween UA and UNEXE, allowing the identification of novel genes. Transcriptomic studies on the fungus have been carried out by UA and CNR, and sequencing results are actually being analysed.

Task 3.3 - Bioactive compounds and their interactions with EBCAs antagonistic to BW and PPN.

Regarding the use of bioactive compounds we have agreed to use chitosan as bioactive compound with potential as modulator of EBCAs against BW and PPN. Our research group has extensive experinece on chitosan biological activity on fungi and plants other than banana. In this task, we have implemented a system 2 in which we have included treatments with chitosan irrigation. Banana plants in pots with sterile peat are irrigated during 30d with two concentrations of chitosan (0.1 [T1] and 1 mg ml [T2]) based on results obtained by Lopez-Moya et al., 2017 with tomato, barley and Arabidopsis. After 30 days plants are hasvested and the growth parameters (root, shoot and corm weight and length) measured. In the following graphs we represent our preliminary results obtained using chitosan in the irrigation system of banana plants (Fig. 55).

Results indicate that banana plants are tolerant to chitosan. Banana plants irrigated with a high concentration of chitosan (> 1mg ml) do not show significant differences respect to untreated controls (Fig. 55). Chitosan irrigation at this concentration reduces ca. 50% growth of tomato plants.

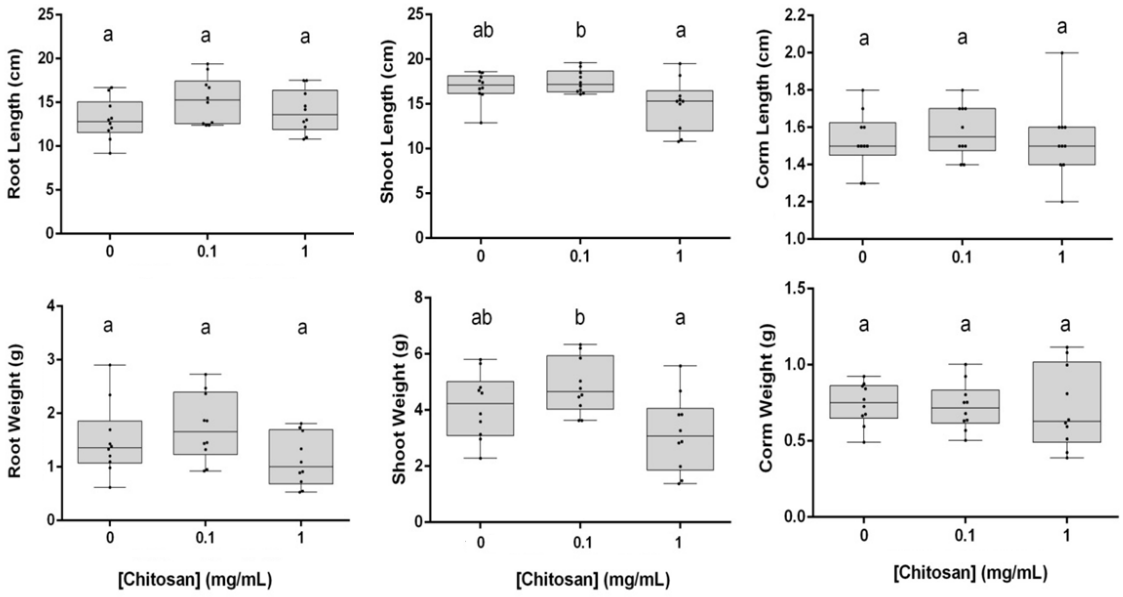

Figure 55. Growth parameter of banana plants irrigated with chitosan in cup experiments (30 days.

UA team is currently designing RNAseq experiments to determine gene expression of Bananna plants exposed treatments with combination of Pc and chitosan in Magenta box system. We want to characterise activation of banana defence genes during early events of Pc root colonisation when it is growing with and without chitosan. We have designed for these experiments the following treatments:

- banana plants in contact with 21 day-old Pc colony on CMA in magenta box 5 days.
- banana plants in contact with 21 day-old Pc colony on CMA amended with chitosan (1mg ml) in magenta box 5 days.
- banana plants in an uninoculated magenta box with CMA for 5 days.
- banana plants in an uninoculated magenta box with CMA amended with chitosan for 5 days.
- magenta box with cellophane of Pc 21 day-old colonies on CMA.
- magenta box with cellophane of Pc 21 day-old colonies on CMA amended with chitosan.

Last two treatments allow extraction of good quality RNA of fungus only RNAseq controls.

UA team is also applying metabolomics to analyse banana plant rhizodeposition. In vitro plants (task 3.2) were placed in SDW overnight in the dark (24ºC, 65% humidity). Rhizodeposition was analysed using fluorescence, NMR and HPLC. A preliminary EEM fluorescence spectrum is shown in Fig. 56.

Fluorecence analysis of banana root exudates generate after PARAFAC principal component analysis two components (C1 and C2). C1 (290-434 nm, 345-434 nm) corresponding to high molecular weight phenolics (fulvic and humic acids type), C2 (230-336nm, 280-336 nm) corresponding to low molecular weight phenolics (amino acids and peptides). NMR and HPLC analyses are in progress.

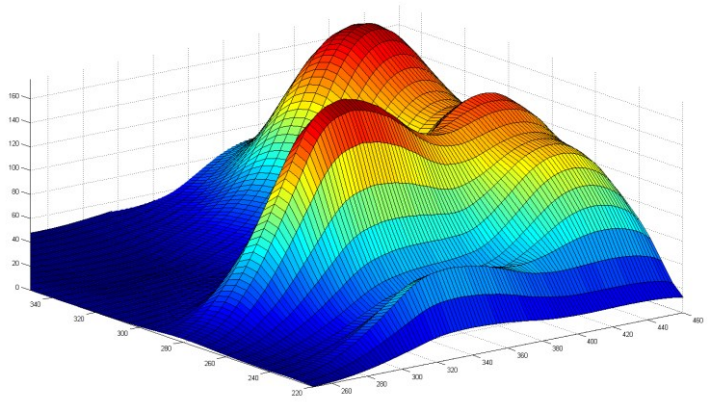

Figure 56. EEM fluorescence analysis of rhizodeposition from *in vitro* banana plants.

A number of experiment is under course at CENSA for the identification and evaluation of bioactive compounds with an impact on BW (subtask 3.3.1) or PPN (subtask 3.3.2.). For the latter, metabolites produced by fungi such as *Acrostalagmus luteoalbus* are under evaluation. An assay has been carried out on the effect of filtrates produced by a number of *Trichoderma* spp. proceeding from Algeria, in collaboration with ENSA (prof. S. Sellami). Filtrates of two isolates tested *in vitro* showed a negative effect on juveniles of *Meloidogyne incognita*. A further assay has been carried out by *in vitro* by CNR using trimethylxhantine (caffeine) as bioactive compound. Exposure of *M. incognita* eggs at a concentration of 4000 ppm completely inhibited eggs hatching after three weeks, although the eggs could complete their embryonic development. A bacteriostatic effect was also observed in *vitro* on egg-associated bacteria.

Task. 3.4 Risk analysis in the banana and ensete defence response

To determine the effect of *P. chlamydosporia* as microorganism enhancer of plant defences UA team studied how the endophytic fungus colonises banana plants in a time-course (1-7 days). We aim to determine by qRT-PCR the transcriptomical response of the fungus during early plant colonisation. In addition, we analyse how this fungus overexpresses genes related with activation of defence response, mainly genes involve in stress hormones homeostasis (like salicylic and jasmonic acid).

Distribution and quantification of Pochonia chlamydosporia in banana roots

Root culturing was used to evaluate the inoculation of *P. chlamydosporia* (e.g. detecting the fungus) in banana roots. *P. chlamydosporia* total and endophytic root colonization were analysed. The spatial pattern of root colonization by Pc123gfp was assessed using laser-scanning confocal microscopy. Ten fragments/root system (5 to 10 mm long) were examined in a Leica DM IRBE2 confocal microscope. GFP fluorescence and root cell autofluorescence were detected. Further detection of *P. chlamydosporia* in roots was performed by PCR. For this purpose, gDNA was extracted from roots of 10 plantlets/treatment (non-sterilized or surface-sterilized). Primers for PCR amplification of *P. chlamydosporia* were derived from Pc SCP1 and Permease1 genes (Escudero et al., 2016, Fungal Biology).

Magenta-box experiments (System 1)

Evaluation of endophytic colonisation of banana plants by P. chlamydosporia *using molecular and physiological analyses for enhance banana plant defense response.*

Banana roots were inoculated with Pc123 isolate transformed with the green fluorescent protein (GFP). We showed Pc colonisation the rhizoplane, root hairs (Fig 57 A,B). However, the fungus also colonises endophytically root cells (Fig. 57 C, D).

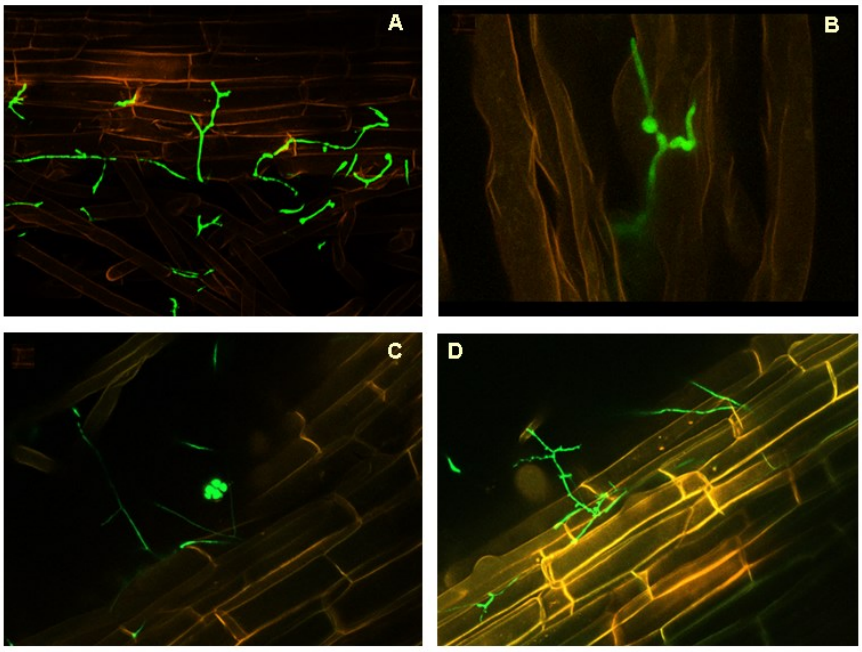

Figure 57. Pc123gfp colonising banana root cells. a) General view. b) Close-up of root hair colonisation. c) and d) Epidermis and cortex banana root cells colonisation.

Quantification of Pc colonization in banana plants by culturing methos.

The UA axenic systems allowed external colonisation of banana roots (Fig. 57). The fungus increased endophytic (internal) colonisation of roots 1.6 fold in 7 days (Fig. 58).

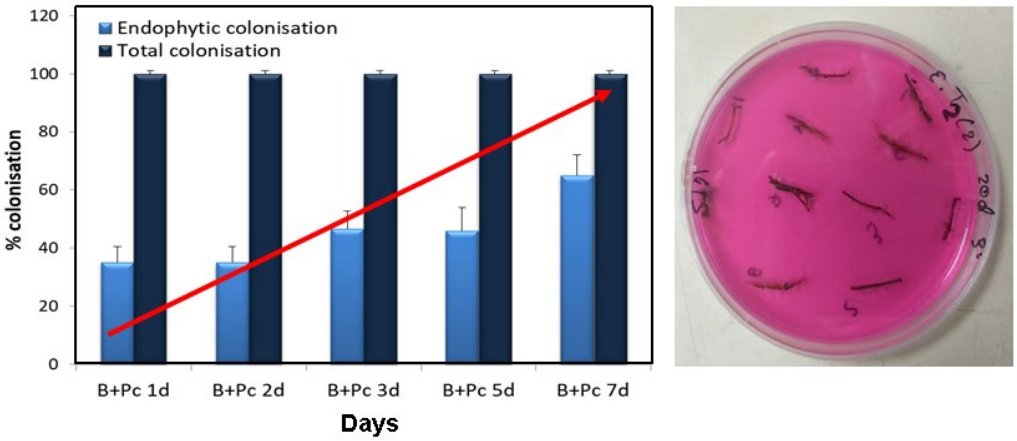

Figure 58. Pc root colonisation from banana plants in axenic Magenta Box (System 1).

Molecular identification of Pc colonising banana roots endophytically

Pc SCP1 clearly reported both endophytic and total root colonisation by Pc (Fig. 59 A, ca. 400 pb band), confirming the amplicon identity by sequencing. Pc permease 1 gene displayed no PCR aspecificities but was less sensitive to detect endophytic root colonisation by the fungus (Fig. 59 B, ca. 250 bp).

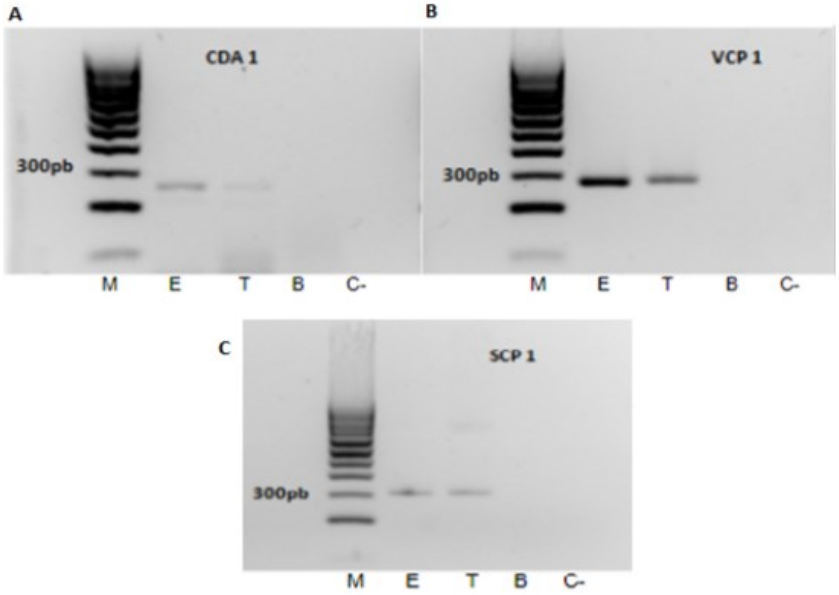

Figure 59. Molecular detection of *P. chlamydosporia* total (T) and endophytic (E) colonisation endophytically of banana root cells using fungal specific primers A) CDA1, B) VCP1, C) SCP1. Lane labels: B= gDNA from uninoculated banana roots. C-= no DNA, negative control. Other lanes include genomic DNA from banana roots inoculated with Pc123.

Colonization of banana roots by *P. chlamydosporia* after 5d in contact with the fungus i was also measured using quantitative PCR, measuring the amout of fungal DNA by evaluating VCP1 gene with specific primers. Banana DNA quntification was performed by amplification of PR1 gene using specific primers. Serial dilutions of isolate Pc123 genomic DNA defined a calibration curve, using three independent calibrations for each DNA sample. After each run, a dissociation curve was acquired to check for amplification specificity. Results are pg *P. chlamydosporia* DNA/ng banana DNA (Fig. 60).

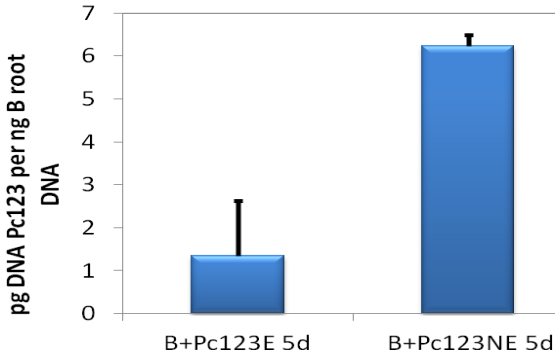

Figure 60. Endophytic and whole *P. chlamydosporia* quantification by qPCR**.**

Data confirm that endophytic colonisation of banana roots by *P. chlamydosporia* is lower (ca. 5 fold less) than total root colonisation.

To characterize transcriptomic response of *P. chlamydosporia* during banana root colonisation, UA team evaluated expression by qRT-PCR of Pc genes after 5 days in contact with banana root in a Magenta, testing genes related to PPN egg infection (SCP1, VCP1 proteases) chitin/chitosan metabolism (chitin deacetylases and chitosanases) and also genes encoding proteins putatively involved in modulation of plant immunity (LysM effectors). The fungus expressed those genes when colonizing banana plants endophyticaly (Fig. 61). Banana plants also showed expression of PR1 and PR3 when fungus is colonizing the rhizosphere (Fig. 62).

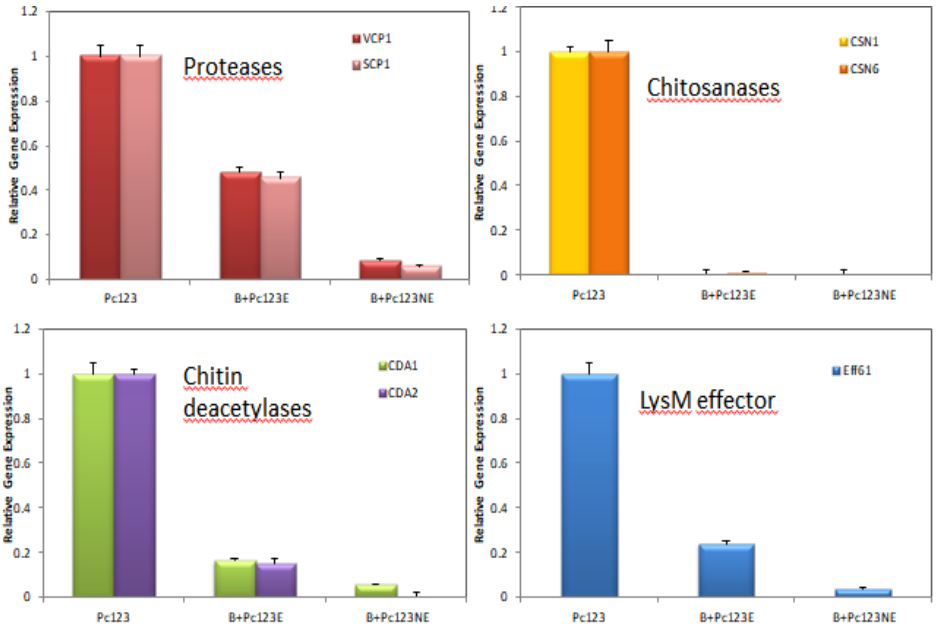

Figure 61. Gene expression of *P. chlamydosporia* proteases, chitosanases, chitin deacetylases and LysM effectors after 5 days colonising banana plants.

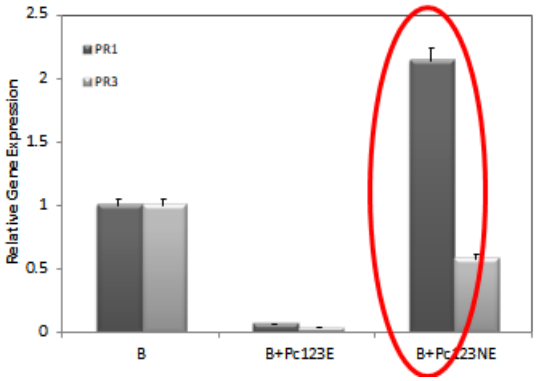

Figure 62. Gene expression of banana pathogenic response proteins (PR1 and PR3) after 5 days in contact with *P. chlamydosporia* in a magenta box.

UA team is currently designing RNAseq experiments to determine the whole gene expression of banana plants exposed to *P. chlamydosporia* in a time-course (1 -7 days)

in the magenta box system, to characterise activation of defense genes during early events of root colonisation. This experiment was designed with three treatments:

- banana plants in contact with 21 day-old *P. chlamydosporia* colony in magenta box for 2, 5 and 7 days;
- banana plants in a uninoculated magenta box for 2, 5 and 7 days;
- cellophanes of *P. chlamydosporia* 21 day-old colonies (see RNAseq in task 3.3).

1.2.4 WP4 - Testing germplasm response for integration with EBCAs (mths: 4-18)

The general objective of WP4 is to test the banana germplasm response against PPN, PD and BW in combination with EBCAs, and to identify the corresponding key genes/molecular pathways induced in the plants during their response. It is, therefore, important to screen different genotypes to identify: a) promiscuous EBCAs, in a compatible interaction with *Musa* spp. germplasm; b) EBCAs able to proliferate on a given genotype; and c) genes/molecular pathways activated in the host response to pests and pathogen attacks when EBCAs are present. The following specific objectives have been established:

- Test a collection of newly isolated and/or commercially available EBCAs for their potential activity as growth stimulators and biocontrol agents in different banana genotypes and environments (greenhouse, nursery and field).
- Detect differentially expressed genes in banana plants showing growth stimulation and biocontrol activity by the EBCAs tested.
- Identify if the growth stimulators and biocontrol agents identified show endophytism and are able to internally colonize banana plant tissues.
- Analyse changes in the rhizospheric microbiota associated to banana plants inoculated with EBCAs.

Task 4.1. Gene expression in tolerant/susceptible bananas under biotic stresses.

In order to find new EBCAs effective against PPN, PD and/or BW in a range of banana cultivars, Real IPM and KU LEUVEN tested commercially available microorganisms on different genotypes and in different environments/conditions (greenhouse, nursery and open field). The microorganisms were provided by Real IPM Company (Kenya) Ltd., and included fungi such as *Trichoderma* or *Glomus* spp., as well as bacteria such as *Bacillus* or *Azospirillum* spp. Their potential as PGPM was tested in banana, as they stimulate growth in other crops acting as biofertilizers and biocontrol agents. In the case of *Trichoderma* and *Bacillus* spp., the potential effect in alleviating drought stress (main abiotic factor limiting banana productions, worldwide) was also tested. Preliminary data from a field trial on cv Williams indicated that *Glomus* spp., *Trichoderma*, *Bacillus* and *Azospirillum* spp. promoted growth in banana plantlets under nursery conditions. Alternatively, *Azospirillum* sp. likely stimulated growth at early stages in post-inoculation.

In a second greenhouse assay, *Trichoderma* sp. was inoculated on different banana genotypes grown either in liquid medium (Gran Enana and Yangambi km5) or in soil (Valery, Pequeña Enana and Yangambi Km5). Two furher trials were also carried out in a tropical environment (Dominican Republic) to evaluate the growth promoting

effect in cv Williams (Cavendish subgroup, AAA) nursery plants of the nitrogen-fixing bacterium *Azospirillum* sp., the arbuscular mycorrhiza fungus *Glomus* sp., the phosphate solubilizers *Trichoderma*, *Bacillus* spp., and a combination of *Trichoderma* + *Bacillus* + *Glomus* spp.

A combination of available resistance in plants with protection conferred by EBCAs under field conditions may lead to increased, durable and sustainable IPM during farming. The interaction between endophytes and hosts are often (with varying degrees) highly specific and might be explained in a gene-for-gene type of interaction. Mechanisms through which EBCAs reduce disease severity may be through direct (same ecological niche, competition for nutrients and antibiosis) or indirect antagonism (reduced disease severity by induced disease resistance). The endophyte-host-pest association is complex and certain species will activate enzymatic host-plant defence mechanisms following inoculation.

A first greenhouse experiment was initiated at KU LEUVEN in December 2017 using *Trichoderma* and *Bacillus* spp., both provided by Real IPM, to explore growth promoting effects. A total of 48 *in vitro* plants of cv Williams were inoculated by root dipping at the moment of transferring from *in vitro* tubes to pots (Fig. 63). Williams was selected being widely used in Latin America and being sensitive to PPN and BW, while resistant to PD.

A total of 48 *in vitro* plants from cv Williams were inoculated by root dipping at the moment of transferring from *in vitro* tubes to pots. Half of them (24 plants) were inoculated with Real Trichoderma at a concentration of 10^5 cfu/mL, while the other half were inoculated with Real Bacillus at 10^6 cfu/mL. Other 48 *in vitro* plants were used as controls (total number of plants in the experiment = 96). Subsequently, two more inoculations were applied by drenching (5% of pot volume) after 2 weeks and after 1 month.

As one month is the approx. time required by banana plants to emerge from the *in vitro* stage, this timing was selected to start a differential irrigation regime. Aim was to test if the inoculated microorganisms could help in alleviating drought stress in banana. A third inoculation by drenching was made with *Trichoderma* sp., ten weeks after starting the differential water supply. Table 14 summarizes the different treatments applied.

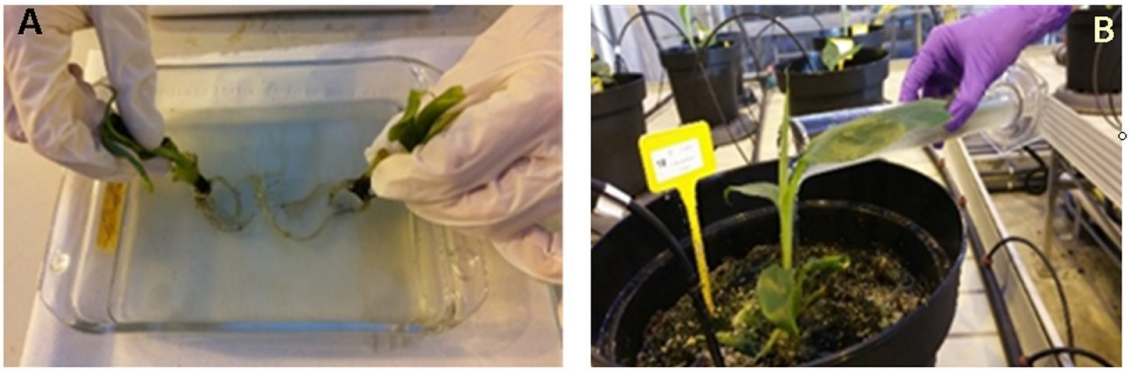

Figure 63. Example of root dipping at the moment of transferring plants from *in vitro* tubes to pots (A), and drench, two weeks after planting (B).

Table 14. Treatments applied to test the growth promoting effects of selected *Trichoderma* and *Bacillus* spp. on cv Williams plants with differential water supply, under greenhouse conditions.

Treatment	Application	Microorganism	Irrigation*	N. plants
1	Water + Tween20	Control	A	12
2	*Trichoderma*	*Trichoderma* sp.	A	12
3	Water + Tween20	Control	B	12
4	*Trichoderma*	*Trichoderma* sp.	B	12
5	Water	Control	A	12
6	*Bacillus*	*Bacillus* sp.	A	12
7	Water	Control	B	12
8	*Bacillus*	*Bacillus* sp.	B	12

* A: optimal irrigation (continuous dripping irrigation; 1 drip per pot). B: suboptimal irrigation (dripping irrigation 3 days per week; 1 drip per pot).

An overview of plants during the differential water supply is presented in Fig. 64 (complete randomized design). The number of newly formed leaves and the pseudostem height were recorded weekly, for each combination of microorganism-irrigation treatment and during the differential water supply. Additionally, the projected leaf area (canopy) of the plants was measured 5 times during this period (Fig. 65 A-G).

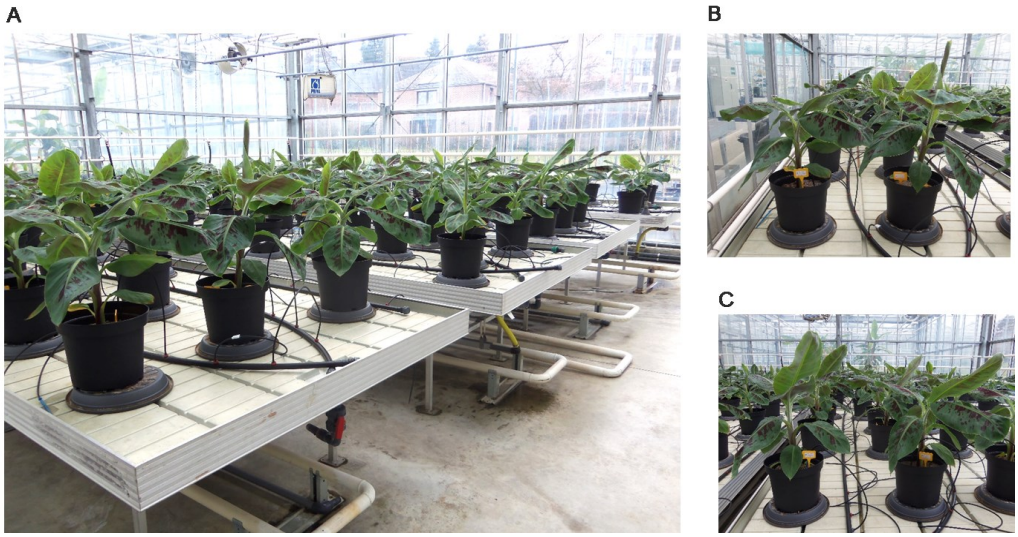

Figure 64. Banana plants of cv Williams (inoculated and control) , nine weeks after starting the differential water supply test (A). Plants inoculated with *Trichoderma* sp. (left) and corresponding control (right) under optimal irrigation (B). Plants treated with *Bacillus* sp. (left) and corresponding control (right) under optimal irrigation (C).

Figure 65. Measured parameters and tissue/rhizosphere sampling at the end of the greenhouse trial. A) number of newly formed leaves; B) pseudostem height; C) fresh weight of the leaves; D) rhizosphere sampling; E) root sampling; F) corm sampling; G) root sampling for RNA extraction.

After 13 weeks of differential irrigation regime, the greenhouse trial was terminated and recording leaf area, number of newly formed leaves, pseudostem height/girth, number of primary roots and fresh weight of the newly formed leaves. Additionally, samples from the different plant tissues (roots, corm, leaves) and from the rhizosphere were collected for gene expression and microbiota analyses, respectively (Fig. 65).

Data from the first greenhouse experiment showed that plants of cv Williams, inoculated with *Trichoderma* sp. and under optimal irrigation regimes, significantly increased the number of leaves, the leaf area and the number of primary roots.

Subsequently, the data were analysed and a significant increase in leaf area and number of primary roots was found for plants treated with *Trichoderma* sp. under optimal irrigation (Fig. 66 A, B). By contrast, no significant results were found in any of the treatments for the newly formed leaves (Fig. 66 C) or pseudostem growth/girth (Fig. 66 D, E). No difference was found with *Bacillus* sp. except for the fresh weigh of leaves, with a significant decreased observed for plants under optimal irrigation (Fig. 66 F).

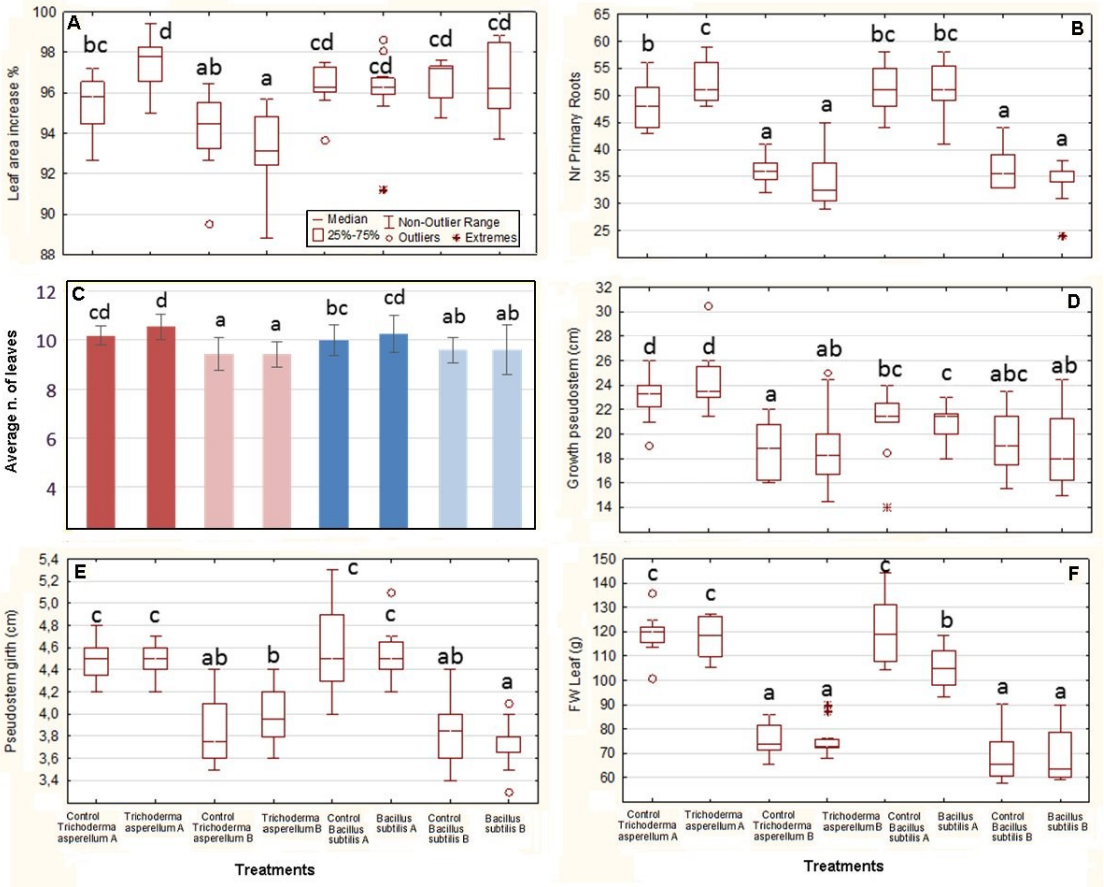

Figure 66. Effect of EBCAs treatments on banana cv Williams plants on: leaf area increase (A), final number of primary roots (B), average number of newly formed leaves (C), pseudostem increase (D) and girth (E), and fresh weight of leaves (F), measured at the end of the differential irrigation test. Group mean comparisons by Fisher's LSD test (P<0.05).

RNA was extracted from the roots collected at the end of treatments 1 and 2. Additionally, primers for 5 banana genes involved in anaerobic metabolism (ADH: alcohol dehydrogenase; PDC: pyruvate decarboxylase; AOX: alternative oxidase; HB: non-symbiotic haemoglobin; ERF1: ethylene response factor 1), 1 gene involved in oxidative stress (SOD: superoxide dismutase) and 2 genes related to endoplasmic reticulum stress (ERO1: endoplasmic reticulum oxidoreductin-1; bZIP17: bZIP transcription factor 17) have been designed/optimized for qRT-PCR analysis. In parallel, the rhizosphere samples collected from treatments 1 and 2 have been processed and sent for metagenomics sequencing to study the microbial composition of the plants with and without *Trichoderma* sp., under optimal irrigation (Task 4.2).

Based on the results of experiment 1, two greenhouse tests using *Trichoderma* (experiments 2 and 3) started in the frame of two MSc projects (see WP10).

Experiment 2 - In October 2018, plants of genotypes Gran Enana and Yangambi km5 growing in liquid medium were inoculated with Real Trichoderma at 10^5 cfu/mL (Fig. 66A). Gran Enana, the most distributed dessert banana cultivar (Cavendish subgroup AAA) has been reported to be sensitive to PPN and BW, while resistant to some Foc lines causing PD. By contrast, Yangambi km5 is a landrace of subgroup Ibota Bota

(AAA), reported as resistant to PPN and PD. This experiment was divided in two independent parts:

1. Sub-experiment 1. Designed to: a) follow the plant growth rate of treated *vs.* control plants over a period of 3 weeks post-inoculation, and b) obtain expression data of banana genes differentially expressed in contrasting genotypes at 3 weeks post-inoculation (8 plants per cultivar and treatment 32 plants in total). The following non-destructive parameters were recorded at the beginning and end of the assay: pseudostem height, leaf area, plant weight, number of newly formed leaves, number of primary roots and root area (Fig. 66B). Additionally, root weight and pseudostem girth/weight were measured at the end and roots and leaves were sampled for gene expression analyses. Currently, the data collected are analysed and root RNA is being extracted.

Sub-experiment 2. Designed to detect genes differentially expressed in contrasting genotypes at two early time points post-inoculation. It consisted of 5 plants per cultivar, treatment and time point (40 plants in total). Root samples were taken 24h and 48h post-inoculation. Currently, RNA from root tips is being extracted and a subset of genes will be amplified by qRT-PCR. If differential expression is observed, whole transcriptomic analysis (RNA-seq) will be performed.

Experiment 3 - At the beginning of November 2018, a third experiment in greenhouse has been initiated in which *in vitro* plants from the genotypes 'Valery', 'Pequeña Enana' and 'Yangambi Km5' were inoculated with Real Trichoderma™ and Asperello™ (*Trichoderma* sp. provided by Biobest Group) at a concentration of 10^6 cfu/mL (Fig. 67). The first inoculation was made by root dipping and drench at the moment of transferring the plants from *in vitro* tubes to pots. The second inoculation was made by drench 2 weeks later, when new leaves started to appear. Valery has been selected for being the standard susceptible check for PPN and it is also susceptible to PD. Pequeña Enana is the most common cultivar in Canary Islands and it also belongs to the Cavendish subgroup, being susceptible to PPN and BW, while resistant to PD.

Figure 67. Plants of Gran Enana (left) and Yangambi km5 (right) growing in trays with liquid medium and air supply in greenhouse conditions. Both genotypes were transferred from *in vitro* tubes to liquid medium at the same time (A). Root picture of Yangambi km5 plant at start of sub-experiment 1, used to calculate root area *via* the webtool DIRT (Digital Imaging of Root Traits) (B).

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In the test with Valery, 30 plants were inoculated with Real Trichoderma, 30 plants with Asperello and other 30 plants were kept as controls for both treatments (in total 90 plants were used, arranged in a complete randomized design). In the test with 'Pequeña Enana' and 'Yangambi Km5', 12 plants per genotype were inoculated with Real Trichoderma, 12 plants per genotype with Asperello and other 12 plants per genotype were kept as controls for both treatments (total: 72 plants, in a complete randomized design) (Fig. 68). The same parameters as for exp. 1 are being measured and tissue/rhizospheric samples will be taken at the end of the assay. The test with Valery will be divided in two different time points (to be determined) for final data collection and sampling.

Figure 68. Start of experiment 3 in greenhouse: plants of Valery, Pequeña Enana and Yangambi Km5 after transferring from *in vitro* tubes to pots and inoculating with Real Trichoderma and Asperello.

Assessing growth promotion under nursery conditions

The following plant growth promotion trials have been performed in the nursery of Cristal Vitro Dominicana (Dominican Republic) using young Williams plants. Trial 1 consisted of 8 microbial treatments with PGPM provided by Real IPM-Biobest and the corresponding control (Table 15). In each treatment, 12 biological replicates were tested.

Table 15. Treatments applied in the nursery trial 1 at Cristal Vitro Dominicana.

Treatment	Application	Microorganism	Concentration	Dose rate	N. Plants
1	PTA001	*Azospirillum* sp.	$4 \cdot 10^8$ cfu/mL	10 mL/L	12
2	PTA002	*Bacillus* sp	$1 \cdot 10^{10}$ cfu/mL	10 mL/L	12
3	PTA003	*Bacillus* sp	$5 \cdot 10^9$ cfu/mL	10 mL/L	12
4	PTA004	*Glomus* spp.	$5 \cdot 10^7$ cfu/L	50 mL/L	12
5	Myc800	*Glomus* sp	800 spores/g	4.24 g/L	12
6	Asperello	*Trichoderma* spT34	10^9 spores/g	1 g/L	12
7	Real Trichoderma	*Trichoderma* spTRC9	10^9 spores/g	1 g/L	12
8	PTA 001 PTA 004 Asperello	*Azospirillum* sp, *Glomus* spp. and *Trichoderma* sp. T34		10 ml/L; 50 ml/L; 1 g/L	12
9	Control	Irrigation water			12

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On August 6th 2018, the treatments were applied by root dipping to six weeks-old plantlets (Fig. 69A) which were subsequently transferred to trays (Fig. 69B).

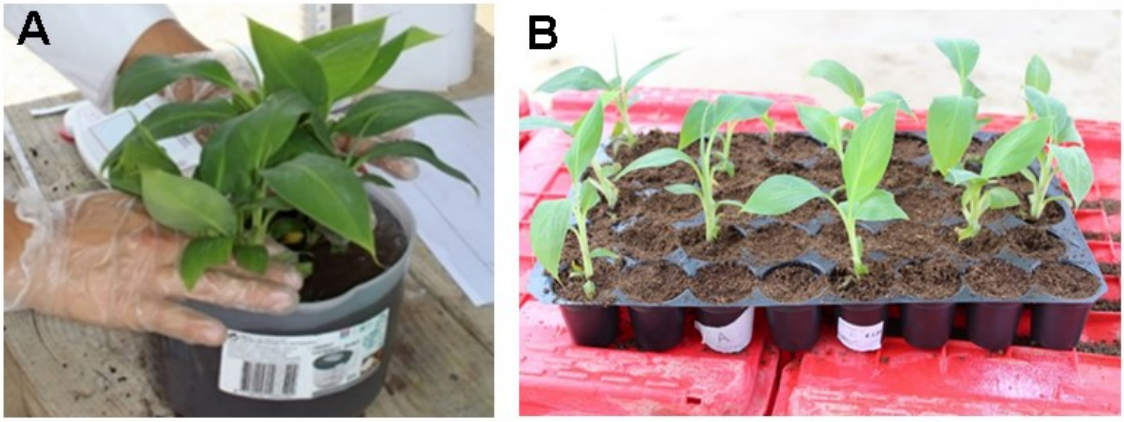

Figure 69. Root bath set-up. Plant roots were submerged for 1 min in the microbial solutions while stirring gently to ensure optimal contact with the product and prevent microbial aggregates to settle (A). Planting trays used to transfer the banana plantlets after root dipping application (B).

Once the plants were transferred to trays, the microbial and control solutions were additionally applied by drenching with 10% of the pot volume. On Aug. 17th, 11 days after the first application, plants were transferred to 1.5 L bags filled with the same substrate to ensure enough physical space for the growing roots. Upon transplant, a second drench inoculation was done with the same treatments and at the same concentrations as indicated in Table 15. The treatments were applied a third time by drenching on Sept. 5th, 4 weeks after first inoculation. During the trial, non-destructive parameters were recorded: pseudostem height, leaf emission rate, pseudostem girth and leaf area (data not analysed yet). Fresh/dry shoot weight and fresh/dry root weight were measured at the end of the trial, 10 weeks after the first inoculations (data not analysed yet). Plants from treatment 4 showed a higher pseudostem than the controls in most of the time points recorded. Plants belonging to treatments 1, 2, 3, 6, 7 and 8 showed a higher leaf emission rate than the controls in the last recorded time point.

In Trial 2, four microbial treatments were compared to the control and each treatment was applied to 10 plants (Table 16). The trial started on Aug. 7th 2018 and the treatments were applied via root bath and drench as described. The plantlets were transferred to 1.5 L bags and were drenched again with a volume of 0.15 L of the microbial solutions or irrigation water. Plants growth after treatments was assessed measuring non-destructive parameters: pseudostem height, pseudostem girth, leaf emission rate, length of the youngest leaf (Fig 65) and leaf area (data not analysed yet). Fresh/dry shoot weight and fresh/dry root weight were measured at the end of the trial, 10 weeks after the first inoculations (data not analysed yet). Preliminary results 9 weeks after starting the trial indicated that Real Trichoderma and Asperello might promote pseudostem growth and increase in lenght of the youngest leaf (Fig. 70).

Table 16. Treatments applied in the nursery trial 2 at Cristal Vitro Dominicana.

Treatment	Application	Microorganism	Concentration	Dose rate	N. plants
1	PTA 003	*Bacillus* sp.	$5 \cdot 10^9$ cfu/mL	10 mL/L	10
2	PTA 004	*Glomus* spp.	$5 \cdot 10^7$ cfu/L	50 mL/L	10
3	Real Trichoderma	*Trichoderma* sp.TRC9	10^9 spores/g	1 g/L	10
4	PTA 003, PTA 004 and Asperello	*Bacillus* sp., *Glomus* spp. and *Trichoderma* sp.T34	$5 \cdot 10^9$ cfu/mL, $5 \cdot 10^7$ cfu/L and 10^9 spores/g	10 mL/L, 50 mL/L and 1 g/L	10
5	Control	Irrigation water	0		10

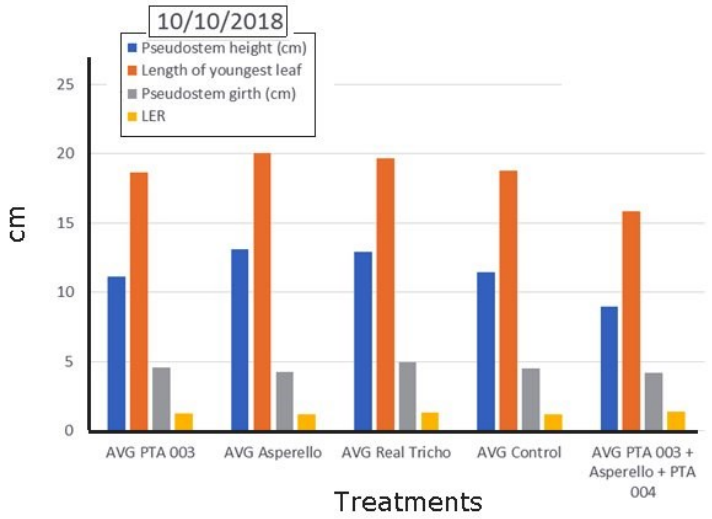

Figure 70. Average value of the parameters recorded nine weeks after inoculating with microorganisms listed in Table 16.

In order to carry out all the activities of the MUSA project in which KU Leuven is involved, a subcontracting was required for the technical development of specific assays on susceptible and tolerant banana varieties in response to biotic stress, with and without ECBAs (task 4.1). Due to the limited expertise and lack of appropriate facilities for Plant Microbiology and Phytopathology tests, the Laboratory of Tropical Crop Improvement (Professor Rony Swennen, KU Leuven) has requested the Laboratory of Integrated and Urban Plant Pathology (Professor Sébastien Massart, University of Liège), to act as a subcontractor with respect to the following services:

1. Advice on any microbiological aspect in the project.
2. Viability tests and quantification of commercial microorganisms.
3. Isolating beneficial microorganisms from soils, banana rhizosphere and/or banana tissues.
4. Contributing to inoculations of microorganisms on banana plants.

5. Selection and development of beneficial microorganisms towards a commercial product.
6. In vitro and/or greenhouse maintenance of banana plants.
7. Joint publications with KU Leuven.
8. Joint work planning with KU Leuven.

This subcontracting started on September 2017 and shall continue over a period of 48 months under an agreement already signed by both parties. The Integrated and Urban Plant Pathology Laboratory of Prof. Sébastien Massart has been selected as the result of a process that respected the subcontracting principles of best value for money and avoidance of Conflict of Interest (Art. 13 of the GA). At the moment of this First Report presentation the subcontracting has not been invoiced yet.

Assessing growth promotion under open field conditions

The field trial was located in Guayubín, Monte Cristi, Dominican Republic (19°35'N 71°24'W) and consisted of 240 banana plants cv Williams distributed in 12 by 20 rows and planted on June 21st 2018 (Fig. 71). The climatic conditions in Guayubín are tropical with a minimal temperature of 19 °C and a maximal temperature of 34 °C. The average precipitation is 693 mm per year and the humid/moist period stretches from April to beginning of December.

Figure 71. Overview of the field trial in Guayubín, Monte Cristi (Dominican Republic) with 240 'Williams' plants.

Bioformulation PTA 001, which contains the nitrogen fixing bacterium *Azospirillum* sp. at $4 \cdot 10^8$ cfu/mL, was used at a final concentration of 10 mL/L. The plants were inoculated for the first time in the nursery on March 17th 2018, 14 weeks before planting in the field. Subsequently, 4 additional drench applications were performed in the field at 2, 4, 9 and 11 weeks after planting. The treatment was applied to one block of 120 plants out of which 20 plants were selected randomly. Simultaneously, a control treatment was applied to 20 plants planted at the same time in the same field but on another block of 120 plants.

Starting from the 5th week after planting, the following parameters were recorded weekly: number of leaves, pseudostem height and pseudostem diameter. Work is in progress.

The outputs considered are summarized as follows:

– Optimized growth of banana plants and optimized protocol for greenhouse, nursery and field inoculation of banana plants with different PGPM.

- Optimized image-based system for the analysis of leaf and root areas in banana plants grown under greenhouse and nursery conditions.
- Set of plant growth parameters recorded in plants treated with different microorganisms under greenhouse, nursery and open-field conditions.
- Root RNA extracted from plants inoculated with *Trichoderma asperellum* in greenhouse.
- Developed primers for qRT-PCR analysis of a specific gene set in cDNA obtained from inoculated and control banana plants.
- Microbial DNA extracted from rhizosphere of plants inoculated with *Trichoderma asperellum* in greenhouse.

The EARTH trail in Costa Rica consists of three banana cultivars that have been already planted in the MUSA field plot as follows: two cultivars of subgroup Cavendish, Grand Naine (AAA) and Williams (AAA), as well as Red Makabu (AAA). The plots are already established for field evaluation of endophytes application for control plant parasitic nematodes (Figure 72).

Figure 72. Establishment of the banana plot for field evaluation of endophytes for biological control of plant parasitic nematodes at EARTH field facility.

Nematodes and germplasm interactions in Cuba. In the Cuban Germplasm Bank at INIVIT, located in the central region (Fig. 73), a survey was carried out to determine the nematofauna associated with the most important banana genotypes and to establish pure populations of nematodes at CENSA. Genotypes sampled and host range assessment are summarized in Table 17.

Figure 73. View of the Germplasm bank at INIVIT, Villa Clara Province (Cuba).

Table 17. List and nematode resistance of genotypes sampled at the INIVIT Germplasm bank.

Genotype	Sample number	Resistance *
Burro CEMSA	M1	RN
ManzanoVietnamita	M2	
PisangCeilan	M3	
CEMSA 3/4	M4	SN
INIVIT PB 2011	M5	
INIVIT PV 2012	M6	
Yangambí	M7	ARR
Grand Naine	M8	SN
Gross Michel	M9	MRR
PisangHariBuaya	M10	RR
Calcuta 4	M11	
FHIA 18	M12	RR
INIVIT PV 0630 (Z30)	M13	SN
Enano Guantanamero	M14	SN
Macho 3/4	M15	SN
FHIA 21	M16	RN
SH3436L9	M17	RR
FHIA 01	M18	
FHIA 01-V1	M19	RR
SH3142	M20	
SH3362	M21	
FHIA 17	M22	

* RN: resistant to nematodes; SN: susceptible to nematodes; MRR: moderately resistant to *R. similis*; ARR: Highly resistant to *R. similis*; RR: resistant to *R. similis*

Potential of commercial EBCAs. Pot trials were established by IITA to study the potential of commercially available EBCAs for promoting growth and control of Fusarium wilt pathogen race 1 (Foc race 1) of banana. The commercial EBCAs were *Bacillus* sp.. and *Trichoderma* sp., sourced from Real IPM. Greenhouse pot experiments were conducted with tissue culture derived plants of the cultivars Grand Naine (some Fusarium resistant) and Mchare (Fusarium susceptible). Endophytes were tested for their growth promoting attributes in resistant cultivar and disease suppression efficacy against susceptible cultivar. Results revealed that *Bacillus* sp. significantly enhanced growth of banana plants by 42% to 82%. *Trichoderma* significantly reduced Fusarium wilt symptoms compared to control and *Bacillus* sp. treatment. Thus, *Trichoderma* has potential for managing Foc race 1, while *Bacillus* has the potential to be used for enhancing banana growth.

Task 4.2 Rhizosphere metagenomics and EBCAs-induced effects on soil microbial communities.

Metagenomic analyses. Using samples collected from banana type Mchare in Tanzania, we investigated the root associated microbiome of healthy and Foc-infected plants. Preliminary results revealed that the root endophytic microbiome in banana is dominated by Proteobacteria, followed by Actinobacteria and Bacteroidetes, irrespective of cultivars and locations. Comparative community analysis of healthy and Foc-infected roots also revealed that PD reduced the abundance of Pseudomonadales and Streptomycetaceae, known for the production of antagonistic compounds against phytopathogens. Further, root microbiome in Foc-infected banana revealed higher abundance of Flavobacteriales and Rhizobiales endophytes, involved in carbohydrate metabolism.
To identify differences in endophytes populations contributing to maintain asymptomatic plants in cultivated banana fields infected by PD, IITA developed. the following activities:

– Identification of optimal banana-cultivated agroecosystems for endophyte isolation and characterization.
– Use of metagenomics for endophyte isolation and biological characterization to detect plants with higher differences in endophyte populations between PD-symptomatic and PD–asymptomatic plants.
– Upon identification of diseased and healthy plants with significant difference in microbiota composition, performing metatranscriptomics to identify differences in expression profiles of plant and microorganism between symptomatic and asymptomatic bananas.
– Integration of data obtained from metagenomics (KU LEUVEN), metatranscriptomics (CNR-IITA), and endophytes microbiological isolation (IITA) analyses to identify new genera/species involved in tolerance to PD.

In a first sampling campaign, three agroecosystem have been selected for sampling around Kampala in Uganda. GPS has been used to record the exact position of each sampled plant. In Kawanda, IITA selected 3 symptomatic and 3 asymptomatic plants for each banana cultivar: Silk (AAA) and Sukali Ndiizi (AAB). Plants were grown in a

field trial with Foc, selected for the semi-controlled infection condition that guaranteed the presence of the pathogen in both symptomatic and asymptomatic plants.

In Luwero, IITA selected 5 symptomatic and 5 asymptomatic banana plants of cultivar Sukali Ndiizi (AAB). This field was selected because plants were embedded in an agroforestry ecosystem with coffee and cassava plants that might have influenced more variability in the endophytic population.

In Kisoga, 5 symptomatic and 5 asymptomatic banana plants of the cultivar Sukali Ndiizi (AAB) were selected. In this field banana was in monoculture and symptomatic and asymptomatic plants were identified in distinct spots in the field.

Collected samples consisted of: (i) bulk soil, collected nearby roots, (ii) rizosphere, (iii) roots, and (iv) corm. Endophytes have been isolated according to the protocol shared within MUSA project and DNA extracted for high throughput sequencing for fungal and bacterial composition analysis (performed by KU LEUVEN). Molecular data analysis did not reveal significant difference in microbiota composition between diseased and healthy plants. However, some families/genera hosting biocontrol agents were identified only in healthy plants when the analysis was conducted on samples collected from young sucker. Indeed even when collected from heavily infected plants, this tissue did not present necroses, which significantly affect the characteristics of the macrobiota.Based on these preliminary, promising results, IITA organised a second sampling campaign in November 2018 returning to the Luwero field only. The second sampling included a preliminary metagenomic analysis. In addition, samples from suckers from either Foc infected mats or aymptomatic mats, were collected from asymptomatic plants. Endophytes were isolated according to the protocol developed by IAS CSIC. DNA was extracted, sequencing the 16S rRNA gene for bacteria and ITS for fungi (analyses in progress). Abundance and diversity analyses are in progress. For this sampling, pairs of plants were identified, one symptomatic and one asymptomatic, located very close to each other. Root and corm samples were collected from young suckers from nine healthy and nine diseased plants in total. Samples are currently under investigation for endophytes isolation and DNA extraction for high throughput sequencing of fungal and bacterial species.

Metagenomics sequence data have been produced for fungi and bacteria from rhizosphere of Pequeña Enana plants and adiacent, uncultivated control soil, proceeding from Tenerife farms sampled in collaboration by Coplaca and CNR (Feb. 2018).

SARI with UNEXE progress in WP 4 (Testing germplasm response for integration with EBCAs) was as follows:

- Established local collaborations in Ethiopia to carry out a comprehensive diagnostic study on nematodes of enset with the College of Agriculture and Veterinary Medicine at Jimma.
- Trained two researchers with field sample management and extraction techniques.
- These activities prepared partners to carry out field sample collections and diagnostic studies on nematodes and *Fusarium* on enset.
- Samples will allow isolation and characterization of EBCAs.

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1.2.5 WP 5 Procedures for EBCAs mass production, storage and application (mths: 16-18)

Methods for large-scale cultivation of EBCAs

Continued mass production of referenced strains of *Pochonia chlamydosporia* var. *catenulata* (IMI SD187) and *Trichoderma asperellum* (*Ta*.13) is performed using a Solid State fermentation Technology developed at CENSA to obtain two commercial products, KlamiC® y SevetriC, respectively. They are used for the experimental assays to evaluate new applications and use as Endophytes and Biological Control Agents (EBCAs).

A different liquid-solid production method for *P. chlamydosporia* production, was developed by SacomLab, divided into the following steps (Fig. 74 A-D):

- Pre-inoculum culturing and subsequent inoculum preparation;
- Liquid State Fermentation (LSF);
- Solid State Fermentation (SSF);
- Downstream mass production.

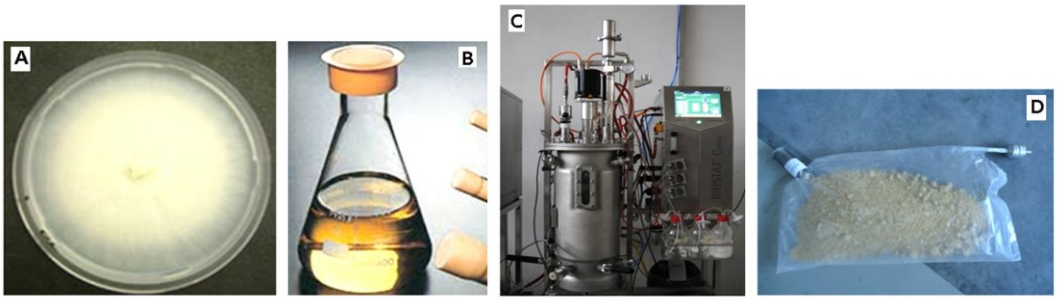

Figure 74. Initial steps followed for the liquid production of the *P. chlamydosporia* inoculum, starting from a petri dish culture (A) used for liquid inoculum preparation (B) and subsequent massal production in liquid (C). Finally, the inoculum is introduction on rice grains-based solid medium in plastic bags, for mass production (D).

The production starts in the laboratory, with *in vitro* cultures (Fig. 74 A) to obtain the fermenter pre-inoculum in a liquid growth medium (Fig. 74 B). The volume of the microbial biomass in liquid is then increased in fermenters (Fig. 74 C). The biomass produced in liquid provides the final inoculum for the subsequent fermentation on a solid medium (Fig. 74 D). Immediately after, the downstream process follows on a solid medium, in controlled environmental, aerobic conditions (Fig. 75). Once the sporulation is completed, the biomass is subjected to an extraction processes for recovery of the chlamydospores amd mycelial matters on a solid substratum. This represents the inoculum used for the preparation of the commercial products, suitably formulated and integrated with other ingredients, in order to preserve their stability, efficiency and shelf-life.

Figure 75. Downstream mass production of *P. chlamydosporia* on solid medium.

The solid-state fermentation (SSF) is a technology employed in several fields of organic production in which the microorganism to develop grows on a solid substratum, unlike the conventional fermentation in liquid. By applying a SSF, microorganisms are grown on natural materials such as agro-food waste, corn, rice, barley etc. The SSF has many advantages, such as the use of simple substrates, the low content of free water (which avoids contaminations), and higher yields, in environmental conditions similar to the ones naturally encounterd by the microorganism, at the time of application.

Entomopathogenic Nematodes (EPN)

In vivo mass production

The method for *in vivo* EPN production, developed by Dutky *et al*. (1964) was modified by CENSA research team for massive use in Cuba, including some quality control steps (Rodríguez Hernández, 2015). The document with the protocol modifications produced by Sánchez *et al*. (2006) has been deposited in the Cuban Copyright Center (Centro Nacional de Derecho de Autor, Cuba, n. 09613-2002) and represents the reference for production of EPN in cottage laboratories in Cuba.This procedure represents a low input technology and labour-intensive process, used in more than 30 Cuban cottage laboratories for EPN production, since the ’90s. The methodology has two main processes. The first one aims at the production of healthy late-instar larvae of the greater wax moth *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). The second step aims at the production of infective EPN.

Production of late-instar larvae of the greater wax moth

Adults of the greater wax moth are placed in glass or plastic containers. Their eggs are then collected starting 2-3 days after mating. The eggs are then introduced in selected substrates (previously sterilized) with honey. After 10 days, the small wax moth larvae are transferred to a large container with the same substrate and kept more than 25 days. The larvae are then manually separated from the substrate and classified, using the more developed for EPN production (Fig. 76, A-D). The smaller larvae are re-introduced in the substrate again.

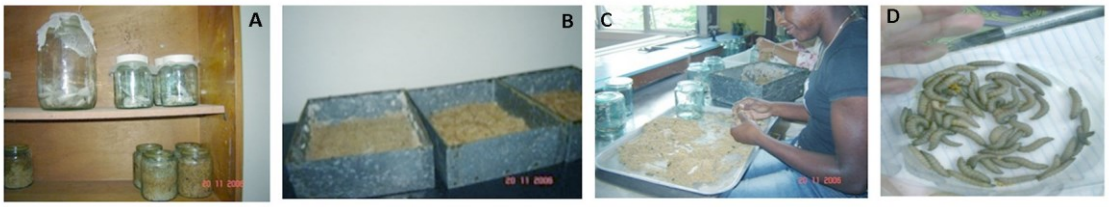

Figure 76. Initial steps in EPC production. Mating of adult moths (A), larvae development in alternative substrates (B), wax moth larvae extraction (C) and collection of healthy late-instars.

Production of EPN

A water-nematode suspension is added to to containers with healthy *G. mellonella* larvae (weigh 0.2 g) followed by incubation for 10-12 days at 25-27 ºC. the *G. mellonella* dead body, with characteristic colour at 72 hrs after inoculation, indicate that the bacteria has developed the phenotypic phase I and good yields of infective juveniles must be expected. The infective juveniles are then harvested using the White's trap and a vacuum pump. The suspension is then cleaned of drebris and residues and the juveniles are formulated in nylon bags, using clean sponges (Fig. 77, A-E).

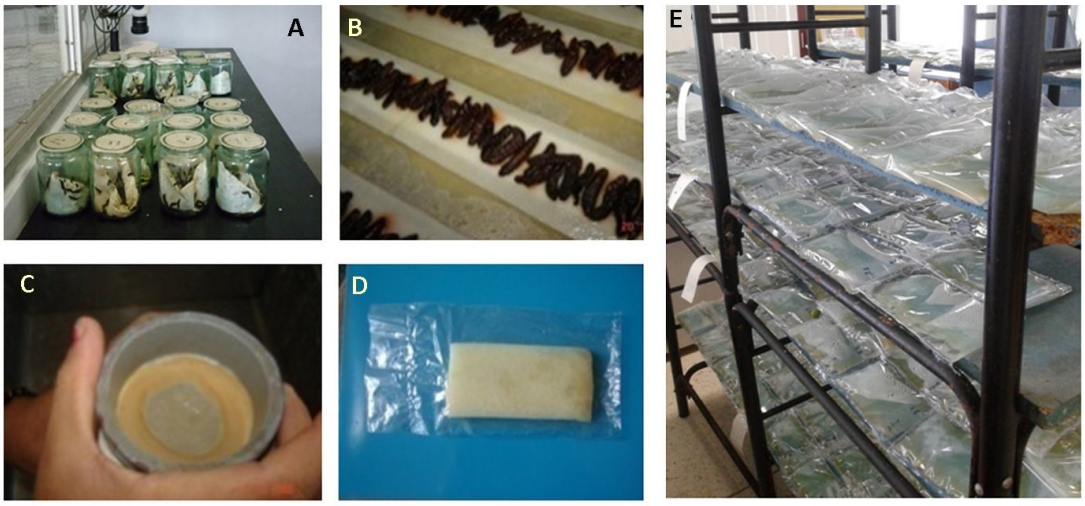

Figure 77. Steps followed in EPN production on *G. mellonella* moth larvae. Incubation of larvae inoculated with EPNs (A). Harvest of nematodes by White's trap, using trays and glass pieces (B). Cleaning and concentration of nematode suspensions using sieves and vacuum (C). Simple formulation of nematodes suspension in distilled water in sponges (D). Storage of bags with EPNs at room conditions (25ºC) for 2.5 months (E).

Using the mass rearing process of *G. mellonella* in local facilities at the Nematology Lab (CENSA) more than 7000 million infective juveniles were produced in the first 18 months of the MUSA Project. The inoculum served for laboratory-field experiments and commercially released to farmers for insect pest management in the Mayabeque and Havana Provinces.

In vitro *mass production*

Due to the characteristic of the symbiotic complex, the bacteria must transform the media before nematode introduction in the system. For *in vitro* mass production, the bacteria must be first introduced in the media (i.e. in plates, Erlenmeyer's flasks or fermenters), to be then followed by the nematode introduction.

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Production of the bacterial inoculum

Dead inoculated *G. mellonella* larvae incubated at room temperature during 24-48 hours, were disinfected with ethanol (90 %) with a brief ignition. The dead bodies are flamed, open with a sterile needle in their ventral area near the head. The haemolymph is then taken with a needle and is used for producing bacterial colonies in McConkey agar media, incubated for 24-48 hours. On this medium the neutral red stain (Fig. 78) is indicative of the Phase I development.

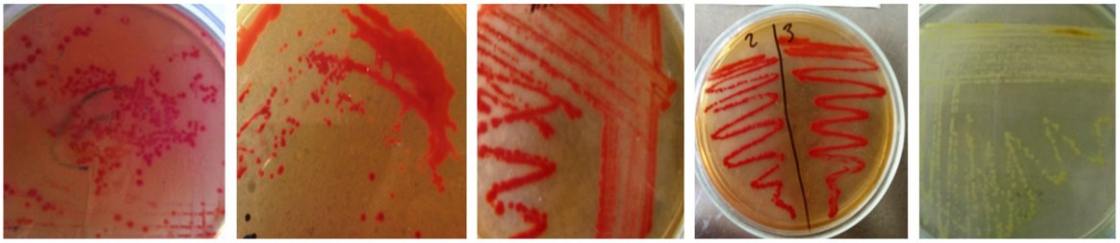

Figure 78. Colonies of *Photorhabdus luminescens* growing on McConkey (red) and nutrient agar (irregular borders and yellow color).

Sterilization of infective juveniles

The nematodes must be sterilized before their introduction in the culture medium for *in vitro* reproduction. Two reagents were evaluated: thimerosal and sodium hypochlorite (0.1 and 0.6 %), with best results obtained using 0.1 % Na hypochlorite.

In vitro *EPN production*

Using the composition of solid culture media described by Sánchez *et al*. (2006, Patent OCPI 882/2006) as a reference, ten further media were evaluated as liquid substrates, (ncluding co-product from animal industries and botanic products, in three trials, using 150 ml Erlenmeyer flask on orbital shakers. Six media did not produce juveniles. One medium was selected, composed by animal and vegetal co-products, that yielded 16 896 IJ/ml (Fig. 79). The yields remain still low, however, compared to those reported by other authors.

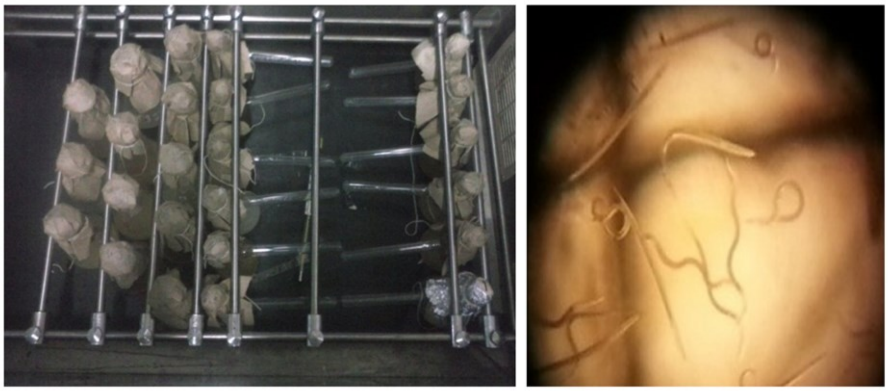

Figure 79. Production of EPN *in vitro*.

Work package 10: Dissemination, communication and exploitation of results (mths 3-18)

*Task 10.1 Production and validation of Communication, Dissemination and Exploitation Plan (CDEP)***.** Task leader: CNR Other Participants: all.

IAS-CSIC was the primary author of Del. D10.1 (Communication, Dissemination and Exploitation Plan, CDEP) and D10.6 (Data Management Plan, DMP). The External Advisory Board (EAB) members and the General Assembly (GA) of partners agreed on the CDEP general principles communicated during the kick-off meeting hold in Canary Island, Spain, on 28-31 August 2017. Each WP leader appointed a reference person to act as conveyor of data and information to and from the CDEP. At each yearly Project meeting, the partners evaluated the progress in relation to milestones, the main messages to be conveyed and the status of the tasks performed, together with the challenges and barriers encountered, the status and timing of dissemination and communication actions, the status of deliverables dissemination, the knowledge generated as relevant to be communicated, and the most appropriate communication routes and target audiences to involve.

Task 10.2 Dissemination Actions. Project websites. Task leader: CSIC, Participants: all.

The MUSA logo (see first page, Del. 10.1), produced by SacomLab, was adopted by the Consortium through an internal call, after a selection among different images. It is instrumental for Project's identity and is considered as a basic component of the dissemination strategy. It represents the aims and target of MUSA, and has been designed based on a banana leaf profile.

Using the designed logo, a number of templates have been prepared for different types of documents and formats (doc, docx, pdf, ppt etc.), allowing easy recognition of MUSA in a uniform way. All templates are available for use by the Consortium partners.

To inform a widest audience about progress, major achievements and events related to the MUSA project, and to increase its visibility, dissemination materials include a *leaflet* with main Project details and partners was distributed in an electronic version, to boost the project visibility and extend related contacts. The leaflet (Fig. 80) was exibited at the REA AgriResearch Conference, in held in Bruxelles on 2-3 May 2018 .

The MUSA project website (http://www.projectmusa.eu/) is up and running and in the process of being populate, including information on project activities posted on the website. Partners agreed that each project participant will write one page notes on activities and progress that will be posted on the webpage.

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Figure 80. The MUSA leafllet.

A link to this site, along with the MUSA logo, is inserted in other web pages as well as repositories, social networking tools (ResearchGate, https://www.researchgate.net/project/Microbial-uptakesfor-sustainable-management-of-major-banana-pests-and-diseases-MUSA); FB (https://www.facebook.com/H2020-Project-MUSA-1735663333397367/) (Fig. 81) and Twitter, https://twitter.com/MusaProject), to disseminate all informations and actions produced by partners, including meetings updates and research advancements. Informations have been updated after meetings and provided along the MUSA time framework. Other secondary domains with the "*musa*" term reference (such as www.musaproject.eu) are reserved, to act as mirror sites or to avoid non-authorized use of the MUSA logo or the Project's name. Partner IITA is carrying out tasks related to domains acquisition and registration, together with implementation of webpages.

Figure 81. MUSA page on Facebook.

Further webpages have been produced by partner Coplaca on its website and Facebook pages (Fig. 82). Coplaca organized a seminar on November 9th, 2018 in Tenerife about the pest and diseases considered in MUSA, with related press and television references, as well as a permanent COPLACA website.

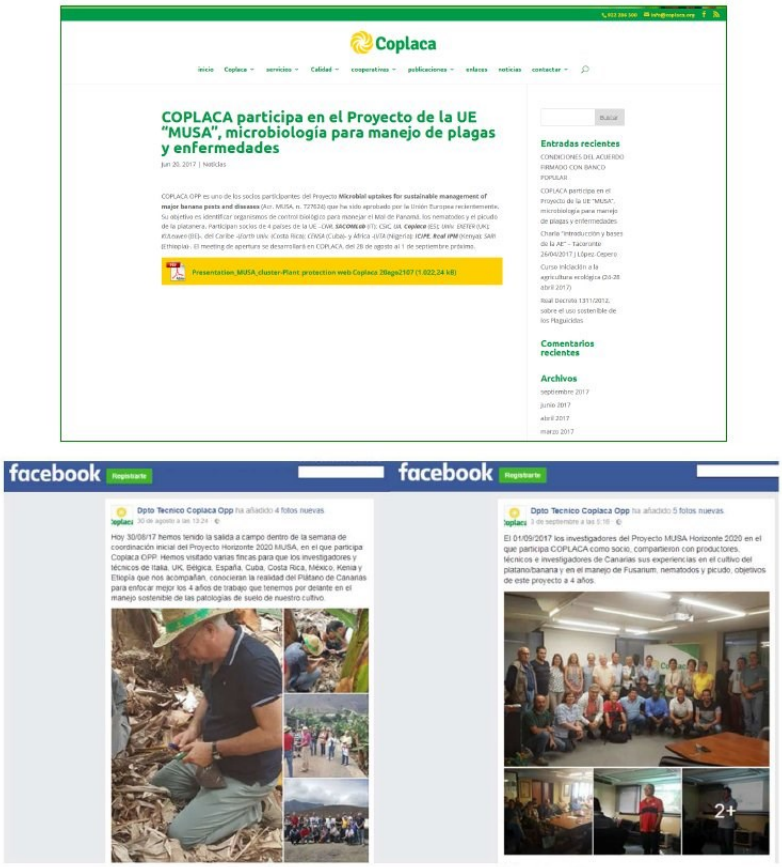

Figure 82. Coplaca web pages related to MUSA activities and kick-off meeting

Partner Coplaca has been involved in dissemination and communication actions, hosting the kick-off meeting and organizing a further workshop with farmers. Further stakeholders, including organizations of producers, scientific institutes and regional organizations have been also involved as external targets of the Communication and Dissemination actions. Coplaca organized a Seminar on BW, PD and PPN on Nov. 9, 2018. The activity was open to all technicians of the banana sector of the Canary Islands, held at the School of Agricultural Training of Tacoronte (Tenerife). It consisted of 6 presentations, since each pathology dealt with its dynamics in the field and its management in the laboratory. The speakers were Carmen Gomez Lama (IAS-CSIC) and Javier López-Cepero (COPLACA), members of the Consortium, and Jessica Perez (agronomist from nematology laboratory of Canarias Explosivos), Dr. Raimundo Cabrera (from University of La Laguna), Dr Ana Piedra Buena (Canary Agrarian Research Institute, also member of the MUSA External Advisory Board committee) and Manuel Moreno, technician with a wide experience in PD banana management. The Seminar was attended by more than 50 stakeholders. After the presentations and discussion a survey was conducted to assess the level and spreading affection of PD, BW and PPN globally in the Canary Islands banana farms, whose results will be

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presented in a future report. Coplaca produced a section on its website (www.coplaca.org) with the most relevant information, news and videos related to MUSA (direct link: www.coplaca.es/proyecto-musa).

Further video records have been produced and made available on the project website and social media to offer a support to MUSA achievements and events, reaching targeted audience such as end users, farmers, students and wider research community. Images of events and interviews were produced during the meetings and/or field activities. A TV service with partners interviews has been produced during the kick-off meeting by MIRAME TV, a regional Canary Islands TV channel (see 7.5.2), and was already broadcasted on 19th Sept. 2017. The link to the TV report can be found at: https://youtu.be/1Ud5_ytd_qE. A press interview has been produced by Coordinator and IITA partners in occasion of the meeting held at Kampala (Uganda) in March 2018.

With all other partners, UA and CNR organized the production of practical abstracts (PA, Del. D10.3 submitted on 7th Nov., 2018), describing the situation of banana pests and diseases in Canary Island. UA directly coordinated and produced three PA in which the situation of PD, BW and PPN affecting banana in Canary Islands was revised. In these documents practical informations for farmers and stakeholders were included, with recommendations and suggestions to manage these pests and diseases.

The titles of the PA (Del. 10.3), produced with the collaboration of all partners, are: 1) *Nematode parasites of banana in Latin America*, 2) *Cultivated bananas and germplasm in Latin America*, 3) *Biology of the banana weevil (Cosmopolites sordidus) in Sub-Saharan Africa*, 4) *Progress by genotyping the enset germplasm*, 5) *Managing nematode parasites of banana and plantain in sub Saharan Africa*, 6) *Fusarium (Panama disease) in banana crops in the Canary Islands: symptoms, damages and management*, 7) *How to manage the banana weevil in the Tropics and* 8) *The black weevil (Cosmopolites sordidus) of the banana tree in the Canary Islands*.

The next annual meeting will be organized by CENSA in Cuba during the planned *International Seminar of Plant and Animal Health* (SISA), scheduled in May 2019. The Consortium agreed in order to have the opportunity to assist SISA activities and expose the Project results in the framework of the event, reinforcing professional and scientific links with the international community of scientists.

Task 10.3 Communication activities. Task leader: IITA Other Participants: all.

All partners but SARI attended the kick-off meeting (Tenerife, Spain). All partners but CSIC, UA, EARTH, CENSA, SacomLab and Coplaca attended the Kampala meeting. All partners but SacomLab and SARI attended the First Annual MUSA Meeting organized by EARTH University, in Costa Rica.

On 20-21th September 2018 several representative members of the Consortium including EAB member prof. R. Manzanilla-Lopez presented a talk at the *International Congress of Biological Control of pests and diseases for sustainable agriculture,* organised by Earth University in Costa Rica.

CSIC personnel presented four communications (two oral and two posters) in different scientific/technical meetings in which activities and some of the results mentioned above were divulgated:

1) "Microorganismos beneficiosos en agro-biotecnología", by Jesús Mercado Blanco (invited speaker), presented at "*Jornada Técnica Agroalimentaria: Innovación como Motor de Negocio en Producción vegetal*", March 13th 2018, IFAPA-Córdoba, Córdoba, Spain:

http://biovegen.org/es/page.cfm?news=210&title=ifapa,-ias-csic,-cajamar-y-biovegen-organizan-la-jornada-innovacion-como-motor-de-negocio-en-produccion-vegetal#.WpkLsWrem71

2) "*Musa acuminata* (cv. Pequeña Enana) roots: an important reservoir of endophytes with potential as biocontrol agents against Panama disease", by Carmen Gómez-Lama Cabanás, Antonio Valverde-Corredor, Javier López Cepero, Jesús Mercado-Blanco (poster presentation at the meeting of COST Action FP1305, Linking belowground biodiversity and ecosystem function in European forests (BioLink) '*Soil biodiversity and European woody agroecosystem*', March 14th-16th 2018, Granada, Spain.

This abstract is also published in the e-book: Grenni P., Fernández-López M., Mercado-Blanco J. (Eds), 2018. Soil biodiversity and European woody agroecosystems. COST Action FP1305 BioLink-Linking belowground biodiversity and ecosystem function in European forests, Proceedings of 2018 Annual Meeting, Granada, 2018, ISBN 978-88-97655-03-9 https://granada-en.congresoseci.com/biolink_2018/abstractbook_biolink_granadauv .

3) "Fusarium en el laboratorio", by Carmen Gómez-Lama Cabanás (invited speaker), presented at "*Jornada formativa*: *tres problemas fitopatológicos edáficos de la platanera,* Fusarium *(Mal de Panamá), los nematodos y el picudo*", Nov. 8th, 2018, Escuela de Capacitación Agraria de Tacoronte, Tenerife (ES), http://coplaca.es/2018/11/14/jornada-divulgativa -proyecto-musa-8-noviembre-2018/ .

4) "La raíz de *Musa acuminata* Colla (cv. Pequeña Enana): un importante reservorio de endófitos con potencial como agentes de control biológico contra el Mal de Panamá", by Carmen Gómez-Lama Cabanás, Antonio Valverde-Corredor, Javier López Cepero, Jesús Mercado-Blanco (poster presentation), presented at *XIX Congreso de la Sociedad Española de Fitopatología*, Toledo, Spain Oct. 8-10, 2018: http://www.congresosef2018.es/librodeabstracts delxixcongresodelasociedadespaaoladefitopatologia .

Finally, activities aiming to the correct internal management and functioning of the research performed at IAS-CSIC were scheduled. These activities mainly consisted on monthly briefings among components of the working team. Communication among other partners (particularly partners 4, 1 and 3) was kept along this period when needed, aiming to coordinate research activities and sampling schedules, as well as to distribute biological material.

IITA organized the MUSA meeting and the following *Banana workshop* held in Kampala, Uganda, on March 27-29, 2018. The meeting was attended by most project participants to assess progress, while additional participants and local stakeholders contributed to the Banana Workshop for communication to project participants about banana agriculture, agronomy, breeding, economics and trade aspects in the region. In addition, participants visited the MUSA field sites and research facilities in Uganda. The project meeting was well covered in the news as well as in the ONTA Newsletter (see the clips below).

– The Observer Newspaper: http://observer.ug/business/57349-researchers-discuss-how-to-biologically-fight-banana-disease.html. Banana farmers blamed for the low banana production in the country March 28, 2018.
– On Television https://urbantv.co.ug/banana-farmers-blamed-for-the-low-banana-production-in-the-country/ .
– The radio story aired and posted by RadioFM90 http://radioonefm90.com/researchers-seek-to-control-pests-diseases-in-bananas/ .
– Heading used to the TV story online: Boosting banana production, banana farmers tipped on how to increase production https://www.youtube.com/watch?v=8TXKZhylTbg
– TV: https://youtu.be/7sVnNvu7FIA .
– ONTA (Organization of Nematologists of Tropical America) Newsletter vol. 48, issue 1, pp. 10-12: *MUSA project and Workshop in Kampala*, *Uganda, 27 – 29 March 2018.*
– The MUSA meeting and Biocontrol workshop held at EARTH University, Costa Rica, 17-21 Sept. 2018 attended by IITA with presentations made for the MUSA meeting and the Biocontrol Workshop.

UA participated in several activities presenting results produced in MUSA: on 13th Sep. 2018 a number of conferences were held at *Casa Mediterráneo* (Alicante, Spain, http://casa-mediterraneo.es/) (Fig. 83). Aim was to introduce public institutions and private enterprise managers, as well as graduates from different university degrees in Mediterranean and related climatic zones, to the main lines of research and specialisation that the Master in Analysis and Management of Mediterranean Ecosystems (MAMME) implements. The main goal was to enlarge and consolidate impact through new academic, scientific, technological and socioeconomic interactions.

Dr Luis Vicente Lopez-Llorca and Dr Federico Lopez-Moya attended the *International Congress of Biological Control of pests and diseases for sustaniable agriculture,* organised by Earth University in Costa Rica. Both researchers presented results from MUSA project to broad audience including stakeholders form Costa Rica and other Central America countries, giving two talks:

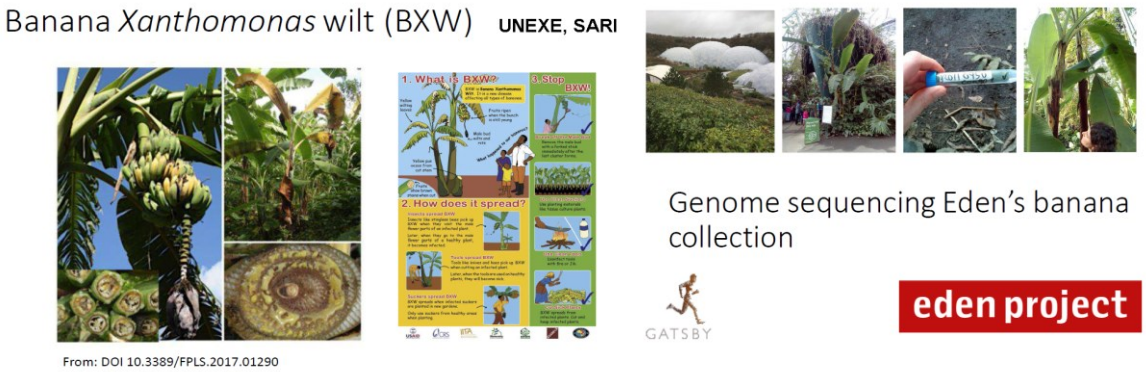
– Dr Luis V. Lopez-Llorca: “Effect of fungal endophyte on plant growth and defenses”
– Dr Federico Lopez-Moya: “Effect of chitosan on fungi and plants”

The talks showed results obtained during the first 18 month of MUSA project.

Currently, UA group is preparing a workshop on the practical mode of action of chitosan on fungi and plants, and contacted several stakeholders interested. The workshop will be open to the Consortium and held by the end of March 2019.

UA partners also submitted a review paper on chitosan biological activity to IJMS journal (https://www.mdpi.com/journal/ijms) which has been reviewed and is currently under manuscript final editorial processing. UA and Coplaca partners are also preparing a review on BW biology and sustainable management by EF, for a Special Issue on fungal-insect interactions organised by *Journal of Fungi* (https://www.mdpi.com/journal/ jof/special_issues/fungal_insect).

Links with other projects



From: DOI 10.3389/FPLS.2017.01290

Completed banana genome sequencing (UNEXE, IITA)

Received as	Source
20011027 *Musa balbisiana*	Eden Project
19982307 *Musa* Sucrier 'Pisang Mas'	Eden Project
20121164 *Musa acuminata* x *balbisiana* 'Calypso'	Eden Project
20121154 *Musa acuminata* ssp. Malaccensis	Eden Project
19990524 *Musa textilis*	Eden Project
20110950 *Musa* 'Congo 2'	Eden Project
20121152 *Musa Eumusa* AB Subgroup Ney Poovan 'Safet Velchi'	Eden Project
20110952 *Musa* 'One Hand Planty'	Eden Project
19992846 *Musa* x *paradisiaca* L.	Eden Project
19990158 *Musa troglodytarum* (F'ei Group) 'Wain'	Eden Project
20121166 *Musa velutina*	Eden Project
Sukali Ndiizi AAB	L. Tripathi, IITA
Pisang Awak (Kayinja) ABB	L. Tripathi, IITA
Gonja Manjaya AAB	L. Tripathi, IITA
Cavendish AAA	L. Tripathi, IITA
Musa balbisiana BB	L. Tripathi, IITA

Two other H2020 running projects at IPSP CNR are PONTE and XF-ACTORS, on invasive pests and *Xylella fastidiosa.*

Task 10.4 Training and formation to boost exploitation of results.

UA PhD student Ms Ana Lozano Soria presented her work "*Isolation and identification of entomopathogenic fungi from banana soils*", showing laboratory results obtained at UA, related to the MUSA project (Fig. 83).

Figure 83. Ms Ana Lozano Soria presenting her work "*Isolation and identification of entomopathogenic fungi from banana soils*" in Casa Mediterraneo, Alicants (ES).

During the European Researchers Night (held at Alicante on Sept. 28th, 2018, https://biblioteca.ua.es/es/cde/noche-europea-de-investigadores-2018.html), the University of Alicante organized activities in which researchers showed their work to the public (Fig. 84). The UA Laboratory of Phytopathology joined with a communication activity on the MUSA projec,t including a poster. UA researchers also showed banana plants and entomopathogenic and nematophagous fungi to general public of all ages including children. People who participated in this activity were Ms Marta Suárez, Ms Ana Lozano and Ms Cristina Mingot (students collaborating with MUSA project).

Figure 84. Images of the Plant Pathology lab stand at UA showing MUSA resources to young attenders to the European Research Nigth, University of Alicante (Sept. 2018).

In March 2018, two Master Thesis topics were announced at KU Leuven to be carried out in the frame of the MUSA project and two students were selected during the academic year 2018-2019. *Topic 1*: "Assessing the effect of plant growth promoting microorganisms in banana (*Musa* spp.): evaluation under nursery and semi-field conditions" (student: Janne Delva). *Topic 2*: "Assessing the effect of plant growth promoting microorganisms in banana (*Musa* spp.): evaluation under greenhouse conditions" (student: Anouk Van den Bergh).

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The Musa field experiments currently in use at EARTH as well as the greenhouse and experimental plots for biological control of PPN have been visited by representatives of several international organization such as:

- Banana producer of Belize in conection with a project financed by the EU and led by IICA and the University of Belize (Fig. 85)
- Delegation of Banana Curators led by Bioversity International
- Delegation of banana producers of Costa Rica

The plots are used by the students of EARTH University for training and as a teaching scenarios for the course of tropical crops, as well as for collecting data on plant growth.

Figure 85. Banana growers from Belize visiting the banana plot of the MUSA project at EARTH University in Costa Rica.

WP 11 Project Management (mths: 1-18).

Task 11.1. Management

The Project Coordinator has been flanked by EAB members prof. A. Bianco, prof. Rosa Manzanilla Lopez and Dr Ana Pietra, that attended the first kick-off meeting in Tenerife. Prof. Manzanilla Lopez significantly followed the project activities and upfdates, contributed to the planning and details of the scheduled work. By attending the Kampala meeting she remained in direct contact with other Consortium members and Coordinator, also joined through email and other communication forms. Thus far no problem occurred among partners, apart of the problem related to the issue of Visa required for some members to attend EU meetings. The information flow is efficient in most cases, and contacts occur almost on a weekly or daily basis.

Task 11.2 Management of innovation and results

The Data Management Plan (DMP) was produced by month 4 by the Consortium. It was set up at kick-off meeting and describes the data management life cycle for all datasets to be collected, processed or generated by the Project. The DMP covers: Open Access, and other similar options for scientific publications (peer-reviewed and others); Intellectual Property Rights for project deliverables subjected to intellectual property;

the data to be collected, processed or generated, including all data, such as: crop yield data and susceptibility to diseases and pests; geo-referenced data for population densities of PPN, BW and prevalence of PD; epidemiologic data and informatins related to EBCAs; data concerning their biology and host interaction; models, modeling data and parameters; sequence data for genes and transcripts; metagenomic data; informations resulting by statistical analyses and taxonomic identifications; microbial species, isolates or populations, in collection or in nature; collection codes and sequence accession codes. A Business Plan was not considered for the MUSA as it aims at satisfying needs of small holder farmers and consumers for more sustainable crops in tropics and subtropics, as well as to produce fruits with higher quality. However, considering marketing perspective the participating SMEs provide their expertise and assistance in the project activities free of a direct economic fall out. However, as they may seek, at the end of the project a possible expansion of market and consequential economic returns, the Consortium will consider if requested a Business Plan to be structured by the participating SMEs, as well as any amendment to GA and Consortium Agreement, if necessary.

The Stakeholders Advisory's Board has not been appointed yet, although a number of partners keep contact with the several stakeholders that follow and provide support.

Task 11.3 Management of dissemination and exploitation activities

Almost all Consortium members participated in the activities and meetings organised by the Consortium to discuss about the practical organisation of the Project. UA partners Dr Luis Vicente Lopez-Llorca and Dr Federico Lopez-Moya attended the kick-off Meeting in Tenerife (September 2017), discussing the EBCAs and germplasm lines used in the experiment. MUSA Consortium decided to include *Musa acuminata* var. Cavendish (Pequeña enana), Gran Enana, Williams and Yangambi km 5. During the kick of meeting we agreed with Partner 4 (Coplaca) to collaborate for soil sampling in banana plantations they supervise. UA, CNR and IAS-CSIC also contacted Cultesa as supplier of banana plants during MUSA project. UA contributed to establish the basis for procedures working with EBCAs, translated in the Milestone M3. After the kick-off of Meeting the Coordinator generated the Kick-off Meeting Report. We contributed to elaborate this document in whcih all the basis for the practical work were established .

UA contributed by generating the 6th month report requested by the Coordinator, with included methodologies implemented until March 2018, basis for EF isolation and conservation (WP2). UA and CSIC also established methods to work with banana plants and EBCAs and Pc inoculation. These methods and results were included in of WP3.

Partners of SARI could attend the Kampala Meeting organized by IITA and by this way start a number of cooperative activities and links with other partners.

Eleven MUSA partners participated in the 1st Annual Meeting held at EARTH University (Partner 13) in Costa Rica. During this meeting the 1st year results were presented, and discussing with other partners and stakeholders. UA agreed with Dr. Luis Pocasangre from Earth University to develop a stay in Alicante University group

for 2019, and agreed with Coordinator to use chitosan as a bioactive compound in combination with EBCAs (WP3 task 3.3).

EARTH activities included: Coordinating and executing of all research activities related to project such as: field sampling, isolation of endophytes, screening of endophyes in the lab, testing endophytes in greenhouse, reproduction of nematodes in the lab, establisment the fiel plot. Coordination of expenditure related to the project. Presenting reports to the general coordinators. Preparing practical abstracts for dissemination of the activities of the project. Organizing the annual meeting in Costa Rica in September 2018, that almost all the partners of the project attended (Fig. 86)

Figure 86. The MUSA Consortium during the annual meeting held at EARTH Campus in Costa Rica, on September 17-19, 2018.

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