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

MUSA activities proceeded during months 19-36 developing the planned work on endophytes and biocontrol agents (EBCAs), with laboratory, greenhouse and field trials. Aim of the work has been to set up with stakeholders a number of innovative management approaches for pests and diseases of banana and enset (*Ensete ventricosum*). The threats considered are: plant parasitic nematodes (PPN), Fusarium wilt (FW) caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) and banana weevil (BW, *Cosmopolites sordidus*). The Action is carried out in the three world regions interested by the Project (EU, in particular Canary Islands, Central America and sub Saharan Africa).

The activities described in WP 2, 3 and 4 were completed and terminated, with knowledge produced on several biological resources, including sequenced and annotated EBCAs genomes (*Pochonia chlamydosporia*) and enset genome annotation. Isolates were identified and characterized, with many resulting suitable for exploitation in the other WPs. Useful traits (prevalence, effectiveness, performance, reaction to bioactive compounds) were also measured in comparison with available commercial bioformulations. A group of isolates showed a potential for inclusion in new bioformulations, tailored vs one or more Foc races (including the production of Synthetic Communities). Similarly, effective strains of *Trichoderma* spp. or *P. chlamydosporia* appeared ready for a pre-commercial development phase, for use in PPN biomanagement. They were tested successfully in different and independent greenhouse or field conditions vs *Radopholus similis* or *Meloidogyne* spp. The fungus *P. chlamydosporia* was also found to produce volatile organic componds (VOCs), active on BW behaviour either as repellent or attractants (UA patent). The studies on the interactions of resistant/tolerant banana *cvs* with EBCAs or pests/diesases were completed in WP3 and 4, allowing the identification of genes selectively expressed in the interaction with one or more EBCAs, either in roots and leaves. New information has been produced on the banana roots and corm microbiomes, in the three regions interested by the Project. Parameters for bio-formulation of most performant EBCAs (i.e. isolate PICF7) were re-evaluated in WP5 and validated. Mass production of *P. chlamydosporia* var. *catenulata*, *T. asperellum* (Ta. 13) and entomopathogenic nematodes (EPN) such as *Heterorhabditis amazonensis* strain HC-1 through liquid fermentations were also deveploped, with a submerged liquid fermentation process for mass production of *P. chlamydosporia* and *Beauveria bassiana* isolates. Field trials were carried out and/or initiated in WP6 and WP7 planning epidemiological data collection forecasted in WP8. Socio-economic factors underpinning successful biomanagement of PPN and BW were investigted in WP9, by means of interviews with farmers, questionnaires, and application of SWOT analyses. The Consortium also carried out several communication and dissemination activities, as planned in WP10.

Considering the complexity of the Project, developed in three world regions targeting three problems (as described in Annex 1), the Consortium identified common ways to keep a consistency of results and fallouts, facing complexity and dispersion risks. These are: *i)* the selection, characterization and testing in controlled, and then in field conditions, of most promising organisms for each targeted problem (including comparison with products/isolates already in commerce); *ii)* the study of local banana clones most commonly used in each area; *iii)* the application of advanced molecular approches (metagenomics, transcriptomics, genomics) allowing a standardization and replication of the information produced; *iv)* the demonstration and dissemination to local stakeholders of results to achieve impact, integrated by *v)* communication initiatives developed in cooperation among beneficiaries in each region interested by the Action.

Progress has been made in the work applied for PPN, BW and Foc sustainable management. A broad amount of data has been produced and several assays set up on isolates, either deposited in the national/regional collections produced during the first Project period, or made available by Consortium partners (through bilateral agreements on exchange of materials and background information, as stated in the Project Consortium Agreement). New EBCAs species and isolates have been identified as deserving further attention for subsequent exploitation, also in comparison with available products. Some isolates already in commercial bioformulations of industrial stakeholders (Real IPM, MSBIO) were also tested and applied for comparative purposes in greenhouse or field trials. Focus and priority were given to endophytes and biocontrol agents such as *Pochonia chlamydosporia* and *Trichoderma asperellum*, as well as to a number of *Bacillus* spp. and strains of *Pseudomonas* spp., including a soil and rhizosphere competent isolate of *P. simiae* (PICF7 from IAS-CSIC).

Progress has been made in the identification of isolates or pathogens, through the production of sequence data, deposited at international Open Access databases (i.e. NCBI). For one EBCA, *P. chlamydosporia*, the whole genome sequence has been re-assembled and its re-annotation has been completed and deposited in NCBI (highlighted as a Project Result). Similarly, for enset, a partial annotation of the genome sequence has been deposited.

A further advancement has been the production of data on the effect of bioactive compounds, i.e. chitosan or essential oils, and on their interaction with EBCAs, produced in controlled conditions. A patent has been also deposited by UA on volatile organic compounds (VOCs) released by *P. chlamydosporia*, affecting the behaviour of BW (highlighted as a Project Result).

Apart of the numerous laboratory and greenhouses results, field data have been obtained in each region interested by the Action. They proceed from demonstration trials showing i.e. effective management of the burrowing nematode *R. similis* by *T. asperellum* ENDO_4 in Costa Rica (highlighted as a Project Result), or from demonstration fields set up by Real IPM in Kenya and IITA in Uganda. Field trials with the EPN HC-1 have been carried out in Cuba vs BW.

Soil and rhizosphere metagenomic studies on bacteria and fungi have been completed, and related sequence databases have been deposited in NCBI (highlighted as a Project Result). Metabarcoding data were produced from banana crops sampled under different stress and climate or agronomic conditions, in each region. Bioinformatic analyses showed that several extra-farm factors affect the composition and structure of the microbiomes investigated. Data have been also produced by SARI on enset in Ethiopia, showing the occurrence in this crop of several concomitant diseases in the sampled regions, including *Xanthomonas* bacterial wilt. This region was minimally explored thus far, and few data were made available on the phytosanitary status of crops like enset, which has a key role in local food security. Samplings also showed occurrence of nematode taxa not previously reported from the region or the rhizosphere of enset. Distribution and characterization of Fusarium wilt isolates produced in Ethiopia revealed a broad biodiversity range, and likely the occurrence of a complex of species or *formae specialis*.

Several initiatives and events have been organized to achieve impact through the dissemination of results and communication of MUSA Project activities. They targeted the scientific community, as well as interested stakeholders and the society at large, in particular addressing low and high grade students. Several MsSc or PhD students have been involved in research work at UA, IITA, CENSA and EARTH. The Project website, Twitter, Facebook and other social platforms (YouTube) have been used to communicate informations at large, together with press articles and announcements provided on local newspapers or by attending local communication initiatives. During the period covered by this report, two Project Annual

meetings have been organized by partners CENSA in Cuba (May 2019) and by Real IPM with ICIPE in Kenya (Nairobi, February 2020). The meetings were integrated by workshops, seminars, and presentations given at national and international congresses (i.e. SISA 2018 in Cuba, ONTA 2019 Annual meeting in Costa Rica and others), or locally organized by partners UA, IITA, Real IPM and IAS-CSIC.

As recommended by the previous evaluation panel, stakeholders have been addressed by selecting and implementing a Stakeholders Advisory Board, led by Coplaca, whose first initiative has been to organize with EARTH an International Conference planned with the participation of SMEs, service providers (i.e. Agribiotecnologias nursery in Costa Rica), national private organisms i.e. Coplaca (Spain) or CORBANA (Costa Rica) and international agencies (including representatives of FAO and of the Ministery of Agriculture of the Republic of Ecuador) They were integrated by organizations of plant and banana producers, together with many other interested stakeholders. The event was planned for April 2020 in Costa Rica, and was locally organized at EARTH University Campus in Limón. Unfortunately, due to the SARS-Cov-2 pandemic and related emergency regulations, the conference had to be cancelled. It was, however, replaced by a web conference (which is not detailed in this report being part of the last year activities), organized by UA and the same stakeholders, held on June 26, 2020. The conference attained an international audience (with an average of 66-70 connections from all over the world) and attracted the active participation of several interested stakeholders (*http://coplaca.es/2020/07/27/reunion-virtual-de-stakeholders-26-jun-2020/*).

Similarly, an international workshop was organized by UA and CNR to set up collaborations and common dissemination and communication events linking with other Projects, either funded by the EU Programmes or not. The workshop was scheduled for the end of April 2020 in Alicante (Spain) and was locally planned by UA with *Casa Mediterraneo*, a regional non-governamental agency. Also in this case the event had to be postponed, likely to April 2021, due to the actual pandemy emergency.

Overview of results

Achievements produced include the construction of national microbial collections and the identification of new microorganisms/isolates exploitable as endophytes and biocontrol agents of all the pests/disease targeted. The discovery of specific components in *P. chlamydosporia* VOCs with an active effect on BW offers a new perspective in management of this pest and in trap applications. Similarly, testing bioactive compounds such as chitosan showed benefits for crop protection, and direct effects on Foc and PPN. New knowledge has been acquired on the biology of beneficial fungi (*Trichoderma* spp. and *P. chlamydosporia*), bacteria (*Pseudomonas*, *Bacillus* spp.), as well as on PPN, BW and Foc.

Genome and sequence data have been produced on *P. chlamydosporia*, banana cvs or *Pseudomonas* spp. genes expressed in the tri-trophic interactions with roots and EBCAs, as well as on enset. Data have been produced on soil and crps microbiome profiles, as well as on the effect of extra-farm factors involved in production. Results include innovative methods and procedures for inoculation of plants with endophytes and biocontrol agents, molecular tools for the identification and detection of strains and populations, and new industrial know-how for mass production of a number of EBCAs and EPN.

Models forecasting FW epidemics offer an opportunity to estimate the disease spreading, that is actually alerting several stakeholders and threathening food security, worldwide. This situation is due to the arrival in South America of Foc virulent strain TR4, for which no resistant/tolerant Cavendish clone is available thus far. Know-how and technologies produced

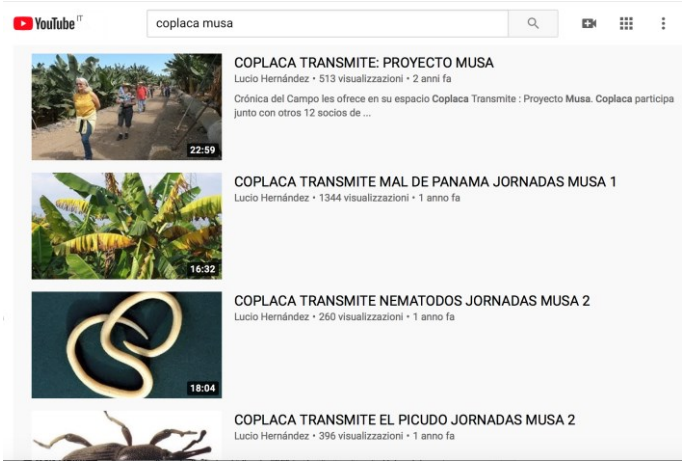
have been transferred to different stakeholders, by means of cooperative research work and through different communication tools and channels.

Summary of deliverables and milestones

The following milestones have been checked in reference to the related deliverables, both inoccasin of the Consortium periodic meetings held during the second Project timeframe, or through bilateral or multilateral meetings.

Verified milestones	*Deliverables*	*WP*
MS11 (by mth 24, Resp. CENSA): Best impact assessment approaches - *Description:* Identification of the most appropriate actions to be deployed and data to be collected to evaluate: the impact of the EBCAs selected on yields; their socio-economic value; the structure of the guidelines to be communicated to improve biomanagement. *Cheking milestone achievements* (accomplished during the Second Annual Meeting, May 2019, Varadero, Cuba): - to evaluate EBCAs impact, the Consortium identified field efficacy through the collection of quantitative data from assays on pests/Foc, such as population density (BW and PPN), EBCAs and Foc prevalence/incidence, to be determined focusing only on best performing EBCAs, as resulting from previous controlled assays. Tested isolates are: *Trichoderma asperellum* ENDO_4 (for PPN); *Pochonia chlamydosporia* (Tenerife isolate), isolates Pc 123 as PGP; *H. amazonensis* for BW; *Pseudomonas* spp. and *Bacillus licheniformis* (Foc management). - Evaluation of socio-economic impact: measuring yields from field assays in the three regions of the Actions; potential for production of "green" bananas for EU markets (feedback to be received through a stakeholders' conference). - Communication guidelines: identifying targets such as producers and other audiences (commercialization, distribution, consumers) interested in organic banana management and products (addressed for feedback through a stakeholders conference).	D9.1 EBCAs impact data, market receptivity and socio- economic data report, with guidelines for crop biomanagement	WP9
MS12 (by mth 30, Resp. CENSA): Programming farming schools - *Description.* Planning of the programs and activities to be carried out for the farming schools (FS) completed, and made available on the web site; identification of teachers and speakers; calendar	D9.2 Farming school activities on EBCAs and IPM	WP9

with sites and dates defined; organizations of farmers contacted and involved in the decision process. *Checking milestone achievements* (during Third Annual Meeting, Feb. 2020, Nairobi, Kenya)*:* - FS general goals have been elaborated with partners EARTH and CENSA as concerns farmers' audience, needs and knowledge to be transferred. FS are planned for November 2020 and will be announced on the MUSA web sites by mid September, with calendars and dates. Locations: Costa Rica and Cuba. Stakeholder CORBANA (http://www.corbana.co.cr) has been involved by EARTH (prof. Pocasangre) in Costa Rica. Activities are not yet confirmed, depending on the local evolution of the Covid-19 epidemic. - A similar meeting (due to legal advice it could not be announced as a "school") with farmers for presentation and transfer of Project's results is scheduled for Canary Islands during October-November 2020 by Coplaca, in collaboration with partners CNR, UA, IAS-CSIC and KUL. Focus is on soil microbiology and BW/Fusarium management. Activities are not yet confirmed, depending on the local evolution of the Covid-19 epidemic.		
MS14 (by mth 24, Resp. IAS-CSIC): Mid-term communication activities achievements - *Description.* The communications activities forecasted will be periodically checked. The milestone corresponds to at least three of the following significant achievements reached: web pages; scientific articles; workshops; seminars and formations actions; started PhD courses at IITA and UA; short visiting scientific missions. *Cheking milestone achievements* (accomplished during Second and Third Annual Meetings): - additional web pages of MUSA implemented on the Coplaca web site *http://coplaca.es/category/proyecto-musa/* apart of the official site *http://www.projectmusa.eu/wp/*. FB page: *https://www.facebook.com/H2020-Project-MUSA-1735663333397367/*. Twitter page: *https://twitter.com/musaproject* - Five scientific papers have been published and four more are in the process of publication, with at least four congress presentations given (see WP10 section). - One workshop organized on chitosan application by UA.	D10.4 Practice abstracts (second and third series)	WP10

- PhD students involved in MUSA work at UA (3), EARTH (4) and IITA (1). One short-term visiting scientist (Ing. Vaniert Ventura from CENSA) spent one month at Istituto per la Protezione Sostenibile delle Piante of CNR, Sede di Torino (Italy, supervisor Dra. Raffaella Balestrini).		
MS15: Achieved communication activities following EAB guidelines - *Description.* Guidelines provided by the EAB members examined by the Consortium; a database of communications activities produced, indicating those already achieved or planned. *Cheking milestone achievements* (during Second and Third Annual Meetings): - Guidelines by EAB members were received after both periodic meetings. - Communication activities achieved: articles on newspapers, videos and web pages, attending public initiatives on science (i.e. Researchers' Night), presentation on SciSoc Newsletters (i.e. ONTA, 2020), leaflets, production of a MUSA 2020 calendar (by Coplaca), communication articles published on local newspapers and press articles (IT, ES, Kenya, Nigeria), radio TV interviews. Database of communication activities drafted, not yet published. - videos available on Coplaca YouTube channel (https://www.youtube.com/watch?v=1Ud5_ytd_qE)  - Communication activities planned: a comics for children has been drafted and will be published in different languages (Spanish, English, Swaili) by November 2020, dealing with biological control of BW and good practices for Foc prevention (researchers involved: L. Stavolone, A. Ciancio, CNR; J. Cepero, Coplaca; G. Muhuku, IITA). - Dissemination activities have been planned with	D10.4 Practice abstract (second series) D10.2 Web pages, scientific publications, presentations and other communication activities, including seminars, workshops and formation actions	WP10 (by mth 36, Resp. CNR; by mth 48, Resp.IAS-CSIC)

Coplaca towards the Canary Islands network of farmers, concerning results of the field assays carried out in Canary Islands (topics: soil microbiology, FOC prevalence, nematodes, BW spread) to disseminate best management options by the Consortium. The planned meetings are not yet confirmed, depending on the local evolution of the Covid-19 epidemic, considering alternative options via web.		

Considering the opinions expressed by the previous evaluation panel on the activities carried out during the first reporting period (months 1-18), the following, additional verifications have been made during the two last Project Annual meetings, in reference to the cited milestones.

Verified milestones	*Verifications, updates or partners reviews*	*Applied to WP*
MS6 - Planning for BW management	Planned assays *vs* BW were carried out in controlled (greenhouse /laboratory) and field conditions: a) VOCs from *P. chlamydosporia* were tested by UA for effect on BW mobility b) isolates of the entomopathogenic nematode (EPN) *Heterorhabditis amazonensis* were tested by CENSA c) *Beauveria* isolates (ICIPE 648, ICIPE 660 and ICIPE 273) were tested by IITA and ICIPE d) isolates V5w2 and WA were tested by IITA *vs* BW, after inoculation of Tissue Cultured (TC) banana plantlets e) Gene expression data were produced by KUL from five banana genotypes in greenhouse: 'Foconah' ITC0649 (AAB, Pome subgroup), 'Yangambi km 5' ITC1123 (AAA, Ibota Bota subgroup), 'Gros Michel' ITC1122 (AAA, Gros Michel subgroup), 'Petite Naine' (AAA, Cavendish subgroup) and 'Enzirabahima' ITC1354 (AAAh, Mutika/Lujugira subgroup), based on reported data on resistance and susceptibility to PPN, Foc and BW. f) Further assays were carried out by CENSA in controlled conditions for	WP 2 to 4

	effect (and interactions) of bioactive essential oils with EBCAs antagonistic to BW (Task 3.3)	
MS7 - Implementing field assays	As field assays in SSA and Caribbean regions require usually a longer preparation time for planning with farmers, their work had to begun earlier. Verified through: a) Field trials were conducted to test ENDO_4 plant colonization and biocontrol of PPN (see WP6). Field evaluation of endophytes was performed to validate *R. similis* biocontrol and increase yields (task 7.1) b) Four field studies completed on banana soil and plants metagenomics in: Tenerife, Costa Rica, Uganda and Tanzania c) Field trial running in Guayubín, (Monte Cristi, Dominican Republic), see Task 6.1.1: Effect of commercial Plant Growth Promoting Rhizobacteria (PGPR) on banana growth under open field conditions d) Field trials with *Heterorhabditis* sp. carried out in Cuba vs BW e) IITA assessed field performance of TC banana plants following single and dual inoculation of fungal endophytes *B. bassiana* (isolate WA) and non-pathogenic *F. oxysporum* (isolate V5w2). Real IPM started field validation for best fungal isolates *vs R. similis*.	WP 6 - 7
MS8 - Planning epidemiological data collection	Tangible, verifiable progress was checked during Second and Third Annual meetings and/or by direct communication with partners (i.e. SARI): a) Regional data on PPN and Foc were produced in Ethiopia on banana and enset, Foc isolates were characterized and PPN identified at genus level	WP 2, 3, 8

	b) Areas were identified and data were produced on PPN species in banana fields in Canary Islands c) Foc epidemiology data produced in Canary Islands and identification of races by means of molecular tools (see Task 8.2)	
MS9 - Implementing analtical approaches	SWOT analysis was developed by partners and detailed report is included in Del. 9.1. Efficacy of EBCAs experimentally evaluated using quantitative approaches: population densities of PPN and BW, Foc growth rates (SARI assays), prevalence data, gene expression data for endophytes in banana roots. Molecular approaches applied for identification of genes expressed in banana-EBCAs interactions. Analysis of soil, root and plant microbiomes by means of NGS metagenomic approaches and bioinformatic analyses.	WP 7
MS10 - Identifying best sampling methods and protocols.	Sampling and survey works have already been described in the First Report. Data therein presented are now integrated with those given in this present report, including reference langitude and latitude data of sampled farms in Canary Islands, and data on location (with other parameters) of sampled farms in Cuba and Ethiopia. Data are kept in replicated SSD and devices in beneficiary labs. Climate models proposed by UNEXE at the First year meeting in Costa Rica consider a stochastic spatio-temporal approach projecting Foc dispersal upon its introduction into tropical regions. They comprise a set of influential spread and survival parameters, including an empirically-derived temperature response function assigning infection risk based on climatic suitability (see task 8.1). Raw and model data are kept in replicated	WP8

	SSD and hardware devices in beneficiary labs. The dregree of bioprospecting diversity and disparity were already considered in Annex 1, in relation to the regional diversity of climate, soil, and farming systems, among the different nationalities represented in the Project. This diversity valorized the data and knowledge produced in such a range of agronomic and social realities.	

Summary of exploitable results

Four results have been highlighted on ECAS, as susceptible of further exploitation:

- use of isolate ENDO_4 of the endophyte and biocontrol fungus *Trichoderma asperellum* for management of *Radopholus similis* on banana in the Caribbean region.
- exploitation of one or more specific components of VOCs produced by the endophyte and biocontrol fungus *Pochonia chlamydosporia*, in traps for BW management.
- re-assembled and re-annotated whole genome sequence of *P. chlamydosporia*, deposited in NCBI, susceptible of exploitation in further studies on the fungus diversity, on production of secondary metabolites and active compounds, i.e. VOCs or antibiotics, enzymes, as well as in root endophytism and PPN eggs parasitism, and industrial microbiology at large.
- soil and rhizosphere metagenomic sequence databases produced on bacteria and fungi from three world regions (deposited as OA in NCBI), susceptible of further exploitation in microbial genetic diversity and comparative studies, soil ecology and microbiology, bacterial and fungal taxonomy, microbial epidemiology.

Further exploitable results are listed as follows.

- Data on the occurrence, diversity and spread of microbial species and pathogens.
- Annotated sequenced genome of enset (deposited as OA in NCBI), susceptible of further exploitation in plant molecular biology and physiology studies, enset biodiversity, crop protection and characterization, germplasm collection and conservation.
- Innovative methods and procedures for inoculation of plants with endophytes and biocontrol agents.
- Molecular toolboxes for advanced identification and detection of microbial strains and populations, with PCR and other related approaches, including primers and target sequence information, exploitable for detection.
- Collections of microorganisms susceptible of commercial exploitation in pest biological control and management, as well as in plant protection and growth promotion, and industrial microbiology at large.
- Know-how on methods and infrastructures required for industrial mass production of EBCAs.

- New knowledge produced on the biology of beneficial fungi and bacteria, as well as on PPN, BW and Foc.
- Models to forecast FW epidemics and effects on yields at different spatial levels, suitable of exploitation by different stakeholders, ranging from farmers' organizations to regional authorities and policy makers.
- OA scientific publications and data.

1.1 Objectives

As described in the First Report, MUSA aims at practical tools for management of FW, BW and PPN of banana and enset. The Action is developed in three world regions: Canary Islands, Sub Saharan Africa and Central America. The enset crop is included through the activities of partner SARI. 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 achieving sustainable and environment-friendly IPM methods, improving the resilience of crops, thus reducing costs and pesticide use.

The Consortium integrates stakeholders such as organizations of producer and SMEs (MSBIO replaced SacomLab that terminated on Dec. 31, 2018; the tasks and resources were transferred to MSBIO as indicated in GA amendment AMD-727624-20). Partners seek innovative IPM products based on novel bioformulations, considering economic sustainability and crop resilience towards climate change. Direct benefits are 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 and/or thermally treated suckers).

The focus is on products and processes improving the efficacy of IPM, as well as the profitability of new methods/resources for stakeholders. Partners target organic as well as conventional farmers, involved or not in trade. In the EU, producers aim at improving the quality and management of crops, reducing pesticide use and sustaining the commerce 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

In more detail, MUSA aims at: *i)* 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) Fusarium wilt (Foc) 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

Specific objectives: 1, 2.1

A large part of WP2 work was performed during the previous reporting period, with Task 2.4 remaining for isolates/plants interaction studies. It was completed during this second evaluation period, through the production of data on EBCAs biology. IAS-CSIC isolated and selected additional EBCAs to develop IPM strategies in WP3 and WP6 based on their exploitation in bioformulations and/or field applications. The isolation task was integrated by production of sequence data for molecular identification and by selection of more promising isolates. The bacterial strains isolated by IAS-CSIC in Canary Islands with Coplaca were characterized by UA to assess their growth kinetics in presence of chitosan and for use in WP3 assays, whereas VOCs released by *P. chlamydosporia* were tested for use against BW. Most isolates showed a satisfactory level of campatibility with banana plants in controlled conditions. A number of selected EBCAs were evaluated *vs* PPN by EARTH, CENSA, and MSBIO. Moreover, new isolates of *Bacillus* spp. were tested by MSBIO for antagonism *vs Fusarium* spp. The isolates and data produced were used in the following WPs, and compared with available bioformulations. Finally, further data were produced by partner SARI during prospections developed in Ethiopia during the previous reporting period, but not yet completed. Deliverables D2.1 and D2.2 were produced by month 20.

Specific objectives: 2.1, 2.2, 3.1

Biocontrol and management trials. Most promising organisms were investigated in WP3 by means of advanced molecular biology methods applied *in planta*, to produce knowledge on their interactions with target pests/pathogens. IAS-CSIC improved the molecular detection both of selected EBCAs and of the pest/disease causal agents, to be applied in ecology and epidemiology field work in WP6. Analyses of culturable and non-culturable banana root endosphere microbiota were performed from three Canary Islands (Tenerife, La Gomera and La Palma). This work was integrated by a metagenomic study carried out by CNR with Coplaca, to investigate the effect of plant and other factors (latitude, soil structure, PPN), on banana microbiome profiles.

Evaluation of banana roots endophytic colonisation by *P. chlamydosporia* was performed using molecular and physiological analyses of the plant defense response. UA team also investigated the use of chitosan, a biopolymer with potential in organic management, checking its effect on gene expression of *P. chlamydosporia* and on root knot nematodes (RKN). UA team also analyzed the effect of chitosan on banana rhizodeposition and soil, analysing its microbiota, with additional greenhouse tests. UA evaluated the *P. chlamydosporia* strain origin in banana growth promotion, and applied a PCR method to estimate rhizosphere colonization and endophytism, showing that chitosan treatments enhanced root colonization by the fungus. These trials were integrated by assays on the insecticidal and behavioral effects of some bioactive plant essential oils on BW, carried out by CENSA, studying the chemical composition of oils for management of BW with EPN and their nematicidal activity *vs Meloidogyne incognita*. Further studies were conducted on the compatibility of EPN, *P. chlamydosporia* and *T. asperellum* with essentials oils, to improve of shelf life of bioformulations with *P. chlamydosporia* and test the effect of different carriers on EPNs.

Two biocontrol trials vs Foc tropical race 4 (TR4) were conducted by CNR with a modified Synthetic Community (SC 1.2) on banana cv. Grand Enana. Moreover, two substrates used in banana nurseries of Costa Rica, provided by a national stakeholder, were evaluated for their suppressiveness *vs* FocTR4 with NGS methods. Greenhouse experiments were carried out by IITA with tissue cultured (TC) banana plantlets *cv* Sukari Ndizi and Mchare in Uganda and Tanzania respectively, both susceptible to Foc R1, testing *in vivo* bacterial endophytes suppressing Foc growth. IITA tested the efficacy of *T. asperellum* isolate TRC 900 (commercial product provided by Real IPM) against *R. similis* penetration in banana TC plants. Assessment of *in vitro* antagonism of three bacteria (isolates 1HR-B1, 1HR-B3 & 1DR-B4) and two fungi (T34 and TRC900) against *R. similis* were also completed. A screenhouse experiment was set up to assess efficacy of bacterial (1HR-B1, 1HR-B3 and 1DR-B4) and fungal (T34 and TRC 900) isolates against *R. similis* in TC plantlets, to establish optimal inoculation dose and time of application. Deliverable D3.1 was produced by month 24.

Specific objective: 3.1

As scheduled, IAS-CSIC selected strains for plant growth promotion assays to be conducted in WP4 by other partners. UA set-up a method for mass production of EBCAs (*P. chlamydosporia*, *B. bassiana* and *M. anisopliae*) based on growth on a solid substrate. The method allows growth and sporulation of the isolates and was used to evaluate VOCs production. KUL optimized protocols for greenhouse, nursery and field inoculation of banana plants with different EBCAs and/or plant growth promoting microorganisms (PGPM), recording growth data in plants treated with different PGPM under greenhouse, nursery and open-field conditions.

Gene studies involved qRT-PCR for amplification of *Musa* spp. paralogs in specific gene families, and evaluation of global gene expression changes associated to plant growth promotion and biotic stress resistance. DNA-based studies were carried out from the rhizosphere of plants inoculated with *T. asperellum* in greenhouse. IITA identified bacterial and fungal communities associated to healthy and diseased plants, located in banana farms subjected to Foc. The team conducted transcriptome analyses on 9 pairs of banana plants, each pair including FW symptomatic and asymptomatic plants, of the same variety, grown next to each other. IITA also carried out metagenomic analyses on roots from FW symptomatic/ asymptomatic plants, to understand the effect of infection on microbiome profiles. Further metagenomic studies were carried out by CNR in Tenerife (in collaboration with Coplaca), identifying factors affecting the root microbiome and related to latitude, PPN and rhizosphere, as compared to soil from adjacent controls without roots. A metagenomic study was also carried

out by CNR in Costa Rica in collaboration with EARTH, in experimental fields subjected to different cropping systems, including fallow and organic management. Deliverables D4.1 and D4.2 were produced by months 34 and 36, respectively.

Specific objective: 4

In WP5 progress was made in the development of fermentation technologies applied to most significant EBCAs. Parameters for bioformulation of *Pseudomonas simiae* isolate PICF7 were re-evaluated and validated. Mass production studies were carried out for *P. chlamydosporia* var. *catenulata*, *T. asperellum* (Ta. 13) and for HC-1 in liquid fermentation. MSBIO proceeded in the development of a submerged liquid fermentation process, followed by a solid state fermentation, for *P. chlamydosporia* and *B. bassiana*. This technology was integrated by UA with a method for mass production of *P. chlamydosporia*, *B. bassiana* and *M. anisopliae*, based on a solid substrate. Deliverable D5.1 was produced by month 30.

Specific objective: 5

Progress was also made by testing a number of isolates in field trials. In WP6 IITA assessed field performance of TC banana plants following single and dual inoculation with an endophytic *B. bassiana* (isolate WA) and a non-pathogenic *Fusarium oxysporum* isolate (V5w2). Plant flowering and yield data were collected on a weekly basis, in addition to data on PPN and BW infestation levels. Field assays were also carried out by KUL using PGPR in the Dominican Republic, by EARTH with *T. asperellum* ENDO_5 in Costa Rica and by Real IPM in Kenya. Deliverables D6.1 and D6.2 were produced by months 26 and 36, respectively.

Specific objectives: 5, 6

Validation of PICF7 in liquid bio-formulation required field trials in different regions and environments, using different R4 susceptible banana cultivars. Experiments in WP7 under field conditions are crucial to confirm its effectiveness both as a preventive and a palliative control measure. Local restrictions on Canary Islands made it impossible to apply non-endemic microorganisms to cultivated fields. Therefore, formulation and mass production of the most promising strain, PICF7, in banana farms infested with Foc at Canary Islands could not be performed so far. In the meantime, risk assessment on EBCAs use and SWOT analyses were accomplished. In WP7 UA tested, in field conditions and in collaboration with Coplaca, repellent compounds C1, C2, C5 and C7 isolated from previous work. A prototype of VOC delivery system was designed and included into traps in Canary Islands fields, testing by capture their effect on BW. Deliverable D7.1 was produced by month 30.

Specific objectives: 6, 7

For application of modeling tools in WP8, CSIC developed molecular approaches for Foc qualitative detection. CENSA studied epidemiologic parameters for FW, BW and PPN, and sampling protocols for EPN isolation. Cooperativew work was implemented on metadata and data analysis (D. Bebber from UNEXE in cooperation with CENSA). UNEXE developed a stochastic spatio-temporal model projecting Foc dispersal upon its introduction into tropical regions. The model comprises a set of influential spread and survival parameters for the pathogen, including an empirically-derived temperature response function that assigns infection risk to regions, based on climatic suitability. From several simulations the disease dispersal appeared most rapid in Africa, in comparison to outbreaks in South America and India, which appeared more contained. Further, sensitivity analyses revealed the relative significance of physical and climatic factors on disease spread, highlighting the importance of road and river networks in Foc dispersal. Deliverable 8.1 was produced by month 28.

Specific objective: 8

Social data collection started in WP9 to assess farmers' receptivity to the innovation produced, with SWOT analyses aiming at improving market and social impacts. Activities included planning trainings through Field Farming Schools to foster IPM adoption, in the regions interested by the Action. Dissemination, Communication and Project Management initiatives have been developed (WP10 and WP11), including the amendments requested to Deliverables D1.1 (Ethics and security issues), to the Data Management Plan (Deliv. D10.6) and the Communication, Dissemination and Exploitation Plan (CDEP, Deliv. D10.1). A second set of Practice Abstracts (D10.4) has been produced, describing results achieved by the Consortium and tested on banana crops. Deliverable D10.4 was produced by month 36.

1.2 Explanation of the work carried out per WP

1.2.1 WP 1 - Ethics requirements

In occasion of the two Annual Meetings held within the second evaluation period, the Consortium discussed requirements concerning: *i)* the presentation of the Due Diligence Act (started by IAS CSIC in Spain), *ii)* the obligations deriving by the application of the Nagoya Protocol rules (concerning movement and exploitation of genetic and biological resources among or within States or regions), *iii)* the amendments applied to Deliverable D1.1 (concerning further risks for personnel security and movement of people, privacy issues, toxicity of isolates, quarantine issues, further safety aspects), and *iv)* the decisions adopted and required to improve Gender balance in research environments (access to facilities, travel opportunities, attending Project meetings and national or international congresses, presenting as MUSA representative or speaker, involvement in communication and dissemination actions, publications and authorship, professional progression and stability).

1.2.2 WP 2 - Isolation and selection of Endophytes and Biocontrol Agents (mths 1-26)

Aim of WP2 has been the collection of isolates and their selection and characterization for managament of the pests/disease considered in MUSA, in the different regions of the Project. These activities are hence reported herein by tasks. The activities carried out in the remaining part of this WP mainly concerned the completion of surveys and the characterization of EBCAs for eventul use on banana and enset.

Task 2.4 - Host range assessment and plant receptivity assays. Task leader: IITA (Resp. D. Coyne). Other Participants: CNR, MSBIO, UA, IAS-CSIC, REAL IPM, SARI, CENSA.

EBCAs isolation and characterization

To increase the microbial collection obtained from banana plants, a second sampling round was carried out by IAS-CSIC in collaboration with Coplaca (Dr. J. López-Cepero and M. Puerta González) in new commercial banana farms from three different Canary Islands. Roots and rhizosphere soil of cv. Pequeña Enana were sampled from farms (Table 1, Fig. 2.1 A,B) in Tenerife, La Palma and La Gomera.

Table 1. Farms surveyed in Tenerife island by IAS-CSIC and COPLACA.

Farm	Name	Latitude (N)	Longitude (W)	Altitude (msl)
F05	Fco Pacheco, Arico, Tenerife	28°10'67"	16°27'64"	188,6
F06	La Caldera, Adeje, Tenerife	28°04'84"	16°43'32"	113,2
F07	Siso, FuencalienteLa Palma	28°28'97"	17°52'58"	28,7
F08	Ortiz, Tijarafe, La Palma	28°41'37"	17°57'01"	299,5
F09	Escuela Capataces, Tenerife	28°29'46"	16°25'15"	299.0
F10	Hermigua, La Gomera	28°10'82"	17°11'08"	123,1
F11	David, San Sebastián, La Gomera	28°06'69"	17°08'68"	82,8

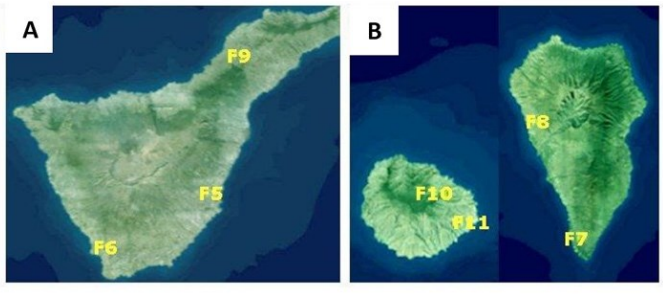


Figure 2.1 Surveyed farms locations in Tenerife, La Gomera and La Palma.

In total, 1095 single/pure isolates (bacteria and fungi) were obtained from the first and second sampling rounds (the latter conducted during this reporting period). Table 2 shows the 29 IAS-CSIC most promising strains, their molecular identification, origin, results for *in vitro* antagonism tests *vs* Foc races, number of positive biocontrol and PGP activities, and potential risks. The six strains in green are IAS-CSIC final selection. A strain of *Serratia marcescens* (in red) was also kept in further bioassays (in spite of association with nosocomial infections) only for scientific purposes, because of enzymatic activities related to biocontrol and PGP.

Table 2. Tested isolates from Canary Island added to those reported in Deliverable 3.1.

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

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SARI surveyed enset and banana fields in Ethiopia, providing new data on most recurrent pathogens, including PPN and Foc, prospecting a total of 1069 farms across major enset producing areas. Data showed that Enset Xanthomonas Wilt (EXW) was the most frequently disease recorded, occurring in 41.2% of farms. Farmer perception of predominant constraint on enset was highly consistent with the frequency of recorded pest and pathogens (Table 3).

Table 3. Comparison of farmers' perception of major enset constraints and observed pests and pathogens.

Farmer reports	Relative pest proportion				
	EXW	Mealybug	Corm rot	Molerat	Porcupine
EXW	0.71	0.29	0.48	0.31	0.42
Mealybug	0.15	0.98	0.31	0.13	0.38
Corm rot	0.07	0.21	1	0.13	0.28
Molerat	0.2	0.45	0.39	0.98	0.47
Porcupine	0.17	0.23	0.47	0.26	0.98

For spatial modelling of pests and pathogens, the niche space for the five major pest and pathogen species was computed with sufficient data and projected into their geographical space (Fig. 2.2). Comparison was made for environmental variables across disease incidence quantiles. Pairwise niche overlap was highest for corm rot and mealybug and lowest for porcupine and mealybug (Table 4). SARI team combined the niches for the five pest and pathogens (Fig. 2.3A), plotted together with the frequency at which multiple pathogens were observed (Fig. 2.3B).

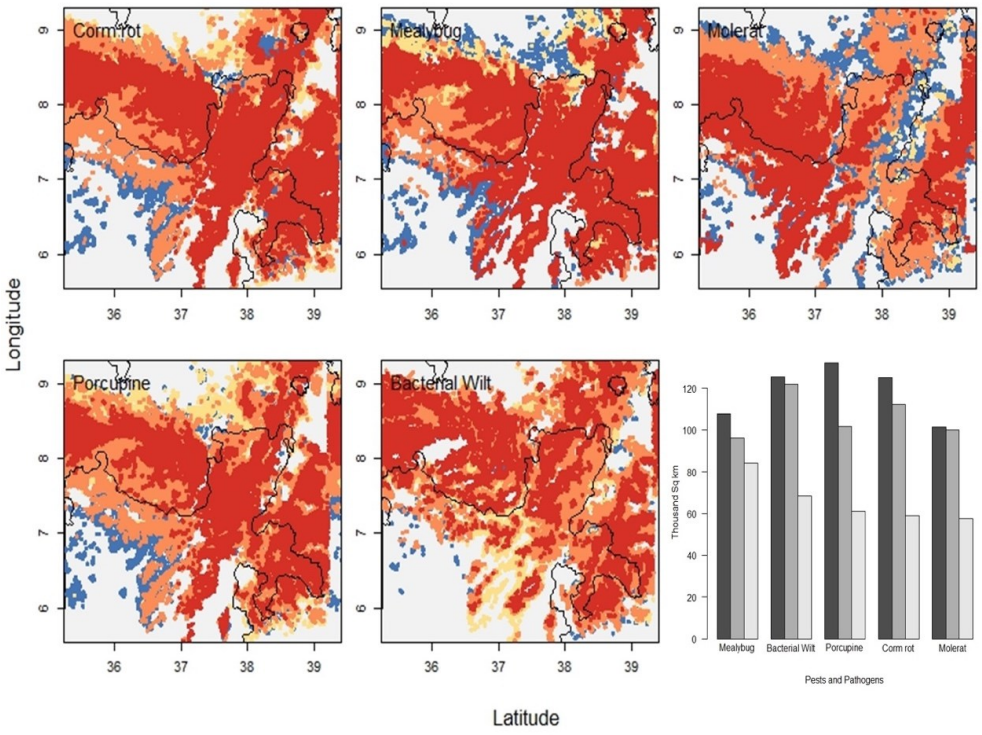

Figure 2.2 Distribution maps of five major enset pests and pathogens present in Ethiopia. Barplot depicts the area of pest and pathogen occurrence, at a range of incidence quantiles.

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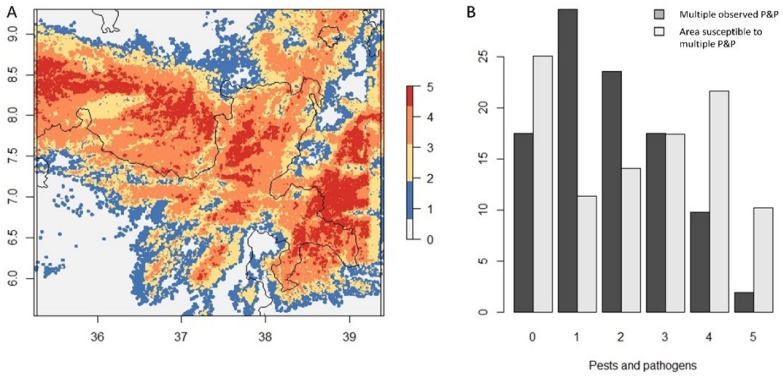

Figure 2.3 Co-occurrence of multiple pests and pathogens in Ethiopia. A) Overlapping distributions of five major pests and pathogens. B) Comparison of modelling-predicted and empirically observed co-occurrence of multiple pests and pathogens on farms.

Table 4. Pairwise overlap (%) across the five major enset pests and pathogens found.

	Corm rot	Mealybug	Molerat	Porcupine
Corm rot				
Mealybug	0.805			
Molerat	0.740	0.801		
Porcupine	0.697	0.700	0.68	
EXW	0.730	0.770	0.724	0.801

In a collaborative study with Kew Botanical Garden, SARI team detected *Xanthomonas vasicola* pv. *musacearum* (Xvm) in diseased enset samples, that displayed the known disease phenotype. A large number of sequence reads mapped to putative Xvm sequence (Fig. 2.4). Surprisingly, a significant number of Xvm reads was also detected in a few asymptomatic, domestic and wild enset samples. Samples collected from plants with EXW symptoms aligned to an average of 96 Xvm contigs. Asymptomatic samples aligned to an average of 15 contigs, but displayed a highly skewed distribution that overlapped with diseased samples (Fig. 2.5).

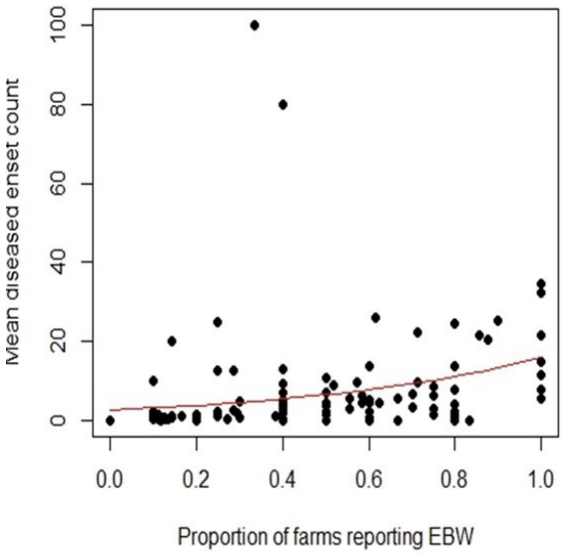

Figure 2.4 Quasi-poisson GLM of the relationship between the proportion of farm clusters reporting EXW and the mean number of diseased enset recorded (unpublished; SARI and Kew Botanical Garden, UK).

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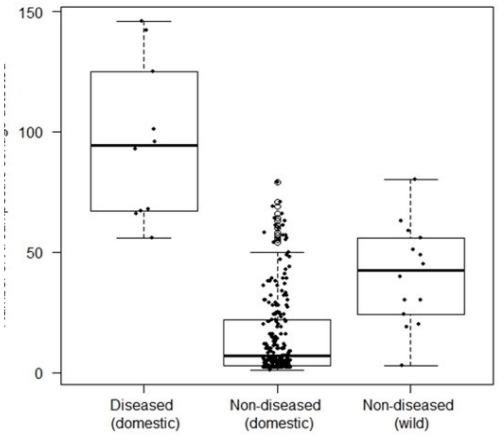

Figure 2.5 Boxplot of the number of *Xanthomonas vasicola* pv. *musacearum* contigs identified by aligning raw reads from wild and domestic enset samples.

A total of 295 banana and enset fields were visited and inspected in Ethiopia for FW, which was detected in 162 fields. The symptomatic samples (by crop type and cultivar) were 22 for enset and 140 for banana. Of a total 162 samples diagnosed in the laboratory, highly presumptive Foc symptoms were found on 66 (55.46%) and 5 (22.7%) samples of banana and enset, respectively, with frequencies of infected samples varying across locations. Media-based laboratory analyses showed strain features highly similar to Foc and assumed as Foc. However, further vegetative compatibility group tests, PCR or genome-based diagnostics are needed to validate the assessment and provide a better insight.

Activities and results concerning the identification of Foc and PPN EBCAs for enset and banana (related to previous tasks 2.2 and 2.3) produced by SARI in Ethiopia are also provided with this report. Samples were collected in the two administration zones of southwestern Ethiopia, viz Kefa and Bench Sheko, from 28 farms visited for collection. 415 samples were taken from leaves, pseudostems, petioles, and roots of 83 plants (13 from banana and 70 from enset).

An initial assessment was carried out to get an overview of the microbial profile of harvested samples, that could potentially be used for detection and identification of EBCAs active against Foc and PPN. Out of 332 plant tissue samples incubated in PDA and NA, 198 bacterial and 153 fungal colonies were observed, respectively (Figs 2.6, 2.7). Bacterial and fungal colonies were observed from petiole (36.9 and 32.7 %), roots (25.2 and 18.3 %), psedostem (22.2 and 20.9 %) and leaves (15.7 and 28.1%), respectively.

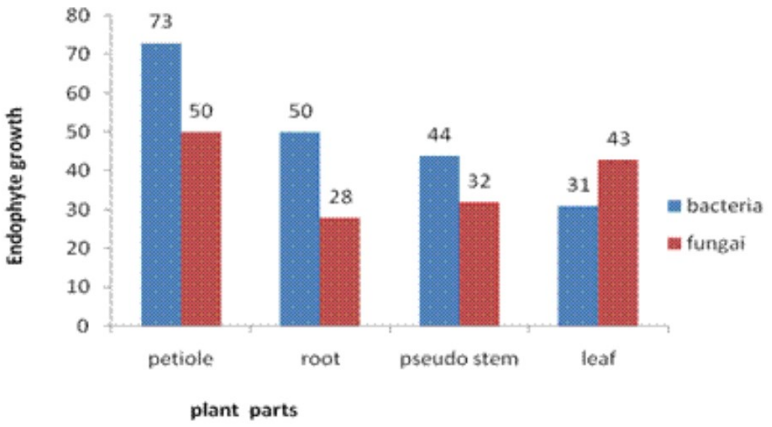

Fig. 2.6 Number of fungal and bacterial endophytes observed on PDA and NA per plant parts.

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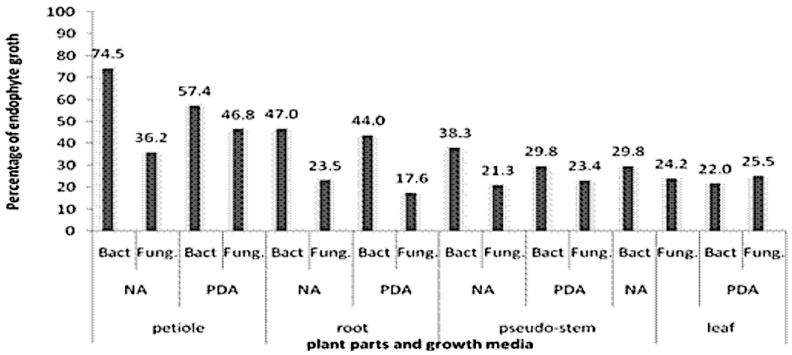

Figure 2.7 Prevalence of bacterial and fungal endophytic isolates grown on PDA and NA.

A total of 170 enset and banana fields were selected and a total 515 soil samples were collected, (320 from enset, 195 from banana fields) for PPN identification and characterization in Ethiopia. The following PPN genera were found in roots and soil, in SNNPR and Oromiya regional states: *Criconema, Helicotylenchus*, *Hemicyclophora*, *Meloidodera, Meloidogyne, Paratrichodorus, Pratylenchus*, *Rotylenculus, Scutellonema*, *Trophurus*, *Tylenchorhychus, Longidorus, Trichodorus*, *Dolichodorus, Hoplolaimus* and *Paralongidorus* (Table 5, Figs. 2.8, 2.9). The most common PPN belonged to genera *Helicotylenchus, Meloidogyne, Pratylenchus, Rotylenculus, Scutellonema,* and *Tylenchorhychus* (Table 5). Rare nematode genera such as *Longidorus*, *Trichodorus*, *Hoplolaimus* and *Paralongidorus* had a distribution/prevalence from 14.2 to 28.5%, with higher abundance only in specific locations.

The most common migratory endoparasites feeding on the root cortex of banana and enset were spiral nematode *Helicotylenchus* spp. and lesion nematodes *Pratylenchus* spp. Sedentary endoparasites of banana and enset included the root-knot nematodes (RKN *Meloidogyne* spp.), and the reniform nematode, *Rotylenchulus* spp.

Table 5. Occurrence (%) of plant parasitic nematodes in soil and roots of enset and banana from 170 sampled fields in Ethiopia (altitude range: 1114-2765 masl).

Genera	Frequency (%)
Criconema	57.14
Helicotylenchus	100
Hemicycliophora	42.85
Meloidera	42.85
Meloidogyne	100
Paratrichodorus	42.85
Pratylenchus	100
Rotylenculus	85.71
Scutellonema	100
Trophurus	42.85
Tylenchorhychus	85.71
Longidorus	14.28
Trichodorus	28.57
Dolichodorus	42.85
Hoplolaimus	28.57
Paralongidorus	14.28

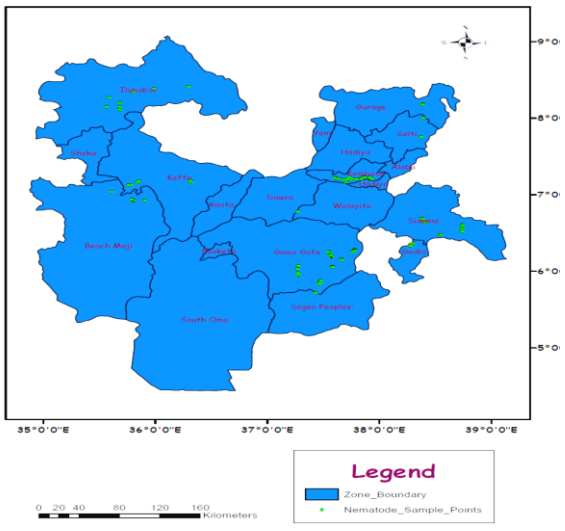

Figure 2.8 Distribution map of sampled areas for nematodes of enset and banana in Ethiopia.

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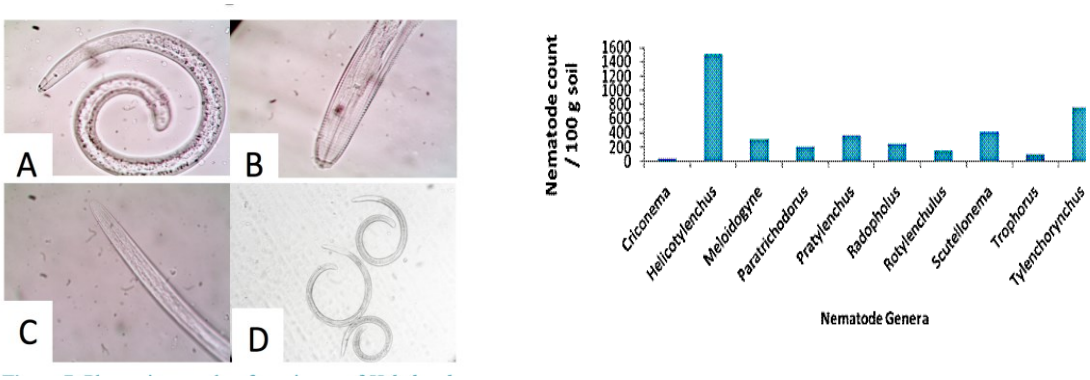

Figure 2.9 Specimens of *Helichotylenchus* (A), *Hemicyclophora* (B), *Longidorus* (C) and *Scutellonema* (D) extracted from enset and banana rhizosphere, and densities measured for most common genera.

EBCAs identification and selection tests

IITA completed in vitro antagonism assays of bacterial endophytes *vs* Foc R1, in Uganda (299 isolates) and Tanzania (102 isolates). The 16S rDNA gene was sequenced and used to identify promising isolates. Out of the 299 bacterial endophytes tested *in vitro* in Uganda, seven showed antagonistic activity against Foc R1 (Fig. 2.10, Table 6). Analysis of 16SrRNA data revealed the endophytic bacteria with antagonistic effect belonged to genera *Burkholderia*, *Enterobacter*, *Herbaspirillium*, *Micrococcus*, and *Bacillus*. Out of the 102 bacterial isolates tested in Tanzania, only two isolates effectively suppressed *in vitro* growth of Foc Race 1 (Fig. 2.11, Table 7). The two isolates were identified as *Bacillus amyloliquefaciens*. This brings the total of endophytic bacteria effective against Foc Race 1 to 18 isolates.

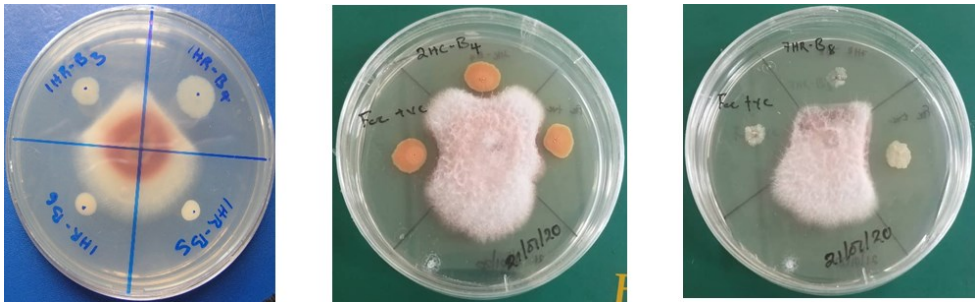

Figure 2.10 Antagonistic activity against Foc race 1 for selected endophytic bacterial isolates

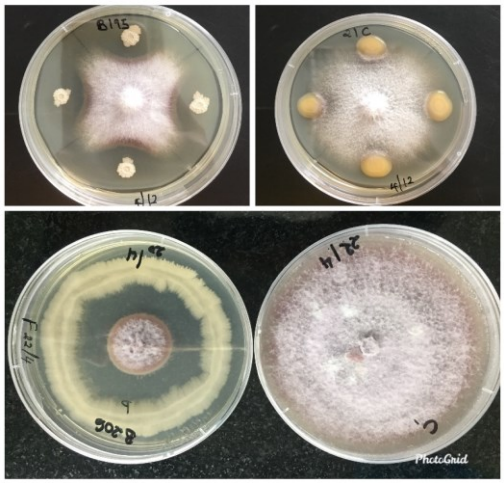

Figure 2.11 In vitro antagonistic activity of a selected bacterial endophyte *vs* Foc Race 1.

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Table 6. Characteristics of the endophytes antagonistic to Foc R1 from Uganda.

Sample	Plant status	Source	Plant tissue	Morpho-characteristics	Id.	Inhibition zone
1DR-B1	Diseased	Root, SN	Roots	flat, dull, irregular, large colonies	*Burkholderia* sp.	4.6
1HR-B3	Healthy	Root, SN	Roots	large diam. (12 mm), cream, flat, shinny, smooth circular margin	*Micrococcus* sp.	4.7
1HR-B16	Healthy	Root, SN	Roots	cream, flat, irregular margins, opaque, diam. 34 mm	*Bacillus* sp.	7.3
2HC-B4	Healthy	Corm, SN	Corm	large diam. (17 mm), cream, flat, rough, ovate, lobed irregular margin,	*Bacillus* sp.	0
7HR-B1	Healthy	Root, SN	Roots	diam. 12 mm large, pink, dome, smooth, shinny, circular, regular, opaque	*Bacillus* sp.	4
7DR-B6	Diseased	Root, SN	Roots	white, dry, irregularly shaped	*Bacillus* sp.	0
7HR-B8	Healthy	Root, SN	Roots	medium diam. (8 mm), cream, smooth, shiny, opaque, regular, circular margin	*Bacillus* sp.	3

Table 7. Identity of bacterial endophytes antagonistic to Foc R1 in Tanzania.

Sample ID	**Foc radius (mm)**	**AUDPC**	**Identification**
A32	0.97 ± 0.12y	5.19 ± 0.66h	*Pseudomonas protegens*
A57	1.81 ± 0.08f	9.63 ± 0.42c	*Pseudomonas* sp.
A64	1.75 ± 0.11g	9.28 ± 0.56d	*Kluyvera* sp.
14C	1.16 ± 0.16	6.15 ± 0.82f	*Pseudomonas protegens*
21C	1.99 ± 0.1e	10.56 ± 0.55c	*Kosakonia oryzae*
25C	2.2 ± 0.11c	11.54 ± 0.58b	*Lelliottia amnigenia*
32C	2.07 ± 0.14d	10.89 ± 0.74c	*Bacillus safensis*
33C	1.88 ± 0.09f	9.95 ± 0.49c	Unidentified
34C	1.88 ± 0.09f	9.92 ± 0.51c	*Enterobacter* sp.
35C	1.87 ± 0.09f	9.86 ± 0.52c	*Pseudomonas* sp.
B129	2.32 ± 0.11b	12.29 ± 0.54b	*Sphingomonas paucimobilis*
B137	1.08 ± 0.11x	5.73 ± 0.59h	*Pseudomonas protegens*
B138	1.02 ± 0.13x	5.42 ± 0.68h	*Pseudomonas* sp.
B140	1.79 ± 0.12g	9.49 ± 0.65c	*Pseudomonas kribbensis*
B195	1.35 ± 0.13h	7.30 ± 0.74e	*Bacillus amyloliquefaciens*

In vitro antagonism assays of isolates from the second Canary Islands sampling were carried out by IAS-CSIC *vs* representative Foc isolates, subtropical race 4 (STR4), race 1/2 (R1/R2) (isolates CAV-095 and CAV-2790, kindly provided by Prof. Altus Viljoen, Stellenbosch Univ., South Africa), and race TR4 (isolate II5, kindly provided by Prof. Antoni Di Pietro, Córdoba University, Spain) following the same protocol used in the first sampling.

Phenotypic characterization was carried out after selection of 150 isolates, showing the highest Foc antagonistic activity. Assays were performed to assess diverse phenotypes, traditionally associated with biocontrol and/or plant growth promotion such as volatiles production (e.g. 2,3

butanediol and hydrogen cyanide), enzymatic activities such as catalase, xylanase, amylase, phytase, protease and β-glucosidase, and siderophore production.

For molecular identification, the most promising isolates were identified by sequencing of several housekeeping genes: *16S rDNA* and *gyrB* genes (bacteria), and Translation Elongation Factor 1-alpha (*tef1*) and Internal Transcribed Spacer (ITS) (fungi). Most prevalent bacterial endophytes from banana roots belonged to genus *Pseudomonas*, with subspecies *piscium*, *aurantiaca* and *aureofaciens* of the *Pseudomonas chlrororaphis* lineage. Other bacterial endophytes were also found such as *Pantoea*, *Rhizobium*, *Flavobacterium* or *Luteibacter* spp. Endophytic fungi were less abundant than bacteria, and included members of genera *Gliocladium*, *Acremonium*, *Metacordyceps*, *Plectosphaerella*, *Fusarium* and *Alternaria*.

Characterization of Foc isolates from Ethiopia

Significant ($p < 0.0001$) differences were observed among Ethiopian Foc isolates for colony growth (CG), at all assessment dates (Table 8). At three, four and five days after incubation (dai), CG ranged from 0.67-2.87 to 0.87-3.33 and 1.23-4.30 cm for isolate KTKGBB24 (lowest) and GaAMZW04 (highest), respectively (Table 8). Mean CG ranged from 0.02 to 0.15 cm/day. Fastest growth on PDA was shown by KTKGA06, and lowest by GaAMZW03 (Table 8).
Foc isolates were culturally characterized for the study areas. Variations were found for colony color, texture, margin and zonation of mycelia (hairy to cottony in texture, circular to irregular in margin and narrow to wider in zonation) (Table 9, Fig. 2.12). All Foc isolates exhibited variability in colony color (front and reverse side), hairy to cottony texture, circular to irregular margins and presence/absence of a narrow to wider zonation on PDA. The aerial mycelium growth turned from white to variable colors, from violet to deep purple. Front and reverse side color varied from light to deep pink and/or pure violet (Table 9). Most (77.42%) Foc isolates had a cottony to hairy texture, whereas a few (22.5%) produced a soft colony. About 54.8 and 45.1% of isolates exhibited a circular and irregular shape of colony margin, respectively. **Table 8.** Variability in colony growth and growth rate of 31 Foc isolates infecting banana and enset from SNNPR and Oromia Regions, Ethiopia.

Isolate	Colony growth (cm) at different days after incubation*			CGR (cm/day)	Crop	Cultivar	Altitude (masl)
	3-days	4-days	5-days				
KTKGJ02	1.80^{fg}	2.00^{d}	2.67^{ef}	0.067^{o}	Banana	White banana	2068
KTKGA06	1.90^{ef}	2.67^{b}	3.60^{b}	0.149^{b}	Banana	White banana	2031
KTKGBB22	1.33^{j}	1.80e	2.40^{gh}	0.078^{j}	Banana	White banana	1977
KTHGM50	1.33^{j}	1.70^{ef}	2.30^{hi}	0.071^{m}	Banana	White banana	1802
KTHGM49	1.70^{gh}	2.40^{c}	3.13^{d}	0.117^{e}	Banana	White banana	1799
KTKBBN59	1.03^{kl}	1.33^{hi}	1.60^{lm}	0.039^{x}	Banana	White banana	2024
KTKGBB24	0.67^{o}	0.87^{j}	1.23^{n}	0.037^{z}	Banana	White banana	1977
KTKGAZ17	0.97^{lm}	1.23^{i}	1.50^{m}	0.038^{y}	Banana	White banana	2026
KTHTLM43	1.33^{k}	1.43^{gh}	1.80^{kl}	0.047^{v}	Banana	White banana	1717
KTHTT32	1.90^{ef}	2.00^{d}	2.67^{ef}	0.061^{s}	Banana	White banana	1605
KTKGZS12	0.77^{no}	0.97^{j}	1.13^{n}	0.025^{z*}	Banana	White banana	2028
KTHTT38	1.80^{fg}	2.47^{c}	3.40^{bc}	0.137^{d}	Banana	White banana	1539
KTHTT37	1.33^{j}	1.80^{e}	2.40^{gh}	0.078^{j}	Banana	White banana	1585
KTKGZS11	1.50^{i}	2.30^{c}	2.87^{e}	0.105^{g}	Banana	White banana	2030
KTHTT29	1.50^{i}	2.10^{d}	2.77^{ef}	0.097^{i}	Banana	White banana	1604
KTKGAZ19	1.33^{j}	1.60^{fg}	2.20^{hi}	0.063^{q}	Banana	White banana	1982
GaAMSM21	2.00^{de}	2.40^{c}	2.57^{fg}	0.045^{w}	Banana	Medium Cavendish	1112
GaAMSM19	2.20^{bc}	2.40^{c}	2.87^{e}	0.055^{t}	Banana	Medium Cavendish	1117
GaAMZW04	2.87^{a}	3.33^{a}	4.30^{a}	0.144^{c}	Banana	Medium Cavendish	1170
GaAMW25	2.30^{b}	2.47^{c}	3.13^{d}	0.073^{k}	Banana	Dwarf Cavendish	1198

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GaMAM37	2.00^{de}	2.47^{c}	2.57^{fg}	0.045^{w}	Banana	Dwarf Cavendish	1233
GaAMW27	2.10^{cd}	2.40^{c}	2.87^{e}	0.063^{r}	Banana	Medium Cavendish	1190
GaAMZW03	1.60^{hi}	1.6^{fg}	1.90^{jk}	0.021^{z**}	Banana	White banana	1191
GaMAOL32	2.00^{de}	2.10^{d}	2.67^{ef}	0.053^{u}	Banana	White banana	1182
GeGDGG27	1.33^{k}	1.50^{gh}	2.10^{ij}	0.069^{n}	Enset	Gonticha	2299
SWGML01	0.97^{lm}	1.70^{ef}	2.40^{gh}	0.104^{h}	Banana	White banana	1730
GeYCAd34	0.87^{mn}	2.47^{c}	3.33^{cd}	0.194^{a}	Banana	White banana	1850
GeGDGG29	0.77^{no}	1.33^{hi}	1.80^{kl}	0.072^{l}	Enset	Astara	2283
SWGML02	1.13^{k}	2.00^{d}	2.67^{ef}	0.115^{f}	Banana	White banana	1720
GeDZTM42	0.97^{lm}	1.80^{e}	2.40^{gh}	0.104^{h}	Enset	Toracho	2143
SKDST18	0.77^{no}	1.23^{i}	1.70^{k-m}	0.065^{p}	Banana	White banana	1787

* On PDA, after five days incubation at 28 ^{o}C. Means in the same column followed by the same letter (s) are not significantly different from each other at $p \leq 0.05$ (LSD); **** Significant at $p < 0.0001$; LSD = Least significant difference; CV = Coefficient of variation.

Table 9. Colony characteristics of 31 Foc isolates from banana and enset samples collected from SNNPR and Oromia regions in Ethiopia*.

Isolate	Front color	Reverse color	Texture	Margin shape	Zonation
KTKGJ02	Deep purple	Pink	Cottony	Circular	Present
KTKGA06	Light purple	Light pink	Cottony	Circular	Present
KTKGBB22	Deep purple	Deep pink	Cottony to hairy	Irregular	Present
KTHGM50	Light purple	Light pink	Cottony	Irregular	Present
KTHGM49	Purple	Deep pink	Cottony to hairy	Irregular	Present
KTKBBN59	Light purple	Light pink	Cottony to hairy	Irregular	Absent
KTKGBB24	Light purple	Light pink	Cottony	Circular	Absent
KTKGAZ17	Purple	Light pink	Cottony to hairy	Irregular	Absent
KTHTLM43	Light purple	Light pink	Cottony to hairy	Circular	Present
KTHTT32	Light purple	Light pink	Cottony to hairy	Circular	Present
KTKGZS12	Purple	Light pink	Cottony to hairy	Circular	Absent
KTHTT38	Purple	Light pink	Cottony to hairy	Circular	Present
KTHTT37	Light purple	Light pink	Cottony to hairy	Circular	Present
KTKGZS11	Light purple	Light pink	Cottony	Circular	Present
KTHTT29	Purple	Light pink	Cottony to hairy	Irregular	Present
KTKGAZ19	Light purple	Light pink	Cottony	Circular	Present
GaAMSM21	Deep purple	Deep pink	Cottony to hairy	Circular	Present
GaAMSM19	Deep purple	Deep pink	Cottony to hairy	Irregular	Present
GaAMZW04	Light purple	Light pink	Cottony to hairy	Irregular	Present
GaAMW25	Light purple	Pink	Cottony	Circular	Present
GaMAM37	Light purple	Light pink	Cottony	Irregular	Present
GaAMW27	Light purple	Deep pink	Cottony	Circular	Present
GaAMZW03	Light purple	Pink	Cottony to hairy	Irregular	Absent
GaMAOL32	Light purple	Light pink	Cottony to hairy	Irregular	Present
GeGDGG27	Deep purple	Pink	Cottony to hairy	Circular	Present
SWGML01	Light purple	Light pink	Cottony to hairy	Circular	Present
GeYCAd34	Purple	Pink	Cottony to hairy	Circular	Present
GeGDGG29	Light violate	Light violate	Cottony to hairy	Irregular	Absent
SWGML02	Light purple	Light pink	Cottony to hairy	Irregular	Present
GeDZTM42	Purple	Deep pink	Cottony to hairy	Circular	Present
SKDST18	Light purple	Light pink	Cottony to hairy	Irregular	Absent

* On PDA, incubated 5 days at 28 ^{o}C.

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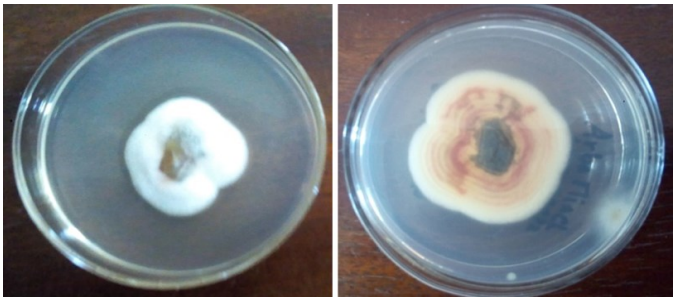

Figure 2.12 Variability in colony color, texture, marigin and zonation of *Foc* isolates from SNNPR and Oromia regions in Ethiopia, grown on PDA medium (five days after incubation at 28 ºC).

Morphological characterization of Foc *isolates in Ethiopia*

Foc isolates were examined in light microscopy for morphological traits such as the presence/absence of septa, shape of micro- and macroconidia and chlamydospores, that showed variability in conidial septation, color and shape (Table 10). Microconidial color varied from hyaline (56% of isolates) to black (41.9%). Most microconidia (56%) had an oval shape, while 41.9% exhibited an oval to reniform shape. Microconidia had 0-1 (54.8%), 0-2 (25.8%) or no septa (19.3%) (Table 10). Significant differences ($p < 0.0001$) were found in the microconidia transverse and longitudinal lengths, with longest lengths shown by GaAMSM21 (1.05 µm) and KTKGJ02 (3.4 µm), respectively. Lowest transverse and longitudinal lengths of microconidia were noted for Foc isolates KTHTT37 (0.7 µm) and KTKGZS12 (1.65 µm), respectively.

Table 10. Variations in microconidial traits of *Foc* isolates (n = 31) from banana and enset samples collected from SNNPR and Oromia Regions in Ethiopia and examined in light microscopy.

Isolate	Color	Shape	Conidial septa	Conidial size (µm)			
				Transverse		Longitudinal	
				Range	Mean	Range	Mean
KTKGJ02	Hyaline	Oval & reniform	0 - 2	0.6-0.9	0.75^{l}	2.7-4.1	3.40^{a}
KTKGA06	Hyaline to black	Oval & reniform	0 - 1	0.7-1.0	0.85^{j}	1.4-2.8	2.10^{n}
KTKGBB22	Hyaline	Oval	0 - 1	1.3-1.4	1.35^{c}	1.4-3.8	2.60^{e}
KTHGM50	Hyaline to black	Oval & reniform	0 - 2	1.1-1.4	1.25^{e}	1.3-3.2	2.25^{k}
KTHGM49	Hyaline	Oval	0 - 1	0.7-0.9	0.80^{k}	2.0-3.0	2.50^{f}
KTKBBN59	Hyaline	Oval	0 - 1	1.0-1.4	1.20^{f}	1.6-2.6	2.10^{n}
KTKGBB24	Hyaline	Oval	0 - 1	1.1-1.4	1.25^{e}	1.5-2.4	2.05^{o}
KTKGAZ17	Hyaline to black	Oval & reniform	0 - 1	1.1-1.6	1.35^{c}	2.1-3.1	2.60^{e}
KTHTLM43	Hyaline to black	Oval & reniform	0 - 1	1.0-1.3	1.15^{e}	1.4-2.8	2.10^{n}
KTHTT32	Hyaline to black	Oval & reniform	0 - 2	1.1-1.4	1.25^{d}	1.8-2.5	2.15^{m}
KTKGZS12	Hyaline	Oval	0 - 1	1.0-1.3	1.15^{g}	1.5-1.8	1.65^{t}
KTHTT38	Hyaline to black	Oval	0 - 1	1.0-1.6	1.30^{d}	2.1-3.8	2.95^{c}
KTHTT37	Hyaline	Oval	0 - 1	0.6-0.8	0.70^{m}	1.6-2.8	2.20^{f}
KTKGZS11	Hyaline	Oval	0	1.0-1.3	1.15^{g}	2.1-2.9	2.50^{f}
KTHTT29	Hyaline	Oval	0 - 1	0.9-1.3	1.10^{h}	1.3-3.4	2.35^{i}
KTKGAZ19	Hyaline	Oval	0 - 1	0.9-1.4	1.15^{g}	1.3-3.1	2.20^{i}
GaAMSM21	Hyaline to black	Oval & reniform	0 - 2	1.2-1.8	1.50^{a}	2.2-3.9	3.05^{b}
GaAMSM19	Hyaline to black	Oval & reniform	0 - 2	1.1-1.5	1.30^{d}	1.3-3.4	2.35^{i}
GaAMZW04	Hyaline	Oval & reniform	0 - 2	1.3-1.5	1.40^{b}	1.1-3.3	2.20^{l}
GaAMW25	Hyaline	Oval	0	1.0-1.5	1.25^{e}	1.3-2.9	2.10^{n}
GaMAM37	Hyaline to black	Oval	0 - 1	0.8-0.9	0.85^{j}	1.9-3.1	2.50^{l}
GaAMW27	Hyaline to black	Oval & reniform	0 - 1	1.2-1.5	1.35^{c}	1.7-2.9	2.30^{j}

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GaAMZW03	Hyaline	Oval & reniform	0 - 2	0.7-1.0	0.85^{j}	1.4-3.4	2.40^{h}
GaMAOL32	Hyaline to black	Oval	0 - 1	1.0-1.6	1.30^{d}	2.3-3.3	2.80^{d}
GeGDGG27	Hyaline to black	Oval & reniform	0 - 1	1.0-1.5	1.25^{e}	1.4-2.3	1.85^{r}
SWGML01	Hyaline	Oval	0 - 2	1.0-1.4	1.20^{f}	1.1-2.5	1.80^{s}
GeYCAd34	Hyaline to black	Oval	0	0.7-0.9	0.80^{k}	1.3-2.8	2.05^{o}
GeGDGG29	Hyaline	Oval & reniform	0 - 1	1.1-1.4	1.25^{e}	1.6-3.0	2.30^{j}
SWGML02	Hyaline	Oval	0	0.8-1.4	1.10^{h}	2.0-2.9	2.45^{g}
GeDZTM42	Hyaline	Oval	0	0.8-1.0	0.90^{i}	1.4-2.4	1.90^{q}
SKDST18	Hyaline	Oval	0	0.9-1.0	0.85^{j}	1.2-2.8	2.00^{p}

Means in the same column followed by the same letter do not significantly differ from each other at $p \leq 0.05$; ****= significant at 0.0001; LSD = Least significant difference; CV = Coefficient of variation.

Variations were also observed for chlamydospores and sporulation, conidial color, shape and formation on hyphal tips (Table 11). Most isolates (51.6%) had hyaline to black chlamydospores, whereas a few showed all hyaline or black colors (25.8 and 22.5%, respectively). Out of all isolates, 51.5% had circular to globose chlamydospores, while the remaining (22.5 and 12.9%) had circular or globse only. About 83.8% of isolates produced a single chlamydospore, with only 16.13% showing a pair.

Table 11. Chlamydospore characterization of *Foc* isolates (n = 31) from banana and enset samples collected from SNNPR and Oromia Regions in Ethiopia.

Isolate	chlamydospores		
	color	shape	formation
KTKGJ02	hyaline	globose	single
KTKGA06	hyaline to black	circular & globose	single
KTKGBB22	black	circular & globose	single
KTHGM50	black	globose	single
KTHGM49	black	circular & globose	single
KTKBBN59	hyaline	globose	single
KTKGBB24	hyaline	circular & globose	single
KTKGAZ17	hyaline to black	circular & globose	single
KTHTLM43	black	circular & globose	single
KTHTT32	black	circular & globose	single
KTKGZS12	hyaline	circular & globose	single
KTHTT38	hyaline to black	circular & globose	single
KTHTT37	black	circular & globose	single
KTKGZS11	hyaline to black	circular & globose	single
KTHTT29	hyaline to black	circular & globose	single
KTKGAZ19	hyaline to black	circular & globose	single
GaAMSM21	hyaline to black	circular & globose	paired
GaAMSM19	hyaline to black	globose	single
GaAMZW04	black	circular & globose	paired
GaAMW25	hyaline to black	globose	single
GaMAM37	black	globose	paired
GaAMW27	hyaline to black	circular & globose	paired
GaAMZW03	hyaline to black	circular & globose	paired
GaMAOL32	hyaline to black	circular & globose	single
GeGDGG27	hyaline	circular	single
SWGML01	hyaline	circular	single
GeYCAd34	hyaline to black	circular	single
GeGDGG29	hyaline	circular	single
SWGML02	hyaline to black	circular	single
GeDZTM42	hyaline to black	circular	single
SKDST18	hyaline to black	circular	single

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Antagonism assays and plant interactions

EBCAs kinetics in presence of chitosan

In vitro assays were carried out by UA to characterize the bacterial strains isolated by CSIC in Canary Islands with Coplaca, and to test their growth kinetics in presence of chitosan. Single cell bacterial colonies were used to start the trial during its exponential growth phase. *In vitro* assays were carried out in 96-multiwell plates. The multiwell plate was incubated at 25°C and the OD (595 nm) was measured each 30 min during 48 h.

Preliminary results are in course of evaluation. Two different behaviors of banana rhizobacteria were found. *Pseudomonas chlororaphis* had an improved growth at low chitosan doses, but was inhibited at higher amounts. B346 was partially resistant to chitosan. PS5, P1A1 and P1C1 showed resistance to chitosan, as only high doses (2 mg/ml) affected their growth (Fig. 2.13).

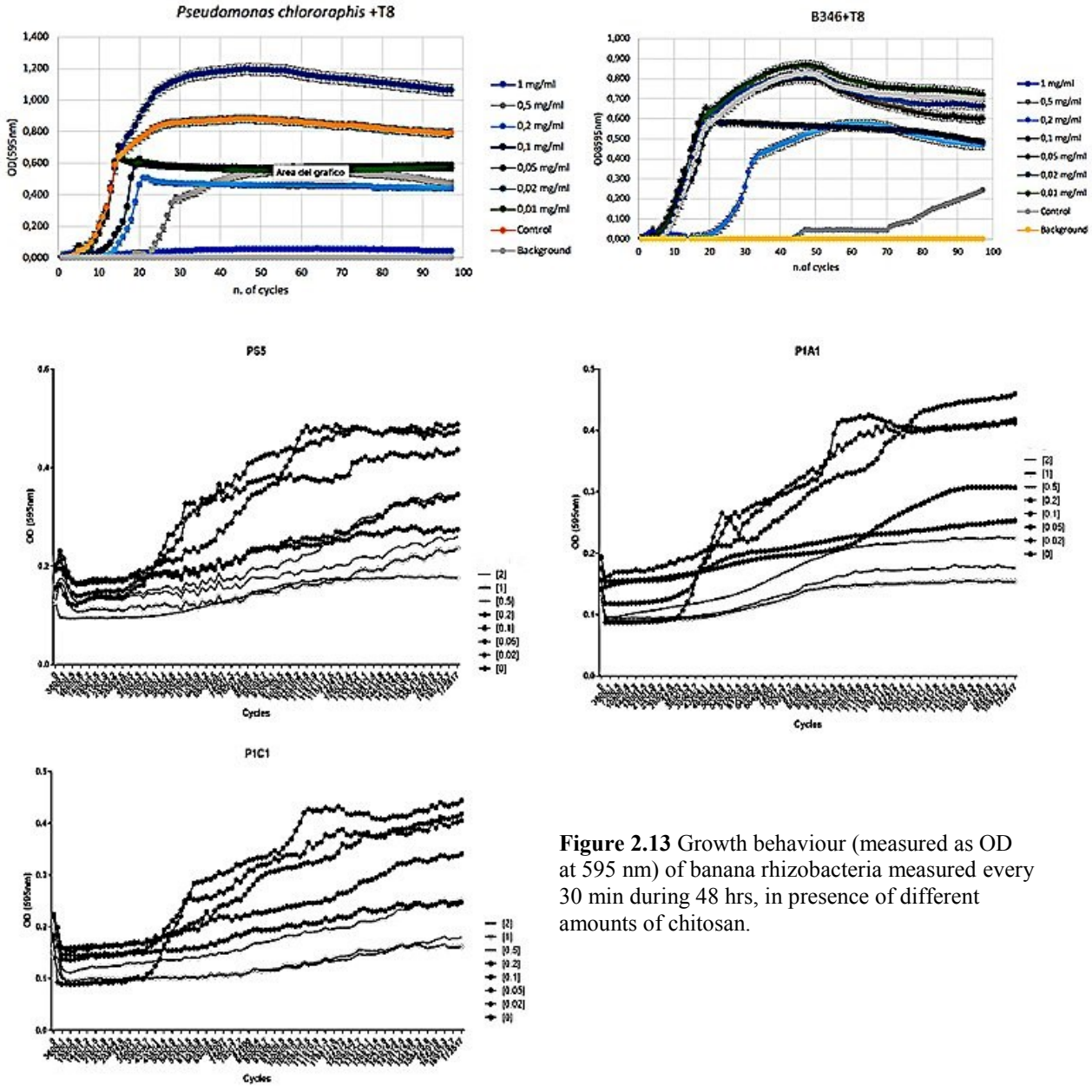

Figure 2.13 Growth behaviour (measured as OD at 595 nm) of banana rhizobacteria measured every 30 min during 48 hrs, in presence of different amounts of chitosan.

Effect of VOCs from EBCAs on BW behavior

A number of VOCs was tested by UA for effects on BW mobility, using a Y-test glass tube device (Table 12, Fig. 2.14).

Table 12. Fungal volatile organic compounds (VOCs) selected for olfactometry bioassays.

Num.	Compound	Ret. Time (min)	Peak Height 10d	Peak Height 20d	Peak Height 30d	Peak Height 40d	Peak Height 50d	Peak Height 60d	Fungal species
C1	Styrene	18.55	-	-	-	-	-	-	Bb1TS11 Bb203
C2	Benzothiazole	31,00	NA	NA	23152	NA	NA	NA	Bb1TS11 (Bb203)
C3	Camphor	29.11	-	-	-	-	-	-	Bb1TS11
C4	Borneol	29,30	96273	24965	NA	31486	20551	NA	Bb1TS11
C4	Borneol	29,30	52467	48687	39535	39445	66449	40861	Bb203
C5	1,3-dimethoxy-benzene	28,75	263989	NA	NA	NA	NA	NA	Ma4TS04
C5	1,3-dimethoxy-benzene	28,75	7233098	509769	3583189	3807170	3046943	308330	Pc123
C6	1-octen-3-ol	22,36	26983	385375	141833	28896	NA	NA	Ma4TS04
C6	1-octen-3-ol	22,36	123365	108363	557934	NA	NA	308377	Pc123
C7	3-cyclohepten-1-one	15,20	50086	475717	306136	120587	NA	233415	Bb1TS11
C7	3-cyclohepten-1-one	15,20	274698	246124	103529	NA	NA	NA	Ma4TS04
C7	3-cyclohepten-1-one	15,20	714704	1027453	NA	NA	NA	NA	Pc123
C7	3-cyclohepten-1-one	15,20	179843	704724	147859	330871	239657	263098	Bb203

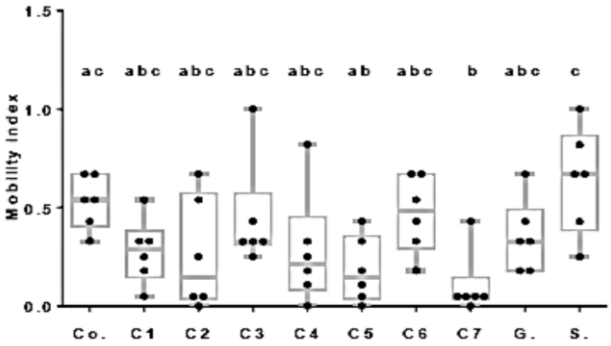

Figure 2.14 Plot of Tukey test data on the TsR showing the effect of fungal VOCs and other compounds (technical repellents) on BW mobility (patented, Spanish Patent Office, n. P201930831).

Biocontrol assays

Several biocontrol assays were performed by IAS-CSIC, in collaboration with Coplaca, *vs* Foc races STR4 and/or TR4. In the first experiment, 7 selected banana endophytes (see Table 2) plus strains PICF7 (see above) and *Paenibacillus polymyxa* PIC73, also from olive rhizosphere (IAS-CSIC collection) were tested. Optical densities (OD_{600}) needed to prepare inocula corresponding to $1 \cdot 10^8$ colony forming units (CFU)/ml for each bacterium were calculated. *In vitro* propagated banana plants (cv. Gran Enana, provided by Coplaca) were transplanted into pots containing the potting substrate described above (Fig. 2.15 A,B). Prior to endophytes inoculation, plants were grown in a growth chamber under control conditions during one month. After that, they were inoculated with the selected endophytes (concentration $1 \cdot 10^8$ UFC/ml) and seven days after, with Foc STR4 ($5 \cdot 10^6$ conidia/ml plus 1.86 mg of pathogen biomass/ml).

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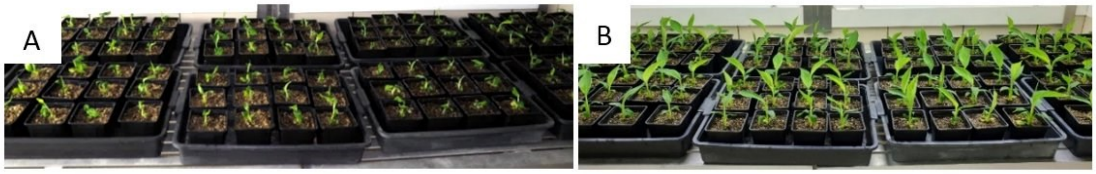

Figure .15. (A) Appearance of banana in vitro-propagated plants at the day of planting. (B) Plants fifteen days after planting.

Results from first experiment showed a disease reduction trend for some EBCA treatments (i.e. IAS-B-364 and IAS-B-1040). However, only plants treated with PICF7 showed a significantly ($P < 0.05$) lower severity of symptoms compared with the control (Figs. 2.16, 2.17).

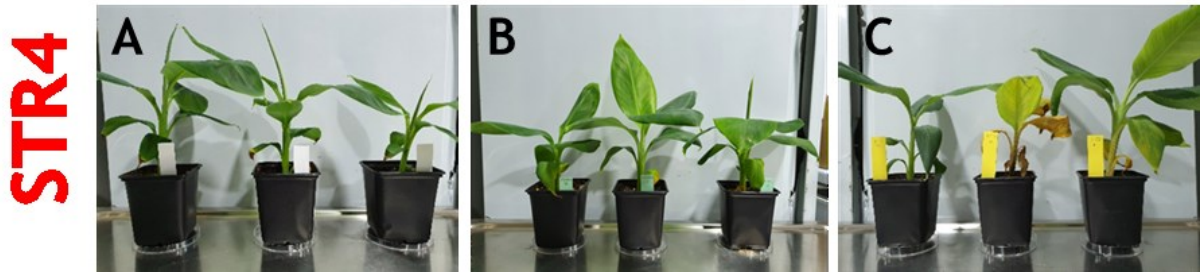

Figure 2.16 Effectiveness of the biological control agent *Pseudomonas simiae* PICF7 against *Fusarium oxysporum* f. sp. *cubense* (Foc) STR4. (A) Control plants; (B) plants inoculated with pathogen and pretreated with the BCA; and (C) disease control plants.

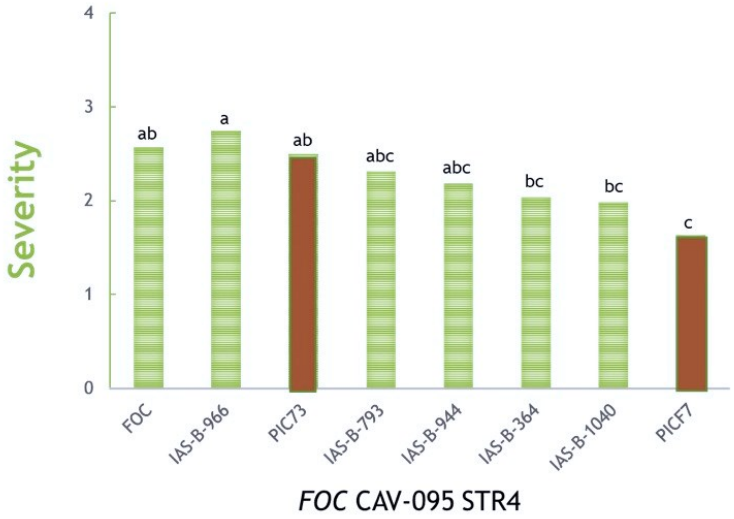

Figure 2.17 Fusarium wilt disease severity on banana plants inoculated with *Fusarium oxyspourm* f. sp. *cubense* CAV-095 STR4 and potential EBCA from banana (strains IAS-B364, IAS-B-793, IAS-B-944, IAS-B-966 and IAS-B-1040; light green bars) and olive (strains PIC73 and PICF7; brown bars) roots.

A second experiment was performed under the same conditions as above to test effectiveness against STR4 of nine selected banana endophytes. In this experiment, no significant difference was found among the tested EBCAs (Fig. 2.18). However, a trend in disease reduction was observed for strains IAS-B-364 and IAS-B-481. Besides, a reduction in necrotic area produced by the infection of Foc in the corm as consequence of treatments with strains IAS-B-364 and IAS-B-481 was observed (Fig. 2.19).

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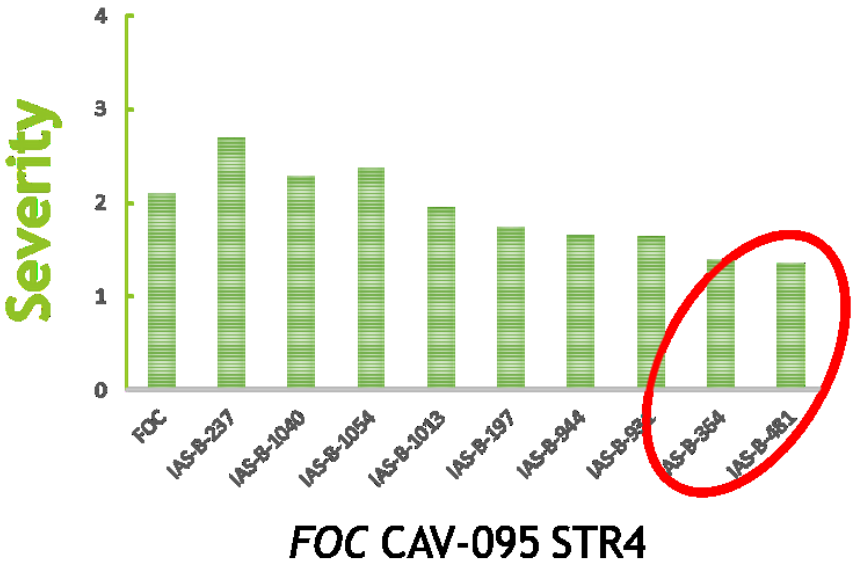

Figure 2.18 FW severity on banana plants inoculated with Foc CAV-095 STR4 and EBCAs from banana roots.

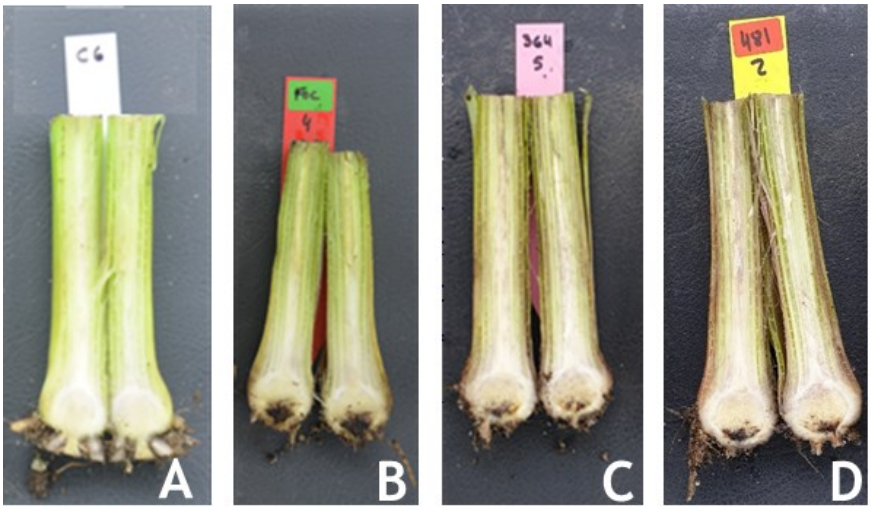

Figure 2.19 Reduction in necrotic area produced by the Foc infection in the corm, due to EBCAs treatment. (A) Control plant, (B) *Foc* STR4 inoculated plant, plants pre-treated with *P. chlororaphis* strains IAS-B-364 (C) and IAS-B-481(D). Images representative of ten replicates per treatment (analyzed with *ImageJ* 1.52).

Finally, strains PICF7 and IAS-B-364, that showed the best results in previous assays, were tested in a third bioassay against *Foc* TR4. Again, PICF7 treatment significantly reduced disease symptoms compared to control plants, while only a slight and not significant reduction was detected in disease symptoms of plants treated with IAS-B-364. Currently, a new bioassay is being carried out in which PICF7 biocontrol effectiveness against *Foc* STR4 and TR4 will be reassessed (Fig. 2.20 A, B).

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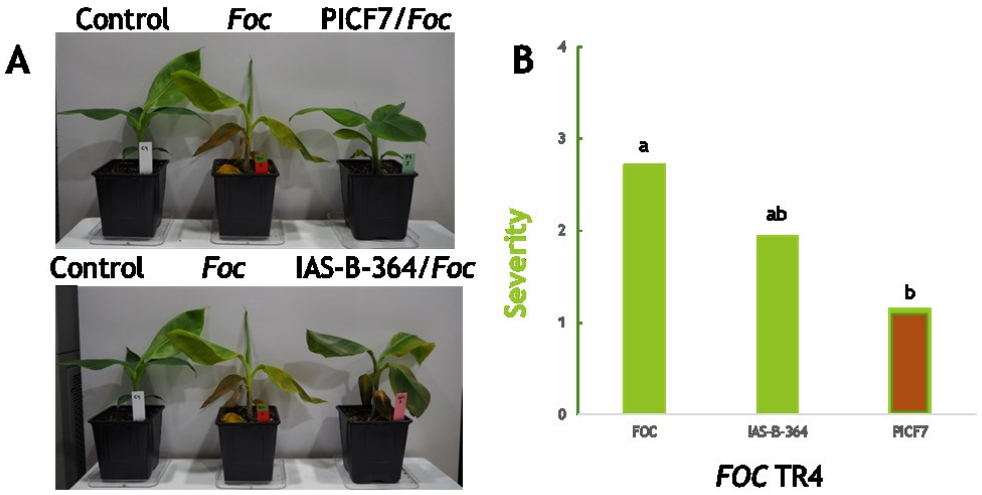

Figure 2.20 Effectiveness (A) and severity (B) of biological control agents from banana (IAS-B-364; green) and olive (PICF7; brown) roots against Foc TR4.

A different approach was used in two biocontrol pot-trials were carried out by CNR team vs FocTR4 on banana cv. Gran Enana, by means of a modified microbial Synthetic Community (SynCom 1.2). Based on previous *in vitro* study on EBCAs - Foc interactions, three isolates were selected for construction of SynCom 1.2, composed by *Trichoderma* sp. T2C1.4, *Pseudomonas* sp. PS5 and *Bacillus* sp. BN8.2 (Fig. 2.21).

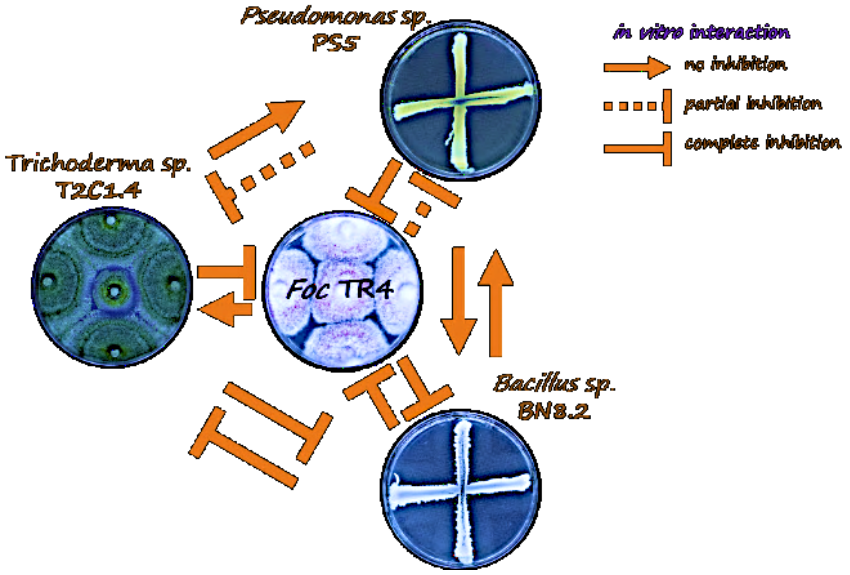

Figure 2.21 Interaction among the three strains selected for construction of the SynCom1.2.

Two biocontrol pot-trials were carried out. The application protocol of SynCom1.2 was set up based on the strains compatibility (Fig. 2.22), and compared with untreated control. In a second trial, the SynCom1.2 was compared with two commercial products retrieved from the literature: PHC BioPak (containing *Bacillus* spp.) and T-Gro (containing *T. asperellum*). Another treatment consisted of SynCom1.2 application plus two additional drenchings with PS5 and BN8.2 isolates, at 7 and 14 days post-transplanting. Therefore, a total of 5 treatments were compared in the second trial: 1) untreated control, 2) SynCom1.2, 3) SynCom1.2 + 2

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drenchings, 4) T-Gro, and 5) PHC Biopak. At 0 and 7 days post-transplanting, the rhizosphere was sampled for a metatranscriptomics analysis. FW biocontrol was higher in the second trial, showing that tailor-made consortia outperform mixtures of beneficial microorganisms. A significant delay in disease progress occurred on banana plants treated with SynCom1.2, both in terms of disease incidence and severity (Fig. 2.23). At 42 dpi, when disease severity was almost at the maximum peak on the untreated control, banana plants treated with SynCom1.2 showed a significantly reduced incidence (Fig. 2.24). However, at later stages of the trial, viz. 63 and 70 dpi, disease severity on treated and untreated plants did not differ, very likely because of the limited volume of soil in the pots. A metatranscriptomic analysis was designed to unveil the mechanisms underlying the biocontrol of FW mediated by SynCom1.2. Analysis of result is still in progress.

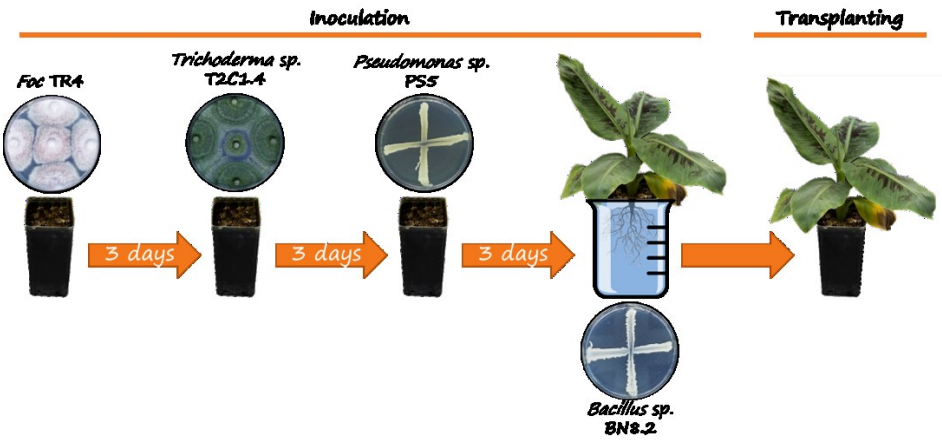

Figure 2.22 Application protocol of SynCom1.2 for Trial B.

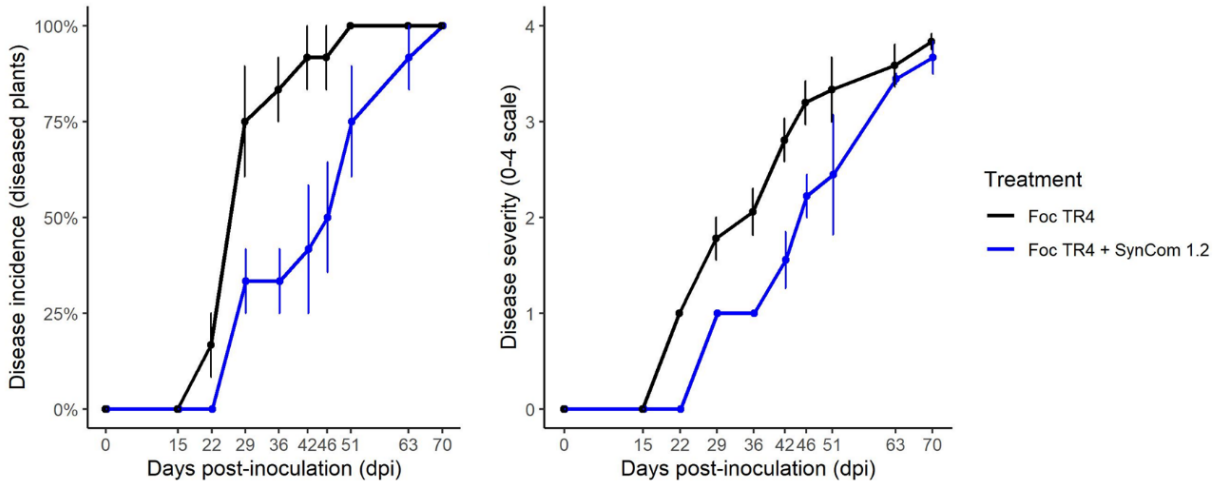

Figure 2.23 Progress of FW on banana plants in first pot trial trial.

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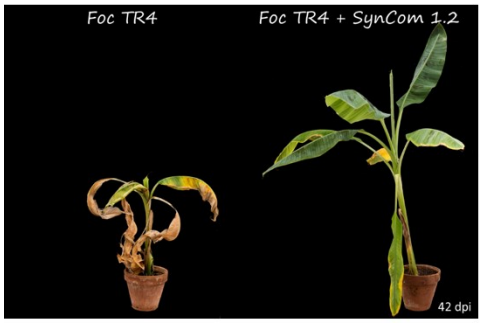

Figure 2.24. Symptoms of Fusarium wilt on banana plants treated with SynCom1.2 or the control in first trial.

Further assays were carried out in Uganda by IITA, using TC plants primed with endophytes that suppressed Foc R1 *in vivo* and challenged with a Foc R1 isolate Foc-0125. Similarly, in Tanzania, endophyte primed plants were challenged with a Foc R1 isolate (Fig. 42). For both assays data analysis for exploitation is in progress.

Evaluation of selected EBCAs vs PPN

Host range tests concerned the main PPN affecting banana in Costa Rica viz. root-knot nematodes (RKN, *M. incognita*), the burrowing nematode *Radopholus similis*, lesion nematodes *Pratylenchus coffeae* or *P. goodeyi*, and the spiral nematode, *Helicotylenchus multicinctus*. Fallow endophyte strain ENDO4 of *Trichoderma asperellum* was selected and tested by EARTH team for biological control of PPN on banana. It was recovered from a commercial banana plantation with 6 years of fallow and was tested in several trials in greenhouse conditions (as well as in the field in WP6) as a biocontrol agent of *R. similis*. Results from greenhouse assays indicated that ENDO4 significantly affected the *R. similis* population.

Tissue cultured cv Grande Naine banana plants were inoculated with 4 endophytes from Costa Rica and two commercial products (Soilset© and Trichomax©, as requested at the issue of first Project evaluation) for biological control of *R. similis* in greenhouse. Results showed that endophyte inoculated plants had a significantly lower population of *R. similis*, compared with control. Best performance was shown by ENDO4 and Soilset (Fig. 2.25).

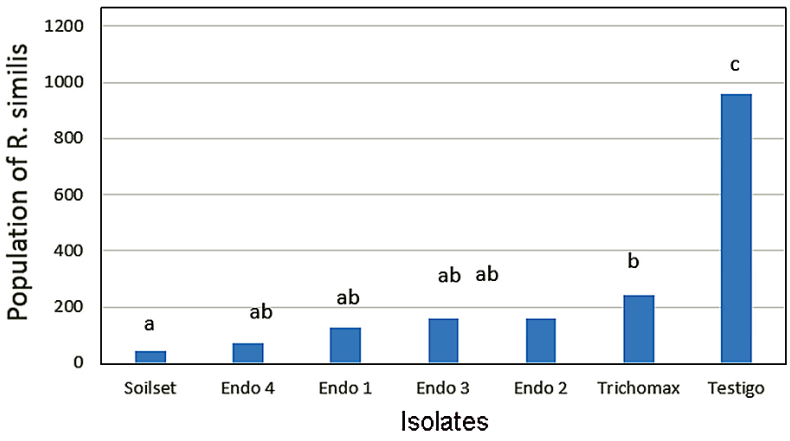

Figure 2.25 Biological control effect of endophytes isolated in Costa Rica on *R. similis* final population, in greenhouse conditions.

Colonization

A criterion selected for application of banana endophytes is that they should be inoculated and re-isolated from internal plant tissues. In addition a precondition is that they should be able to colonize the tissues where the attacks of the target pathogen/pest mostly occur. Studies on plant

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colonization were conducted in greenhouse conditions as well as in the field (see WP6), to determine if the fungus selected (ENDO4) was able to establish in the plant.

ENDO4 was reproduced in sterile corn seeds in the laboratory. Tissue-cultured plants of Grande Naine were inoculated by introducing three corn seeds in three holes in the pot, around the plants. At 15 dai, colonization was assessed in root, corm and pseudostem. Results showed that the fungus colonized the internal plant tissues, including roots, corms and pseudostem (polar colonization) (Fig. 2.26).

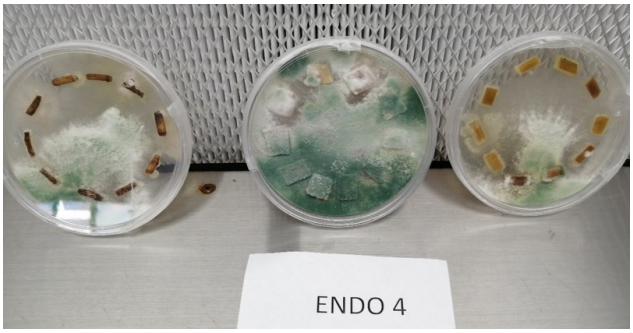

Figure 2.26 Polar colonization of *T. asperellum* ENDO4. From left to right: roots, corm and pseudostem of banana cv Grand Naine, at 15 dai with fungal corn seeds in greenhouse.

In vitro assessment of B. licheniformis *SGB413 nematocidal activity*

Isolate SGB413 from the MSBIO collection was grown in Petri dishes until sporification. The spores were collected in sterile distilled water (SDW) and pasteurized at 80 °C for 10 min. Initial concentration was determined by serial dilutions and brought to $1 \cdot 10^5$ CFU / ml. Juveniles (J2) of *Meloidogyne* sp. were obtained in an Erlenmeyer flask from aerated roots galls in water, to achieve a final concentration of 50 J2 and 15 eggs / ml. The test was prepared in glass tubes in 3 replicates, incubated at 27 °C for 1 week, testing 4.5 ml J2 suspension + 0.5 ml spore suspension in SDW. Control was 4.5 ml J2 suspension + 0.5 ml SDW. Every 24 h one ml of the J2 suspension was taken for light microscopy countings, checking motile and not motile J2 and unhatched eggs, in a Hawksley counting chamber. Results are shown in Fig. 2.27.

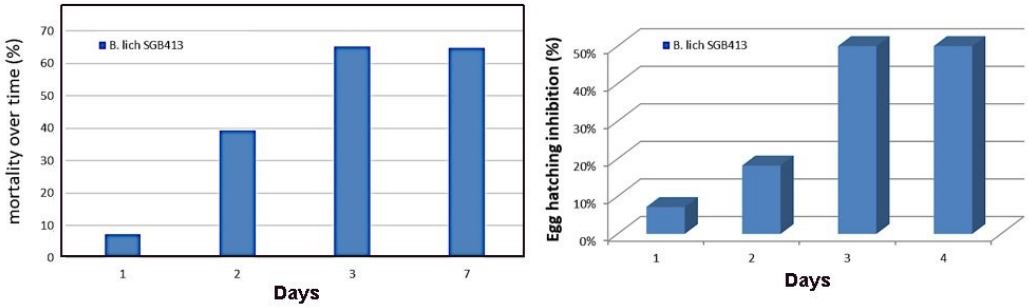

Figure 2.27 Effect of *Bacillus licheniformis* SGB413 on *Meloidogyne* sp. J2 mortality and eggs hatching inhibition.

EBCAs vs Fusarium oxysporum

MSBIO also tested 50 *Bacillus* spp. isolates in collection, for biocontrol of phytopathogenic fungi, insects and nematodes. The isolates were stored as glycerinates and in criovials at - 20 °C and were subjected to quality screening and genetic characterization. The selected isolates were subjected to mass production tests. Best isolates were deposited for patenting purposes at DSMZ International Deposit Authority (AID) (Germany) and Istituto Zooprofilattico Sperimentale - Lombardia and Emilia Romagna (IT). Isolates SGB100Z (*Bacillus velezensis*/*amyloliquefaciens* subsp. *plantarum*, deposit n. DPS RE RSCIC19) and SGB0013

(*Bacillus amyloliquefaciens*, deposit n. DPS RE RSCIC20), showed highest antagonism vs *F. oxysporum* in *in vitro* assays. SGB413 (*B. licheniformis*, deposit n. DSM33310), was effective *in vitro* for its nematocidal activity. *In vivo* tests, however, had to be stopped due to Covid-19 pandemic.

Banana germplasm colonization and response

For BW management, CENSA completed morphological studies for new EPN strains, measuring host penetration range in one-to-one tests, with biochemical characterization of the simbiotic bacteria.. For morphological studies of new EPNs, the latter were fixed in TAF and stored for morphometric and biochemical characterization of simbiotic bacteria.

Selection of new P. chlamydosporia *isolates*

Based on data produced on biological parameters of the isolates, including basal mycelial growth (diameter of the colony), growth rate (mm/day) and sporulation (chlamydospores/mm^2), CENSA selected 15 new monosporic isolates.

Characterization of Trichoderma asperellum

Although the genus *Trichoderma,* included *T. asperellum*, has been extensively investigated, on the basis of our knowledge no data were previously reported about endosymbiotic bacteria hosted inside the fungus. Among the isolates of soil fungi produced by CNR, hyphae of IPSP_MA_1 (proceeding from tomato roots collected at Ragusa, Italy) were found to host a non-parasitic bacterium. Morphological examination and sequence analysis of the ITS 1 - 5.8S - ITS2 ribosomal gene region and RNA polymerase II subunit (RPB2) gene confirmed the hosting IPSP_MA_1 isolate as *Trichoderma asperellum* (99.8% BLAST identity). Optical examination with light, fluorescence and electron microscopy confirmed its endophytic nature and the presence of a further endosymbiotic microorganism within young hyphal tips. The endosymbiotic bacterium hosted in *T. asperellum* was isolated and cultured, growing with convex colonies, round and smooth in shape with undulated margins, yellow-green in color (Fig. 2.28). DNA extracted from a pure culture produced a fragment of about 1500 bp with 99% sequence identity with *V. paradoxus* 16S rDNA and other 26 *V. paradoxus* deposited sequences.

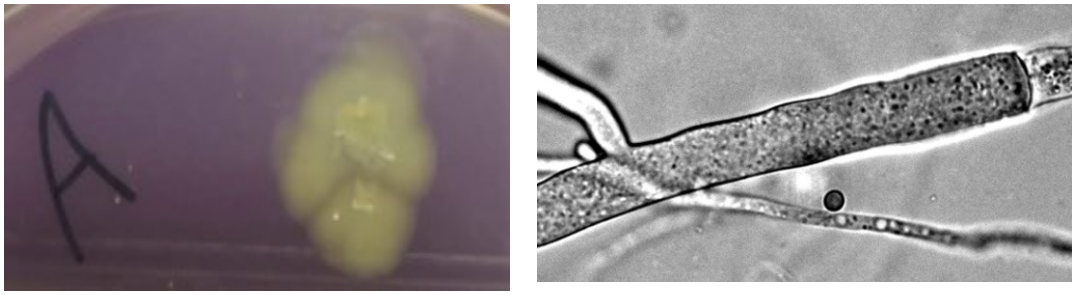

Figure 2.28 Colony of *Variovorax paradoxus* isolated from *Trichoderma asperellum* isolate IPSP_MA-1 (left) and hyphal colonization by the bacterium endosymbiont.

Tissue-cultured banana plants cv. Grand Naine, planted in pots with sterile soil, were inoculated with a rice grain formulation with *T. asperellum* IPSP_MA_1 (30 g/plant), in three replicates. Plants applied with rice grains without *Trichoderma* served as control. Plant tissue colonization by the endophytic fungus was assessed 4 to 7 weeks after treatments. The emergence of the *Trichoderma* isolate from roots was confirmed by the characteristic conidia, conidiophores and phialids, and percent colonization was assessed. Presence of *V. paradoxus* inside the mycelium

of recovered *T. asperellum* IPSP_MA_1 colonies was also assessed by microscopic examination, and confirmed by molecular techniques.

EBCAs vs *BW*

For three *Beauveria* isolates (ICIPE 648, ICIPE 660 and ICIPE 273) tested by IITA and ICIPE, the highest cumulative BW mortality was observed in weevils exposed to the highest spore concentration (1.0×10^8 spores ml^{-1}) (Fig. 2.32). The lowest BW mortality was observed in weevils sprayed with the lowest spore concentration (1.0×10^6 spores ml^{-1}). Across treatments, maximum mortality was obtained by day 10 following exposure of the weevils to the respective fungal treatments. Egg-laying capacity of adult BW was significantly reduced by spraying of the weevils with the BCAs.

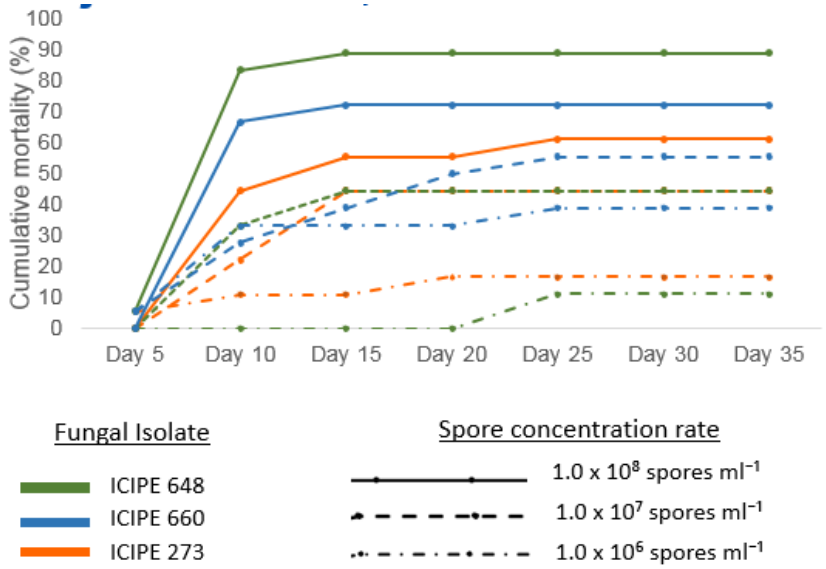

Figure 2.32 Cumulative mortality rates of *Cosmopolites sordidus* following exposure to the *Beauveria bassiana* isolates ICIPE 648, ICIPE 660 and ICIPE 273, at three different spore concentration rates.

1.2.3 WP3 - EBCAs biology in plants, pests and pathogens interactions (mths 19-28)

Task 3.1 - Molecular biology of EBCAs. Task leader: CSIC (Resp. J. Mercado Blanco). Other Participants: MSBIO, CNR, UNEXE.

Defense gene response in plants during interaction with PICF7

In vitro propagated banana plants cv. Gran Enana (provided by Coplaca) grown one month were inoculatied with strain PICF7, by adding 85 ml of the bacterial cells suspension ($1 \cdot 10^8$ CFU/ml). Non-inoculated plants received the same volume of solution. Roots and leaves were sampled at 0 h, 24 h, 48 h, 7, 15 and 56 days (4 plants/time point/treatment) after PICF7 inoculation. RNA was extracted from root tissues to monitor expression of several defense genes (e.g. ascorbate peroxidase, catalase, superoxide dismutase, phenylalanine ammonia lyase, polyphenol oxidase, pathogenesis related protein or ethylene responsive factor 1) during the banana-PICF7 interactions. This experiment has been considerably delayed by the Covid-19 pandemic, with personnel involved forced to domestic confinement during March-May 2020. We expect to collect and report results during the last reporting period.

Plasmid detection in T. asperellum *endosymbiont*

The *V. paradoxus* strain isolated from *T. asperellum* IPSP_MA_1 was also studied with molecular analyses. *Variovorax* spp. have a broad range of diverse metabolic capabilities targeting a wide variety of compounds. CNR team hypothesized the presence of extra-chromosomal replicons inside the *V. paradoxus* endosymbiont, likely playing a role in its metabolism and adaptation to different trophic or environmental niches. Although none of the complete genomic sequences of *V. paradoxus* strains, currently available in GenBank (NCBI), include plasmids, there are four reports on *V. paradoxus* plasmids in the literature. To ascertain plasmid presence into the *V. paradoxus* isolate, bacteria were grown on lysogenic broth media at 30 °C. Plasmid DNA was then isolated and visualized by electrophoresis. Gel analysis confirmed the presence of plasmids in the bacterium, whose nucleotide sequencing and characterization is in progress.

Pochonia *genome*

UNEXE, in collaboration with UA, carried out bioinformatic analyses for re-assembling, validation and re-annotation of *P. chlamydosporia* isolate 123 genome, later deposited in NCBI.

Task 3.2 - Root biology with endophytic EBCAs in PPN and banana root interactions. Task leader: UA (Resp. L. V. Lopez Llorca). Other Participants: CSIC, CNR, EARTH University.

Greenhouse assays were carried out by CNR to test the capability of *P. chlamydosporia* to elicit, in the interaction with Foc or PPN, a systemic expression of defense genes such as *PAL 5*, *PIN II*, *PR1* and *LOX D*. Roots of plants cv Pequeña Enana were inoculated or not in pots with *P. chlamydosporia* Pc21 (DSM 26985) and artificially infected with isolate NRRL36114 (CBS 102025) of *F. oxysporum* f. sp. *cubense* TR4. In a second assay, banana plants were inoculated or not with DSM 26985 and a total 3000 J2 and eggs of *Meloidogyne incognita.* A further assay was carried using adults and juveniles of the lesion nematode *Pratylenchus goodeyi* on vitroplants in Magenta boxes, maintained at 26 °C. RT-PCR data on gene expression from the different assays showed various activation patterns of the defense genes in leaves. In the first assay, RT-PCR showed that PR1 was significantly up regulated 7 dai only in leaves of plants inoculated with *P. chlamydosporia* DSM 26985 (Fig. 3.1A). PIN II was differentially over expressed at 10 dai in leaves of plants inoculated only with the FocTR4 isolate (Fig. 3.1B). In the RKN assay, PR1 was differentially up regulated only in presence of *P. chlamydosporia* at 4 and 7 dai. This gene showed a different behaviour in plants inoculated with RKN and *P. chlamydosporia*, being differentially down regulated at 4 dai and up regulated at 10 dai (Fig. 3.2A). PIN II was significantly up regulated in leaves but only at 10 dai, in plants with RKN and DSM 26985 (Fig. 3.2B).

In the *P. goodeyi* assay, PR1 and LOX were up regulated at 21 dai only in presence of *P. chlamydosporia* DSM 26985, whereas PIN II was down regulated in both treatments with *P. goodeyi*, irrespective of the endophyte presence (Fig. 3.3A). At 21 dai PAL was also differentially down regulated in treatment with *P. chlamydosporia* DSM 26985 (Fig. 3.3B). Defense gene expression in leaves suggests the occurrence of upward systemic signals induced by *P. chlamydosporia* for PR1, LOX, and PIN II. This link confirms a specific relationship between host plants and *P. chlamydosporia*, functional to the systemic and selective activation of defense genes, in presence of biotic stress factors.

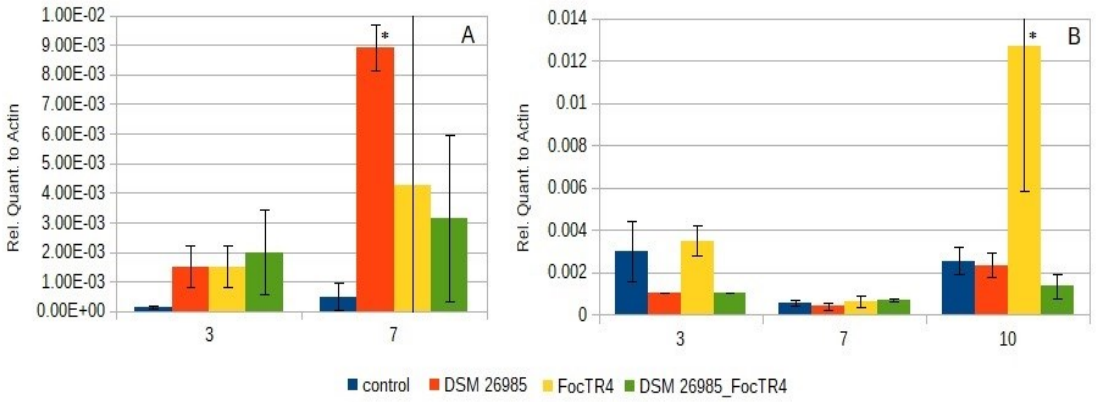

Figure 3.1 PR1 (A) and PIN II (B) expression in leaves of Pequeña Enana plants, 3, 7 and 10 dai with FocTR4 isolate NRRL36114 (CBS 102025) and *P. chlamydosporia* DSM 26985. Asterisk shows significant difference from untreated control ($p < 0.05$).

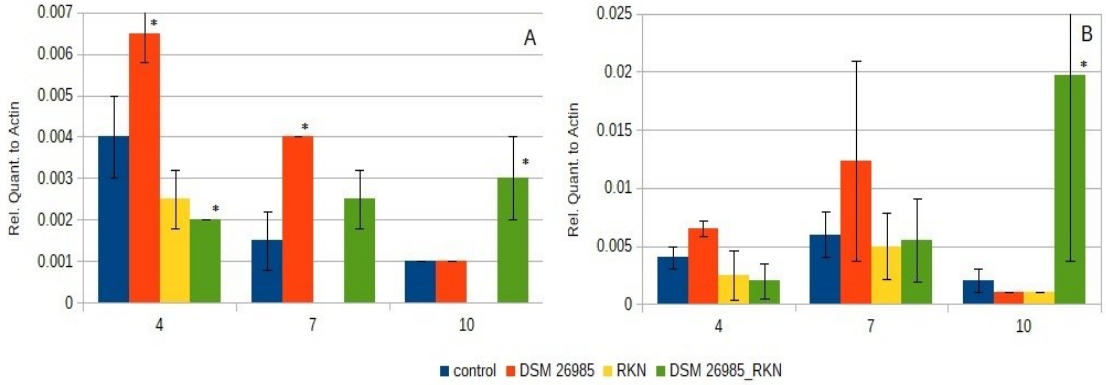

Figure 3.2 PR1 (A) and PIN II (B) expression in leaves of Pequeña Enana plants at 4, 7 and 10 dai inoculated with RKN *M. incognita* L4 and/or *P. chlamydosporia* isolate DSM 26985. Asterisks show significant difference from correponding controls (LSD test, $p < 0.05$).

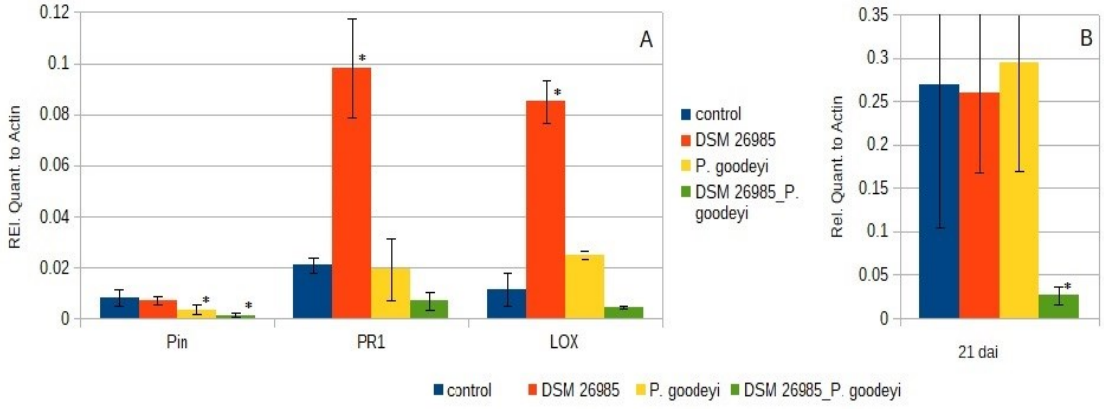

Figure 3.3. Gene expression of PIN II, PR1, LOX (A) and PAL (B) at 21 dai in leaves of Pequeña Enana plants inoculated with *P. goodeyi* and/or *P. chlamydosporia* isolate DSM 26985. Asterisks show significant difference from untreated controls (LSD test, $P < 0.05$).

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Real IPM conducted a greenhouse trial at Kichozi farm (located at Thika, Kenya, lat. 1° 1' 33.5'' S) using *Beauveria bassiana* (Bb02), *T. asperellum* (TRC900), *T. atroviride* (TRC902) and *T. hamatum* (TRC901), as water formulated products with at least 1×10^7 viable conidia/ml. *In vitro* propagated banana plantlets (Williams and Grand Naine) were root dipped separately in different spore suspensions for 10 min (controls dipped in water only) and planted in trays. The plantlets were then transferred into acclimatization chamber and allowed to harden for a period of 8 weeks, before inoculation with 1000 J2 of *R. similis* reared on carrots discs. Two additional inoculations were done at two weeks interval post inoculation through soil drenching. Banana plants were harvested 8 weeks post inoculation, and nematode reproduction was determined.

The *B. bassiana*, *T. asperellum* and *T. hamatum* isolates reduced nematode reproduction and development in cv Williams, while the *T. viride* isolate was not effective. Only *T. hamatum* reduced nematode reproduction and multiplication in cv Grand Naine (Fig. 3.4).

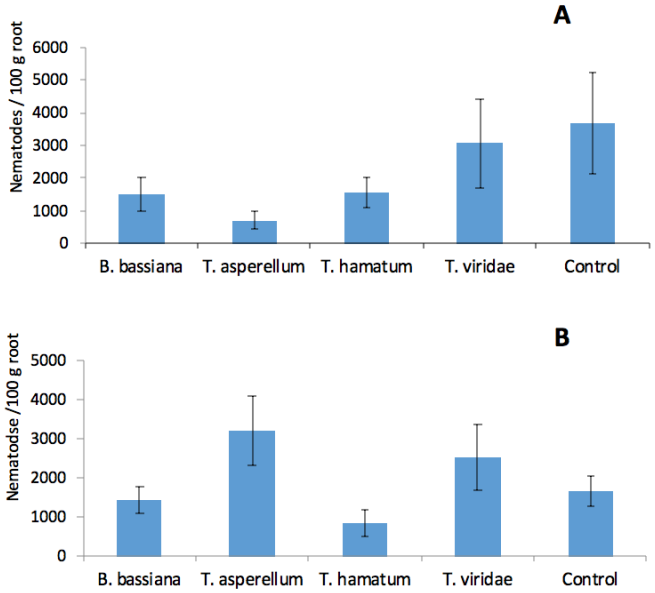

Figure 3.4 Densities of *R. similis* following inoculation of banana plants cvs Grande Naine (A) and Williams (B) with isolates of *B. bassiana*, *T. asperellum*, *T. hamatum* and *T. viride*.

Efficacy of Isaria fumosorosea *and* Purpureocillium lilacinum *vs* R. similis

Microbial isolates of *Isaria fumosorosea* and *Purpureocillium lilacinum* proceeding from Real IPM Ltd were tested as water formulations with at least 1×10^7 viable conidia/ml. The experiment aimed at screening their effect on *R. similis* in TC cv Grand Naine and William. Inoculation of the two EBCAs significantly reduced reproduction of nematodes in both cvs (Figs. 3.5A, B). The nematode reproduction was reduced in TC plants pre-inoculated with the two EBCAs, compared to those challenged with nematodes and then inoculated with the fungus.

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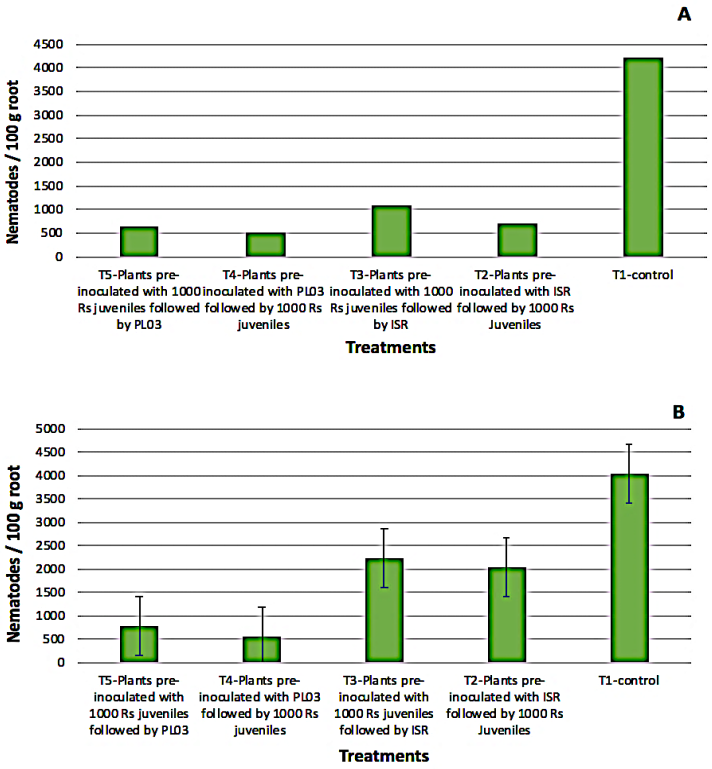

Figure 3.5. Nematode counts per 100 g root weight following inoculation of banana plants of Cv Grande Naine (A) and Williams (B) inoculated with *Purpureocillium lilacinum* and *Isaria fumosorosea* and challenged with 1000 *R. similis* per plant.

In a further assay IITA and ICIPE teams studied enhancement of TC plantlets with the fungal endophyte TRC 900 (provided by Real IPM) *vs R. similis*. The endophyte significantly reduced nematode roots penetration by 52 and 93%, for plants grown in soil and coconut peat media, respectively (Fig. 3.6)

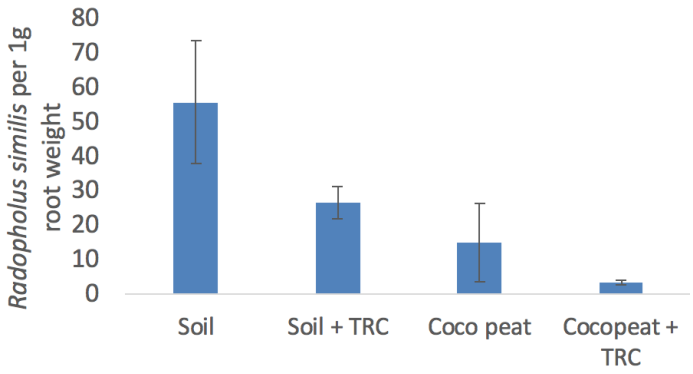

Figure 3.6 *Radopholus similis* penetration (mean ± SE) in banana roots 14 days after challenge of banana TC plantlets with nematodes.

IITA team also tested *B. bassiana* isolate WA and *F. oxysporum* non-pathogenic isolate V5w2, alone or in combination, on TC plantlets, followed by inoculation with *R. similis*, reducing infestation levels by 28, 56 and 44% respectively.

Exposure of *R. similis* to other EBCAs filtrates showed 2.3 - 20.0% paralysis for the bacterial and 7.0 – 18.3% for fungal isolates. Percent mortality of *R. similis* was highest (47.8%) for the bacterial isolate 1HR-B4, followed by 1HR-B3 (30.65%). The fungal isolates TRC900 and T34 recorded nematode mortalities of 21.5 and 26.2%, respectively (Fig. 3.7).

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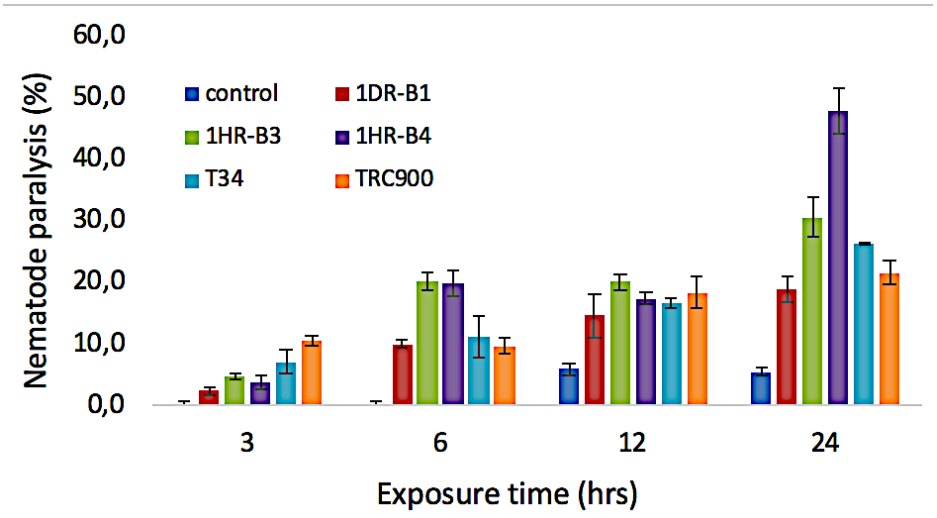

Figure 3.7 Percent paralysis (3, 6 and 12 hrs) and mortality (24 hrs) of *R. similis* mixed stages in culture filtrates of bacterial (1HR-B1, 1HR-B3 & 1DR-B4) and fungal (T34 & TRC900) isolates.

The two isolates were also effective *vs* BW. Inoculation of TC plantlets with either V5w2 or WA alone resulted into a 15% reduction in BW damage to the banana corm, while their inoculation in combination resulted into a 10% reduction in BW damage (data not shown).

ICIPE tested three doses (1.0×10^6, 1.0×10^7 and 1.0×10^8 spores ml^{-1}) of two candidate fungal endophytes (ICIPE 697 and ICIPE 700) for the management of *R. similis* infection in TC banana plants. And set up a screenhouse experiment to assess the efficacy of bacterial (1HR-B1, 1HR-B3 & 1DR-B4) and fungal (T34 TRC 900) isolates against *R. similis* development in TC banana plantlets, to establish optimal inoculation dose and time of application. Enhancement of the banana TC plantlets with the highest dose (1.0×10^8 spores ml^{-1}) of fungal endophyte resulted into 51 and 73% reduction in *R. similis* infection for ICIPE-697 and ICIPE-700 respectively. The lowest dose (1.0×10^6 spore ml^{-1}) yielded the least reduction (20 and 26%) in nematode infection for the isolates 697 and 700, respectively.

3.2.1. Microscopy analysis of the interaction of *P. fluorescens* PICF7 with PPN

This study was required to assess banana root colonization by *Pseudomonas simiae* (formerly *Pseudomonas fluorescens*) strain PICF7. IAS-CSIC previously identified and characterized the rhizobacterium (*P. simiae* PICF7) from olive roots, as an effective EBCA of Verticillium wilt of olive. Moreover, this bacterium is able to endophytically colonize roots and persist in many plants, such as olive, wheat and barley. Therefore, independent experiments were needed to check through a fluorescently-labeled derivative if PICF7 could colonize banana roots endophytically. Consistent microscopy evidence showing that PICF7 efficiently colonizes the interior of banana roots require further assays. Nevertheless, PICF7 was found to be localized on specific spots of the banana roots surface, and some limited events of endophytic colonization were observed (Fig. 3.8).

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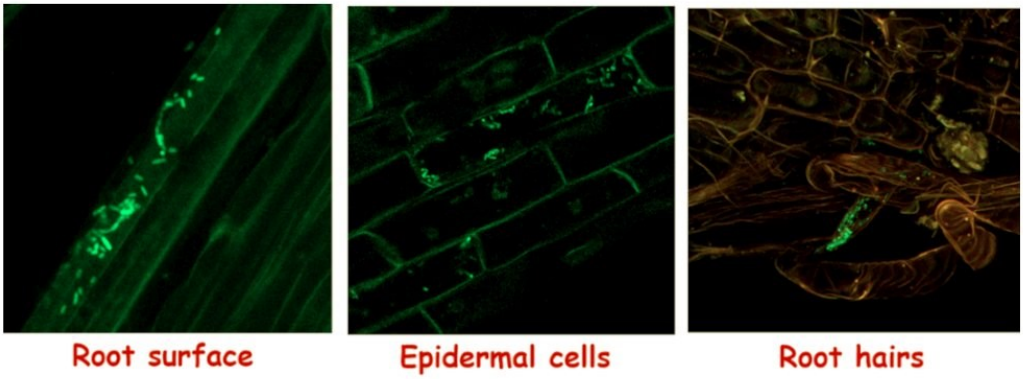

Figure 3.8 Confocal laser scanning microscopy images visualizing colonization of banana roots by the endophytic olive rhizobacterium *Pseudomonas simiae* PICF7. Pictures show events of root surface, epidermal cells and root hairs colonization.

3.2.2. Rhizosphere competence

Analysis of the banana root endosphere-associated microbiota

After a thorough surface disinfection procedure, banana root samples collected in the second sampling round by IAS-CSIC were used to study the whole endophytic communities (bacteria and fungi) by DNA (*16S rDNA* gene for bacteria and the ITS for fungi) amplicon sequencing (Fig. 3.9). DNA samples from roots were analysed to shed light on the structure and composition of their endophytic microbiota and possible correlations with soil type, management, geographical origin (island), or plant phenology.

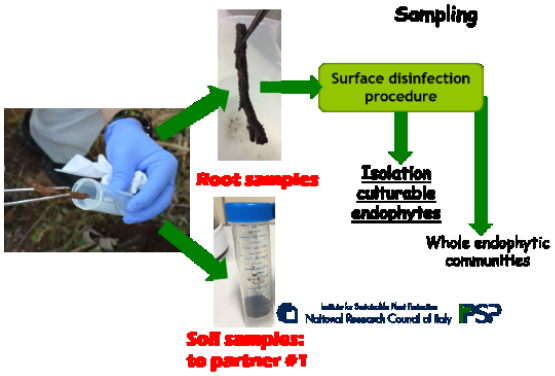

Figure 3.9. Scheme showing manipulation and repartition of banana root samples.

Overall, culturable (WP2) and non-culturable approaches showed a low microbial diversity in banana roots endosphere in fields sampled at Canary Islands. For bacteria, genera *Pseudomonas, Rhizobium, Streptomyces* and *Actinophytocola* showed the highest relative abundance, while for fungi *Ophioceras, Cyphellophora, Plecosphaerella* and *Fusarium* were dominant. When comparing microbial communities from mother and sucker plants some significant differences were found in α-diversity for both the bacteriota and the mycota.

For β-diversity, slight differences among islands and farms were also observed for both microbial communities. Since only a few significant differences were observed the plant genotype appeared crucial for recruiting its associated microbiota. It is worth mentioning the very low number of genera (*Pseudomonas*, *Streptomyces* and *Rhizobium*) that constitute the 'Pequeña Enana' core bacteriome for the 3 surveyed islands (Tenerife, La Gomera and La

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Palma). The core bacteriome shared by mother and sucker banana plants is formed of these same bacterial genera. The sequences of these genera represent more than 38% of total classified sequences. Individual core bacteriomes of Pequeña Enana plants at Tenerife, La Gomera and La Palma islands were made up of 3, 7 and 10 bacterial genera, respectively. The core bacteriome of mother plants consisted of a much higher number of genera than that observed in sucker plants.

For fungal communities, a similar situation was observed. Only 2 genera (*Ophioceras* and *Cyphellophora*) appeared as Pequeña Enana core mycobiome, and 3 (*Ophioceras*, *Cyphellophora* and *Alternaria*) constituted the core shared by mother and sucker plants representing approx. 33 and 38% of the classified sequences, respectively. In this case, the core mycobiome of La Gomera showed the highest number of genera (7), followed by La Palma (5) and Tenerife (3). However, mother and sucker core mycobiomes were nearly similar. Mother mycobiome showed four genera while sucker mycobiome included just three (Fig. 3.10).

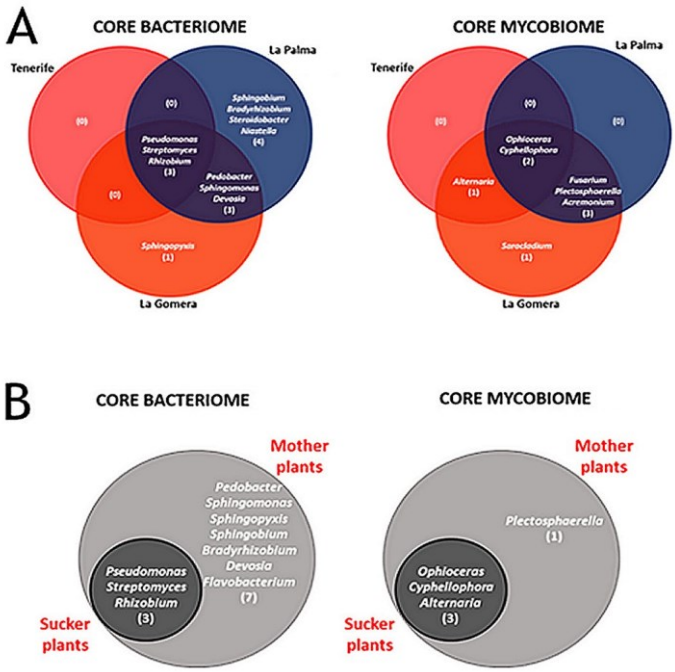

Figure 3.10 Core bacteriome and mycobiome of the cultivar 'Pequeña Enana' root endosphere at Canary Islands (Tenerife, La Gomera and La Palma) (A); Core bacteriome and mycobiome shared by mother and sucker Pequeña Enana plants (B).

Influence of phenology on network topologies of root endosphere communities

Microbial networks based on Spearman's rho correlation were separately constructed for each plant phenological stage (mother plants and suckers). Co-occurrence networks analysis revealed that microbial community interactions in banana roots differ, depending on the plant phenology stage. Different network topologies were observed when comparing mother plants and suckers networks (Fig. 3.11). The network was significantly more complex for suckers and showed higher modularity and Geodesic Distance than shown by mothers.

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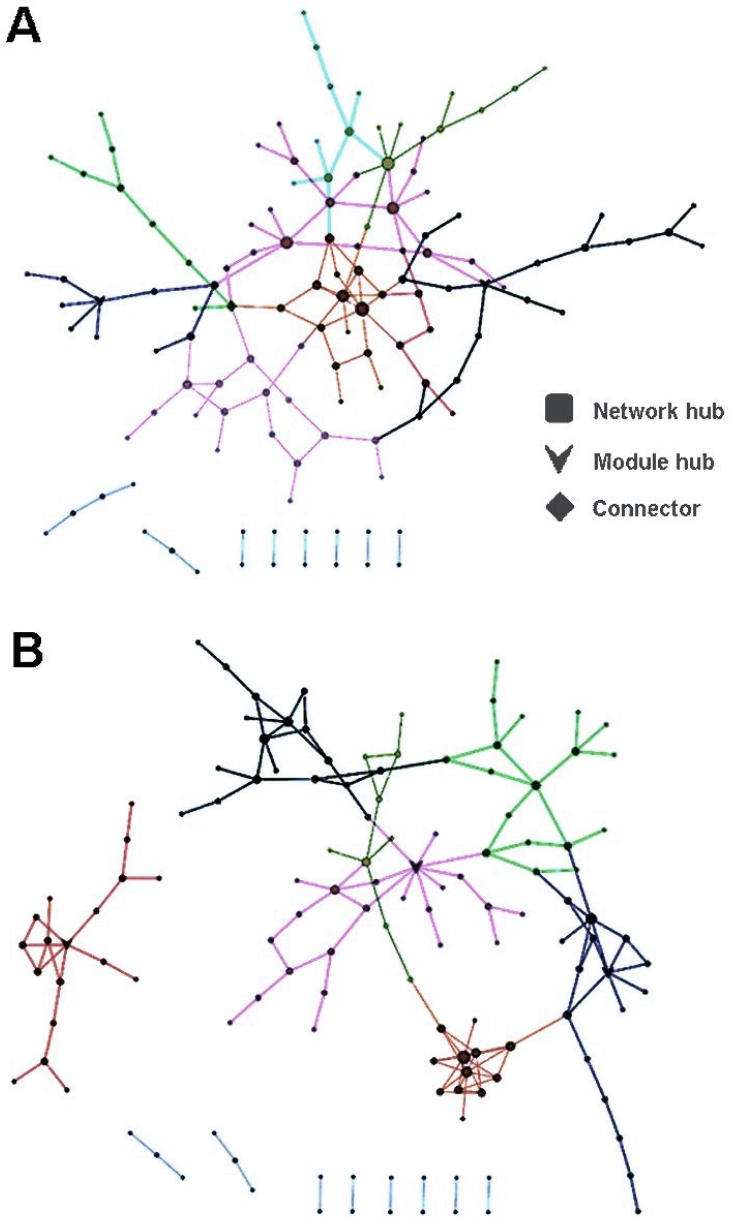

Figure 3.11 Co-occurrence networks of microbial communities from the banana root endosphere of mother plants (A) and suckers (B) at Canary Islands. Each color represents different modules in the networks. Modules with less than 5 nodes are drawn in grey.

Foc *pathogenicity*

To assess FW symptoms development under controlled conditions, new pathogenicity tests were performed by IAS-CSIC evaluating two different substrates and two pathogen inoculation methods. Satisfactory results were obtained by both root-dip and drenching inoculation methods, although disease symptoms by root-dipping appeared at earlier times were more severe. Concerning the potting substrate used, the best results were obtained by using a substrate made of equal volumes of peat:sand:vermiculite (1:1:1) (substrate #1, Fig. 3.12).

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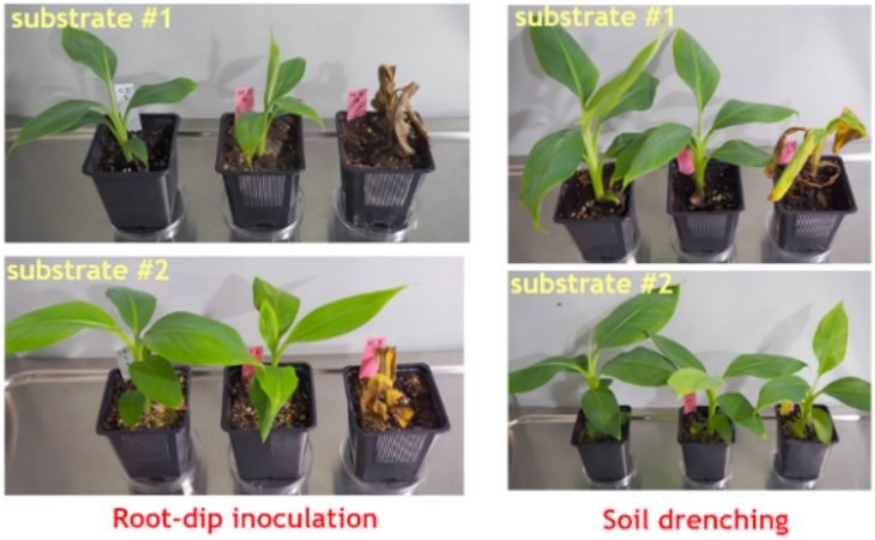

Figure 3.12 Different experimental condition used in Foc pathogenicity tests, comparing two different substrates for plant growth and two pathogen inoculation methods.

Effect of chitosan on banana rhizodeposition

In collaboration with KU Leuven, UA evaluated the effect of chitosan in two different experiments, studying the effect of this biopolymer in liquid or encapsulated formulations, on banana root exudates. For the first experiment, fluorescence analyses were performed showing components (Fig. 3.13), which putatively corresponded to: indole acetic acid (IAA, Component 1) (Em 368/ Ex 290; Em 368/ Ex 255); salicylic acid (SA, Component 2) (Em 418/ Ex 330; Em 418/ Ex 265; Em 418/ Ex 245) and aromatic amino acids (peptides, Component 3) such as tyrosine (Em 314/ Ex 275) and tryptophan (Em 314/ Ex 235).

Chitosan effect on substrate properties for plant development

The effect of chitosan on the physicochemical and microbiological properties of a sandy and a peat substrate was studied by UA. The microbiological analysis was carried out by means of the dilution technique in a soil plate, measuring the CFU/g of cultivable microbiota in two different culture media, per treatment. In the experiment with sand without banana roots, it was found that the microbiota significantly increased in treatments with dissolved chitosan (1 mg/ml), compared to the untreated controls. This could be due to the appearance of microorganisms resistant to chitosan. Fungal colonies preliminarly identified as *Paecilomyces/ Purpureocillium* spp. and macroscopically equal (cottony and white) appeared in plates at very high dilutions (1/10000) in chitosan treatments, but were not observed in plates with equal dilutions in untreated sand. X-ray diffraction of the untreated sand revealed a high content of quartz and dolomite, being therefore much less reactive than the peat.

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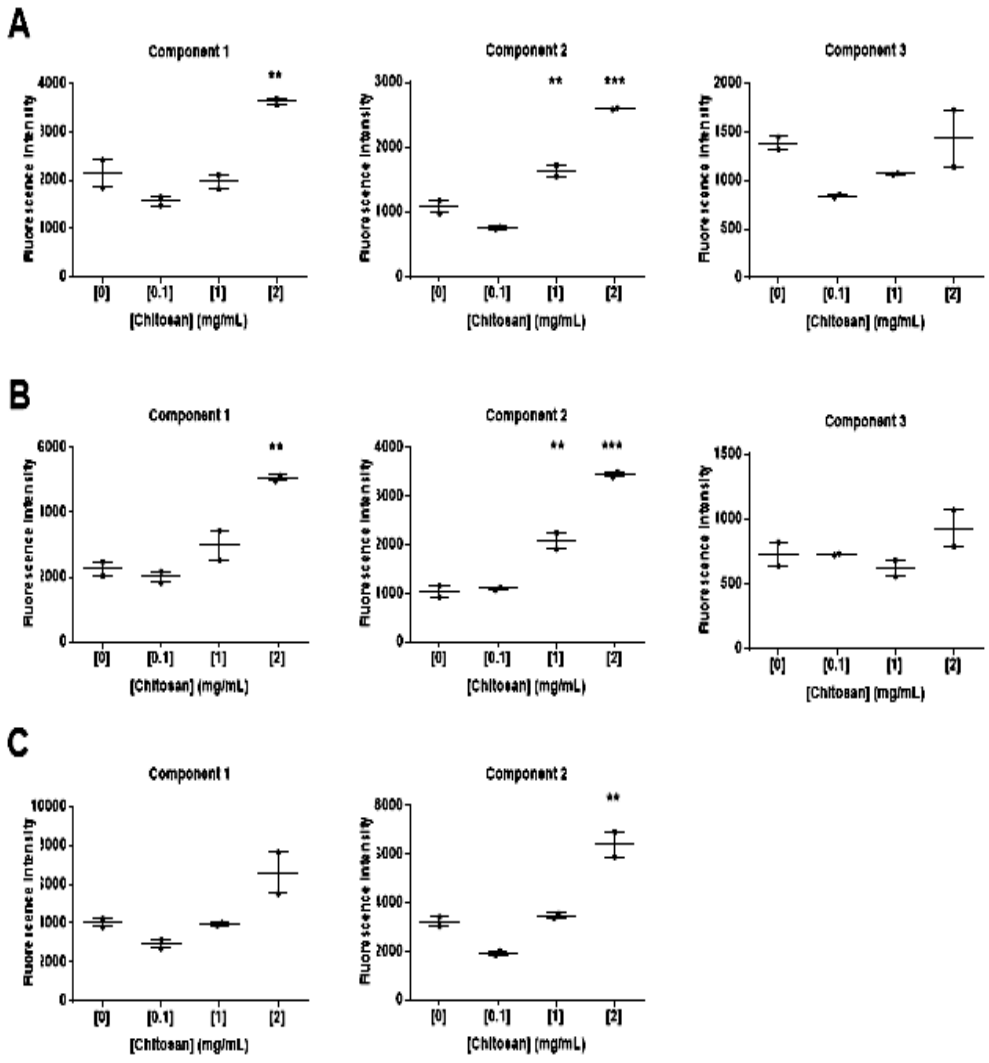

Figure 3.13. Chitosan modified banana (AAA, Petite Naine) roots rhizodeposition. Analysis at 1 (A), 3 (B), and 5 days (C). Chitosan increased the fluorescence intensity of components 1 and 2 in the three times studied. Asterisks show significant differences from control for $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****).

Greenhouse experiments

As *P. chlamydosporia* promoted the plants development in growth chamber, UA team also tested its effect in greenhouse to check growth promotion effects in a long-term experiment. Root, corm, pseudostem and leaf weights and lengths were scored per plant. Number of leaves was also determined, measuring the root colonization with molecular and cultural techniques. UA team observed that flood inoculations of Pc 123 increases plant growth in 105 day-old plants, as the fungus remained in the rhizosphere for a long time (75 days) (Fig. 3.14).

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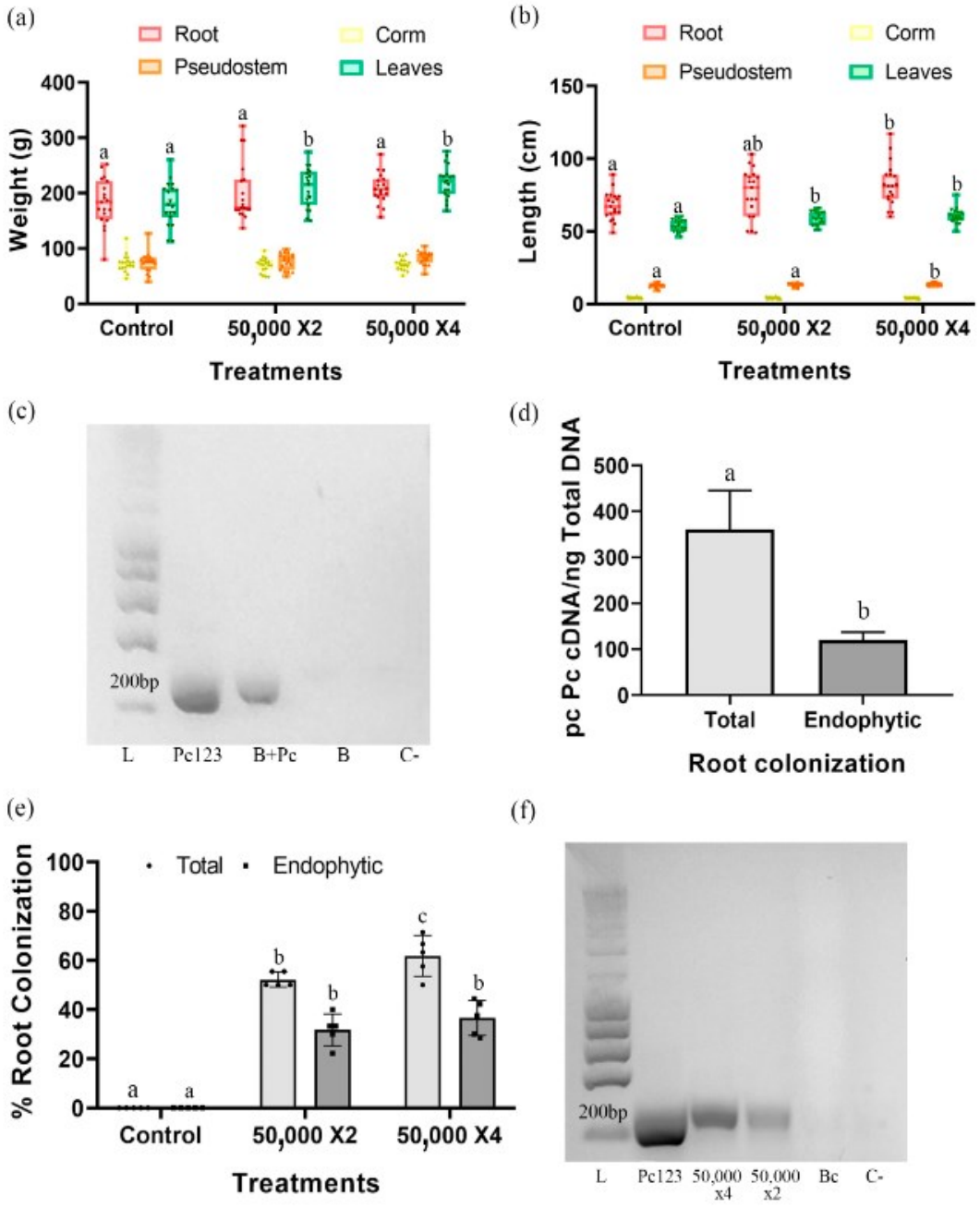

Figure 3.14 Effect of *P. chlamydosporia 123* (Pc) on growth of banana plants. Treatments: (50,000 x2) two spore inoculation plants; (50,000 x4) four spore inoculation plants. Control: plants without inoculation. (a) Maximum root, leaf, pseudostem and corm length. (b) Fresh root, leaf, pseudostem and corm weight. (c) Total and endophytic root colonization. In the parameters analyzed individually, treatments with different letters indicate significant differences ($p < 0.05$). (d) Quantification by qPCR of *Pc* colonization of roots in 30 days old plants. (e) Molecular detection of root *Pc* colonization 30 dpi. (f) Molecular detection of banana root colonization by *Pc* in 105-days-old plants (PCR of the *Pc vpc1* gene). Abbreviations: (M) Ladder; (Pc) DNA extracted from *Pc* mycelium; (B + Pc) DNA extracted from 30 dpi roots of the plant (B) inoculated with *Pc*; (B) DNA extracted from the root of a 30-day banana plant without inoculation; (50,000 x4) DNA extracted from four spore inoculation 105 days old plants; (50,000 x2) DNA extracted from two spore inoculation, 105-day-old plants; (Bc) DNA extracted from the root of a 105-days old banana plant without inoculation; (C-) negative control without DNA.

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Root colonization and plant growth promotion by other P. chlamydosporia *isolates*

To check the genetic variability among *P. chlamydosporia* strains isolated worldwide, UA tested their inoculum in banana plants to measure differences in root colonization and growth promotion. All strains promoted plant growth. Pc21 from Italy (provided by CNR) was the best colonizer and growth promoting strain. Primers designed from the Pc123 *vcp1* gene sequence could amplify the gene from other isolates, apart Pc123 (Fig. 3.15).

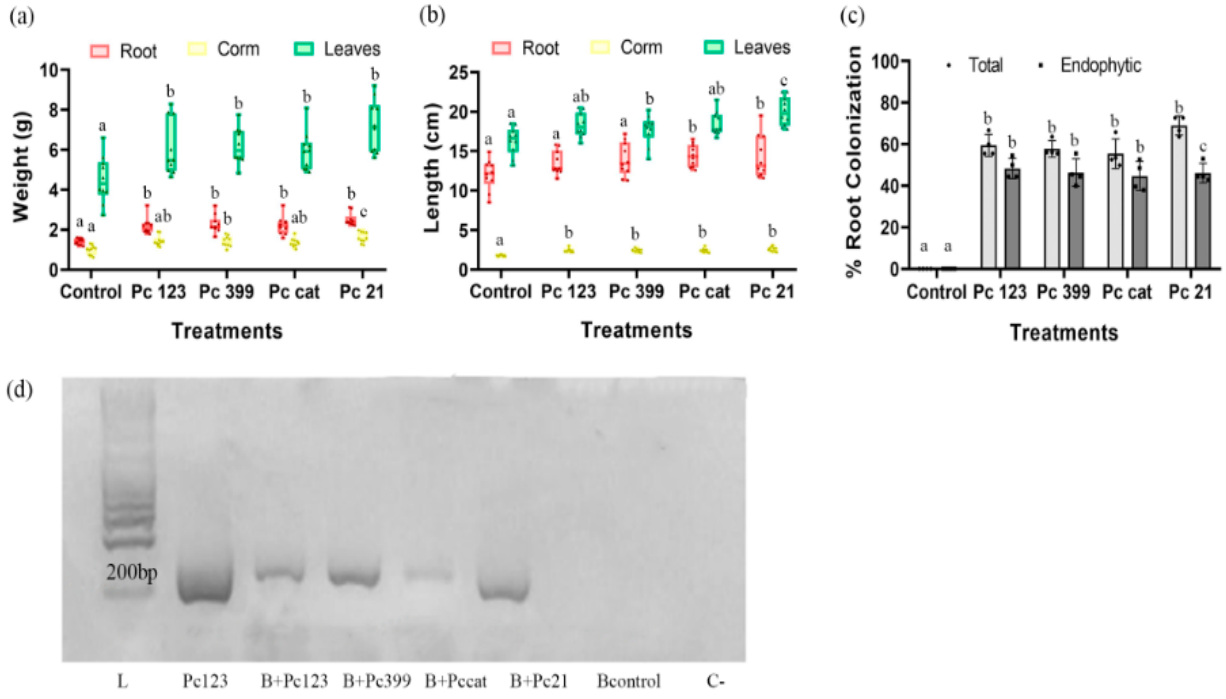

Figure 3.15 Effect of *P. chlamydosporia (Pc)* diversity on the growth of 30-day-old plantlets. Treatments: inoculation of 50,000 conidia and chlamydospores suspension of each *Pc* strains (Pc123, Pc399, Pccat and Pc21). (a) Root, Leaf and Corm Length. (b) Fresh Root, Leaf and Corm Weight. (c) Total and Endophytic Root Colonization. Growth indicators analyzed individually, the treatments with different letters show significant differences (p-value $\alpha = 0.05$). (d) Molecular detection of *Pc* strains colonizing banana roots, using the *vpc1* gene. Abbreviations: (M) ladder; (B+Pc123, B+Pc399, B+Pc cat, B+Pc21) DNA extracted from 30 dpi banana roots with different strains of *Pc*; (Bcontrol) DNA extracted from 30-day plant *(M. acuminata)* roots without *Pc*; (C-) negative control without DNA.

3.2.3 Soil receptivity

Soil properties and banana root endosphere microbiota

IAS-CSIC studied the effect of soil structure on root associated microorganisms. Soil from each of the surveyed orchards were analysed to determine physical and chemical characteristics. Rhizosphere soil samples were collected (5 to 20-cm depth) following plant roots. At each sampling site (mother plant and sucker), two digs were performed. Soil samples from each farm were mixed (1 kg) and analysed by a service provider. Constrained Analysis of Principal Coordinates (CAP or dbRDA) with weighted UNIFRAC distances for bacterial and Bray-Curtis dissimilarities for fungal genera were performed, with ANOVA tests. Finally, the correlation between the significantly different parameters and the genera with $\geq 0.1\%$ relative abundance were computed.

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Overall, physico-chemical soil parameters showed no significant contribution to the microbial profiles found. Although some physico-chemical parameters had highly different values among farms (i.e. pH, organic matter, electrical conductivity and some cations) differing by an order of magnitude, none of them was a good predictor of the root endosphere microbial communities composition in banana plants. Indeed, the statistically significant parameters roughly explained 10% of the samples distribution in the CAP plot. Furthermore, no bacterial genera significantly correlated to any of these parameters and only one fungal genus, *Pyrenochaetopsis* (*p*-value $1.92 \cdot 10^{-8}$), was negatively correlated (*rho* -0.62) to electrical conductivity.

Testing banana cultivation substrates for FocTR4 receptivity

Two substrates largely used in nurseries of Costa Rica were evaluated for suppressiveness against FocTR4. The substrates were provided by Agribiotecnologias, a Costa Rica nationwide micropropation laboratory and nursery, in collaboration with partner EARTH and CNR. The substrates, namely S5 and S6 (based on soil, manure and coconut fibers), were artificially inoculated with FocTR4 whose population density was monitored over time for 14 days, in quarantine controlled conditions in Italy. It was ascertained that S5 and S6 did not contain *Fusarium* spp. before the experiment. Results demonstrated that, once inoculated, FocTR4 was not able to proliferate within S5 and S6 but it was not deactivated (Fig. 53). It was concluded that S5 and S6 were free from *Fusarium* spp. However, if contaminated by FocTR4, they were not suppressive towards the pathogen which could hence infect TC transplanted banana plants.

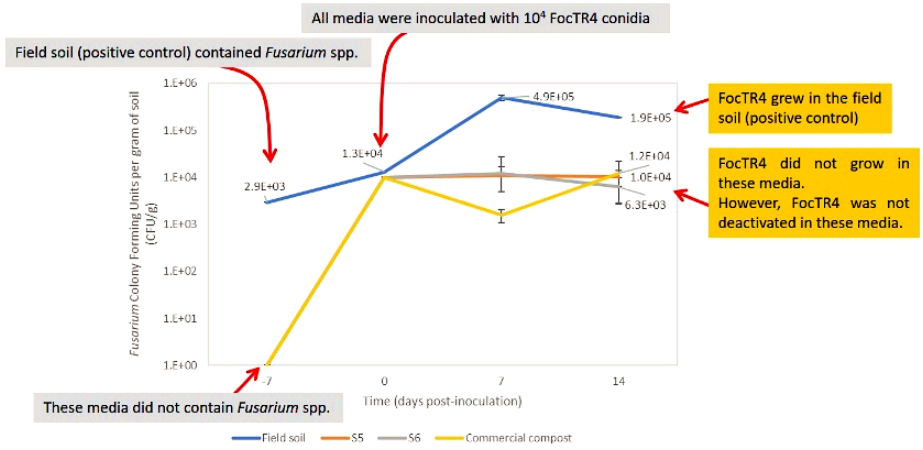

Figure 3.16 Progress over time of Foc TR4 population artificially inoculated in the nursery cultivation substrates S5 and S6.

Receptivity of sterile and non sterile soil to P. chlamydosporia

UA carried out membrane assays to test the capability of *P. chlamydosporia* to colonization sterile and non-sterile soil, by a membrane test in Petri dishes. The surface colonized on the membrane in sterile soil was higher than in non-sterile soil conditions, indicating a competition or fungistatic effect of the indigenous microflora on the fungus (mean colonized surface in sterile and non-sterile soil: 3.3 *vs* 3.9 cm^2, respectively; perimeter of colonized area: 37 *vs* 47 cm, respectively; measures analysed with *SeaScape*). In a different assay, total root colonization (100%) was achieved *in vitro* either for banana root surface and endophytically, after inoculation with conidia or by direct contact with mycelium.

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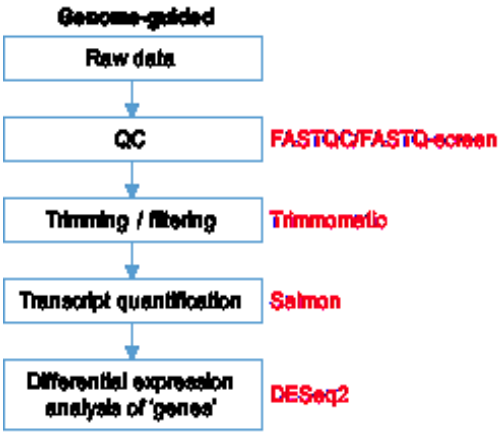
3.2.4 Identification of *P. chlamydosporia* genes involved in plant growth promotion and PPN parasitism

In this task the gene response of *Pochonia chlamydosporia* to the infection of RKN eggs and in response to the biopolymer chitosan was studied by UA in collaboration with UNEXE. For that goal, RNAseq analyses were performed. Three replicates of liquid cultures were performed in four treatments (incubated for 4 days):

- *P. chlamydosporia* (control, Pc)
- *P. chlamydosporia* with 0.1mg/ml chitosan T8 (PcQ)
- *P. chlamydosporia* with *Meloidogyne javanica* eggs (1 egg / µliter, PcRKN)
- *P. chlamydosporia* with 0,1mg/ml chitosan T8 and *M. javanica* eggs (1 egg / µliter, PcRKNQ).

Log_2 fold change values were obtained from Salmon TPM values after mapping RNA sequence libraries against Pc123 re-predicted genome, with the following workflow:



Using this strategy, a number of candidate genes were obtained (Fig. 3.17).

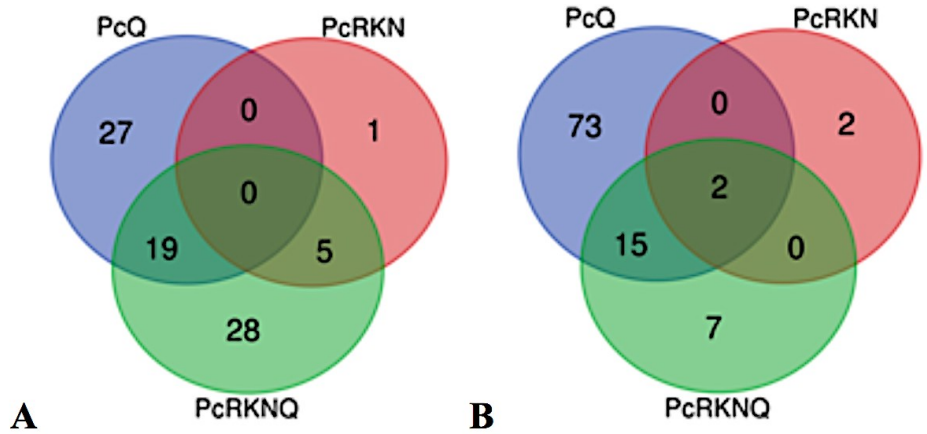


Figure 3.17 Venn diagrams showing a total of 80 up regulated (A) and 99 down regulated (B) genes, with a threshold of ± 2 in log_2 fold change and adjusted p-value (44.7% vs 55.3%). Treatments: PcQ = *P. chlamydosporia* with 1 mg·ml^{-1} chitosan, for 4 days; PcRKN = *P. chlamydosporia* in contact with 1 *M. javanica* egg · µl^{-1}, for 4 days; PcRKNQ = *P. chlamydosporia* in contact with 1 egg·µl^{-1} *M. javanica* and 1 mg·ml^{-1} chitosan, for 4 days.

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Results indicated that chitosan produced a greater stimulus in Pc123 than the mere presence of RKN eggs. The GO enriched functions (Biological Functions category) in the up-regulated genes are shown in Fig. 3.18. Oxidation-reduction and polysaccharide catabolism were the most upregulated processes in chitosan treatments, together with proteolysis. Transmembrane transport was the GO annotation present in all treatments (unique in PcRKN). Functions present in both chitosan treatments, although less represented than the previous ones, were: carbohydrate transport and carbohydrate derivative metabolic process. These processes likely reflect the degradation of chitosan by the fungus.

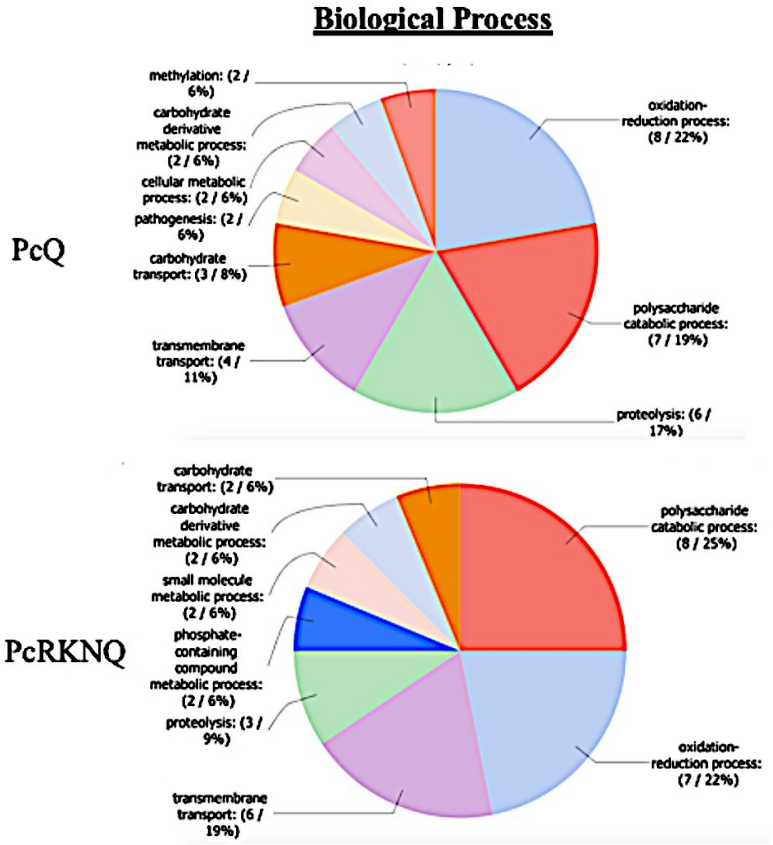

Figure 3.18 GO enriched functions (Biological Functions category) of *P. chlamydosporia* up regulated genes, in the different conditions tested.

A median profile of 180 genes showed 9 clusters. Clusters 1, 2 and 3 had similar behaviors, as in all three cases chitosan activated the expression of genes associated with each cluster. In the first case, chitosan activated these genes in the same way, regardless of the presence of nematodes in the treatment. In cluster 2, the presence of nematodes mitigated the increase in expression, while in cluster 3 the nematodes activated the expression of genes in addition with chitosan. The increase in cluster 3 for treatment without nematodes was very slight. Clusters 4, 5 and 6 included genes with low levels of expression for PcRKN treatment. In this case, chitosan affected the *P. chlamydosporia* treatment, causing a decrease in the expression of genes associated with these clusters. For PcRKN treatment, genes did not modify their expression when chitosan was added. Cluster 7 was opposite to cluster 1, and chitosan completely repressed the expression of all genes associated with this cluster. Cluster 8 contained genes overexpressed in presence of RKN, but adding chitosan it decreased or maintained its expression. Finally cluster 9 included genes overexpressed when chitosan was present, but RKN mitigated their expression.

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In order to find genes of interest in both parasitism and endophytism, the intersection with genes expressed by Pc on barley was determined (Fig. 3.19). Data from the PHI database and genes differentially expressed (DEG) with a log2 fc > 2 were also used. In total, 8 genes were common to all cases:

- RZR66026.1 (secreted aspartic proteinase precursor)
- RZR62042.1 (Helix-loop-helix DNA-binding protein)
- RZR63158.1 (Peptidase A1)
- RZR64159.1 (Major facilitator superfamily domain, general substrate transporter)
- RZR62046.1 (Polyketide synthase)
- RZR61625.1 (Cytochrome P450 ClCP1)
- RZR69240.1 (APSES transcription factor)
- RZR61845.1 (Glycoside hydrolase family 75 protein)

Currently, qPCR data are being analysed to determine the behaviour of Pc in the banana roots colonization, amended or not with chitosan (0.1 mg/ml).

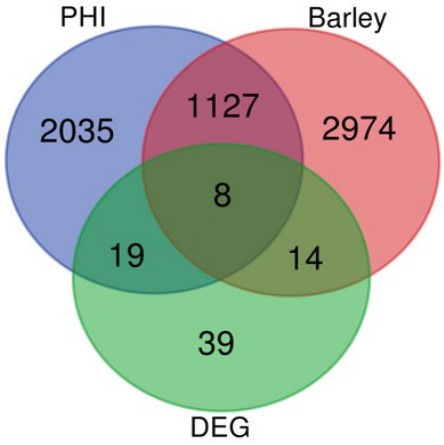

Figure 3.19 Venn diagram showing the intersection of genes expressed when *P. chlamydosporia* colonizes barley. Data from the PHI database and genes differentially expressed (DEG) in this study.

Task 3.3 - Bioactive compounds and their interactions with EBCAs antagonistic to BW and PPN. Task leader: CENSA. Other Participants: UA, ICIPE, Real IPM.

3.3.1 BW bioactive compounds

Insecticidal and behavioral effects of some plant essential oils on BW and chemical composition

All essential oils tested by CENSA caused 100% mortality by topical application of 2 µl of the undiluted oil to each insect (even after 30 min) and at 1 µl after 2, 12, 4 and 48 hours of treatment. At the 1 µl dose per insect, oils 115, 118 and 123 showed the highest toxicity to BW, similar to Cypermethrin, at 30 min, followed by oil 124 at 1 hour. Nevertheless, at the 0.5 µl dose per insect, only essential oils 115 (since 12 hours) and 118 (24 hours of evaluation) killed all treated insects. The essential oils 115 and 118 showed the highest insecticidal activity by topical application, showing the first one a faster effect. Oils 115, 123, 114 and 118 showed a repellent effect at all concentrations and times evaluated, except for oil 118 at the lowest concentration at 12 and 24 hours, that was classified as neutral.

Results indicate that the application as a repellent would be more promising for oils 115 and 118. The effect of oil 124 was classified as attractant, as it attracted a greater number of BW adults at concentrations of 50 and 5%. Oil 116 repelled *C. sordidus* adults at 50% and attracted them to lower concentrations. This oil showed the greatest influence of concentration and time on BW behavior. The presence of oxygenated monoterpenes as main components in 115 and 118 likely underpinned their insecticidal and repellent effects.

Effect of H.amazonensis *HC1 on BW adults*

Strain HC1 of *H. amazonensis* caused highest BW adults mortality (Fig. 3.20). This strain was selected as a reference in studies relative to characterization of new EPNs isolates from banana/plantain fields in western Cuba. The LC_{50} was estimated in > 1800 infective juveniles (IJ) per BW adult. Estimated LC_{90} was > 6000 IJ/adult. Around 50% of BW adults died at 417 hours (~17days) and 90% at 693 hours(~29 days). The HC1 strain caused significant mortality on BW, with highest mortality in the treatment with highest concentration (5000 IJ/BW adult).

Efficacy of different EPNs isolates against BW

Symbiotic bacteria associated to the five *Heterorhabditis* sp. strains (PR-C1, PR-C2, PR-CSJ, PR-C4, and PR-C5, deposited at NL-CENSA Collection) were characterized by CENSA using API and several growth media. All bacteria belonged to *Photorhabdus* spp. EPN caused significant mortality on BWs. The highest mortality (30 %) was caused by *H. amazonensis* HC1 strain. *Heterorhabditis* sp. isolates from Cuban banana/plantain also caused BW mortality, in some cases over 15% (Fig. 3.20).

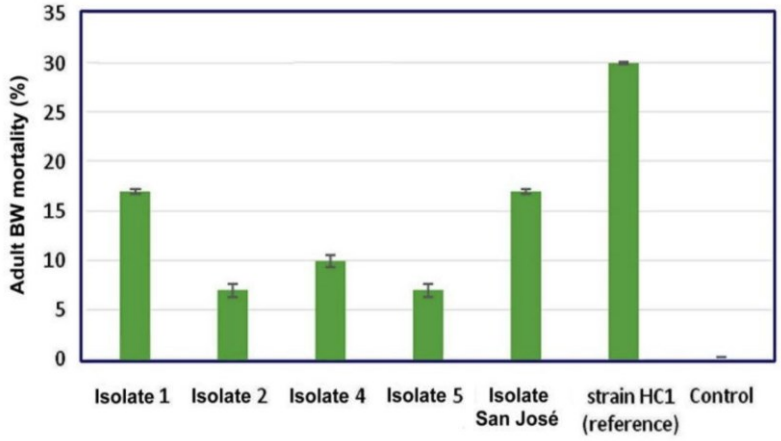

Figure 3.20 EPN induced mortality on BWs (negative control = distilled water).

3.3.2. PPN bioactive compounds

Nematicidal activity of plant essential oils vs Meloidogyne incognita

CENSA studied the effect of essential oils (VenAgro products) vs PPN. The compounds (identified as oils 138, 132, 110, 107, 119, 95, 116, 143 and 108) showed nematicidal activity when compared to the control. The essential oils 138, 132, 110, 107 and 119, killed up to 100% of *M. incognita* J2. For the oils 116, 143 and 108, the mortality values recorded at the second assessment (48 h) increased, compared to values recorded at first assessment (24 h). The activity of these oils against *M. incognita* provide alternative methods for nematode control. The chemical composition of compound 132 is characterized by a high content of oxygenated molecules, mostly alcohols (linalool, α terpineol, geraniol) that are related to a biological activity. The nematicidal action is associated with the presence of alcohols, specifically linalool,

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although other minor components may also contribute. This oil has a strong nematicidal activity and spectrum of action, indicating potential for PPN management.

Compatibility of EPN, P. chlamydosporia *and* T. asperellum *with essentials oils*

The highest viability of *H. amazonensis* strain HC1 was observed in distilled and boiled water. The pH 7 was the most favourable for nematodes, and optimum temperature range was 22 ± 2ºC. The best results were obtained when the IJ were preserved in a mix of methyl paraben (0.18 %) and propyl paraben (0.02 %), and streptomycin sulphate (0.05 %). Ascorbic acid affected the viability and infectivity of IJ and was compatible with the VenAgro product. Triton X-100 at 0.5% had low toxicity over HC1 and can be used in chemical compatibility tests on this EPN strain. The essential oils 99 and 114 and the components canfeno, p-cimeno and piperitone, according to the parameters evaluated, appeared compatible with *H. amazonensis* HC1, and their combined use can represent an effective alternative for pest management.

For *P. chlamydosporia* and *T. asperellum* bioformulations it is recommended to use tensoactives at concentrations that promote sporulation, without significantly affecting vegetative growth, i.e. Tween-20 (1.5%) and Tween 80 (3%).

Chitosan enhancement of banana root colonization by P. chlamydosporia.

A new *P. chlamydosporia* strain was isolated from the rhizosphere of banana plants in Canary Islands. In collaboration with partner Coplaca, UA studied root colonization by this strain and others using chitosan. Chitosan enhanced endophytic colonization of all strains (Fig. 3.21).

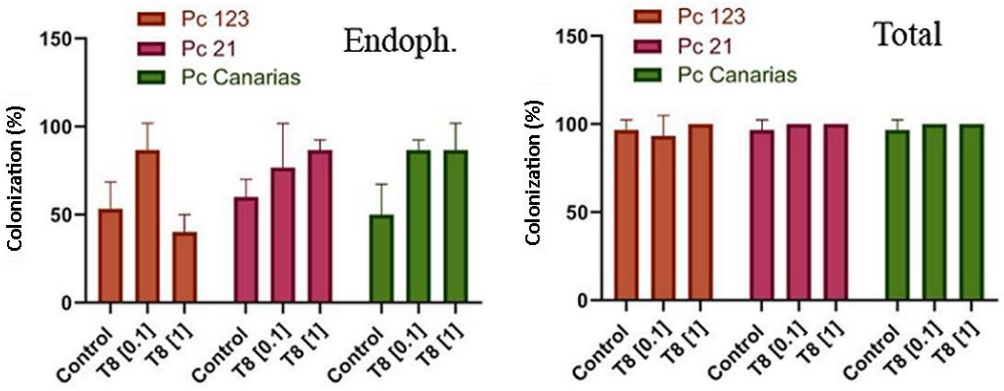

Figure 3.21 Effect of chitosan on endophytic colonization of banana roots by different isolates of *P. chlamydosporia*.

VOCs production by P. chlamydosporia

The fungal strain used by UA in this work was *Pochonia chlamydosporia* Pc123. Conidia from Pc123 were inoculated in four 25 ml Erlenmeyer flasks with hydrated rice. Two different fungus ages were measured: 30 and 70 days after inoculation (dai). To one flask for each dai 10 ml of a 1 mg · ml^{-1} chitosan (T8) dissolved were added, and 10 ml of sterilized distilled water were added to controls (30 and 70 dai, respectively). Finally, the samples were analysed with GC-MS to identify the VOCs profile of each sample (Fig. 3.22). Furthermore, two flasks of hydrated rice, a tube of sterilized distilled water and a tube of liquid T8 were also measured.

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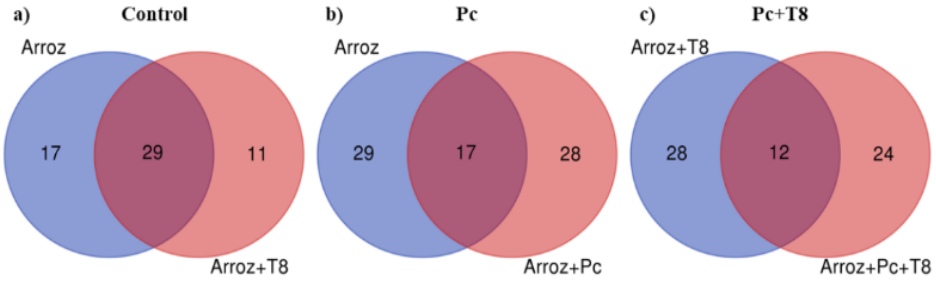

Tabla 2. Tabla de presencia (X) y ausencia de los MVOCs y mVOCs (Pc = *Pochonia chlamydosporia*, T8 = quitosano, RT = Tiempo de retención).

Nº	Compuesto	Producido por: Arroz+Pc	Producido por: Arroz+Pc+T8	RT (min)
	MVOCs			
	Cetonas			
1	DC002	X	X	15.15
2	DC008	X	X	22.18
	Terpenos			
3	DC025	X		29.13
4	DC036		X	35.44
5	DC041	X	X	37.92
	Alcoholes			
6	DC009	X	X	22.34
	Éteres			
7	DC022	X	X	27.92
	Ésteres			
8	DC007		X	22.01
	Compuestos de flúor			
9	DC021	X		27.91
	mVOCs			
	Alcanos			
1	DC037	X		35.45
2	DC040	X		37.15
	Alquenos			
3	DC015		X	24.30
4	DC024		X	29.13
	Alcoholes			
5	DC014	X		24.29
6	DC033	X	X	32.48
7	DC034	X		32.49
	Cetonas			
8	DC001	X	X	6.90
9	DC004	X	X	16.88
	Bencenoides			
10	DC026		X	29.44
	Éteres			
11	DC006		X	21.68
12	DC010		X	22.99
13	DC012	X		23.62
14	DC016	X		24.39
15	DC017	X		24.40
16	DC018	X		24.76
	Terpenos			
17	DC011	X	X	23.16
18	DC038	X		35.73
	Nucleosidos purínico			
19	DC031		X	31.00
	Compuestos de nitrógeno			
20	DC013		X	23.86
	Compuestos de azufre			
21	DC027	X	X	30.20
22	DC032	X	X	31.00
	Compuestos de silicio			
23	DC020		X	26.49
24	DC028	X		30.53

Figure 3.22 VOCs production by *P. chlamydosporia* Pc123 in presence of chitosan.

Task 3.4 - Risk analysis in the banana and enset defense response. Task leader: UA Other Participants: CSIC.

A Risk analysis assessment was carried out by IAS CSIC, through molecular identification of potential human, plant or insect pathogens among the banana root endophytes isolated at Canary Islands, to rule out those posing potential biological risk to humans and the environment. Potential human (e.g., *Serratia marcescens*, *Mycobacterium peregrinum*, *Chrysobacterium indolegens, Alternaria alternata*), plant (e.g., *Alternaria alternata, Erwinia* spp., *Fusarium oxysporum* f.sp. *dianthi, F. proliferatum, Plectosphaerella cucumerina*) and insect (e.g., *Verticillium insectorum*) pathogens were identified as natural inhabitants of the root endsophere. Despite the fact that some of them showed phenotypes related to biocontrol, PGP and *in vitro* antagonistic ability *vs Foc* races, they were discarded for field application.

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Toxicological test were carried out by CENSA for *Trichoderma asperellum* strain 13 and 78. Assays aimed at evaluation of different parameters and biological traits, as follows:

Aimed effect/ assay	Result
Toxicity / pathogenicity	Not toxic or pathogenic to fish
Toxicity / pathogenicity	Not toxic or pathogenic to earthworms
Administered orally to rats, in high concentration and at a single dose	Did not produce mortality, did not induce the appearance of signs or toxic alterations, nor caused infectivity or pathogenicity in the conditions of the assay.
Assessment of acute toxicity/ dermal pathogenicity in rabbits.	Did not produce mortality, did not induce the appearance of signs or toxic alterations and did not cause pathogenicity in rabbits administered by dermal route with a limit concentration.
Evaluation of ophthalmic irritability.	Did not present an irritant or ophthalmic corrosive potential.
Evaluation of dermal irritability.	Did not present corrosive potential or primary dermal irritant.

Elements for EPN risk analysis in Cuba

CENSA reported about the registration process of biocontrol agents in Cuba, whose distinctive elements are the active participation of different Ministries for the analysis of each product. All biopesticides are evaluated by the National Director of Environmental Health in Public Health Ministry and by General Director on Plant Heath Center (in the case of biopesticides for agricultural purposes). The process is described in "Procedimiento para el registro de plaguicidas biológicos (Procedure for biological pesticides register)". This process produces the biopesticide publication in the Official List of Authorized Pesticides. In Cuba, as in the UE, EPN do not require registration. In Cuba their import - export are, however, regulated by Res. 180/2007, Annex12 and 13 (Min. Science and Technology - CITMA, 2007). Therefore EPNs are no-risk organisms.

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

Task 4.1 Gene expression in tolerant/susceptible banana and enset, under biotic stresses

Effect of commercial and non-commercial PGPM on gene expression and/or plant growth

Plant growth promotion assays were carried out by KUL in collaboration with project partners Real IPM/Biobest and/or IAS-CSIC. A number of trials were developed on plant growth promotion with strains previously selected. Seven banana endophytic bacteria (IAS-B-197, IAS-B-364, IAS-B-793, IAS-B-931, IAS-B-944, IAS-B-966 and IAS-B-1040) and two bacterial BCA isolated from the olive roots (PICF7 and PIC73) were used. As mentioned above, optical densities (OD_{600}) have been calculated for each bacterium to prepare inocula (10^8 CFU/ml) as cell suspension for each bacteria (Fig. 4.1). These strains have been transferred to KUL after the signature of a corresponding MTA.

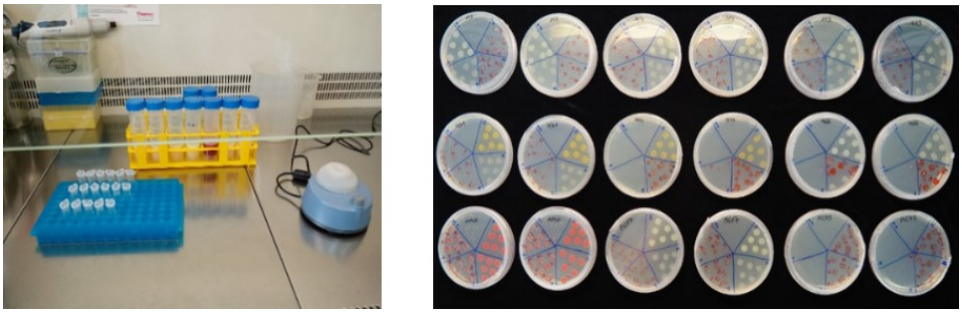

Figure 4.1 Estimation of CFU/ml for each strain to be used in bioassays.

A second plant growth promotion assay was carried out by KUL (Dr. Jassmine Zorrilla) with seven selected strains of banana endophytic bacteria (IAS-B-197, IAS-B-364, IAS-B-793, IAS-B-931, IAS-B-944, IAS-B-966 and IAS-B-1040). Significant differences in plants treated with IAS-B-364 were found for pseudostem height, projected leaf area, number of primary roots and fresh root weight parameters. Additionally, reference strain PICF7 promoted dry root and fresh leaf weights, 10 weeks after inoculation.

To evaluate the response at the gene expression level and/or the growth promotion effect of PGPF *T. asperellum* and five different PGP bacterial endophytes (PGPBE), greenhouse experiments were carried out by KUL.

Experiment 1

Two banana genotypes were used: Gran Enano ITC1256 (Cavendish subgroup, AAA) and Yangambi km 5 ITC1123 (Ibota Bota subgroup, AAA). They were chosen for being contrasting genotypes based on previously reported data on resistance and susceptibility to plant parasitic nematodes (PPN), Fusarium wilt (Foc) and banana weevils (BW). *In vitro* plants provided by International Transit Center (ITC: https://www.biw.kuleuven.be/biosyst/plantenbiotechniek/tropical/international-transit-centre) were transferred to greenhouse and air pumps were connected to avoid hypoxia. Two weeks later, half of the plants were inoculated with a known concentration of spores of PGPF *T. asperellum* strain TRC900 (marketed as Real Trichoderma and provided by Real IPM). Finally, root and leaf samples were taken for further gene expression analyses at 24 and 48 hours post inoculation (hpi) (Fig. 4.2). A subset of 21 genes of interest were analysed by qRT-PCR with primers designed to amplify all paralogs in each gene family (Table 13), selected based on previously reported data with other PGPM.

Fig. 4.2 Hydroponic system assay for *Trichoderma asperellum* TRC900 inoculation of banana plants. Left: 'Gran Enano' and 'Yangambi Km 5' plants growing in hydroponics with aeration. Middle: size of the plants at the end of the experiment. Right: root samples for gene expression analyses.

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Table 13. List of 21 selected genes for qRT-PCR analyses together with their abbreviations and metabolic processes in which they are involved.

Gene	Abbreviation	Metabolic process
1-aminocyclopropane-1-carboxylate oxidase	*ACO*	Ethylene biosynthesis
Ethylene responsive factor 1	*ERF1*	
Peptidylprolyl Cis/Trans Isomerase NIMA-Interacting 1	*PIN1*	
Tryptophan aminotransferase	*TAA1*	Auxin biosynthesis, transport or signalling
Indole-3-acetaldehyde oxidase	*AAO1*	
Amidase 1	*AMI1*	
Auxin response factor 1	*ARF1*	
Non-symbiotic class-1 hemoglobin	*HB1*	Anaerobic respiration
Alternative oxidase	*AOX*	Alternative respiration
Alcohol dehydrogenase	*ADH*	Fermentation
Lactate dehydrogenase	*LDH*	
Pyruvate decarboxylase	*PDC*	
ER Oxidoreductase	*ERO1*	Endoplasmic reticulum stress
Transcription factor bZIP17	*bZIP17*	
Ascorbate peroxidase	*APX*	ROS scavenging
Catalase	*CAT*	
Glutathione reductase	*GR*	
Superoxide dismutase	*SOD*	
Pathogenesis-related 1	*PR1*	Plant defense
Phenylalanine ammonia lyase	*PAL*	
Polyphenol oxydase	*PPO*	

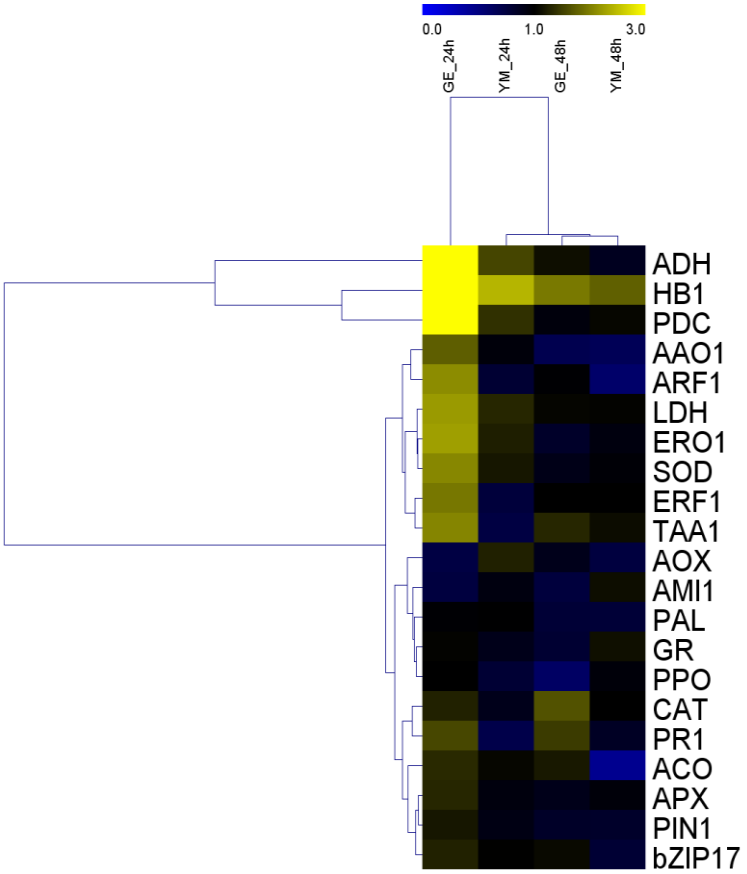

Figure 4.3 Hierarchical clustering representation of the differential expression in 21 genes quantified by qRT-PCR in roots after *Trichoderma asperellum* TRC900 inoculation. Euclidean distance and complete linkage clustering were used to group the selected genes and genotype-time point combinations. Roots were collected at 24 and 48 hpi. Each cell represents the fold expression average of 5 independent biological replicates of each time point, and is relative to the control collected in each time point. GE: Gran Enano; YM: Yangambi Km 5. Gene abbreviations according to Table 13. Yellow: upregulation, blue: downregulation, black: no significant change ($p>0.05$).

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Differential gene expression analyses showed that 7 out of the 21 tested genes were up regulated in Gran Enano at 24 hpi. Those involved in hypoxia (*ADH, PDC, LDH, HB1*) were the most significantly up-regulated, followed by genes involved in ROS scavenging and endoplasmic reticulum stress (*SOD, ERO1*), or auxin biosynthesis (*TAA1*) (Fig. 4.3). The up regulation of genes related to hypoxia/anaerobic metabolism indicates a lack of O_2 inside the root tissue, caused by an increased metabolic rate. Remarkably, the only gene up regulated in all conditions was a non-symbiotic class-1 hemoglobin (*HB1*), involved in anaerobic respiration (Fig. 4.3). Clustering of the genotypes and treatments showed that the response in Gran Enano at 24 hpi was stronger and significantly different from that at 48 hpi, or from response in Yangambi km5 at 24 or 48 hpi (Fig. 4.3).

Experiment 2

In this assay, the effect of two strains of the PGPF *T. asperellum* on PGP and gene expression was evaluated separately. Banana plants genotype Valery ITC0048 (Cavendish subgroup, AAA), provided by the International Musa Transit Center, were inoculated with *T. asperellum* strain TRC900 or *T. asperellum* strain T34 (marketed as Asperello) in a greenhouse experiment (Fig. 4.4). Real Trichoderma, (isolated from tomato plants from a greenhouse in Kenya) was provided as pure spores by Real IPM. Asperello (isolated from peat soil in the south of Spain) was provided by Biobest as a formulated powder containing spores. Both strains were inoculated at a known spore concentration as CFU/mL by root dipping and drenching at the start of the experiment, with a second drench repeated 2 weeks later with the same inoculum concentration (Fig. 4.4).

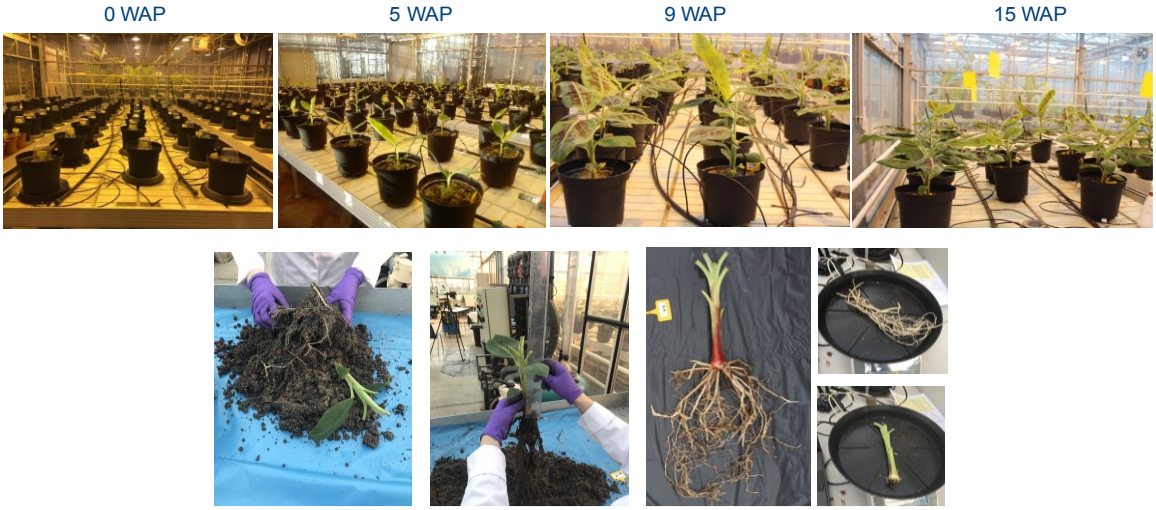

Figure 4.4 Overview of the plant growth promotion experiment in pots in which Cavendish type banana plants were inoculated with *T. asperellum* strains TRC900 and T34. Top: Valery plants at different growing stages during the experiment. Bottom: examples of destructive parameters measured at the two selected time points (9 and 15 WAP).

Plants inoculated with TRC900 or T34 showed significant increases when compared to controls for the following plant growth parameters: PsH, NL, LA, NR, FRW, FSW and FLW, with increases rates usually similar for both strain at each time point. Fig. 4.5 shows significant differences as increase rates, over control plants for PsH and LA (above-ground), NR and FRW (below-ground).

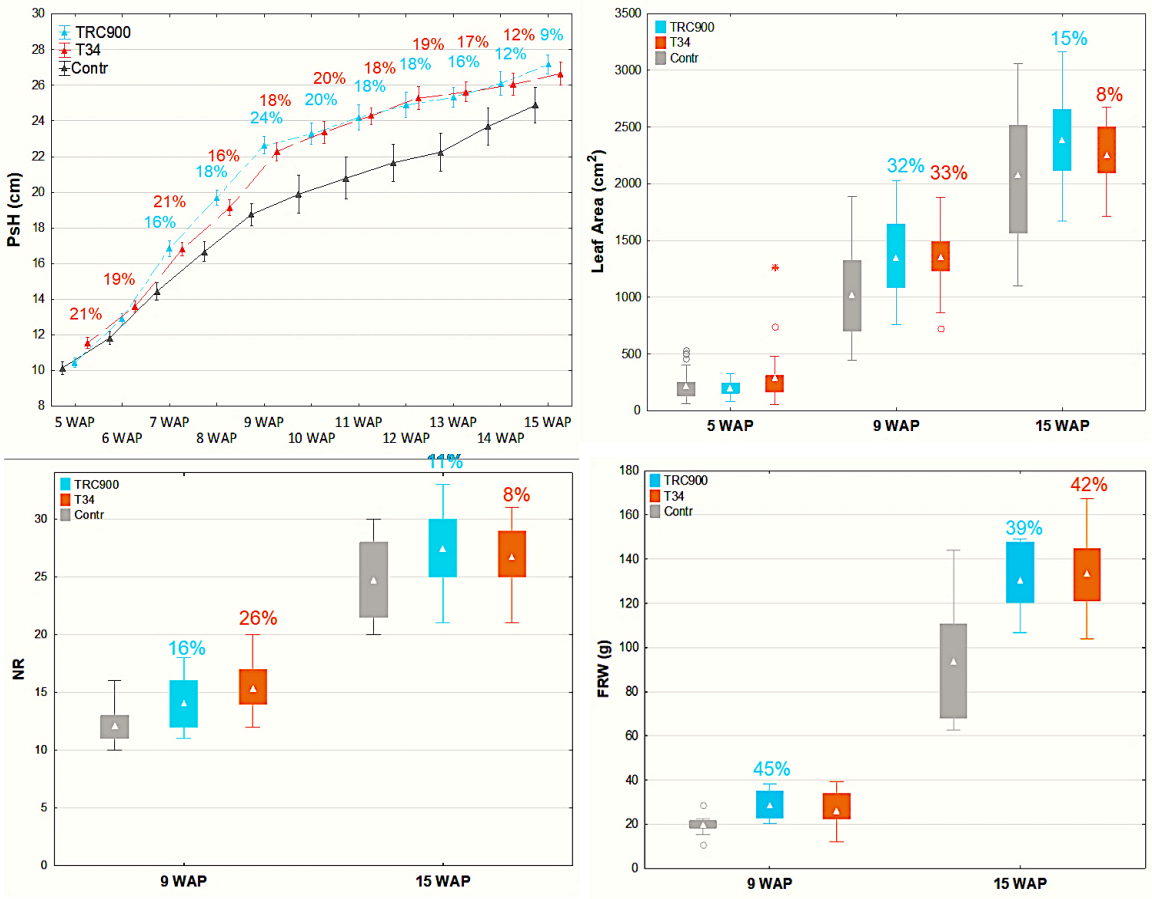

Figure 4.5 Examples of above- and belowground plant growth parameters evaluated after inoculating with *T. asperellum* strains TRC900 or T34. PsH: pseudostem height, LA (projected leaf area), NR (number of primary roots) and FRW (fresh root weight). N. of plants per treatment and time point =15. Percentage of significant increase respect to control is indicated for the corresponding treatments. WAP: weeks after planting.

At 9 and 15 WAP, samples of vegetative tissues (root, leaf and corm) were collected for gene expression analyses. A subset of 28 genes of interest were analysed by qRT-PCR with primers designed to amplify all paralogs in each gene family (Table 14).

Table 14. List of 28 selected genes for qRT-PCR analyses, together with their abbreviations and metabolic processes in which they are involved.

Gene	Abbreviation	Metabolic process
1-aminocyclopropane-1-carboxylate oxidase	*ACO*	Ethylene biosynthesis
Ethylene responsive factor 1	*ERF1*	
Peptidylprolyl Cis/Trans Isomerase NIMA-Interacting 1	*PIN1*	
Tryptophan aminotransferase	*TAA1*	Auxin biosynthesis, transport or signalling
Indole-3-acetaldehyde oxidase	*AAO1*	
Amidase 1	*AMI1*	
Auxin response factor 1	*ARF1*	
Non-symbiotic class-1 hemoglobin	*HB1*	Anaerobic respiration
Alternative oxidase	*AOX*	Alternative respiration
Alcohol dehydrogenase	*ADH*	Fermentation
Lactate dehydrogenase	*LDH*	

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Pyruvate decarboxylase	*PDC*	
ER Oxidoreductase	*ERO1*	Endoplasmic reticulum
Transcription factor bZIP17	*bZIP17*	stress
Ascorbate peroxidase	*APX*	
Catalase	*CAT*	ROS scavenging
Glutathione reductase	*GR*	
Superoxide dismutase	*SOD*	
Pathogenesis-related 1	*PR1*	
Phenylalanine ammonia lyase	*PAL*	Plant defense
Polyphenol oxydase	*PPO*	
Abscisic stress-ripening protein 3	*ASR3*	Abiotic stress
Transcription factor YABBY5	*YABBY5*	Cell expansion
9-cis-epoxycarotenoid dioxygenase 3	*NCED3*	ABA biosynthesis
ATP binding casette G25	*ABCG25*	ABA transport
Horcolin-like	*Horcolin*	Induced by gibberelins
Caffeic acid 3-O-methyltransferase	*COMT*	Lignin biosynthesis
CASP-like protein 4D1	*CASP*	Construction of cell wall

As shown in Fig. 4.6, and based on the differential gene expression patterns of the 28 selected genes, clustering reflected tissue type rather than the inoculated strain, with root and leaf tissues grouping together while the corm clustered separately.

When evaluating the effect of each strain in the three vegetative tissues, TRC900 induced a higher response in the leaf with 6 genes differentially expressed (*PAL, ARF1, AAO1* and *PDC* down regulated; *TAA1* and *NCED3* up regulated) followed by the corm with 5 differentially expressed genes (*APX, bZIP17, AMI1* and *TAA1* down regulated; *PR1* up regulated), while none of the tested genes was significantly up or down regulated in roots (Fig. 4.6).

Strain T34 induced even a higher response in the leaf with 18 differentially expressed genes (*SOD, CAT, GR, ERO1, bZIP17, ASR3, PAL, ARF1, AAO1, AMI1, PIN1, ABCG25, YABBY5, ADH, PDC* and *AOX* were down regulated; *TAA1* and *NCED3* were up regulated), followed by the corm with 6 genes differentially expressed (*APX, bZIP17, PAL, AMI1* and *TAA1* were down regulated; *PDC* up regulated), and 2 genes differentially expressed in root (*PAL* was down-regulated; *PDC* was up-regulated) (Fig. 4.6).

Globally, *T. asperellum* induced genes related to hypoxia and plant defence in corm and root tissues, while modifying the expression of ROS-scavenging and hormone biosynthesis/ transport genes in leaf at 9 WAP. Both *T. asperellum* strains induced a higher response in leaves than in the other two vegetative tissues indicating a bottom-up signal (from roots to leaves).

To investigate if the application of *T. asperellum* strain TRC900 or T34 on banana plants cv Valery leads to changes in rhizosphere microbial communities and root endophytes under greenhouse conditions, a total of 33 root and 33 rhizosphere soil samples were collected from both time points (9 and 15 WAP) for further molecular analyses at University of Liège.

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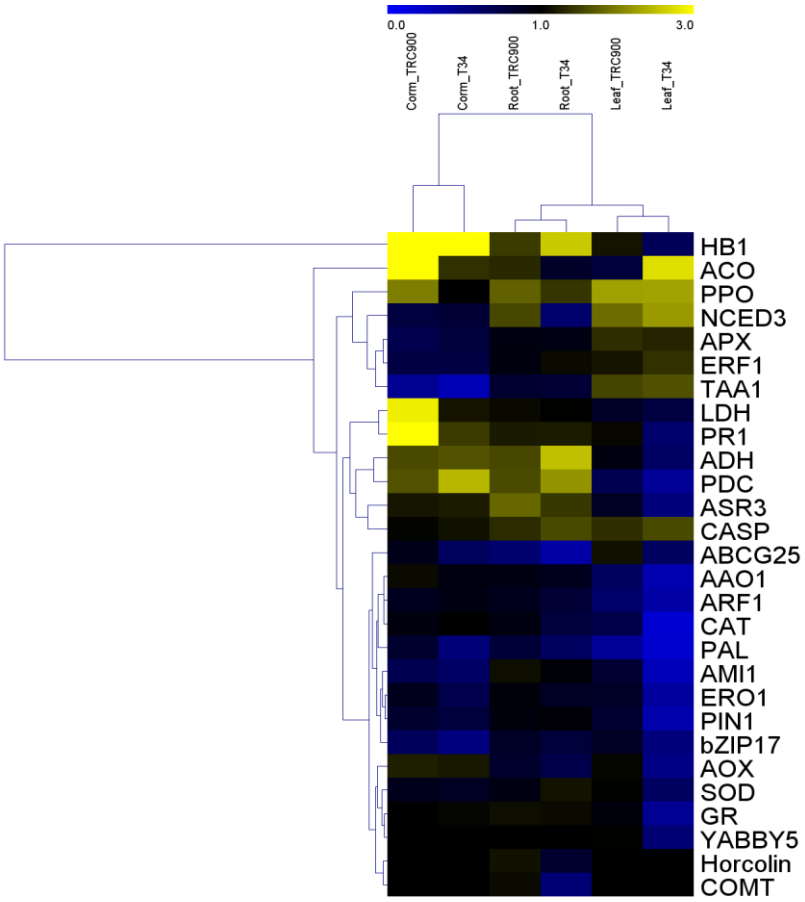

Figure 4.6 Hierarchical clustering representation of the differential expression in 28 genes quantified by qPCR in vegetative tissues after *T. asperellum* strain TRC900 or T34 inoculation. Euclidean distance and complete linkage clustering were used to group the analysed genes and tissue types collected at 9 WAP. Each cell represents the fold change expression average of 5 independent biological replicates of each time point, and is relative to control. Gene abbreviations according to Table 14. Yellow: up regulation, blue: down regulation, black: no significant change ($p > 0.05$).

Experiment 3

This assay started in March 2020 in collaboration with IAS-CSIC to test growth promotion effect of five bacterial endophytes from banana or olive roots, belonging to *Pseudomonas chlororaphis*, *P. protegens*, *P. fluorescens* and *Paenibacillus polymyxa* (Table 15).

Table 15. Root endophytic bacterial strains and root systems from which they were isolated.

Molecular ID of bacterial strains	**Isolates**	**Native root system**
Pseudomonas chlororaphis	IAS-B-364	Banana
Pseudomonas chlororaphis subsp. *piscium*	IAS-B-966	Banana
Pseudomonas protegens	IAS-B-793	Banana
Pseudomonas fluorescens	PICF7	Olive
Paenibacillus polymyxa	PIC73	Olive

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The three banana root endophytes were isolated from plants located on farms in the Canary Islands (Tenerife, La Palma and La Gomera) and showed *in vitro* antagonism against isolates of Foc R1 and STR 4. Additionally, they exhibited *in vitro* activities typically associated to biocontrol and growth promotion, such as phytase, catalase, siderophores and other phenotypic traits. *Pseudomonas fluorescens* PICF7 was tested positive for phytase, catalase, protease and phosphatase activities, siderophore production and weak xylanase and glucanase activities. *Paenibacillus polymyxa* PIC73 showed catalase, protease, chitinase, amylase and xylanase activities, siderophore production, and weak phosphatase and glucanase activities.

At the start of the experiment, *in vitro* banana plants cv Grande Naine ITC 0180 (Cavendish subgroup, AAA), were inoculated with the selected strains in greenhouse (Fig. 4.7) and drenched either with the same known concentration of the bacterial inoculum or the control solution. The first non-destructive measurements started at 5 WAP and continued for 5 weeks until the end of the experiment at 10 WAP. At 10 WAP, samples were collected from root, leaf and corm for dry weight measurements and for gene expression analyses (Fig. 4.7).

Figure 4.7 Overview of the plant growth promotion experiment in pots in which Cavendish type banana plants were inoculated with five different root bacterial endophytes (strains IAS-B-364, IAS-B-793, IAS-B-966, PICF7 and PIC73). Top: Grande Naine plants at different growing stages during the experiment. Bottom: examples of destructive parameters measured at 10 WAP.

Plants inoculated with strains IAS-B-364, IAS-B-966, IAS-B-793 or PIC73 showed significant increases when compared to controls in the plant growth parameters: PsH and LA (IAS-B-364, IAS-B-793), NR (IAS-B-364, IAS-B-793 and PIC73), FRW (IAS-B-364) or NL (IAS-B-966). On the contrary, plants inoculated with either strain IAS-B-793 or PIC73 showed a significant reduction in NL. Fig. 4.8 shows significant differences as rate of increase over control plants, for above-ground and below-ground parameters.

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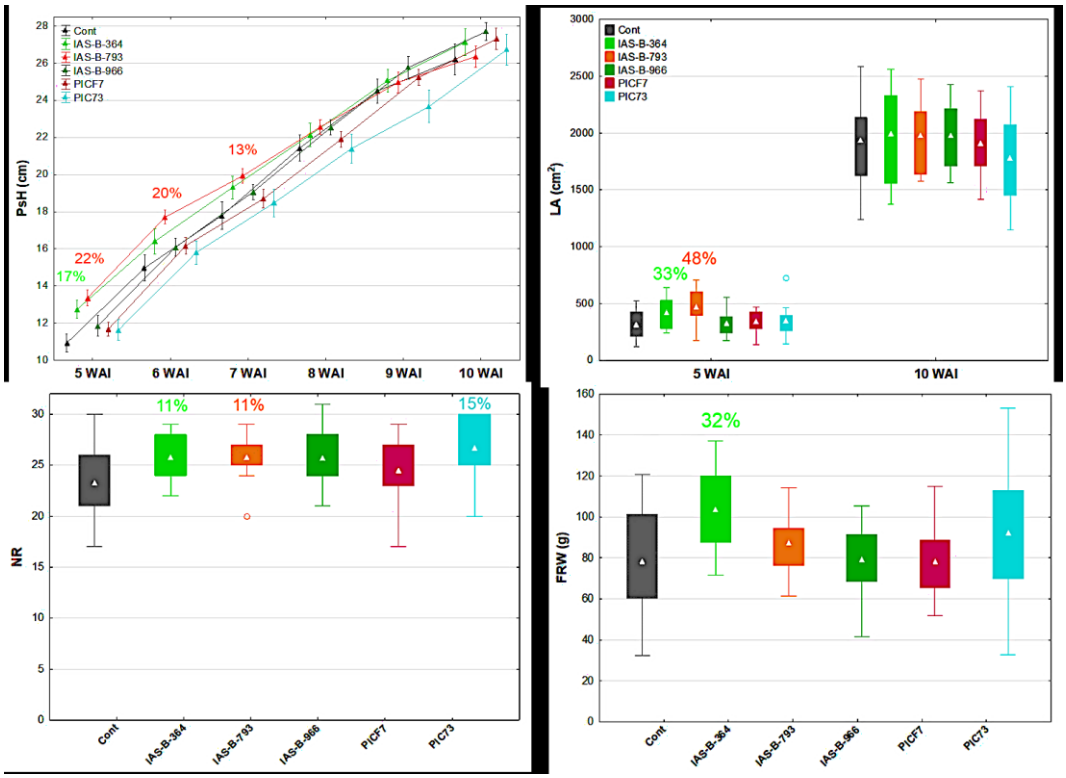

Figure 72. Examples of above- and below-ground plant growth parameters evaluated after inoculating with five different root bacterial endophytes (strains IAS-B-364, IAS-B-793, IAS-B-966, PICF7 and PIC73). PsH: pseudostem height, LA (projected leaf area), NR (number of primary roots) and FRW (fresh root weight). Nr plants per treatment: N=15. Percent of significant increase respect to control is indicated for corresponding treatments (WAP= weeks after planting).

Evaluation of growth promotion effects

Experiments on *Trichoderma asperellum* and *Azospirillum brasilense* were carried out by KUL in Dominican Republic, Cristal Vitro Dominicana nursery (https://cristalvitrodominicana.business.site/).

Experiment 1

In this trial, the effect of the PGPF *Trichoderma asperellum* strain TRC900 or T34 (provided by Real IPM/Biobest) on growth of banana plantlets cv Williams (Cavendish subgroup, AAA) was evaluated. At the start of the experiment, 6 weeks old plantlets were inoculated and transferred from the planting tray to 1.5 pots and the treatments were applied by submerging the roots in the microbial solutions with a known spore concentration in CFU/ml (Fig. 4.8). Plants inoculated with *T. asperellum* TRC900 or T34 showed significant increases when compared to controls in all measured parameters, except number of leaves (Fig. 4.9).

Figure 4.8 Overview of the plant growth promotion experiment in nursery in which Cavendish type banana plants of the genotype 'Williams' were inoculated with the PGPF *Trichoderma asperellum* strains TRC900 or T34.

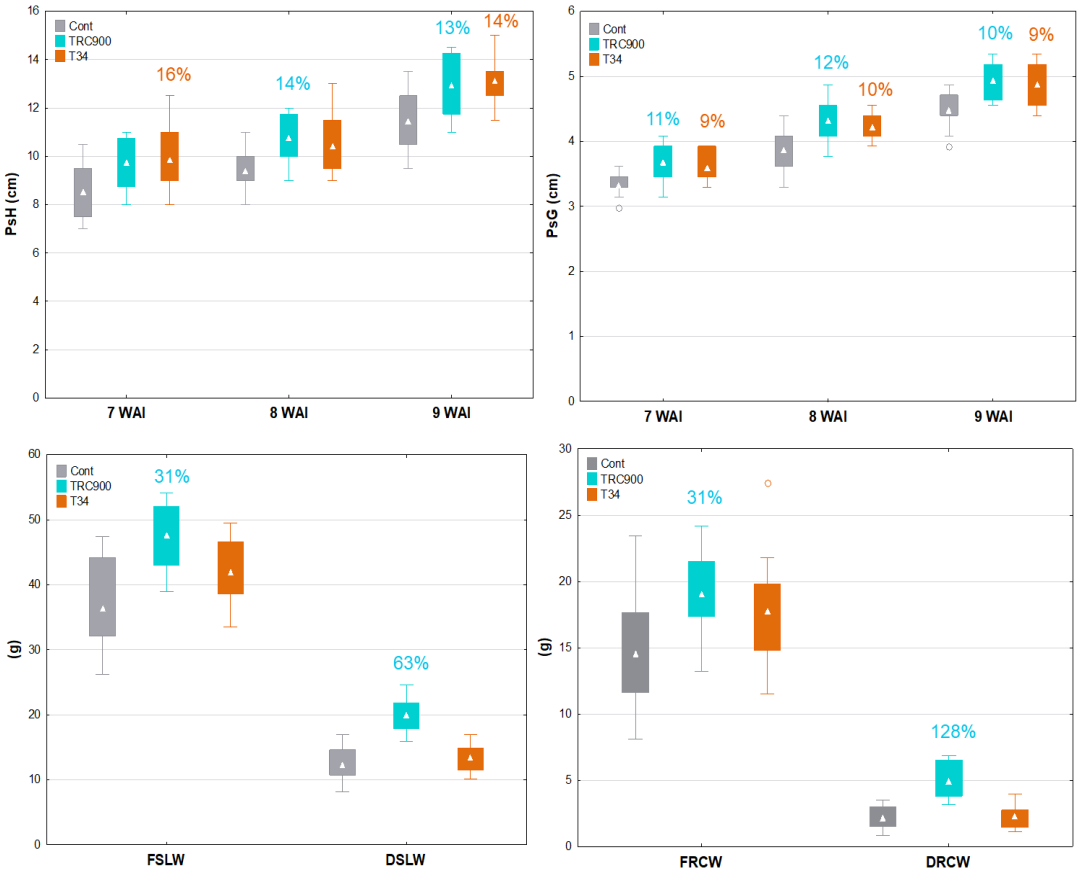

Figure 4.9 Above- and below-ground plant growth parameters evaluated in *Trichoderma asperellum strain TRC900* or *T34* treated plants at 7-9 WAP (non-destructive parameters) or 10 WAP (destructive parameters). PsH (pseudstem height), PsG (pseudostem girth), FSLW (fresh shoot and leaf weight), DSLW (dry soot and leaf weight), FRCW (fresh root and corm weight), DRCW (dry root and corm weight) (Nr plants per treatment, N=10). Percent significant increase respect to control is shown for the corresponding treatments.

Experiment 2

In this trial, the effect of the PGPR *Azospirillum brasilense* (PTA 001, provided by Biobest) on growth of banana plantlets cv Williams was evaluated. *T. asperellum* was applied by submerging the roots of the plants in the microbial solution with a known bacterial concentration in CFU/mL (Fig. 4.10). Plants inoculated with *A. brasilense* showed significant increases compared to controls in most belowground growth parameters (Fig. 4.11). On the contrary, the treated plants showed a significant reduction in LA.

Figure 4.10 Overview of the plant growth promotion experiment in nursery in which Cavendish type banana plants cv Williams were inoculated with the PGPR *Azospirillum brasilense* (PTA 001).

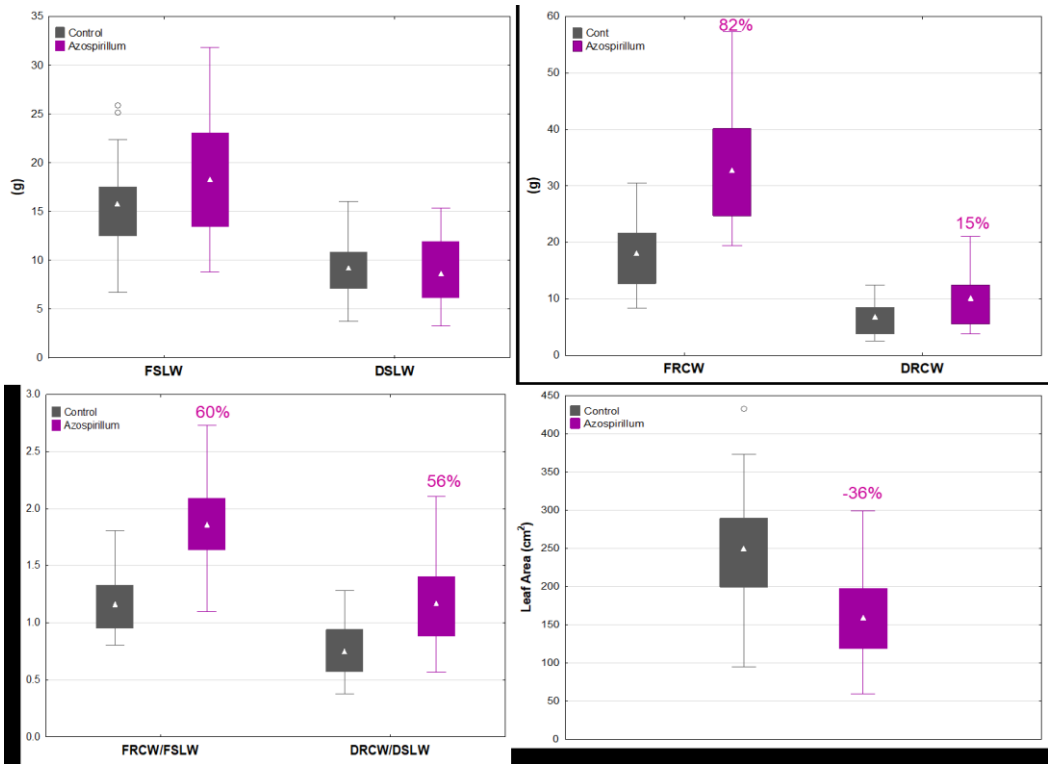

Figure 4.11 Above- and below-ground growth parameters evaluated in *Azospirillum brasilense* treated plants at 10 WAP. LA (projected leaf area), FSLW (fresh shoot and leaf weight), FRCW (fresh root and corm weight), DSLW (dry soot and leaf weight), DRCW (dry root and corm weight). Percent of significant increase respect to control is indicated for the corresponding treatments (20 replicated plants).

Effect of chitosan on gene expression in different banana genotypes

The experiment, in collaboration with UA, evaluated different banana genotypes Foconah ITC0649 (AAB, Pome subgroup), Yangambi km 5 ITC1123 (AAA, Ibota Bota subgroup), Gros Michel ITC1122 (AAA, Gros Michel subgroup), Petite Naine (AAA, Cavendish subgroup) and Enzirabahima ITC1354 (AAAh, Mutika/Lujugira subgroup). They were chosen for being contrasting genotypes based on previously reported data on resistance and susceptibility to PPN, Foc and BW. *In vitro* plants originally provided by the International Transit Center (ITC) were treated with chitosan (elicitor of plant defences) at a known concentration, and root/leaf tissues were sampled for further gene expression analyses (Fig. 4.12). Root RNA was extracted at 72 h and cDNA synthesized. A subset of 18 genes of interest were analysed by qRT-PCR with primers designed to amplify all paralogs in each gene family (Table 16).

Figure 4.12 Hydroponics system assay for chitosan application on banana plants. Left: banana plants from five different genotypes growing in hydroponics with aeration. Right: plants at the end of the experiment and used for root sampling.

Differential gene expression analyses and hierarchical clustering grouped the genotypes into two main groups: 1) Foconah with Yangambi km 5 and 2) Gros Michel, Petite Naine and Enzirabahima (Fig. 4.13), in agreement with their phylogenetic relationships. The selected genes also clustered into two distinct groups: 1) mostly up regulated: *HB1, AOX* and *PR1;* and 2) mostly down regulated: genes related to oxidative stress (*CAT, SOD, GR, APX, ERO1*), auxin biosynthesis (*ARF1, PIN1, AMI, AAO1, TAA1*) and fermentation (*ADH, PDC, LDH*). Remarkably, *PR1* was strongly upregulated in Foconah and Yangambi km 5, while *HB1* was strongly up-regulated in Gros Michel, Petite Naine and Enzirabahima. *PPO* was strongly down-regulated in the root of all genotypes 72h after chitosan treatment (Fig. 4.13).

Table 16. List of 18 selected genes for qRT-PCR together with their abbreviations and metabolic processes in which they are involved.

Gene	Abbreviation	Metabolic process
Peptidylprolyl Cis/Trans Isomerase NIMA-Interacting 1	*PIN1*	Auxin biosynthesis, transport or signalling
Tryptophan aminotransferase	*TAA1*	
Indole-3-acetaldehyde oxidase	*AAO1*	
Amidase 1	*AMI1*	
Auxin response factor 1	*ARF1*	
Non-symbiotic class-1 hemoglobin	*HB1*	Anaerobic respiration
Alternative oxidase	*AOX*	Alternative respiration
Alcohol dehydrogenase	*ADH*	Fermentation
Lactate dehydrogenase	*LDH*	
Pyruvate decarboxylase	*PDC*	
ER Oxidoreductase	*ERO1*	Endoplasmic reticulum stress
Ascorbate peroxidase	*APX*	ROS scavenging
Catalase	*CAT*	
Glutathione reductase	*GR*	
Superoxide dismutase	*SOD*	
Pathogenesis-related 1	*PR1*	Plant defense
Phenylalanine ammonia lyase	*PAL*	
Polyphenol oxydase	*PPO*	

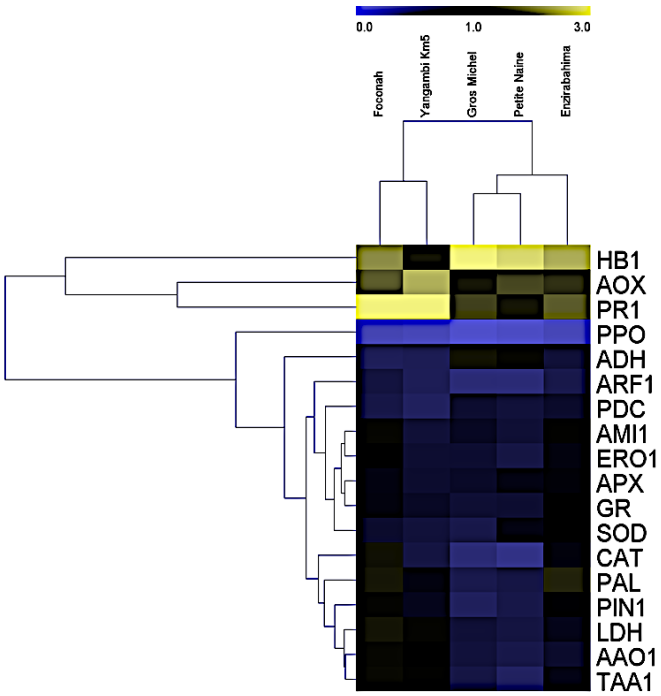

Figure 4.13 Hierarchical clustering for the differential expression of 18 genes quantified by qRT-PCR in roots after chitosan application. Euclidean distance and complete linkage clustering were used to group the analyzed genes and genotypes. Roots were collected at 72 h after applying chitosan. Each cell represents the fold expression average of 6 independent biological replicates of each time point, and is relative to control. Gene abbreviations according to Table 16. Yellow: upregulation, blue: downregulation, black: no significant change (p>0.05).

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Task 4.2 Rhizosphere metagenomics and EBCAs-induced effects on soil microbial communities. Task leader: CNR Other Participants: IITA, CENSA, EARTH University.

The greenhouse assay initially foreseen in this task was replaced by field metagenomic studies carried out in Canary Islands and Costa Rica in collaboration with stakeholder Coplaca and EARTH, and integrated in the metagenomic study already foreseen in SSA (Uganda). Metagenomic data were produced in the three Project regions on microbiomes associated with banana genotypes grown under different production systems, also considering the effect of physical and chemical soil properties on microbial abundance and other extra-farm factors (climate, latitude, soil structure, pests). Metagenomic datasets were produced from EU (Canary Islands), SSA (Tanzania and Uganda) and Central America (Costa Rica).

Microbiome composition of healthy and FW diseased banana plants

First prospections in Uganda (Feb. 2018) and downstream analyses were carried out by University of Liège (subcontracted by KUL). Aim of this study was to analyse the variation in the microbial composition inside the corm and roots of healthy and diseased banana plants due to the presence of Foc in SSA fields. The detection of taxa that modulate their presence following the pathogen infection, could lead to the development of new biocontrol strategies based on the natural microbiota of healthy plants under Foc pressure in the field. For this purpose, 18 banana plants were analysed, divided in asymptomatic and symptomatic, based on the absence / presence of external FW symptoms. The plants were collected in Luweero, Uganda, from a field intercropped with coffee and cassava. Sampling was carried out following external and internal visualization of symptoms. In each plant, corm and root tissues were collected, obtaining 36 different samples, to study the variation of the microbiome between healthy and diseased plants. A total of 32 plants from 3 different farms were selected, after screening several farms for the criterion of getting at least 3 plants in each modality (H/V: seemingly Healthy and asymptomatic or Vigorous, I/W : Infected and symptomatic or Weak). The number of plants in each farm was considered as the replicate number for each modality and sample type. The position of each plant was recorded using a GPS and metadata were collected for description of the environment (cropping system, associated crop, soil texture or humidity). Epiphytes and endophytes isolations were carried out. Taking into account the high risk of environmental contamination from the work space, DNA extracted from a pure culture of a *Xanthomonas campestris* strain (kindly provided by Dr. Valentine Nakato, IITA) was used as control for all DNA samples. The first set of sequencing was run on the DNA samples firstly shipped from Uganda, targeting the endophytes. The second sequencing will be run at a later stage for epiphytes.

Epicoccum nigrum, *Fusarium oxysporum* and *Thielaviopsis musarum* OTU were the core microbiome at 100% level, according to ITS sequence analysis. Added to thems there are OTUs of Cyanobacteria (order Streptophyta), as well as OTU of Proteobacteria (order Pseudomonadales), according to 16S sequence analysis.

Assigned taxonomy for 16S sequences showed 1.3% of unassigned sequences in total, with OTU belonging to Proteobacteria representing 71% of total, followed by Cyanobacteria (18.8%), Bacteroidetes (5.1%) Actinobacteria and Firmicutes (1.3% each) (Fig. 4.14).

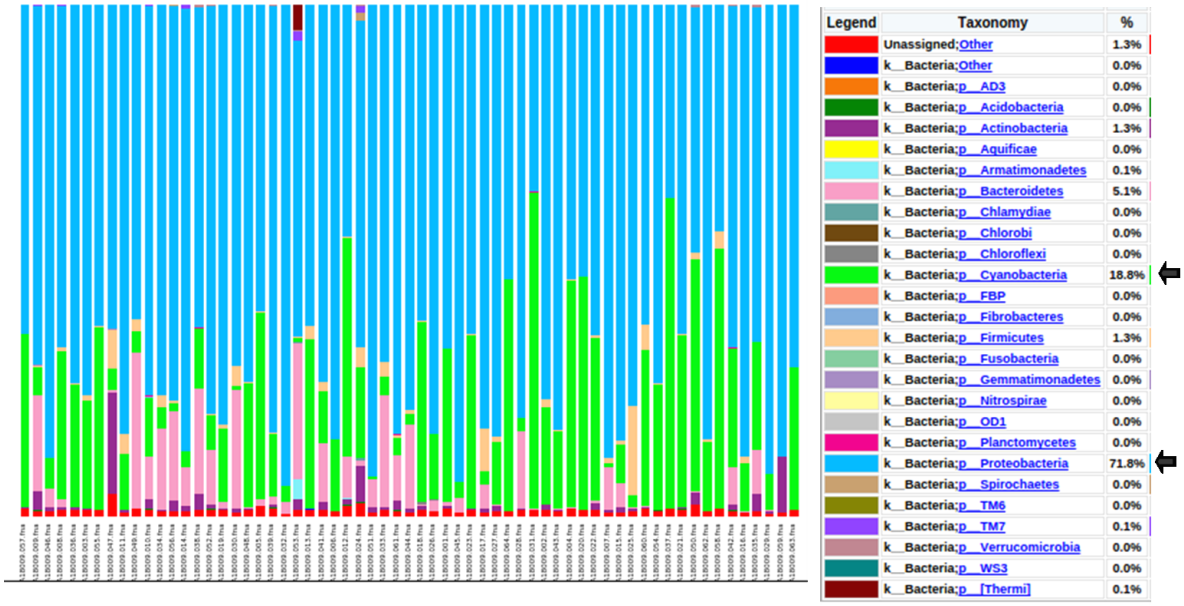


Figure 4.14 Taxa summary plot from of all samples 16S data. Dominant OTU at the phylum level are indicated with arrows on the legend.

Significant differences (Kruskall-Wallis test, FDR-p <0.05) were observed between the three plantations for 38 out of 9221 assigned OTU at the genus level (data not shown). At the phylum level, the OTU grouped in phyla Proteobacteria (order *Xanthomonadales, Enterobacteriales, Pseudomonadales, Burkholderiales, Legionellales*), Actinobacteria, Cyanobacteria (*Streptophyta*) and Bacteroidetes (*Saprospirales*) (Fig. 4.15).

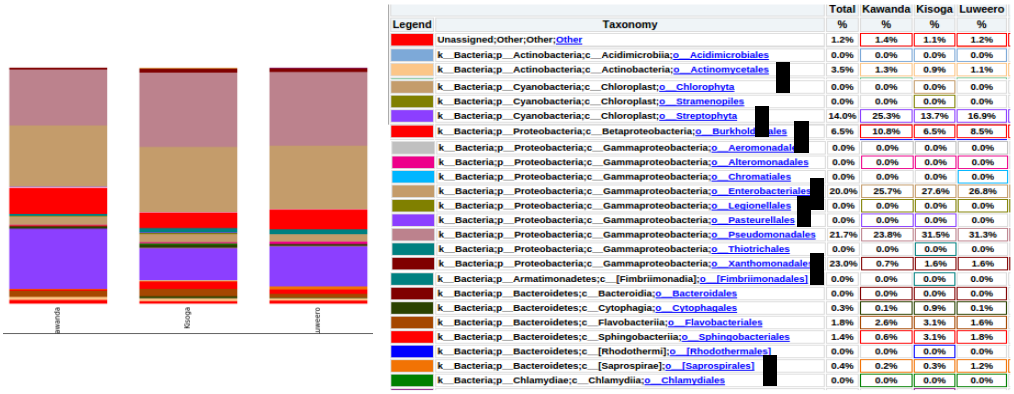
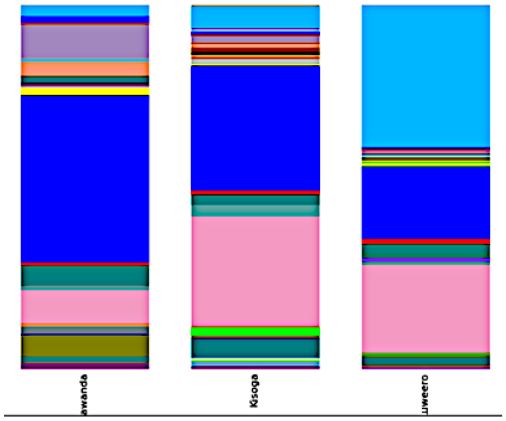


Figure 4.15 OTU relative abundance in the banana plantations. Significantly different OTU between the farms are marked with an asterisk (*) on the extract of the original legend containing 130 assigned OTU at the order level.

The majority of assigned ITS OTUs showed a relative abundance ≤ 1% in all samples (data not shown). Although 197 species were identified, the 3 banana plantations were significantly different by 8 out of 197 OTUs, taxonomically affiliated to *Cercospora zeae maydis*, *Filobasidium magnum*, *Eutypella caricae*, *Cladosporium halotolerans*, *Ophiostoma novo ulmi*, *Trametes versicolor*, *Ganoderma adspersum*, *Armillaria gallica* (Kruskall-Wallis test, FDR-p <0.05; Fig. 4.16).



Legend	Taxonomy	Total %	Kawanda %	Kisoga %	Luweero %
	Aaosphaeria_arxii;Other	0.0%	0.1%	0.0%	0.1%
	Acremonium_brunnescens;Other	0.0%	0.0%	0.0%	0.0%
	Cercospora_zeae-maydis;Other *	0.0%	0.0%	0.0%	0.0%
	Eutypella_caricae;Other *	0.1%	0.0%	0.0%	0.0%
	Filobasidium_magnum;Other *	0.1%	0.0%	0.0%	0.0%
	Flavodon_flavus;Other	0.0%	0.0%	0.0%	0.0%
	Fomitopsis_meliae;Other	0.0%	0.0%	0.0%	0.0%
	Fomitopsis_pinicola;Other	0.0%	0.0%	0.0%	0.0%
	Fusarium_avenaceum;Other	0.0%	0.0%	0.0%	0.0%
	Fusarium_begoniae;Other	0.0%	0.0%	0.0%	0.0%
	Fusarium_culmorum;Other	0.2%	0.0%	0.0%	0.8%
	Fusarium_delphinoides;Other	0.0%	0.2%	0.0%	0.0%
	Fusarium_equiseti;Other	2.9%	5.4%	2.5%	3.7%
	Fusarium_incarnatum;Other	0.0%	0.0%	0.1%	0.0%
	Fusarium_lateritium;Other	0.2%	0.1%	0.1%	0.3%
	Fusarium_nectrioides;Other	0.0%	0.0%	0.0%	0.0%
	Fusarium_nematophilum;Other	0.8%	0.8%	1.0%	1.3%
	Cladosporium_halotolerans;Other *	0.0%	0.0%	0.0%	0.0%
	Ophiostoma_novo-ulmi;Other *	0.2%	0.0%	0.0%	0.0%
	Trametes_versicolor;Other *	0.4%	0.0%	0.1%	0.0%
	Ganoderma_adspersum;Other *	0.9%	0.0%	0.2%	0.0%
	Armillaria_gallica;Other *	0.4%	0.0%	0.0%	0.0%

Figure 4.16 OTU relative abundance in the banana plantations**.** Significantly different OTU between the farms are marked with an asterisk (*) on the extract of the original legend containing 197 assigned OTU at the species level.

For the second prospections (Nov. 2018) and downstream analyses, the samples were collected from Luweero, Uganda (0°44'18.0"N 32°30'43.0"E). All plants belonged to cv Sukari Ndizi (AAB) and were intercropped with cassava and coffee. Samples from roots and corms were collected, and different areas of the root system were used to have the maximum completeness of bacteria and fungi composition. Several slices were taken from younger roots, located closer to the corm, others from older roots, far from the corm, and the rest of the plant. However, all root samples collected were primary roots. In total, 36 samples were used: nine healthy plants (H), with samples from both corm and root (C, R) and nine diseased plants (D), with samples from corm (C) and roots (R).

Amplification of the 16S rRNA gene for the bacteria characterization led to huge biases due to the amplification of chloroplast and mitochondrial genomes. Discarding mitochondrial and chloroplast hits led to a drastic drop of sequences. In fact, from a total of 538749 reads, the remaining ones were 103541, meaning that sequences from endophytic bacteria were 1/5 of the total. Therefore, the data were considered unsuitable to further analysis, requiring to other primers to minimize the bias.

Fungi ITS1 amplification and sequencing produced a total of 768793 reads, with a median of 22105 hits per sample, and a total of 537 OTUs. The positive control, obtained from the isolation of pure culture from root samples, was sequenced as well and it was identified as a strain of *Rhodosporidiobolus* spp. With a 100% of hits for this genus, it was a confirmation of the sequencing accuracy. All samples belonged to four different categories: healthy corm, diseased corm, healthy root and diseased root, for a total of 36 samples.

Beta diversity was inferred using the Bray-Curtis dissimilarity matrix and plotted as Principal coordinates analyses (PCoA) that showed a clear clustering of data by the "sample_type" category (Fig. 4.17). ANOSIM p-value, calculated under this category was equal to 0.001, indicating that this result had a strong statistical significance.

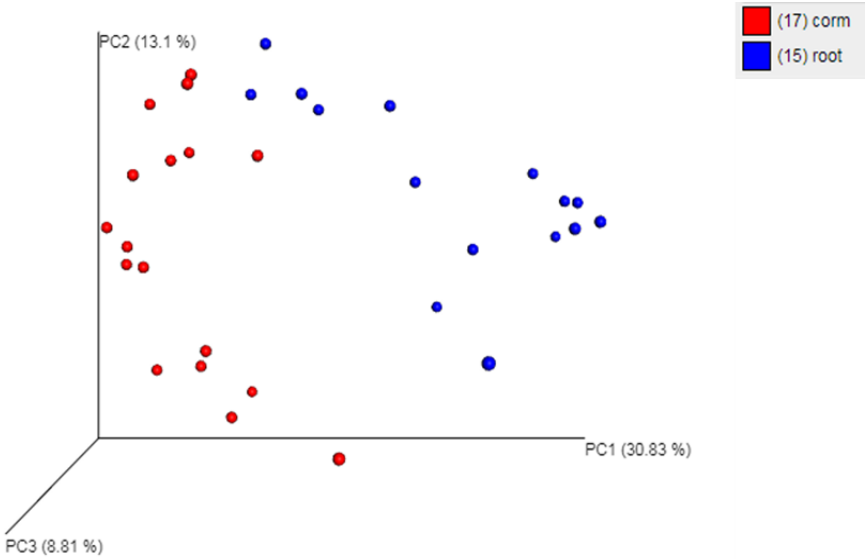


Figure 4.17 PCoA plot showing the clustering of samples under the "sample_type" category. 17 "corm" samples (red), 15 "root" samples (blue).

By using Bray-Curtis dissimilarity matrix and PCoA plots visualization, samples could also be grouped by the "Description" category, being divided according to both tissue and presence of the disease (Fig. 4.18). Samples from healthy and diseased roots formed clear separated groups, as expected, given that these two categories are significantly different in the microbial composition, as proven by the ANOSIM test. Samples from diseased corms and healthy corms showed a partial overlapping, suggesting that there were less differences in the microbial communities between these two groups.

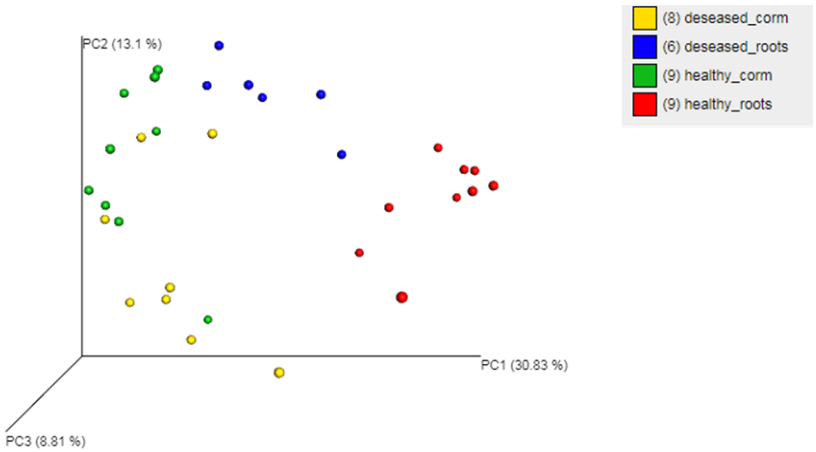


Figure 4.18 PCoA plot showing the clustering of samples in the four categories: healthy roots (red), diseased roots (blue), healthy corm (green) and diseased corm (yellow). Healthy roots and diseased roots formed independent clusters, while clusters from corm overlapped.

Samples from corms and roots were then analysed separately, to test if the populations changed following the "plant sanitary status" characteristic inside the same "sample type" category. Nine asymptomatic and seven symptomatic samples were analysed and visualized

with PCoA plot (Fig. 4.19). The clustering of samples in two big groups is usually the consequence of significant differences between the microbial communities inside them. This difference could be related to the presence/absence of Foc. The same approach was used to visualize differences inside corm tissue (Fig. 4.20).

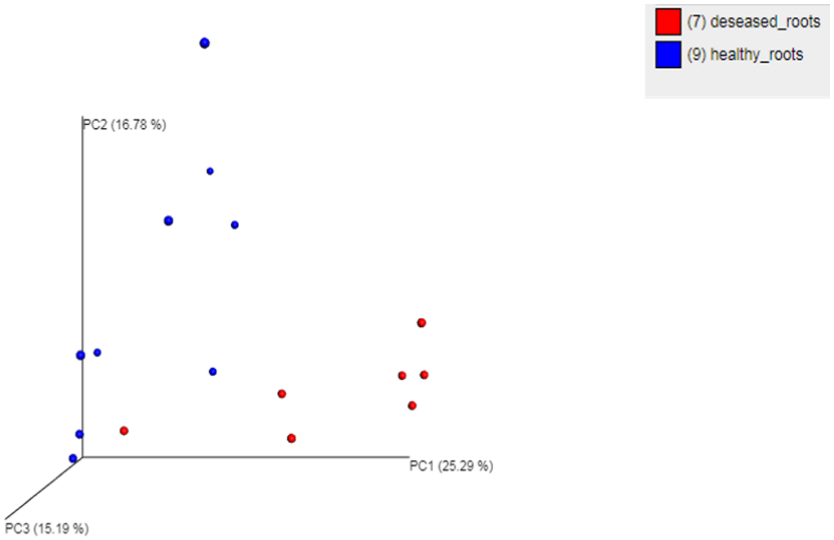


Figure 4.19 PCoA plot for root samples under the "plant sanitary condition" category.

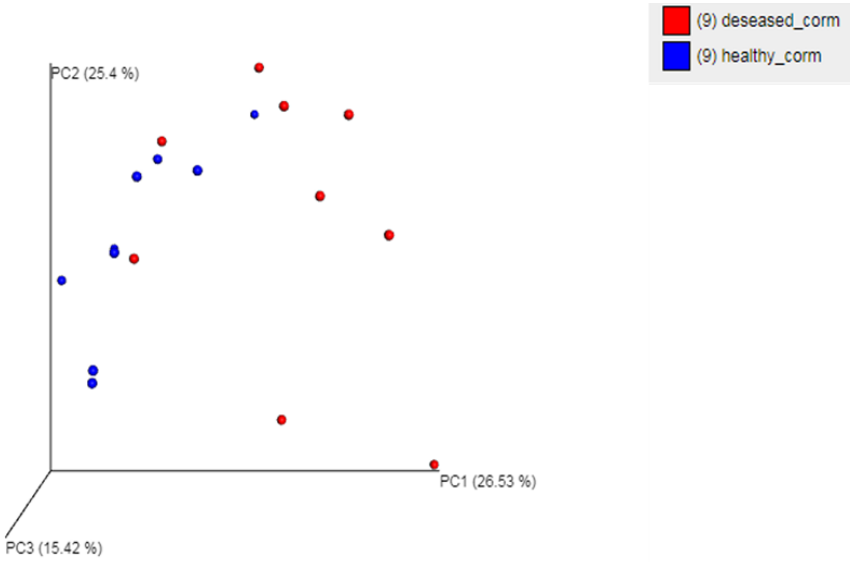
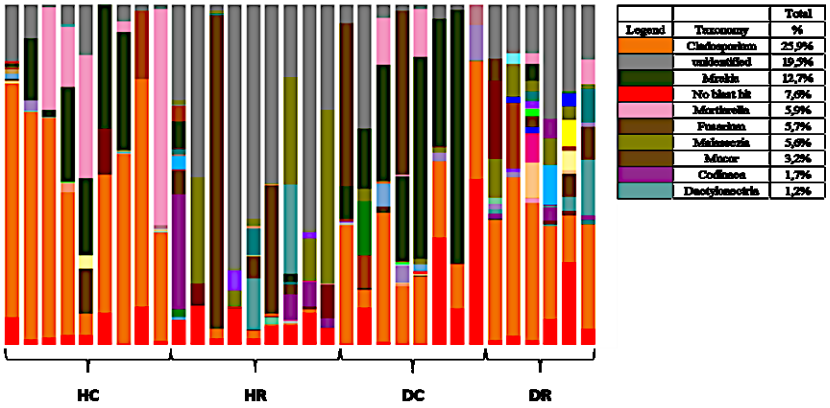


Figure 4.20 PCoA plot for corm samples under the "plant sanitary condition" category.

Taxonomic diversity

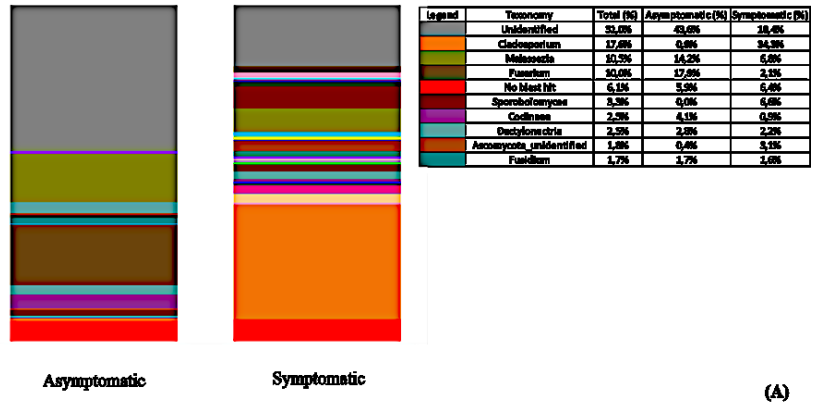
A taxonomic differentiation in all samples could be observed (Fig. 4.21), however, unidentified and "no blast hit" sequences together amounted to 27.1% of total number of sequences. *Fusarium* showed a relatively low percentage (5.7%), while a genus like *Cladosporium* had a high presence (25.9% of the total).



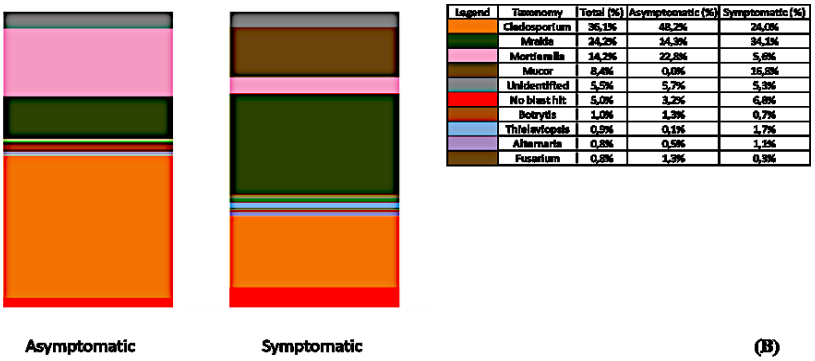
Legend	Taxonomy	Total %
	Cladosporium	25,9%
	unidentified	19,5%
	Mrakia	12,7%
	No blast hit	7,6%
	Mortierella	5,9%
	Fusarium	5,7%
	Malassezia	5,6%
	Mucor	3,2%
	Codinaea	1,7%
	Dactylonectria	1,2%

Figure 4.21 Histogram showing the taxonomic diversity inside all samples. The first ten most abundant genera inside all samples are listed in the table on the right.

The presence of *Fusarium* was not as abundant as expected, especially in symptomatic samples, being only 2.1% of the total hits in diseased root samples and even lower in diseased corm samples (0.3%). Moreover, since the *Fusarium* characterization is limited to the genus level, it was not possible to know if the strains observed in the sequenced samples belonged to the *F. oxysporum* species or not. In root samples (Fig.4.22A), the most abundant genera were *Cladosporium* (17.6%), *Malassezia* (10.5%), *Fusarium* (10%), *Sporobolomyces* (3.3%), *Codinaea* (2.5%), *Dactylonectria* (2.5%) and *Fusidium* (1.7%). In corm samples the most abundant genera were *Cladosporium* (36.1%), *Mrakia* (24.2%), *Mortierella* (14.2%), *Mucor* (8.4%), *Botritis* (1%), *Thielaviopsis* (0.9%) and *Alternaria* (0.8%) (Fig. 4.22B).



Legend	Taxonomy	Total (%)	Asymptomatic (%)	Symptomatic (%)
	Unidentified	33,0%	43,6%	18,4%
	Cladosporium	17,6%	0,8%	34,3%
	Malassezia	10,5%	14,2%	6,8%
	Fusarium	10,0%	17,9%	2,1%
	No blast hit	6,1%	5,9%	6,4%
	Sporobolomyces	3,3%	0,0%	6,6%
	Codinaea	2,5%	4,1%	0,9%
	Dactylonectria	2,5%	2,8%	2,2%
	Ascomycota_unidentified	1,8%	0,4%	3,1%
	Fusidium	1,7%	1,7%	1,6%



Legend	Taxonomy	Total (%)	Asymptomatic (%)	Symptomatic (%)
	Cladosporium	36,1%	48,2%	24,0%
	Mrakia	24,2%	14,3%	34,1%
	Mortierella	14,2%	22,8%	5,6%
	Mucor	8,4%	0,0%	16,8%
	Unidentified	5,5%	5,7%	5,3%
	No blast hit	5,0%	3,2%	6,8%
	Botrytis	1,0%	1,3%	0,7%
	Thielaviopsis	0,9%	0,1%	1,7%
	Alternaria	0,8%	0,5%	1,1%
	Fusarium	0,8%	1,3%	0,3%

Figure 4.22 Histograms of the most numerous genera from roots and corm samples by their sanitary condition. Healthy and diseased samples are named as “asymptomatic” and “symptomatic, respectively. A) Root samples. B) Corm samples.

Metagenomic analysis of banana roots from Foc symptomatic and asymptomatic plants

IITA assessed the diversity, structure, and assemblage of bacterial and fungal communities associated with banana plants with and without Foc symptoms. Sukari Ndizi (Musa subgroup AAB) is a popular dessert banana cultivar in east Africa susceptible to Foc. The rhizosphere, roots, and corm of asymptomatic and symptomatic plants were targeted for sampling. Symptomatic banana plants exhibited yellowing leaves with brown streak discoloration inside the pseudostem and corm, and asymptomatic plants had green leaves with no discoloration inside the pseudostem and corm (Fig. 4.23). A total of 12 composite samples were collected from two locations in Tanzania i.e., Arusha (3°22' 29.6" S, 36° 48' 16.8" E) and Kilimanjaro (3° 14' 14.6" S, 37°15' 3.7" E) from three random locations around a single plant at a distance of 15 cm.

Figure 4.23 Banana plants under non-stressed (asymptomatic) and disease stressed conditions (symptomatic) caused by Fusarium wilt.

Bacterial and fungal operational taxonomy units (OTUs) were identified in the rhizosphere, roots, and corm of the host plant. Results revealed that bacterial and fungal microbiota present in roots and corm primarily emanated from the rhizosphere. The composition of bacterial communities in the rhizosphere, roots, and corm were different, with more diversity observed in the rhizosphere and less in the corm. However, distinct sample types i.e., without (asymptomatic) and with (symptomatic) Fusarium symptoms were the major drivers of the fungal community composition. Considering the high relative abundance among samples, we identified core microbiomes with bacterial and fungal OTUs classified into 20 families and colonizing distinct plant components of banana. Our core microbiome assigned 129 bacterial and 37 fungal genera to known taxa (Fig. 4.24).

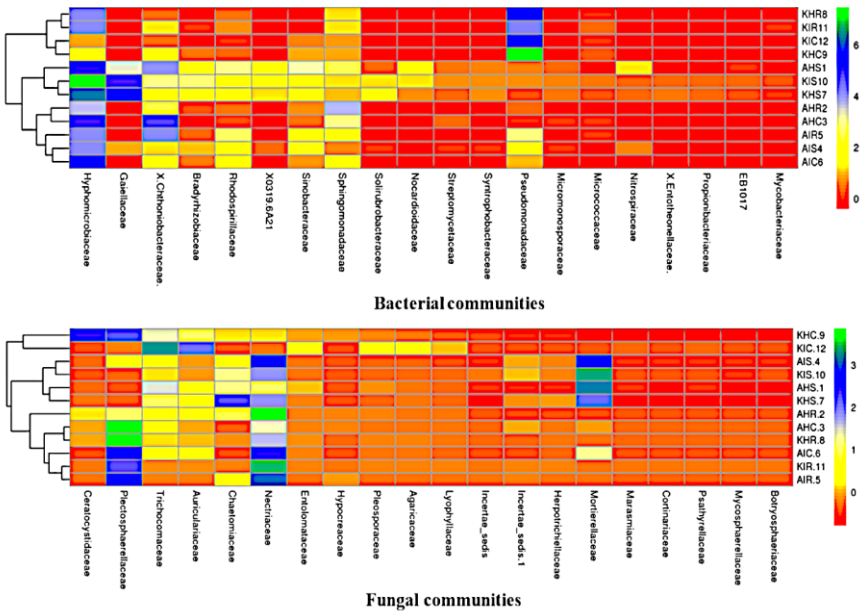


Figure 4.24 Distinct core colonizers pattern of banana samples. Heatmap of distribution pattern of core OTUs across sample types, based on relative abundance (column z-score) of top 20 core OTUs (family level). Samples were hierarchically grouped based on the pairwise distances. A: Arusha; C: Corm; H: Asymptomatic; I: Symptomatic; K: Kilimanjaro; R: Roots; S: Rhizosphere.

Data support the hypothesis that distinct plant components play a key role in engaging bacterial communities, irrespective of location and sample type. IITA team studied banana plants in smallholder's fields, where banana is a perennial crop and successive crop cycle is established from suckers. Hence, through this vegetative multiplication, the sucker is automatically infected with the same bacterial and fungal communities of the preceding cycle. In addition, farmers transplant suckers from the main plant to establish new fields and thus not only possibly transfer soil borne pests and diseases, but also the associated bacterial and fungal communities. Good practices, however, require suckers paring before planting to reduce PPN and BW. Therefore, the banana corm might be the major carrier of bacterial and fungal communities in small holder farming systems. Thus, IITA findings support the notion of niche-mediated host microbiome.

Data showed that the rhizosphere had the greatest diversity of bacterial and fungal communities in the banana plant but that they change towards the roots and corms. Most of the bacterial and fungal communities present in roots and corm samples were also prevalent in rhizosphere samples, in both locations. This suggests that bacterial and fungal communities present in roots and corm are colonized from the rhizosphere. As host plants selectively promote colonization by communities from the rhizosphere, it was found that the Fusarium wilt infected (symptomatic) samples had a lower bacterial and fungal diversity than the asymptomatic ones. This may be due to the disease stress, which is directly linked to the decreased value of available C for microbes in the rhizosphere, responsible for specific microbial groups selection, thus regulating the community compositions.

Banana plant resistance and Fusarium aggressiveness in different plant components also contribute to bacterial and fungal prevalence. In both locations, bacterial and fungal OTU counts were maximum in the rhizosphere followed by root and corm. Thus, microbiome communities in these specific plant components either become enriched or depleted. However, stable core communities (>92.8% of total relative abundance) linked with the

rhizosphere, roots, and corm of sample types. We observed core bacteria and fungi in sample types that were preferential colonizers but differed in each plant component with respect to OTU distribution. Notably, these identified core bacteria and fungi had a higher abundance of genera specifically related to host plant growth and development.

Metagenomic analysis of banana farms in Canary Islands

To understand the effect that banana crops have on the associated microbiota, metabarcoding studies were carried out in Tenerife. Soil samples were collected by CNR and Coplaca, from banana rhizosphere and adjacent control sites (without banana roots) to carry out metagenomic analyses of bacteria and fungi. The study showed that some bacteria phyla were enriched in the banana rhizosphere, compared to non-rhizosphere controls. A higher representation was observed for phyla Proteobacteria, Acidobacteria and Chloroflexi in the banana rhizosphere, whereas Actinobacteria were more prevalent in the adjacent control, non-rhizosphere sites. The most prevalent bacteria classes in the rhizosphere were Alphaproteobacteria, Clostridia, Solibacteres and Anaerolineae, whereas in non-rhizosphere Actinobacteria were more prevalent. At the order level, a higher frequency of Rhizobiales and Solibacterales occurred in the banana rhizosphere, while Actinomycetales were more prevalent in control soils. For fungi, no clear distinction was observed. However, the dominant phyla were Ascomycota, Basidiomycota, Zygomycota, Rozellomycota and Chytridiomycota with varying distributions. Several known biocontrol agents, including *Metarhizium*, *Beauveria*, *Trichoderma* and other *Fusarium* spp. where identified in these samples.

PPN were found mostly in banana rhizosphere in Tenerife. *Pratylenchus goodeyi* was present in 75% of samples from Northern farms, at 200-1750 specimens/100 cc soil, with lower prevalence (22%) and densities in Southern fields. *Helicotylenchus* spp. included *H. multicinctus*, found in Northern and Southern farms, and *H. abunaami*. The correlations among all variables examined are shown in Table 17.

Table 17. Correlation coefficients among soil variables in samples from Tenerife farms, with corresponding significance levels (*p*, italics). Significant correlations are shown in bold (n=36).

	P. goodeyi	Predatory nem.	*Helico-tylenchus*	Free liv. + Aphelenchids	pH	% sand	% loam	% clay
P. goodeyi		*0.59213*	***0.00116***	*0.14411*	*0.55280*	***0.00031***	***0.00735***	***0.00026***
Predatory nem.	-0.090		*0.10610*	*0.93726*	*0.30108*	*0.24468*	*0.73659*	***0.00932***
Helicotylenchus	**0.507**	-0.266		***0.02984***	*0.18466*	***0.00000***	*0.00364*	***0.00001***
Free liv. + Aphelenchids	0.242	0.013	**0.353**		*0.34169*	*0.00077*	*0.00059*	*0.08825*
pH	-0.099	-0.172	0.220	0.159		*0.47186*	*0.97493*	*0.39637*
% sand	**- 0.554**	0.193	**- 0.670**	-0.522	-0.120		***0.00000***	***0.00000***
% loam	**0.428**	-0.056	**0.460**	0.532	-0.005	**-0.87657**		***0.00055***
% clay	**0.560**	**- 0.416**	**0.666**	0.280	0.142	**-0.81552**	**0.53408**	

The most predominant bacteria belonged to phyla Proteobacteria, Bacteroidetes, and Actinobacteria, irrespective of banana production systems or variety cultivated. Regarding fungal communities, the most abundant were Ascomycota, Basidiomycota and Mortierellomycota. Venn diagrams showed a core microbiome of 1681 OTUs, either classified or not, from 190 classified genera, of which 114 were in common for all samples. A higher OTUs prevalence could be observed in Northern samples, which also showed the highest numbers of unique OTUs (Fig. 4.25 A, B).

Differences among the groups of samples were shown by Principal component (PCA) (Fig. 4.26A) and MDS analyses (Fig. 4.27), with clusterings reflecting changes in the microbiome composition, accounting for crop presence and for an effect of latitude. A more homogeneous clustering was observed on the PCA plots at the genus level, indicative of a higher similarity among samples at a lower taxa level (Fig. 4.26B).

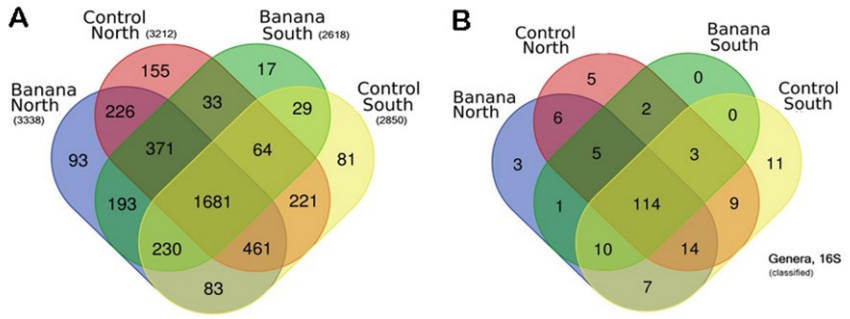


Figure 4.25 Venn diagrams showing the distribution of OTUs (A) and classified genera (B), among samples grouped by crop and latitude. Numbers in parentheses show total OTUs.

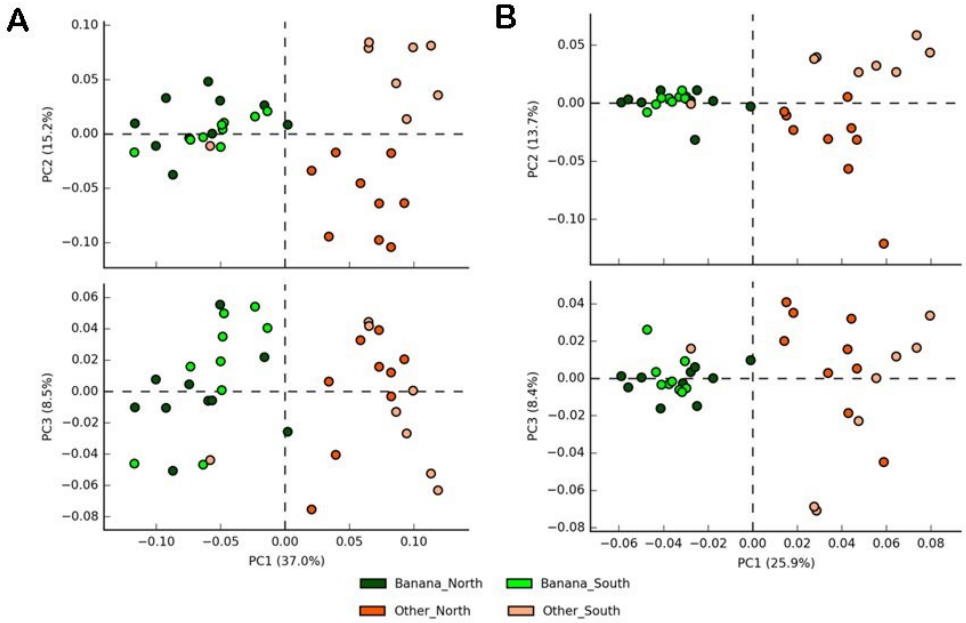


Figure 4.26 PCA plots by crop and latitude, on the three first components plans, showing clustering at the family (A) and genus (B) levels (all samples except N1).

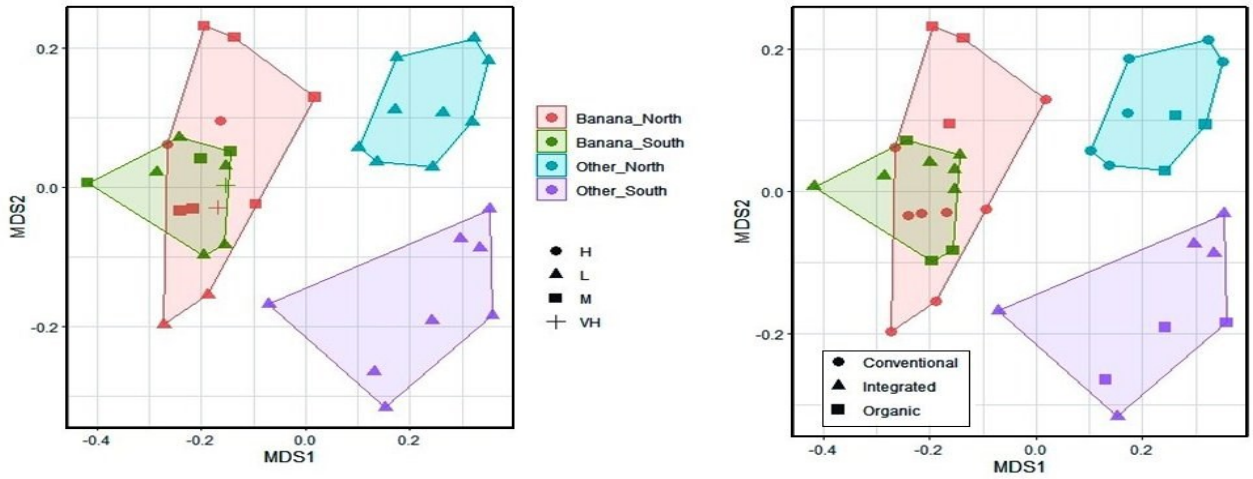


Figure 4.27 MDS plots of all samples based on latitude and density levels of *Helicotylenchus multicinctus* (A), and crop type (B). Nematode density classes (expressed as adult and juvenile nematodes · 100 cc soil $^{-1}$) are: L= low or absent (0 to 290), M = medium (291 to 804), H= high (805 - 1319), VH = very high (> 1391) (mean density and SD = 290 ± 514 nematodes · 100 cc soil $^{-1}$).

Distinct sample clusterings were also shown by MDS when analysis was limited to specific taxa, such as Proteobacteria by crop and latitude (Fig. 4.28A), or Rhizobiales, by latitude and soil pH (Fig. 4.28B).

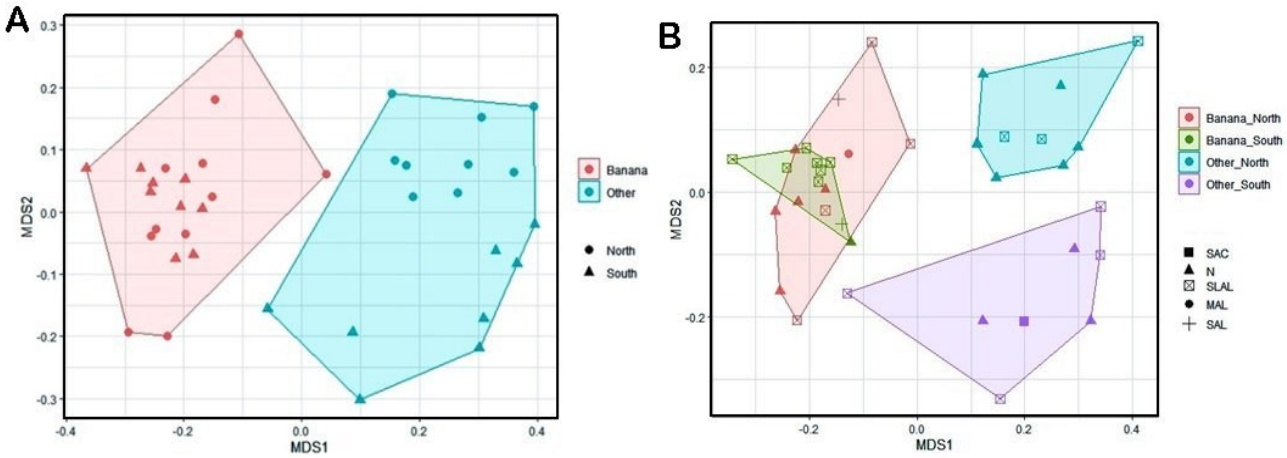


Figure 4.28 MDS plots of all samples considering only Proteobacteria, latitude and crop (A). MDS plots of only Rhizobiales (B) with crop, latitude and soil pH. SAC = slightly acid (6.1 - 6.5), N = neutral (6.6 - 7.3), SLAL = slightly alcaline (7.4 - 7.8), MAL = moderately alcaline (7.9 - 8.4), SAL = strongly alcaline (8.5 - 9.0).

Hyphomicrobiaceae and Solibacteraceae were more prevalent in banana rhizophere samples, whereas Bradyrhizobiaceae were more prevalent in control sites (Table 18).

Table 18. Relative abundance (%) of bacterial families accounting for differences among banana rhizosphere samples and control samples collected in adjacent, crop-free control sites in Tenerife, and classified by latitude.

Families*	Banana North	Banana South	Control North	Control South	P	Bonferroni P	FDR P
f_Geodermatophilaceae	0.013	0.018	0.074	0.062	2.470368e-05	0.00027	0.00027
f_Bradyrhizobiaceae	0.011	0.014	0.052	0.023	1.058206e-04	0.00116	0.00058
f_Solibacteraceae	0.092	0.089	0.035	0.022	1.656480e-04	0.00182	0.00060
f_Hyphomicrobiaceae	0.114	0.116	0.039	0.036	1.671030e-04	0.00183	0.00045
f_Phyllobacteriaceae	0.056	0.048	0.015	0.030	2.181649e-04	0.00239	0.00047
f_Sphingomonadaceae	0.026	0.031	0.075	0.044	5.658774e-04	0.00622	0.00103
f_Micromonosporaceae	0.017	0.029	0.079	0.018	6.404280e-03	0.07044	0.01006
f__Nocardioidaceae	0.033	0.032	0.045	0.076	8.032507e-03	0.08835	0.01104
f_Micrococcaceae	0.013	0.016	0.027	0.079	6.517616e-02	0.71693	0.07965
f__Unclassified	0.242	0.275	0.189	0.218	7.785347e-02	0.85638	0.08563
f__Rhodospirillaceae	0.065	0.065	0.048	0.064	4.070025e-01	4.47702	0.40700

* 16S metagenomic data analyzed in R Studio with library mctoolsr; P values based on Kruskal-Wallis tests, with Bonferroni and FDR corrections (rarefaction = 4500 sequences per sample; total n. of samples retained = 37).

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

Transcriptomics of Foc infected banana tissues

IITA conducted transcriptome analyses on 9 pairs of banana plants, each including one FW symptomatic (D) and another asymptomatic (H) plant of the same variety, grown next to each other. Bioinformatic analyses confirmed the expected number of reads (exceeding 60 M per each sample), with 80% of the sequences mapping to the banana genome. Fold change calculated on gene expression levels indicated clear differences within each couple, but H and D samples separate only partially in two different clusters. To further analyze the transcriptomes of banana samples these were divided in the two groups of plant roots: HR and DR. From the total 46760 transcripts, 243 transcripts were overexpressed in HR compared to DR with FC >2 (max FC 7.61) and p value ≤ 0.05 corresponding to 118 annotated functions in Uniprot. Some transcripts as follows have a potential role in plant defense against Foc and will be further analysed in other sample couples:

- *Caffeoylshikimate esterase* which is an esterase involved in the biosynthesis of lignin located at the plasma membrane;
- *Pectin methylesterase (PME) inhibitor* and is an extracellular or secreted protein;
- *Endochitinase:* defense against chitin-containing fungal pathogens
- *Cytochrome P450*
- *Bifunctional pinoresinol-lariciresinol reductase 2:* this is a reductase involved in lignan biosynthesis
- *Protein EXORDIUM-like 2*: plays a role in a brassinosteroid-dependent regulation

On the other hand, 20 transcripts were down regulated in HR compared to DR with fold change < -2 (min FC -6.64) and p value ≤ 0.05, corresponding to 7 annotated functions in Uniprot.

Generating genomic data for agronomically important enset landraces

In collaboration with UNEXE and other partners, SARI generated genome sequence data for 17 accessions composed of wild and cultivated agronomically important enset landraces. Three of them viz. Bedadeti, Derea and Onjamo were assembled de novo. Using the genome-wide sequence data from the 17 enset accessions high-confidence single nucleotide polymorphisms were inferred that could be utilized for developing markers for genotyping large number of enset landraces. The generated genomic data will serve as a basis to exploit genetic diversity and in improvement studies for enset and related pests.

Evaluation of endophytes against FOC

SARI carried out a preliminary study on the endophytic role of identified media-based bacterial and fungal microbes. After repeated purification through observing the morphology of bacteria (colony color and shape of bacteria) and fungal (the mycelia color and conidia), 94 endophyte were selected. Potential activity against Foc pathogen was evaluated using the paired culture technique and microbes with greater inhibition zone in the Foc plated media were considered for endophytic activity. As a result, five bacterial isolates from four banana plant and one enset landraces were obtained with endophytic activity tested in dual cultures. In addition, eleven potential endophytic fungal against FOC were distinguished from eight enset clones.

1.2.5 WP 5 - Procedures for EBCAs mass production, storage and application (mths: 16-32)

Mass production assays

Isolates subjected to mass production tests by MSBIO were *Bacillus velezensis* SGB100Z, *B. amyloliquefaciens* SGB0013 and *B. licheniformis* SGB413. A submerged liquid fermentation (SLF) process was developed, with the following phases:

- Pre-inoculum preparation: each isolate was grown in flasks containing a suitable sterile liquid substrate, the flasks were incubated in an orbital shaker at 30 ° C up to the exponential growth phase, measured by spectroscopic analysis (OD600).

- The preinoculum transfer to a bioreactor (Sartorius Stedim Biostat C Plus, 30L) containing the liquid culture substrate, previously sterilized *in situ*.

- Growth at temperature adjusted to 30 ° C with pH 7.2.

The trend was followed for each tested substrate during fermentation (monitoring O_2 consumption and changes in pH), applying quality controls carried out periodically on samples by visual analysis with an optical microscope and by measuring the OD600. Fermentation was stopped when the maximum spore concentration was achieved, recording the growth curves obtained. The final broth culture was stabilized through acidification and pasteurization *in situ*. For each bacterial isolate, different formulations of the liquid culture substrate were evaluated, based on soy peptone, yeast extract, mineral salts and vitamins, obtaining three substrates indicated with M1, M2 and M3. The best liquid substrate was considered as the one determining the highest yield in spores. The following scheme shows some results of the spore concentration of the isolates grown in the three substrates:

Liquid Media	Spore concentration (CFU/mL)		
	SGB100Z	SGB0013	SGB413
M1	3,50E+09	4,10E+09	7,20E+08
M2	2,70E+09	2,30E+09	9,60E+08
M3	8,30E+08	9,20E+08	3,70E+09

For SGB100Z and SGB0013, the greatest spore yields were obtained with substrate M1, whereas M3 was best substrate for SGB413 (Fig. 5.1).

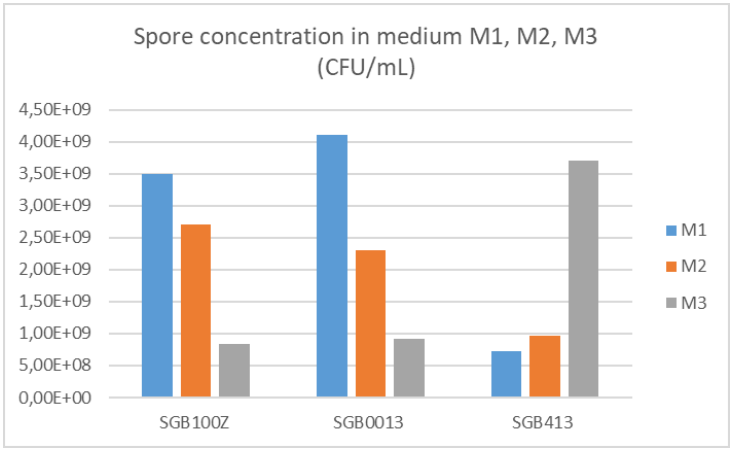


Figure 5.1 Spore concentrations achieved for the three isolates in liquid growth media.

The isolates *P. chlamydosporia* SGB P11 (deposit number DSM26985), and *Beauveria bassiana* SGB7004 (deposit number DSM32859), were used for mass production tests by MSBIO, with a submerged liquid fermentation process (SLF) followed by a Solid State Fermentation (SSF). SLF and SSF phases were the following:

- Each isolate was grown in flasks containing suitable liquid culture substrate previously sterilized, then incubated in an orbital shaker at 26 °C for 24h.
- The pre-inoculum was transferred to the bioreactor (Sartorius Stedim Biostat C Plus, 30L) containing the liquid culture substrate, previously sterilized *in situ*.
- The temperature was adjusted to 26 ° C (pH 6.0). For each test substrate, the trend was followed during fermentation (O_2 consumption, change in pH) and quality controls were carried out, taking a sample of broth culture periodically for visual analysis with an optical microscope, to detect the possible presence of contaminants. Fermentation was halted at the maximum exponential growth phase.
- Aliquots of the obtained broth culture were transferred into bioreactors for SSF, in polyethylene bags containing a previously sterilized solid substrate.
- The bags were shaken and incubated inside the SSF boxes, kept in controlled temperature and humidity chambers, until the complete sporification phase. Samples were periodically taken and subjected to quality control, through visual microscopic analysis, in order to evaluate the presence of contaminants and the spore density.
- Upon obtaining the maximum concentration of spores, the biomass was transferred to the drying chambers, up to final relative humidity below 4%. Samples were taken every day for the determination of RH, measured with a thermobalance.

For each isolate different formulations of the solid culture substrate were evaluated, based on rice, corn and soy, allowing selection of two substrates indicated as A and B. For each isolate and growing substrate, the following pHs were evaluated: 5 - 5.5 - 6 - 6.5, according to the following scheme:

SGB11P	5	5.5	6	6,5
A	PochA5	PochA5.5	PochA6	PochA6.5
B	PochB5	PochB5.5	PochB6	PochB6.5

SGB7004	5	5.5	6	6,5
A	BeauA5	BeauA5.5	BeauA6	BeauA6.5
B	BeauB5	BeauB5.5	BeauB6	BeauB6.5

For each replication of treatment 250 bags were prepared with the solid substrate. For each treatment and isolate, the biomass was dried at complete sporification and subjected to the spore count (CFU/g), yielding the following results:

SGB11P	thesis:	CFU/mL
A	PochA5	5,30E+08
	PochA5.5	9,70E+08
	PochA6	**1,30E+09**
	PochA6.5	8,50E+08
B	PochB5	5,70E+08
	PochB5.5	8,80E+08
	PochB6	9,10E+08
	PochB6.5	8,70E+08

SGB11P	thesis:	CFU/mL
A	BeauA5	6,80E+07
	BeauA5.5	6,90E+07
	BeauA6	9,80E+07
	BeauA6.5	2,60E+08
B	BeauB5	7,80E+08
	BeauB5.5	1,10E+09
	BeauB6	3,50E+09
	BeauB6.5	**5,10E+09**

For isolate SGBP11, the greatest spore yield was obtained with solid substrate A at pH 6.0, and for isolate SGB7004 with substrate B, at pH 6.5 (Fig. 5.2).

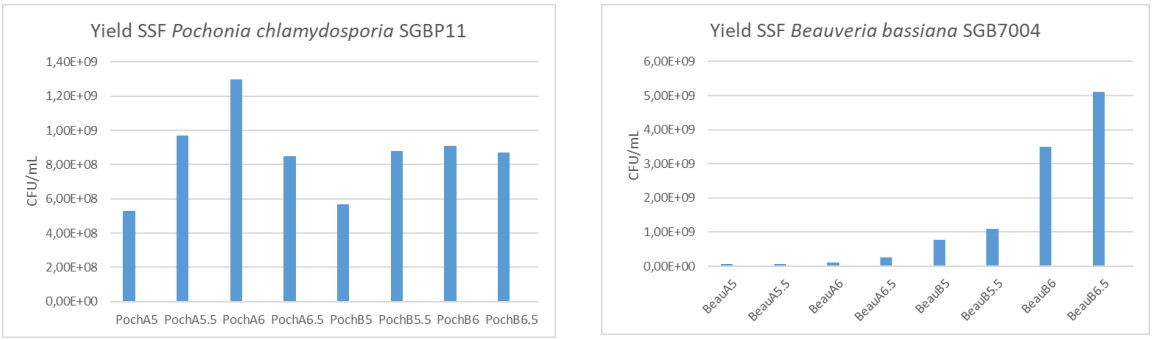


Figure 5.2 Spore yields for isolates SGBP11 and SGB7004, on solid substrates A and B, respectively.

UA set-up a method for mass production of EBCAs (*P. chlamydosporia*, *B. bassiana* and *M. anisopliae*) based on growth of a solid substrate. The method allowed good growth and sporulation of the 3 isolates tested and was used to evaluate VOCs production by EBCAs. Moreover, PICF7 bio-formulation parameters were re-evaluated and validated by IAS-CSIC. Several well-characterized bacterial strains isolated from the root endosphere of banana plants sampled at three Canary Islands were evaluated for their potential as biocontrol agents. Besides, the olive rhizobacteria *Pseudomonas simiae* (formerly *P. fluorescens*) PICF7, a biocontrol agent of Verticillium wilt of olive, was included in these experiments as reference. Results have so far shown that strain PICF7 is the most effective BCA against *Fusarium oxysporum* f. sp. *cubense* race 4 (Foc4) (see activities in WP3).

Funded by the Horizon 2020 Framework Programme of the European Union

Since good niche colonization ability is necessary for successful biocontrol, IAS-CSIC conducted two assays using a fluorescently-labeled derivative of PICF7 that demonstrated this bacterium was able to endophytically colonize banana roots (see activities in WP3). Nevertheless, the ability to colonize the interior of banana roots was lower than observed for olive roots. A good formulation is key to achieve agricultural and commercial success of EBCAs. A bio-formulation usually contains an EBCA plus other ingredients aiming to improve and or facilitate: *i)* survival and effectiveness (high enough concentrations to provide effective and consistent control of the target disease) of the applied microorganism; *ii)* its stability under various conditions during production, distribution and storage steps; *iii)* its application; and *iv)* its protection from environmental factors.

An effective bio-formulation requires a thorough knowledge of the BCA, the pathogen, the target crop, the environment, and the interactions with other organisms. From a stakeholder-user standpoint, it is necessary to understand common application practices and equipment, as well as customers' interests for the handling and managing the bio-formulation. Moreover, accompanying ingredients must be safe and acceptable for regulatory agencies in all areas where the product will be used.

In this context, IAS-CSIC, in collaboration with Bio-Iliberis R&D, a BIO CSIC spin-off biotechnology-based company (*http://www.bioiliberis.com/en*), and within the Recupera2020 project framework (MINECO/CSIC, co-funded by ERDF of UE), had developed a strain PICF7-based non-commercial bio-formulation prototype. Several PICF7 bio-formulation parameters were re-evaluated and validated within MUSA project activities, verifying results previously obtained, and concerning: culturing media for PICF7 inoculum production, determination of the best bio-formulation carrier, persistence of PICF7 properties related to biocontrol and plant growth promotion (i.e. phytase and protease activities, siderophores production, phosphate solubilization) after developing the bio-formulation, minimal number of PICF7 CFU/ml to ensure root colonization, PICF7 survival in bio-formulations, storage and application.

Three types of PICF7 bio-formulations were evaluated: *i)* liquid, *ii)* freeze-dried, and *iii)* inorganic solid carriers. For all bio-formulations developed, the persistence of several biocontrol and PICF7 properties was monitored. For the liquid formulation different culture media and carbon sources were assayed. In order to ensure bacterial cell survival during the freeze-drying process different lyoprotectors (i.e. inositol, threalose and skimmed milk) were tested. Lyoprotectors were selected based on the best resurrection index, PICF7 viability and maintenance of its PGP and biocontrol properties.

Finally, in a third approach IAS-CSIC team tested a formulation in which PICF7 cells were mixed with inert solid carriers (different types of clays) in two different proportions (v/v) for each one of them. Monitoring of PICF7 cells viability in the different formulations and carriers was carried out. Results showed that PICF7 did not respond well to the freeze-drying process regardless of the lyoprotector used. The best solid formulation was based on a PICF7-sepiolite combination, stored at room temperature. Even though an initial decrease of viable CFU/ml after mixing was observed, the population recovered one week later at room temperature. Both liquid formulation and freeze-drying preparations (in this latter case for the surviving bacterial cells) did not affect biocontrol and PGP properties.

A protocol for the easy and effective application of a PICF7-based bio-formulation during the nursery propagation process was also developed. The aim was to set the minimum effective dose needed to ensure effectiveness. Since PICF7 was originally isolated from the roots of nursery-produced plants, pilot experiments have been initially performed using olive plants. Three different ways to apply PICF7-based formulations were assayed: drenching the liquid formulation, drenching a mixture of water and the clay-based carrier, and mixing the latter one with peat. Eventually, PICF7 population density remained more stable when liquid formulation was used. Regarding to biocontrol effectiveness of this latter bio-formulation, strain PICF7 proved to be effective against Verticillium wilt of olive corroborating earlier results. These results, together with those obtained from biocontrol of Foc4 in banana plants during the MUSA project framework, encourage the use of PICF7-based liquid formulations as a preventive treatment during the nursery-propagation phase of banana plants.

Informations on dose, application method, storage and compatibility of the prototype formulation designed by IAS-CSIC/Bio-Iliberis R&D are detailed below.

Dose and method of application:

- Orchards: Dilute 1L of liquid formulation in 100L of water. Drench with the mix each seedling after transplant into a pot.

- Large cultivation extensions: drip and spray irrigation, apply at 1% or 10L/ha.

Storage: conserve in closed original container in a dry place. Do not expose the container to direct sunlight and temperatures above 40°C. Shake before use. Under these conditions, a cell density of 10^6 CFU / ml for is guaranteed for at least one year.

Compatibility: with most fertilizers and phytosanitary products used except acid mixtures (pH < 5).

In vitro *antifungal activity of chitosan against(Foc STR4 and TR4 in combination with selected EBCA from banana roots.*

Chitosan is a polymer widely used in agriculture due to its antimicrobial activity against many fungal pathogens. Chitosan also displays an antibiotic activity against some bacteria. However, different biocontrol agents are resistant to chitosan. If beneficial microorganisms are resistant to chitosan they may be formulated and encapsulated with this polymer. With this final purpose, the combined effect of chitosan and nine selected banana root EBCA and our reference strain PICF7 were tested in *in vitro* assays against two Foc races (STR4 and TR4) in collaboration with UA. A synergistic inhibitory effect was observed (reduction in fungal colony diameter) when chitosan was combined with EBCAs (Fig. 5.3).

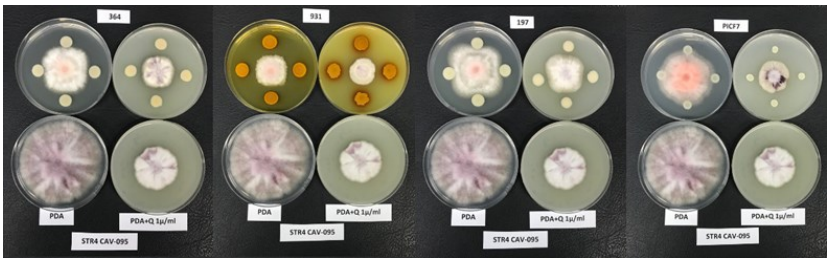


Figure 5.3 Examples of *in vitro* antagonism assays of selected EBCA against *Fusarium oxysporum* f. sp. *cubense* (*Foc*) subtropical race 4 (STR4) in PDA supplemented or not with chitosan.

Chitosan formulation T8 vs Foc TR4 on banana plants

Bioassay in Magenta boxes were carried out to test the effect of chitosan (T8) on Foc banana root colonization. Assays were:

- Preventive experiment: 50 ml of agar (0.05%) + T8 (0.1-1 mg/ml), after 3-5-7 days plants were inoculated with 10^6 spores/ml of Foc. Control plants were left uninoculated.
- Curative: 50ml of Agar (0.05%) + 10^6 spores/ml of Foc during 1 day. Then plants were trespass to other Magenta Box with 50ml of Agar (0.05%) + T8 (0.1-1 mg/ml) during 3-5-7 days. Control plants were left uninoculated.

Liquid and encapsulated T8 experiment:

- 50ml of Agar (0.05%) + 10^6 spores/ml of Foc during 1 day. Then plants were trespass to other Magenta Box with 50ml of Agar (0.05%) + T8 liquid (2 mg/ml) during 5 days.
- 50ml of Agar (0.05%) + 10^6 spores/ml of Foc during 1 day. Then plants were trespass to other Magenta Box with 50ml of Agar (0.05%) + T8 encapsulated (2 mg/ml) during 5 days.
- Controls were left uninoculated.

Proportion of root infection was determined using a culture method (Fig. 5.4).

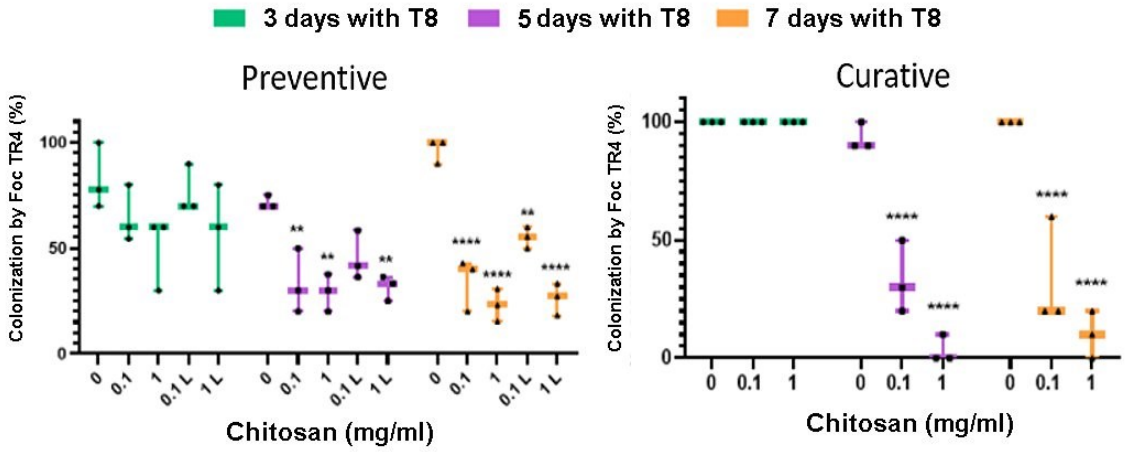


Figure 5.4 Effect of preventive and curative applications of chitosan on banana Foc infection.

Chitosan reduced banana root infection by Foc. Preventive treatments were more effective than curative ones. High concentration of liquid chitosan inhibited most root infection. Chitosan and encapsulated chitosan also inhibited Foc germination (Fig. 5.5).

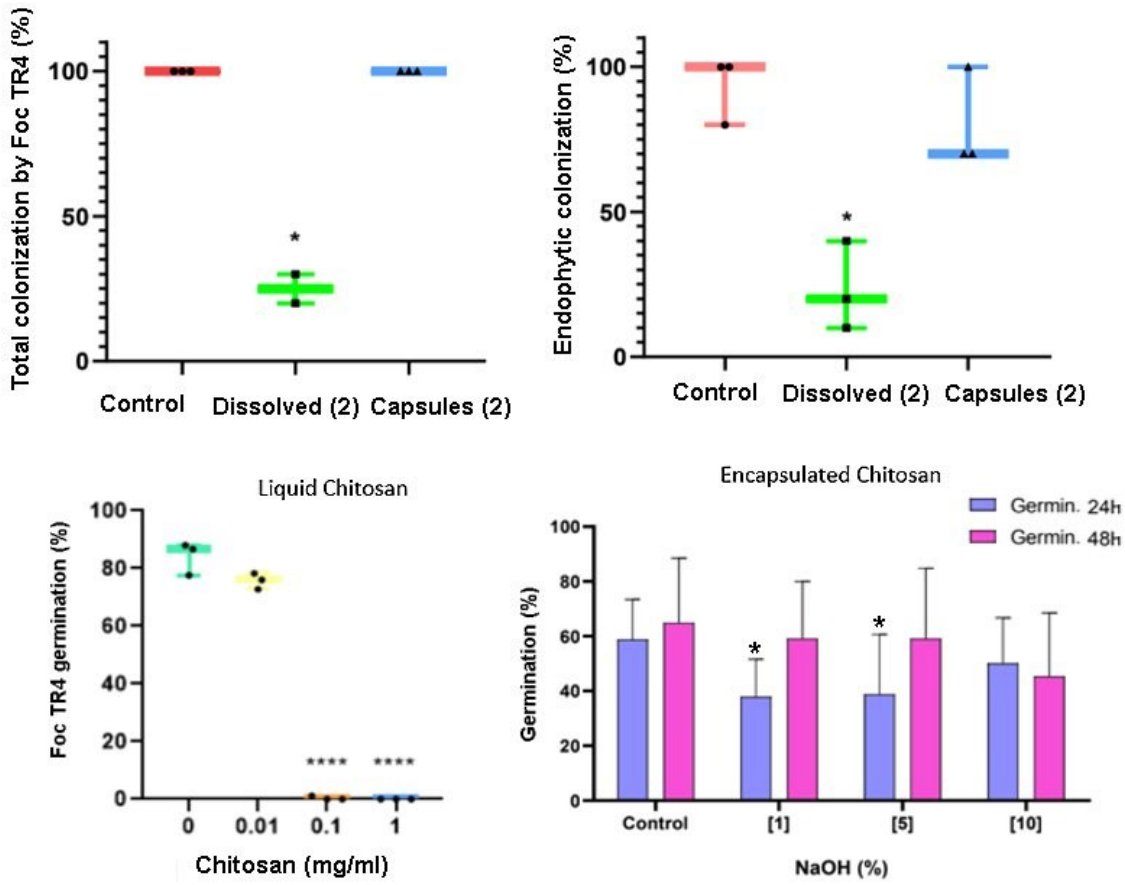


Figure 5.5 Effect of concentration of liquid and encapsulated chitosan on root infection by Foc TR4.

Mass production of P. chlamydosporia *var.* catenulata*,* T. asperellum *Ta. 13 and* HC1 *liquid fermentation*

Continued mass production of referenced strains of *P. 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® and SevetriC, respectively. For EPNs the method for *in vivo* production of Dutky et al. was modified by CENSA for massive use in Cuba, including quality control steps (Fig. 5.6, 5.7). The document with the protocol modifications produced by Sánchez et al. has been deposited in the Cuban Copyright Center (Centro Nacionalde Derecho de Autor, Cuba, n. 09613-2002) and represents the reference for production of EPN in cottage laboratories.

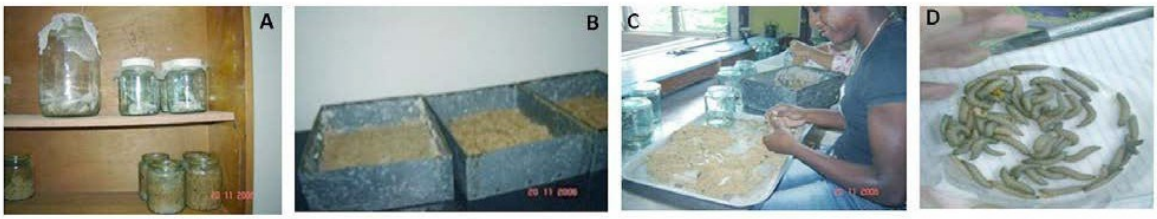


Figure 5.6 Initial steps in EPN production. Mating of adult moths (A), larvae development inalternative substrates (B), wax moth larvae extraction (C) and collection of healthy late-instars.

Figure 5.7 Steps followed in EPN production on *G. mellonella* moth larvae. Incubation of larvae inoculated with EPNs (A). Harvest of nematodes by White's trap , usingtrays and glass pieces (B). Cleaning and concentration of nematode suspensions using sievesand 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 solid culture media described by Sánchez et al. in 2006 as a reference (Patent OCPI-882/2006), ten further media were evaluated as liquid substrates, including co-product from animal industries and botanic products, in three trials, using 150 ml Erlenmeyer flask on orbital shakers. Six media did not produce IJ. One medium was selected, composed by animal and vegetal co-products, that yielded 16 896 IJ/ml.

Management of BW using EPN

The BW adults were susceptible to the HC1 strain of *H. amazonensis*, as the percentage of mortality increased when the concentrations increased. From these results, studies have been planned in semi-controlled conditions and field, to determine the effectiveness of the strains, for incorporation in IPM of banana and plantain crops in Cuba.

Improvement of shelf-life for P. chlamydosporia *product*

Modeling indicated that the best results may be obtained using between 30 and 40% zeolite for filling and storing the product at 15 °C. It was estimated that viability of *P. chlamydosporia* will rapidly decline at temperatures above 25 °C. For all treatments the humidity percentages had to be maintained at an adequate level (below 10%), which indicated that the zeolite contributed to the drying of the spores, maintaining the moisture content in an interval considered appropriate for conservation of the product.

Effect of different carriers on EPNs

As part of pre-formulation studies, the effect of three products (carriers 1, 2 and 3 respectively), were evaluated on the viability of the HC1 strain of *H. amazonensis*. Transparent polyethylene bags with 10, 20 and 30 g of each carrier were prepared and sterilized. Three replicates of each one were compared with the control (polyurethane sponge bags). The concentration used was 10^6 JI. High mortality of IJ occurred with the three carriers evaluated, without greatly impacting the weight of the packaging. This study should continue

after a method is determined to bring juveniles to the state of anhydrobiosis, which allows them to be formulated in solid media avoiding high mortality rates.

WP 6 - Field integration of EBCAs-based IPM and safe propagation material, and impact on yields (months 20-42)

Task 6.1: Regional field assays for IPM based on plant germplasm and EBCAs applications. *Task leader*: CENSA. *Other Participants:* Coplaca, IITA, KU Leuven, EARTH, Real IPM, SARI.

The main objective of this task was to evaluate the efficacy of (non) commercial EBCAs and PGPM in field assays including different *Musa* germplasm, to validate results previously obtained under more controlled conditions (greenhouse, nursery), considering successful treatments for future IPM strategies in banana producing areas.

The Project supported SARI's ex-situ national germplasm, containing about 900 enset landraces maintained as a living collection. Landraces of agronomical merit is being utilized for the project activities in testing candidate EBCAs in WP2 against Foc. There are project's ongoing trails aiming at resistance against nematodes on enset landraces. Furthermore, a number of landraces are being multiplied that will be used for large-scale field evaluation and distribution.

Effect of commercial PGPR on banana growth under open field conditions

This activity was carried out by KUL in collaboration with Real IPM/Biobest. The plant growth promoting effect of the PGPR *Azospirillum brasilense* (PTA 001, provided by Biobest) was tested on the plantation *Paradise produce* of Fresh Fruit Holdings (https://freshfruitholdings. business.site/). 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. Williams was selected for being a widely used cultivar in production areas in Latin America (Cavendish subgroup, AAA). The cultivar has been reported sensitive to PPN and BW, while resistant to *Foc* R1. The climatic conditions in Guayubín are tropical with a minimal temperature of 19 °C and a max temperature of 34 °C. Isolate *A. brasilense* PTA 001 was first applied in the nursery *via* a drench at a known concentration, 14 weeks before planting in the field. Significant increases were observed between 6 and 8 WAP (Fig. 6.1).

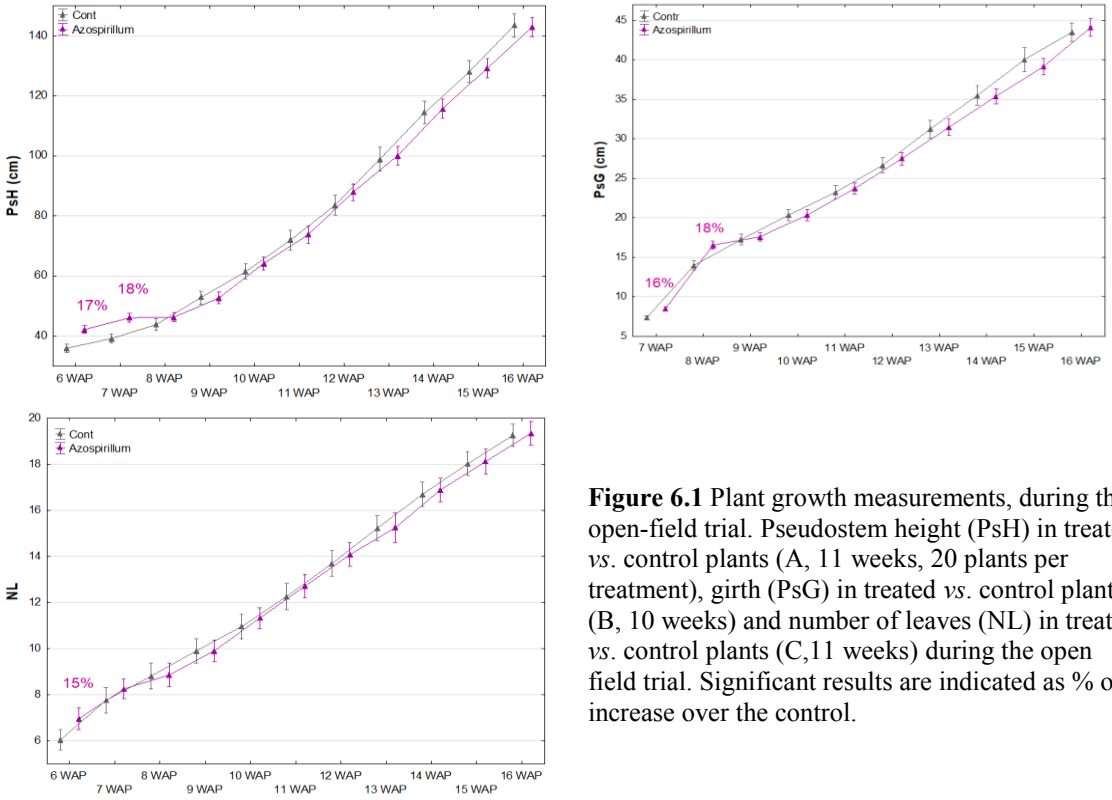


Figure 6.1 Plant growth measurements, during the open-field trial. Pseudostem height (PsH) in treated *vs*. control plants (A, 11 weeks, 20 plants per treatment), girth (PsG) in treated *vs*. control plants (B, 10 weeks) and number of leaves (NL) in treated *vs*. control plants (C,11 weeks) during the open field trial. Significant results are indicated as % of increase over the control.

IITA Assessed field performance of tissue cultured (TC) banana plants following single and dual inoculation of *B. bassiana* (isolate WA) and *F. oxysporum* (isolate V5w2). Plant flowering and yield data were collected on a weekly basis, in addition to data on nematode and weevil infestation levels for every toppled, snapped, flowered and harvested plant. Single inoculation of plants with *F. oxysporum* V5w2 reduced nematode infestation levels at three months post transplanting into the field, *B. bassiana* WA or a combination of both V5w2 and WA did not reduce nematode infestation three months post transplanting (Fig. 6.2).

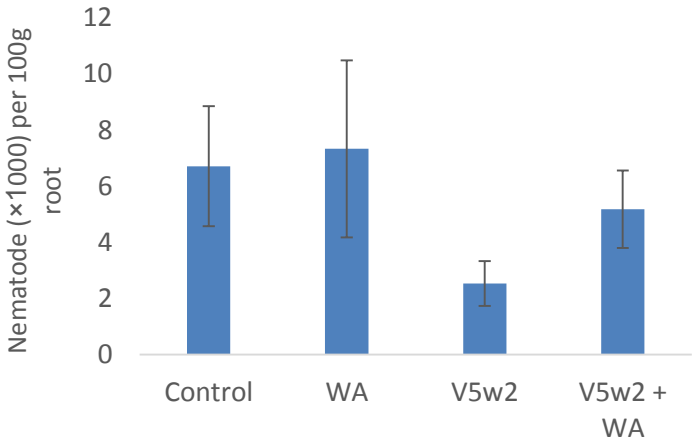


Figure 6.2 Nematode counts per 100 g root following inoculation of TC banana plantlets with *F. oxysporum* V5w2 and *B. bassiana* WA, three months post transplanting into the field.

There was no difference in nematode infestation levels at 6 and 9 months respectively. Preliminary results suggest no effect of the individual or combined endophytes on weevil damage at both flowering and harvest stages respectively. The banana field has attained 91%

flowering, and 69% of the plants have been harvested, with 7.2% of plants dead at pre-flowering. Plant death pre-flowering was attributed to nematode (70%) and weevil (30%) infestation, respectively. Inoculation of plants with the individual isolates of V5w4 and WA improved plant survival beyond flowering stage by 4 and 5% respectively, in comparison to control plants. We are yet to assess effect of the BCAs on yield.

Task 6.2 Analysing plant response to novel IPM approaches.
Task leader: IITA (resp. D. Coyne) Other Participants: Coplaca, ICIPE, SARI, CENSA, Real IPM.

Three demonstration sites have been identified in three banana growing zones in Kenya, namely Muranga County, Embu County and Kirinyaga County, to determine efficacy of *Purpureocillium lilacinum* and *Isaria fumosorosea* in PPN control at field level (Fig. 6.3-6.4). Additionally, Real IPM collaborated with banana plantlet propagators who provided sites for demonstration and to evaluate field performance of EBCAs pre-inoculated plants.

Figure 6.3 Transplanting EBCAs pre-inoculated plants in the field to validate greenhouse results at Muranga County, Kichozi Farm, Kenya.

Figure 6.4 Field Trial to test efficacy of *Purpureocillium lilacinum* and *Isaria fumosorosea* vs PPN at Kichozi (demo site farm).

Task 6.3 Plant safety issues and provision of propagation material. Task leader: IITA (resp. D. Coyne) Other Participants: KU Leuven, Coplaca, CENSA, SARI.

CENSA applied *P. chlamydosporia* and *T. asperellum* in the process of adaptation of banana vitroplants. Real IPM identified commercial mass TC propagating institutions/nurseries and demonstration sites to create awareness on EBCAs integration at nursery level, promoting banana plant growth and induce systemic acquired and induced resistance.

WP7 – Validation for transfer to stakeholders of locally adapted and effective IPM technologies

Task 7.1 Validating bioformulations with farmers. *Task leader*: Coplaca *Other Participants:* IITA, SARI, CENSA, EARTH

UA tested in the filed, in collaboration with Coplaca, repellents C1, C2, C5 and C7 isolated from WP2-3 in banana field conditions, and designed a prototype of VOCs delivery system which was included into BW traps in banana fields. UA also tested (by captures) the effect of those repellent against BW.

Field evaluation of endophytes for biocontrol of nematodes

In a random block design in the experimental fields of partner EARTH, four endophytic isolates previously identified as *Trichoderma asperrellum* and one identified as *T. atroviride* and tested in greenhouse assays in WP2, were evaluated for their afficacy as biocontrol agents of burrowing nematodes. The endophytes were injected in follower suckers of 1.75 m heigh, in two banana cultivars, Grande Naine and Williams. The inoculation consisted in an injection of 10 ml of conidia and propagules suspension of each endophytes at sucker pseudostem level, with a concentration of $1 \cdot 10^7$ CFU /ml. The control was not injected with any endophyte. The data collected in four bimonthly samplings indicated that the endophytes reduced significantly the population density of *R. similis* in comparison to the control, in both cultivars (Fig. 7.1 and 7.2).

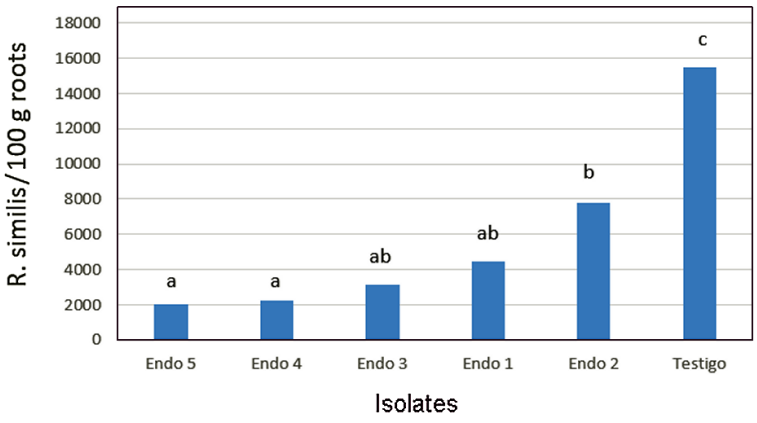


Figure 7.1. Effect of endophytes on the population of *R. similis* on the cultivar Grande Naine after 4 bimonthly samplings in EARTH experimental field.

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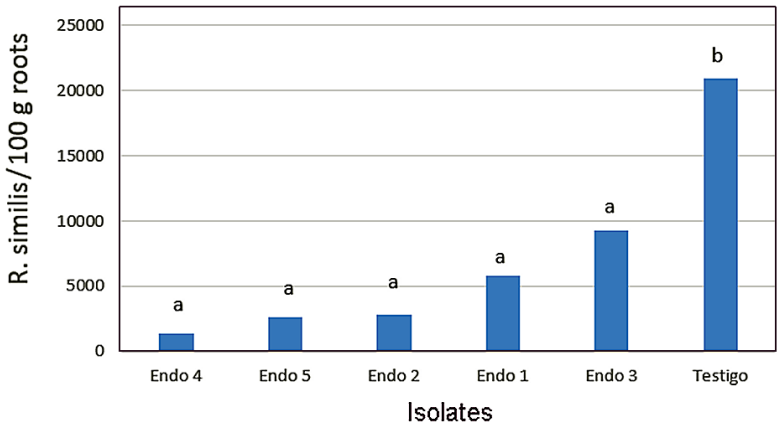


Figure 7.2. Effect of endophytes on the population of *R. similis* on cultivar Williams after 4 bimonthly samplings in EARTH experimental field.

Effect of the endophyte on production. After one year of plantation and more than one year in production, inoculated plants with endophytes presented statistically significant heavier bunches, in comparison with the control, in both commercial cultivars evaluated. In the case of Grande Naine, data from one year of production in Grande Naine indicated that the bunch weight was 2 kg higher in plants protected with the isolate ENDO4, registering on average bunch weight 23.78 kg in comparison with 21.86 kg in the control (Fig. 7.3). In the case of Williams, a mean 21.1 kg bunch weight was obtained in plants protected with ENDO4, in comparison with 19.96 kg in the control (Fig. 7.4). A bunch of 36 kg in the cultivar Grande Naine is illustrated in Fig. 7.5.

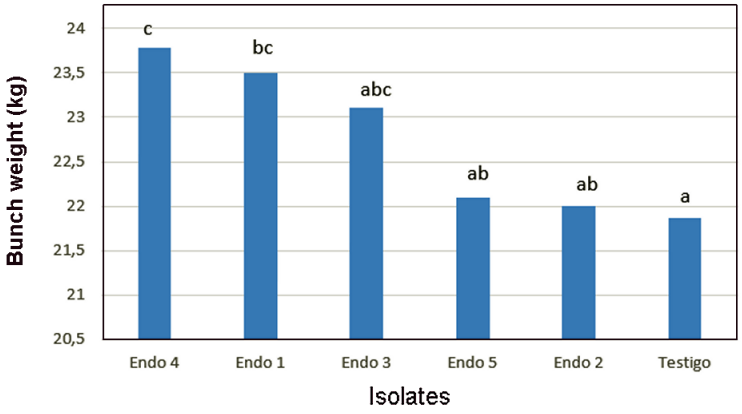


Figure 7.3. Effect of the endophyte on the bunch weight on the cultivar Grande Naine.

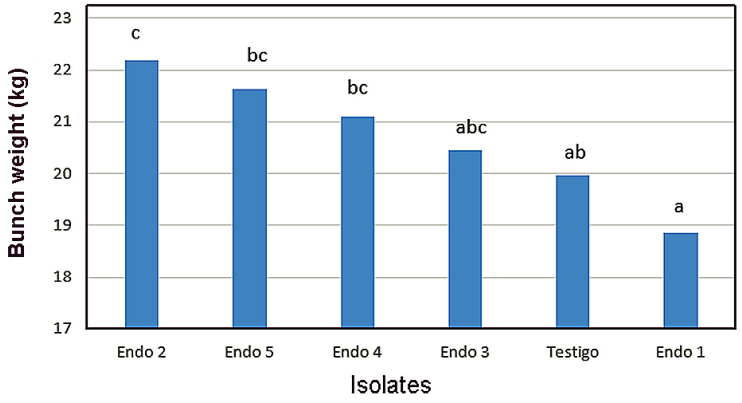


Figure 7.4. Effect of the endophyte on the bunch weight on cv Williams.

Figure 7.5. Banana cv Grande Naine plant inoculated with isolate ENDO4 bearing a bunch measuring 36 kg in weight.

Colonization studies in the field

Following suckers close to the flowering were inoculated with ENDO4 at 30 cm above the soil level, making a 1 cm diam. hole and introducing a corn seed impregned with mycelium and spores of the isolate (Fig. 7.6). 15 days after inoculation, in vitro tests were carried out on plant samples, in order to verify if the fungus colonization occurred. Results confirmed a polar colonization as the fungus could be isolated from roots, as well as the pseudostem at 10 and 50 cm height from the soil surface (Fig. 7.7).

Figure 7.6. Inoculation of *T. asperellum* isolate Endo 4 using corn seeds introduced in the pseudostem at 30 cm from the soil surface, in the field.

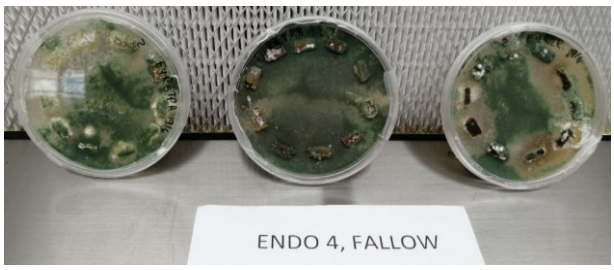


Figure 7.7 Polar colonization of *T. asperellum* isolate Endo4. From right to left: roots, stem at 10 cm height and stem at 50 cm height. Samples collected 15 days after field inoculation.

Field data indicated that the fungus grew in the whole plants including roots, corm, and psuedostem. Base on results, fallow strain ENDO 4 of *T. asperellum* showed potential for development as a commercial product by stakeholders aiming at bioformulations to control PPN.

Plant parasitic nematodes and host weeds at the Cuban National collection of Musa *spp.*

The dominant populations correspond to the nematodes belonging to genera *Meloidogyne* (root knot nematode), *Pratylenchus* (lesion nematodes) and *Helicotylenchus* (spiral nematodes), in descending order. The presence of these populations is due not only to the capacity as hosts of these nematodes of most of the genotypes preserved in the collection, but also to the existence of weed plants that act as alternative hosts, such as *Commelina diffusa* (Fig. 7.8). In roots of most *Musa* genotypes, the PPN *Helicotylenchus, Pratylenchus, Radopholus, Meloidogyne* and *Tylenchorhynchus* dominated. In soils of the national *Musa* spp. collection at INIVIT, 16 PPN genera were found. CEMSA ¾ genotype, one of the most distributed in the country, presented the highest populations of *Helicotylenchus* and *Pratylenchus* in soil. FHIA 01 had the largest populations of *Radopholus* and FHIA 18 of *Meloidogyne*.

Figure 7.8 Galls, females and egg masses of *Meloidogyne* sp. in roots of *Commelina diffusa* from National *Musa* collection in Cuba

Farmers' perception and SWOT analysis

SWOT analysis was performed on field application of MUSA results (see Deliverable7.1 and task 9.2). The dynamics and behavior of BW and the availability of bioactive compounds, either repellent and attractant, are considered by stakeholders as a *strength.* Both sustain the actual use of pheromone repellents or traps, since chemical substances (pesticides) to have an effect must come into contact with the insect during its nocturnal habits. Low adult movements, together with larval development inside the plants, are unfavourable conditions for any application or treatment. However, one of the *weaknesses* is given by the difficulties and obstacles, often encountered in the registration of EBCAs or of other sustainable products, for use in agriculturae, although differences exist among the regions interested by the Action. As for *threats*, apart of recent epidemics, it can be added that EBCAs take time to show results and farmers prefer to apply near, available chemical inputs with immediate effects. For the *opportunities*, it should be noted that in a situation of market competition, the type of pest management solutions proposed by the Consortium, not based on pesticide applications, is an added value for production, trade and consumers.

Task 7.2 Testing farmers' contribution to IPM profiles. Task leader: CENSA Other Participants: Coplaca, IITA, CSIC, EARTH University.

Programmed activities have been integrated in the application of a questionnaire and interviews with farmers in Cuba applied for production of Deliverable D 9.1 (EBCAs impact data, market receptivity and socio economic data report with guidelines for crop biomanagement) in WP9, provided on July 30, 2020.

Task 7.3 Demonstration fields. Task leader: IITA Other Participants: EARTH University, CENSA.

Effect of *P. chlamydosporia* var. *catenulata* and *T. asperellum* (alone and in combination), on PPN populations and the development of *Musa* spp. plants were studied in the field by CENSA. The results showed that one single application after 45 days did not significantly decrease the number of nematodes when comparing plants with biological products applied with the untreated control. However, the plants treated with these BCAs showed greater cluster weight ($p = 0.0375$) and circumference ($p = 0.0402$) of the mother plant, compared to those without application.

From the results of these evaluations derives the need to assess higher application doses and design a field demonstrative experiments with other alternatives and combinations, for example:

- Application of BCAs from the hardening phase in the biofactory + mycorrhizae
- Use, in that phase of biochar + organic fertilizers + oils or plant material with effect vs nematodes analyzed in banana / plantain + mycorrhizae
- In case of use of corms for planting introduce the heat treatment and ten use of essentials oils
- Soil preparation + solarization + infection
- Application of BCAe in the transplant hole
- Application of biochar “loaded” in the hole
- Use of treatments with coverage plants of those informed with nematicidal effect
- Growth promoting bacteria

Application of P. chlamydosporia *and* T. asperellum *in vitroplants adaptation process*

The radical and foliar mass increased when *in vitro* banana plants were inoculated with isolates of *T. asperellum* and *P. chlamydosporia*. Although both endophytes are compatible, it is important to take into account the time of application, since competition could occur during the saprotrophic activity of both fungi. Both EBCAs can be incorporated into the system of banana vitroplants, achieving a better adaptation and stimulating their growth, while contributing to the early protection of the planting material. Application directly into the substrate is recommended for higher effectiveness.

Demonstrative fields were organized by Real IPM, IITA, CENSA and EARTH, for demonstration activities and for the Field Farmers Schools programmed.

Figure 7.9 Collaboration visit at Grow Tech Company at Nakuru with Real IPM personnel. Real IPM demo site at Grow Tech Nurseries in Nakuru county (Kenya) showing banana seedlings treated with *T. asperellum* (left) *vs* untreated control (right).

WP 8 – Ecological and crop data as planning tools in sustainable banana productions, forecasting climate threats to sustainable IPM

Task 8.1 Measuring and forecasting climate change effects (by UNEXE)

Towards a global map of banana production by remote sensing, a severe constraint to any monitoring and assessment of threats to production systems is the lack of accurate and high resolution information on the location and extent of plantation area, globally. Existing global maps (e.g. MapSPAM and crop-mapper.org) of banana growing areas suffer from large errors, coarse spatial resolution and are infrequently updated. This limits their utility for monitoring and assessing risks from dynamic threats, such as disease spread and extreme weather events. For example, with the recent incursion of Foc TR4 into Colombia, there is a major need to understand how and where the disease could spread locally, at a country scale and regionally. This requires detailed maps of plantation areas (i.e. host availability), ideally at a resolution of tens of meters, rather than tens of kilometers. Further, regularly updated high resolution maps are required to track observed spread of diseases and assess the efficacy of containment measures. Similarly, when considering impacts of extreme weather events, such as flooding caused by hurricanes/cyclones/typhoons, a considerable amount of damage to banana cultivation can be the result of relatively smaller, spatially dispersed flooding events. Here, the lack of detailed and up-to-date maps of plantation areas could result in unsufficient flood damage assessment, with planning for flood mitigation overlooking a sizeable fraction of actual damage, and importantly, impacts on small and medium sized farms.

To address the absence of usable distribution maps UNEXE developed a prototype remote sensing-based mapping method to map and track global banana production areas at high spatial-temporal resolutions. We postulated that the typical structure of a banana plant, with its upright stature and large leaves, combined with continuous production systems of plantations, yield an identifiable high backscatter signature, with low temporal variability relative to other landcover classes in Synthetic Aperture Radar (SAR) data. We identified this characteristic signature in SAR data from the European Space Agency's (ESA) Sentinel-1 satellite platform (Fig. 8.1). UNEXE team then leveraged this clear signal in combination

with hyperspectral satellite data from ESA's Sentinel-2 platform, to build a machine learning-based classifier (random forest) for banana plantations at a resolution of 10-30 meters. This method was developed using Google Earth Engine, which allows for rapid prototyping of methods without the need for additional high performance computing (HPC) infrastructure.

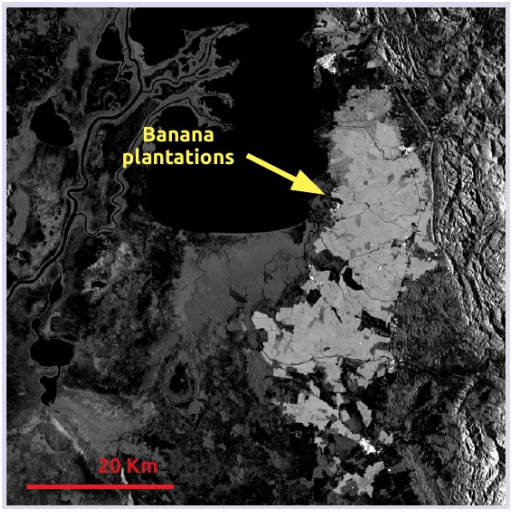


Figure 8.1. Sentinel-1 image of Antioquia, Colombia showing the distinct areas of banana plantations

To create robust and frequently updated mapping products useful in production area monitoring, there was a need for training and validation data of banana plantation locations from actual ground based observations, as well as upscaling the mapping method onto HPC infrastructure. A pilot funding for this work was secured in cooperation with the UK Science and Technology Facilities Council Food Network+ (SFN+) programme. It allowed the development of a formal collaboration with the Costa Rica government-supported banana research organisation (CORBANA), from whom we sourced ground truth data for existing plantations distribution in Costa Rica. In addition, UNEXE also entered a formal collaboration with the Department for Astrophysics at the University of Sussex, holding expertise in HPC based processing of large geographic datasets. Through this collaboration (Fig. 8.2) ground truth data provided by CORBANA were fed into a prototype method for additional calibration. Classification parameters were then communicated to the University of Sussex, who implemented the same method using ESA's Sentinel Application Platform (SNAP) toolbox, running on its HPC infrastructure, with the additional feature of continuous and automated ingestion of additional satellite data, as and when available. Errors in mapping outpust were communicated back to the UNEXE team for modification to the methods eventually required.

Funded by the Horizon 2020 Framework Programme of the European Union

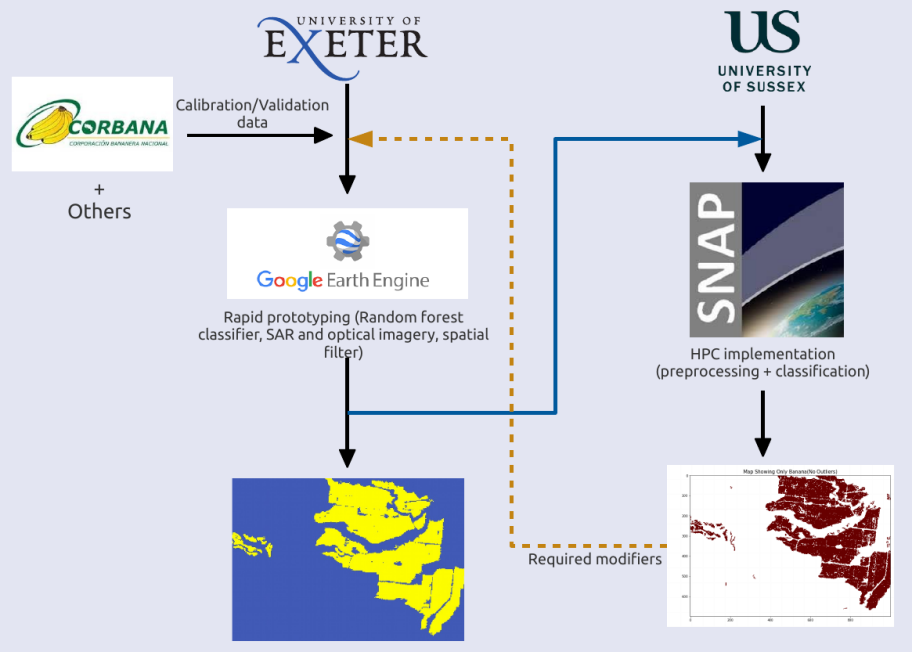


Figure 8.2 Workflow for the development and testing of mapping methods for banana production systems in Costa Rica. Ground truth data provided by CORBANA (Costa Rica partner) was fed into UNEXE mapping algorithm using the Google Earth Engine platform. Prototype methods and parameters were communicated to collaborators at the Dept for Astrophysics, Univ. of Sussex for HPC-based processing of satellite data. Errors in mapping were iteratively minimised with further development of the algorithm at UNEXE, based on output from University of Sussex.

Table 19. Confusion matrix for Costa Rica banana plantation area mapping. Considering errors from false positive and false negative classifications, overall accuracy of banana plantation area mapping was 98%.

Predicted class \ Ground truth class	Banana	Other Crops	Built area	Water	Forest
Banana	**0.99**	0.006	0.005	0	0
Other Crops	0.012	**0.95**	0.01	0	0.031
Built area	0.003	0.011	**0.98**	0	0.01
Water	0	0	0	**1**	0
Forest	0.008	0.035	0.021	0	**0.94**

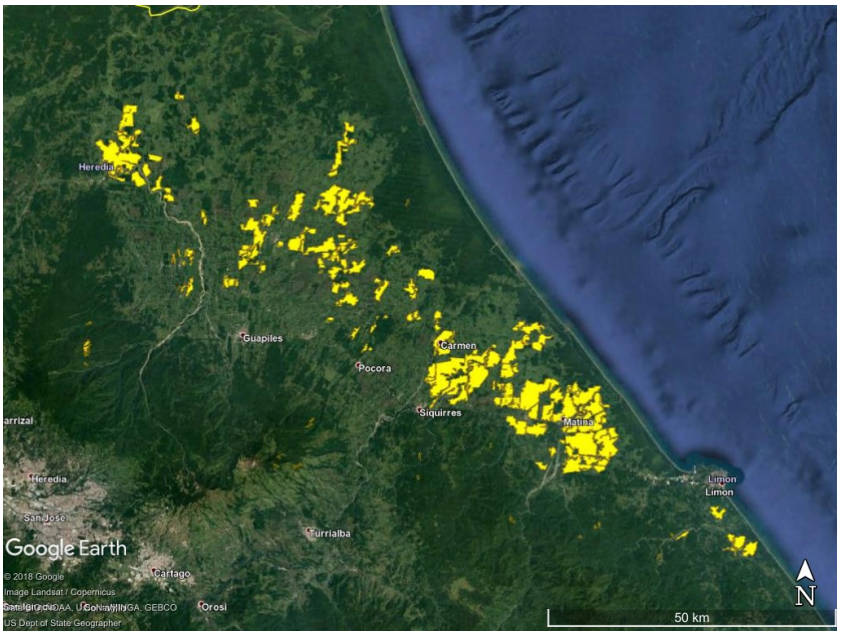


Figure 8.3 Map of Costa Rica banana plantation area in 2019 at a 30 m resolution.

The iterative process resulted in a robust mapping method for the entire production landscape for Costa Rica (Fig. 8.3) with an overall accuracy of 98% (Table 19). Results thus far showed that the developed mapping methods can produce high accuracy and resolution banana plantation distribution maps. However, for more widespread application we needed to assess the feasibility of 'projecting' the algorithm that is trained on a relatively small dataset to larger geographic areas, and eventually at a global scale. This functionality is important as it is unlikely that training datasets will be available for all countries that grow bananas. Hence, as a test we projected our algorithm to 15 other major producing countries spread across Latin America and the Caribbean (LAC), Africa, South and South-East Asia. Visually estimating accuracy of the mapping results using available aerial photos from Google Earth suggests a mapping accuracy > 90% in the regions of LAC, South Asia and South-East Asia, but much poorer accuracy in African production systems (approx. 70%). These are

preliminary estimates, and work is currently underway to compare results to actual ground truth data.

To demonstrate the utility of the mapping method we have also applied the method to map the current and past distribution of plantations. Results illustrate its capability to track the change in banana production area at high resolutions (see Fig. 8.4 for an example of Bocas del Torro region, Panama). Mapping outputs such as these can find a ready use in epidemiological modelling of established and emerging banana diseases, and as a monitoring tool for spread of pathogens.

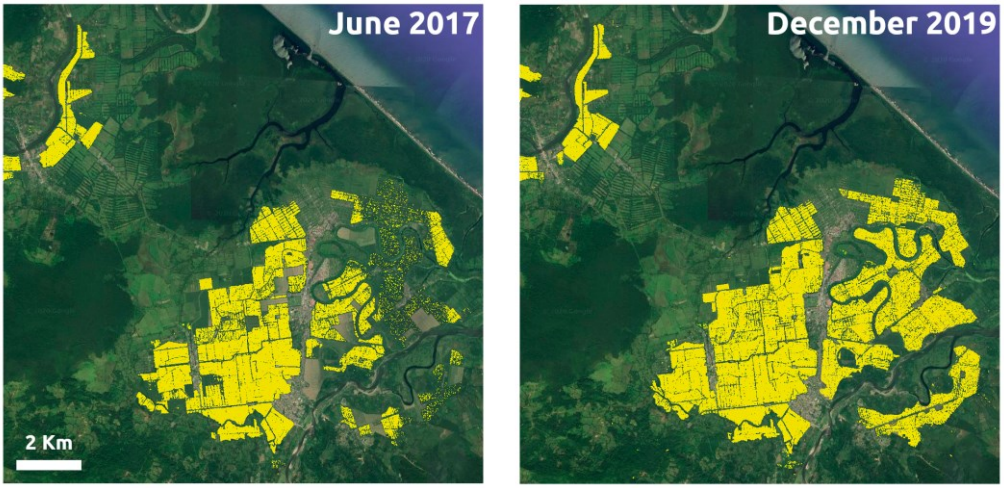


Figure 8.4 High resolution maps showing change in banana planting area for Bocas del Torro region of Panama between June 2017 and December 2019.

The mapping method described let us assess the impact of extreme weather events on production systems. In a collaboration with ETH Zurich and Banalin – a local growers association in the Dominican Republic – UNEXE utilised the method to map and quantify impacts to the country's main banana growing region in the wake of hurricanes Irma and Maria. These were category five hurricanes that grazed the northern coast of the Dominican Republic within a two week period in September 2017. We have successfully mapped hurricane-related flooding (Fig. 8.5) and pre-hurricane distribution of banana plantations in the affected area (Fig. 8.6), identifying farms affected by hurricane damage. We were then able to quantify the rate at which affected farms recover over time. While time to recovery appears to be approximately 9-12 months, on average, we have identified considerable variability in recovery rates. Ongoing work aims to relate this variability to socio-economic drivers using data directly from farmers collected by collaborators.

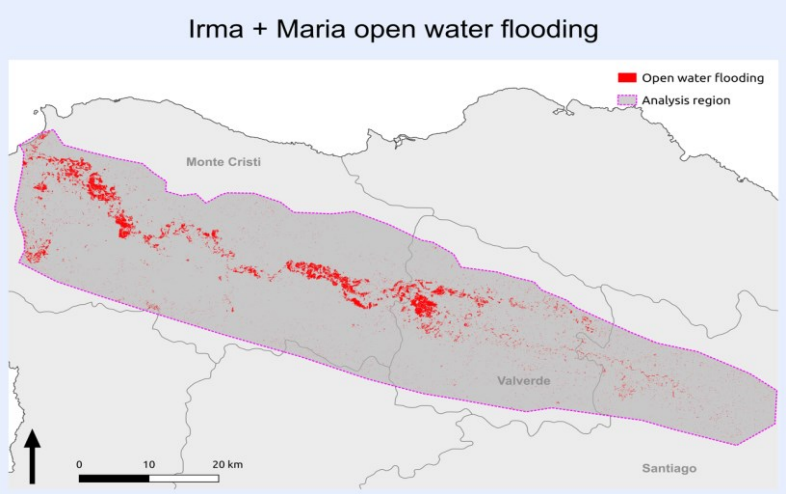


Figure 8.5 Flooding affected regions of Dominican Republic after hurricanes Irma and Maria in Sept. 2017.

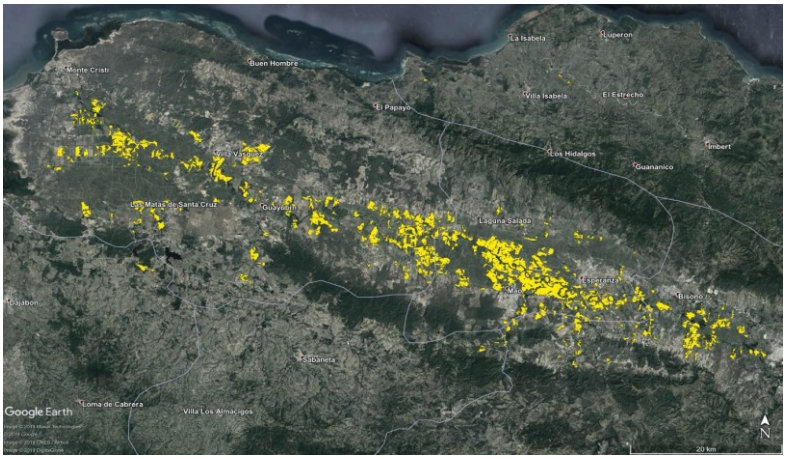


Figure 8.6 Distribution of banana plantations in northern provinces of Dominican Republic in August 2017.

The mapping method and spatial products that it can generate will be of general utility across stakeholder groups in the banana value chain, as well as researchers, governmental and intergovernmental organisations. Hence, efforts will continue to expand the network of collaborators who can contribute training and validation data to improve the mapping algorithm, in order to achieve consistent levels of accuracy, globally.

Task 8.2 Pests population dynamics and Foc epidemiology

Molecular epidemiology of Foc races. It is currently possible to perform specific and reproducible diagnostic assays of Foc isolates based on conventional PCR protocols targeting secreted in xylem genes (*SIX*), using as template DNA extracted from pure Foc cultures (Carvalhais *et al.* 2019). An available set of primers specifically amplify regions of the *SIX6* gene in *Foc* race 1, the *SIX1* gene in TR4, the *SIX8* gene in subtropical race 4, the *SIX9/SIX10* genes in *Foc* VCG 0121, and the *SIX13* gene in *Foc* VCG 0122. These assays include PCRs, with additional restriction digestion steps applied to amplification products of genes *SIX1* and *SIX13*. Thus, these 'molecular tool box' is able to reliably and accurately detect R1, STR4, and TR4, as well as two VCGs (0121 and 0122) causing Fusarium wilt in bananas (Table 20).

One of the aims of the MUSA project was the development of molecular tools enabling the identification and detection of the different Foc races. Since the procedure developed by Carvalhais *et al.* (2019) have been recently published, and claimed to be efficient, race/VCG specific, accurate and reasonably fast, we decided to test and challenge these specific primers and the PCR protocols using our collection of several *formae speciales* of *Fusarium oxysporum*, including Foc from different origins, as well as to qualify *Fusarium* banana endophytes obtained during the sampling campaigns in WP2 using a rapid procedure avoiding DNA extraction, based on the HotShot method reported by Truett *et al.* (2000, Biotechniques, 29, 52–54. doi: 10.2144/00291bm09) with minor modifications.

Table 20. The 'molecular toolbox' evaluated in MUSA activities. Primer sequences (based on *SIX* gene sequences) were designed by Czislowski *et al.* (2018). Full information can be found in Carvalhais *et al.* (2019).

Race	VCG	Targeted *SIX* gene	Product length (bp)	Restriction assay
All *Foc*	All	*SIX9a*	260	No
R1	0123, 01210, 01217, 01218, 0124, 0124/5, 0124/22, 0125, 0128, 01220	*SIX 6b*	210	No
TR4	01213/16	*SIX1a*	266	HpyAV
STR4	0120, 0120/15, 0129, 01211, 01215 0126	*SIX8b*	206	No
R4	0121	*SIX10a*	309	No
R4	0122	*SIX13c*	343	EagI

Assessment of the Fusarium oxysporum genes by the Fo CLOX PCR protocol. The primer pair CLOX1/CLOX2 was used to check whether the HotShot protocol carried out with *F. oxysporum* isolates, including *Foc*, used in MUSA and present in IAS-CSIC collection was good enough for subsequent PCR. All *Foc* isolates amplified the expected amplicon (600 bp) except CAV-2728 strain for which the whole procedure had to be repeated and/or purified DNA must be used as template (see summary of PCR results shown in Table 21). The 600-bp fragment was also amplified in isolate IAS-B-65 (*F. oxysporum* from a healthy banana plant), used as template in PCR experiments (Table 21).

Identification of Foc isolates by the SIX9_Foc PCR protocol. The primer set 'SIX9_Foc' targets homologues of the *SIX9a* gene. According to currently available data, this gene seems to be present in *Foc* isolates but absent in other *formae speciales* of *F. oxysporum* (Czislowski *et al.* 2018). The amplicon with the expected length (260 bp) was obtained in all *Foc* isolates tested except CAV-2728, which failed in all reactions performed in our study (Table 21).

Identification of Foc *R1 isolates by the SIX6_210 PCR protocol.* The primer set 'SIX6b_210' was designed to specifically detect R1 isolates. As expected, this primer set amplified the 210-bp fragment in CAV-183 (Table 20). One isolate classified as R1 and belonging to VCG 0125 (CAV-2790) did not yield the expected PCR product, and therefore must be reassessed. Carvalhais *et al.* (2019) also reported that this primer set did not produce the expected amplicon in some R1 isolates (Table 20).

Unravelling differences in Fusarium oxysporum *f.sp.* cubense *R4*

Identification of Fusarium oxysporum f.sp. *cubense STR4 isolates by the SIX8_206 PCR protocol.* The primer set 'SIX8b_206' was designed to amplify exclusively STR4 isolates (Table 4). A 206-bp product was amplified in all tested STR4 isolates, and no false positives were obtained (Table 21).

Identification of Fusarium oxysporum f.sp. *cubense R4/VCG 0122 isolates by the SIX13_343 PCR protocol (and EagI restriction analysis).* This protocol was designed to target the *SIX13* gene and flanked a R4-VCG 0122-specific recognition site for the restriction enzyme EagI (Carvalhais *et al.* 2019) (Table 20). The expected 343-bp product was amplified in isolates *Foc* CAV-183, *Foc* CAV-2790, *Foc* 54006II5 (TR4 reference strain) (Table 5). After digestion with the enzyme EagI, any expected fragments were obtained (Table 5).

Identification of Fusarium oxysporum f.sp. *cubense R4/VCG 0121 isolates by the SIX10_309 PCR protocol.* The primer set which targets the *SIX10* gene in R4 belonging to VCG 0121 ('SIX10a_309') did not yield the expected PCR product (309 bp) in none of the isolates tested (Table 21).

Identification of Fusarium oxysporum f.sp. *cubense TR4 isolates by the SIX1_266 PCR protocol (and HpyAV restriction analysis).* The primer set 'SIX1a_266' was designed to target conserved regions within *SIX* homologs "a", "b" and "c", which are specific for TR4, R4-VCG 0121 and R4-VCG 0122, respectively (Table 20). These primers flanked a recognition site of the restriction enzyme HpyAV, which is only present in the *SIX1* gene homolog "a" which is unique to TR4 according to Czislowski *et al.* (2018, *Mol. Plant Pathol.*, 19, 1155–1171). The 266-bp PCR product was amplified in *Foc* 102025 isolate (kindly provided by UA). Subsequence digestion with HpyAV confirmed this strain belongs to TR4 race. However, the TR4 reference isolate *Foc* 54006II5 was not amplified using specific primers for this race and therefore must be reassessed using pure DNA as PCR template.

Table 21. Validation of the 'molecular tool box' developed by Carvalhais *et al*. (2019) using the IAS-CSIC collection of *Fusarium oxysporum* (several *formae speciales*) and other *Fusarium* species isolates. A rapid HotShot/PCR procedure was implemented (see text for details).

STRAIN	SIX9a Foc 260bp	SIX13c R4 0122 343bp	EagI 102bp 241bp R4 0122	SIX6b R1 210bp	SIX8b STR4 206bp	SIX1a TR4 R4 0121 R4 0122 266bp	HpyAV TR4 124bp 142bp	SIX10a R4 0121 309bp	CLOX Fo ~600bp
Foc CAV-020	+	-	NT	-	+	-	NT	-	+
Foc CAV-050	+	-	NT	-	+	-	NT	-	+
Foc CAV-095	+	-	NT	-	+	-	NT	-	+
Foc CAV-183	+	+	-	+	-	-	NT	-	+
Foc CAV-2728	-	-	NT	-	-	-	NT	-	-
Foc CAV-2790	+	+	?	-	-	-	NT	-	+
Foc 54006II5	+	+	-	-	-	-	NT	-	+
Foc 102023	+	-	NT	-	+	-	NT	-	+
Foc 102025	+	+	-	-	-	+	+	-	+
Foc 3B	+	-	NT	-	+	-	NT	-	+
Foc 3G	+	-	NT	-	+	-	NT	-	+
Foc 7I	+	-	NT	-	+	-	NT	-	+
Foc 7II	+	-	NT	-	+	-	NT	-	+
IAS-B-54	-	-	NT	-	-	-	NT	-	-
IAS-B-65	-	-	NT	-	-	-	NT	-	+
IAS-B-67	-	-	NT	-	-	-	NT	-	-

IAS-B-69	-	-	NT	-	-	-	NT	-	-
IAS-B-505	-	NT	NT	NT	NT	NT	NT	NT	NT
IAS-B-918	-	-	NT	-	-	-	NT	-	-

Symbols '+' and '-' indicate positive and negative results, respectively; NT, not tested. Orange colour, isolate kindly provided by Altus Viljoen; brown colour, isolate kindly provided by Universities of Córdoba, Salamanca and Alicante (Spain); blue colour, isolate from diseased banana plants sampled at Canary Islands; green colour, endophytic *Fusarium* spp. isolated from healthy banana plants at Canary Islands. Other different *formae speciales* of *F. oxysporum*, non-pathogenic *F. oxysporum* and a *F.chrysanthemi* isolated from different plants or crops were included in this analysis as negative controls (data not shown).

Overall, the 'molecular tool box' developed by Carvalhais *et al.* 2019 (Front Plant Sci, 10:547. doi.10.3389/fpls.2019.00547) was validated to differentiate R1, STR4 and one of TR4 (*Foc* 102025) isolates used in MUSA activities. However, the reference isolate *Foc* 54006II5, classified as TR4, present in our collection could not to be validated using the Carvalhais *et al.* (2019) diagnostic method, since no amplification was observed with primer set SIX1a.

All recent *Foc* isolates obtained in surveys from Canary Islands were identified as STR4, confirming historical reports (Regalado Guijarro and Hernández Hernández, 1998, *Acta Hortic.* 490, 315–321. doi: 10.17660/ActaHortic. 1998.490.31). In addition, the HotShot protocol can be used in combination with the Carvalhais *et al.* (2019) diagnostics procedure. HotShot reactions have been successfully applied with fungal biomass grown on PDA plates. It is rapid, economic and easily automatable to prepare fungal gDNA (which is released by heating samples at high temperature). The DNA can be directly used, in most cases, as template in PCR assays, with minor adjustments in PCR conditions. Since the Carvalhais *et al*. (2019) 'molecular tool box' aimed to detect *Foc* races using as template DNA extracted from pure *Foc* cultures, yet the *in-planta* and in-soil diagnosis of *Foc* remain to be developed. As conclusion of these activities, we encourage the use of this diagnostics procedure to (re)assess *Foc* collections available in research groups working in Fusarium wilt of banana.

Biocontrol assays under tropical and subtropical conditions.

Biocontrol assays against Foc STR4 were performed. Plants were randomly distributed in two growth chambers under artificial lighting (day/night 14/10h) with tropical (26-28°C, humidity 80 %) or subtropical (24-20°C, humidity 65 %) conditions, respectively. Effectiveness of 9 selected banana endophytes (IAS-B-197, IAS-B-237, IAS-B-364, IAS-B-481, IAS-B-981, IAS-B-944, IAS-B-1013 and IAS-B-1040 and IAS-B-1054) and reference strain PICF7 *vs* Foc STR4 was tested in this assays. Overall, although disease severity was slightly lower in subtropical conditions, there were no significant differences for the *Foc* STR4 strain used when comparing the results of the experiments in tropical and subtropical conditions. Results from experiments showed a disease reduction trend for some of the EBCA treatments (i.e., IAS-B-364, IAS-B481 and IAS-B-1040).

Protocols for soil sampling and isolation of entomopathogenic nematodes (CENSA)

Several banana and plantain fields were visited and surveyed. In each site, numerous data from the soil samples must be taken, and additional information must be generating with collaboration of Soil Laboratory from National Research Institute of Agricultural Science (INCA), according to soil characterization (type, texture, pH, organic material content, electrical conductivity and other desired soil parameters among other details).

Example of data taken, for each sample N.	Sampling date	Farm	Municipality	GPS data	Observations
1	02.02.2018	X	Pinar del Rio	22°224' 58.5'' N 83° 41' 10.4'' W	Plantain area : Cv: Macho With > 10 years in production No problem with banana weevil, or other soil insects. Heavy infestation of weeds. See info in annex with data on weed species.

WP 9 – Socio economic factors and FFS-based approaches to foster IPM adoption

Task 9.1 Data gathering for socio-economic analyses
Task leader: CENSA Other Participants: IITA, SARI, EARTH University.

Semi-structured questionnaires began to be applied to farmers and other stakeholders related to banana/plantain production. They were useful to determine the knowledge of those involved in this crop production about i) PPNs and their management and ii) EPNs and their use in BW management. The database in ACCESS was prepared for data collection.

Task 9.2 SWOT analyses to improve market and social impacts. Task leader: IITA Other Participants: SARI, EARTH University, CENSA.

SWOT analysis reports

Strengths

• Generation of a microbial collection including more than 1,000 banana root endophytes (80% bacteria, 20% fungi), with more than 100 isolates (mostly *Pseudomonas* spp.) showing in vitro antagonistic capacity against several *Fusarium oxysporum* f.sp. *cubense* (*Foc*) races and phenotypes traditionally associated to biocontrol and plant growth promotion.

• Setting up and adjusting conditions for conducting bioassays, under controlled conditions, to test selected endophytes and biocontrol agents (EBCA) with higher potential to control Fusarium wilt of banana (FWB) (growth chamber) and evaluation of their plant growth promotion capacity (greenhouse).

• Understanding banana plant´s response to the presence of EBCA: molecular characterization of genes and pathways affected.

• Molecular tools for pathogen (*Foc*) diagnostics to assess the effect of the presence of selected EBCA in the colonization/infection process of *Foc*

• Wide knowledge of the banana root-associated microbiome in Canary Islands, including co-occurence networks.

• Communication to banana farmers and producers of the knowledge acquired and create awareness of the problems associated to a possible new *Foc* race emergence in Canary Islands and other banana producing areas.

• Promotion of containment measurements to avoid pathogen emergence in banana producing areas.

• Promotion of the use of EBCA as a sustainable solution in banana farming systems, less harmful for the environment and for the grower´s and consumer´s health.

Weaknesses

• Intensive and extensive use of Cavendish monoculture resistant to *Foc* R1 and R2 but susceptible to the most virulent *Foc* race (TR4), an emerging more virulent lineage that is steadily spreading across the world.

• As demonstrated in the past for the *Foc* R1 epidemic, the host genetic resistance is the best way to tackle the virulent *Foc* TR4 race. Nevertheless, until now, no commercial varieties displaying an effective resistance against *Foc* TR4 are available.

• Biological control suffers from inconsistent results over the seasons and the environments, probably due to interacting variables (e.g., environmental, genetic, physiological, etc.) present in any given agro-ecosystem, and that are difficult to control and understand. Particularly, we observed inconsistences in the results obtained in biocontrol assays carried out under controlled conditions (growth chambers).

• Low efficacy of chemical fungicides.

• EBCA may reduce disease incidence but do not completely eradicate it.

• Legal issues to formulate, mass-produce and apply non-endemic EBCA in banana growing areas. For example, impossibility to apply the biocontrol agent *Pseudomonas fluorescens* PICF7 strain (isolated from olive rhizosphere in the south of Spain) in Canary Islands and that has revealed as one of the best candidates to control Foc.

• Growers being reluctant to apply living microorganisms in cultivation areas as well as consumers to buy food treated with bacterial and fungal organisms.

Opportunities

• Knowledge of the world´s banana producing areas and their cropping systems, percentage of FWB affected areas and FWB management/control (including the use of EBCA in some banana producing areas around the world).

• Knowledge of Foc TR4 and other Foc races expansion in different banana producing areas of the world.

• Knowledge of the regulation to produce and apply EBCA in different regions of the world.

• Discovery of potential EBCA against Foc and their plant growth capacity in banana.

• Collaborations with Alicante University (combined effect of EBCA and chitosan to test possible synergistic effects against Foc), KU Leuven (plant growth promoting capacity of selected EBCAs), CNR (biochemical and phenotypic characterization of EBCA), COPLACA (sampling of asymptomatic plants in farms where Foc is currently present or it was present in the past).

Threats

• Nowadays, FWB is considered one of the most destructive maladies affecting banana (estimated affected area of 100,000 ha and around US $ 2 billion losses). Since banana (including plantains and other cooking bananas) is the most produced fruit on Earth (148 million tons produced in 2016 in 135 countries), this staple food for some 400 million people worldwide is seriously compromised. Likewise, world banana marketing and trading will be threatened, particularly because of the TR4 expansion (see below).

• Currently, the biggest threat to banana production is the emergence of Foc TR4. It constitutes a serious threat for areas where this pathogen is not present yet (for example, Canary Islands). Consequences of being introduced in TR4-free areas will be worsened due to the genetic uniformity of cultivated banana.

• Cavendish cultivar was used to replace universally the Gros Michel cultivar due to *Foc* R1 epidemics that decimated Gros Michel plantations in Latin America during the 1950s. Currently, *Foc* TR4 is present in 19 of the 135 countries producing bananas. *Foc* TR4 could devastate susceptible cultivars in a similar way as the Foc R1 epidemics. The race 4 affects not only "Cavendish," but also R1 and R2 susceptible varieties. However, the economic impact of a second *Foc* epidemic would be more dramatic than the first one.

Task 9.4 Field farming school-based training to foster IPM. Task leader: EARTH University Other Participants: IITA, SARI, CENSA.

FFS will be organized in Cuba and Costa Rica by partners CENSA and EARTH, provided the actual evolution of the Covid-SARS-2 virus will improve. Programmes definition is in course.

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

Main goal of the WP is to communicate results to the society at large, to inform regional stakeholders, training growers, farmers, technician and young researchers. Furter goals are to develop communication actions and production of related tools fot other stakeholders and the society at large.

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

Dissemination materials and visits have been developed by Real IPM (Fig. 10.1) to aid in transfer of information/knowledge produced to farmers including banana propagators,

scholars and researchers.

Figure 10.1 MUSA dissemination leaflets produced by Real IPM.

Censa personnel gave several talks about the MUSA project, organized the Second Annual MUSA Meeting in parallel to the III Annual Meeting SISA, printing of different materials/leaflets targeted to local producers. IITA contributed to the organization of the MUSA 2020 review and planning meeting that was held at ICIPE, Kasarani, Kenya on February 24-28, 2020.

Outreach activities have been carried out by UA, CSIC, CNR and KUL for dissemination of research results and participation in conferences and congresses, also publishing results in OA and pre-print journals.

The Deliverable CDEP has been amended as requested and represented again.

Task 10.2 Dissemination Actions. Project websites. Task leader: CSIC, Participants: all. Project website has been further developed, including pdf files of presentations given by beneficiaries at the different Annual Meetings of the Project, and OA publications.

Publications, dissemination in meetings, webpages and social networks

Kaushal, M., Mahuku, G., Swennen, R. (2019). Endophytes: turning the tide for plant immunity. Poster presented during the R4D meeting at IITA, Ibadan, Nigeria November 15-23, 2019.

Kaushal, M., Mahuku, G., Swennen, R., Coyne, D. (2018). Exploration of endophytes for growth promotion and biological control of *Fusarium* wilt in banana. Poster presented during the R4D meeting at IITA, Ibadan, Nigeria November 15-23.

Kinalwa, N., Kisaakye, J., Coyne, D. and Shahasi, A. (2019). Utilization of fungal and bacterial endophytes for the management of the banana nematode *Radopholus similis* in East African highland bananas. MSc proposal presented to the Dept. of Botany, College of Natural Sciences, Makerere University, Uganda

Kisaakye, J. Cortada, L., Coyne, D. (2019). Effect of dual fungal endophytes, *Beauveria bassiana* and *Fusarium oxysporum* on *Radopholus similis* infection of East African Highland bananas. Poster presented during the R4D meeting at IITA, Ibadan, Nigeria November 15-23.

Coyne, D., Kidane, S. (2020) Presented a brief Educational Presentation : 'Plant parasitic nematodes in sub-Saharan Africa and enset' to a visiting group of high school children, 10 March, at Norwegian University of Life Sciences (NMBU), Norway.

Coyne, D. (2020) Guest Presentation entitled 'Nematodes and food security in sub-Saharan Africa' highlighting activities including MUSA2020, 11 March at Norwegian University of Life Sciences (NMBU), Norway.

Kaushal, M., Swennen, R., Mahuku, G. (2020). Unlocking the microbiome communities of Banana (*Musa* spp.) under disease stressed (Fusarium wilt) and non-stressed conditions. *Microorganisms* 8, 443. doi:10.3390/microorganisms8030443

Kaushal, M., Mahuku, G., Swennen, R. (2020). Metagenomic insights of the root colonizing microbiome associated with symptomatic and non-symptomatic bananas in Fusarium wilt infected fields. *Plants* 9, 263, 1-18.

Zorrilla-Fontanesi Y, Pauwels L, Panis B, Signorelli1 S, Vanderschuren H, Swennen R. *Fusarium wilt: an opportunity to revise agrosystems and breeding in banana*. Perspective article under revision in Nature Food.

Ventura-Chávez V., D. Hernández-Ochandía, B. Peteira Delgado-Oramas, M.G. Rodríguez Hernández. *Commelina diffusa* Burm. F., new host of *Meloidogyne* sp. in Cuba. *Rev. Prot. Veg.* 34 (2).

San-Blas E., R. Campos-Herrera, C. Dolinski, C. Monteiro, V. Andaló, L.Leite, M. Rodríguez, P. Morales-Montero, A. Sáez, C. Cedano, J. C. López, E. Del Valle, M. Doucet, P. Lax, P. Navarro, F. Báez, P.Llumiquinga, J. Ruiz-Vega, A. Guerra-Moreno, P.Stock. (2019). Entomopathogenic nematology in Latin-America: Brief history, current research and future perspectives. *J. Inv. Pathol.* 165, 22–45.

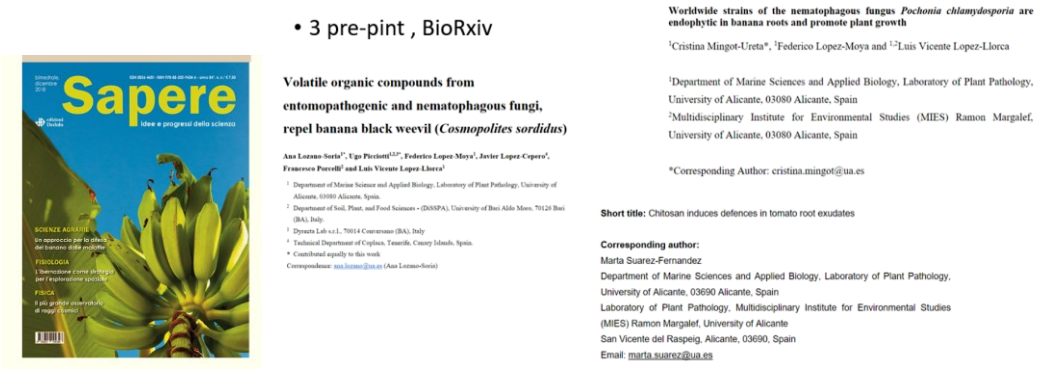
Miranda I., Dairis García-Perera, M. G. Rodríguez Hernández. (2019). Meta-analysis of the strategies for management of Cosmopolitis sordidus Guermar in *Musa* spp. *Rev. Prot. Veg.* 34 (2).

Research article by IAS CSIC personnel entitled "The banana root endosphere microbiota: Unravelling differences between mother plants and suckers and assessing agro-biotechnological potential" has been submitted to a high impact, Open Access journal, and it is currently under review.

Research article by CNR, CENSA and IITA personnel entitled "Functional diversity of soil nematodes in relation to human impact, including agriculture" has been produced for a high impact, Open Access journal, and it is currently under completion.

Publication in the ONTA NEWSLETTER (ORGANIZATION OF NEMATOLOGISTS OF TROPICAL AMERICA), 2020 of the Annual Meeting minutes of MUSA PROJECT, Nairobi (Kenya) 24-28 February 2020. Vol 50: 7-14 (https://ontaweb.com/wp-content/uploads/2020/07/june2020.pdf)

https://www.biorxiv.org/content/10.1101/2020.06.10.144550v1
https://www.biorxiv.org/content/10.1101/2020.07.03.186429v1
https://www.biorxiv.org/content/10.1101/2020.06.09.142653v1



An OA article on MUSA was published on the divulgative journal *Sapere* available at http://www.edizionidedalo.it/articoli-sapere/un-approccio-ecosostenibile-per-la-difesa-del-banano.html More than 100 copies were distributed in secondary schools in the province of Bari.

In collaboration among MUSA partners a review paper has been published in the journal:

Bubici G, Kaushal M, Prigigallo MI, Gómez-Lama Cabanás C, Mercado-Blanco J (2019) Biological control agents against Fusarium wilt of banana. *Frontiers in Microbiology*, 10:616. doi: 10.3389/fmicb.2019.01290.

https://www.frontiersin.org/articles/10.3389/fmicb.2019.00616/full

This review article achieved a big impact that can be fully consulted at: https://frontiers.altmetric.com/details/58565769, and at:

http://loop-impact.frontiersin.org/impact/article/445720#totalviews/views .

Two collaborative manuscripts with MUSA partners and other external collaborators have been prepared focusing on spatial mapping of major disease of enset and screeing of enset landraces for resistance against the bacterial wilt disease caused by *Xanthomonas vasicola* pv. *musacearum*. The first one is finalized for submission and the later one is already submitted to *European Journal of Plant Protection* and current under review and archived on bioRxiv http://dx.doi.org/10.1101/736793

Spatial distribution of the major pests and pathogens of Enset (*Ensete ventricosum*), a domesticated banana relative in Ethiopia has been ready for submission**.** In collaboration with Kew Botanical Garden and Bioversity International.

Sadik M., Alemayehu C., Bezuayehu T., David J. Studholme, Murray G., Zerihun Y., Temesgen M. Evaluation of 20 enset (*Ensete ventricosum*) landraces for response to *Xanthomonas vasicola* pv. *musacearum* infection.
bioRxiv http://dx.doi.org/10.1101/736793.

Scientific Meetings

18/5/2019 - Oral presentation of MUSA activities at the "Fascination of Plants Day" (F. Cillo, CNR) Matera, Italy
16-18/9/2019 - Oral and poster presentation at the XXV National Congress of the Italian Phytopathological Society (G. Bubici).

2-5/12/2019 - Two poster presentations at the International Symposium of Microbe-Assisted Crop Production (miCROPe): opportunities, challenges and needs. Vienna, Austria (G. Bubici, I. Prigigallo).

Oral talks

- Dr. Jassmine Zorrilla (KU Leuven) presented an oral talk in a seminar organized by Dr. Mercado-Blanco at IAS-CSIC on April 12th 2019, in the frame of the MUSA project. The title of the talk was *"Assessing the effect of plant growth promoting microorganisms in banana (Musa spp.) at the phenotypic and gene expression level"*.
- Dr. Jassmine Zorrilla (KU Leuven) presented an oral talk on May 9th 2019, at the International Seminar on Agricultural Health - SISA 2019, organized by CENSA in Cuba, titled *"Assessing the effect of plant growth promoting microorganisms in banana (Musa spp.) under greenhouse, nursery and open-field conditions"* and it was included in session number 3: development and application of bioproducts.
- Coordinator, Dr Aurelio Ciancio (CNR), presented an oral talk on May 10, 2019 at the International Seminar on Agricultural Health - SISA 2019, organized by CENSA in Cuba, titled "*Estado actual del control biológico en la agricultura / Current status of biological control in agriculture*".
- ONTA Meeting 2019, San José, Costa Rica. On oral talk was given by Coordinator, Dr Aurelio Ciancio (CNR),
- Dr Solveig Haukeland (ICIPE) personnel attended the 19th horticultural Association of Kenya (HAK) workshop, November 25th – 29th 2019, Kericho, Kenya.
- Dr Solveig Haukeland (ICIPE) personnel presented project results and finding during the annual IITA R4D week, November 18th – 23rd 2019, Ibadan, Nigeria.
- Giovanni Bubici (CNR) gave an oral presentation on MUSA activities at the "Fascination of Plants Day", Matera, Italy on 18/5/2019
- Giovanni Bubici (CNR) gave an oral and poster presentation at the XXV National Congress of the Italian Phytopathological Society, Milan (IT) 16-18/9/2019.
- Giovanni Bubici and Isabella Prigigallo (CNR) gave two oral poster presentations at the International Symposium "*Microbe-Assisted Crop Production (miCROPe): opportunities, challenges and needs*", held in Vienna, Austria, on 2-5/12/2019.
- On February 2020, SARI team attended the International workshop and conference titled "*Research on Enset and its Agri-system*" hold in Wolkite (Ethiopia), with an oral flash presentation entitled: *Current challenges, achievements and opportunities of enset research in Ethiopia.*

Trainings

- On June 4th 2019, two Master Theses defences took place at KU Leuven where all the results presented were obtained in the frame of the MUSA 2020 project:
- Ms Janne Delva presented her work entitled *"Assessing the effect of plant growth promoting microorganisms in banana (Musa spp.): evaluation under greenhouse, nursery*

and field conditions". Guidance was carried out by Professor Rony Swennen (promoter, KU Leuven), Dr. Lieselot Van der Veken (co-promoter, Biobest Group N.V., partner 11) and Dr. Jassmine Zorrilla (supervisor, KU Leuven).

- Ms Anouk Van den Bergh presented her work entitled *"Assessing the effect of the plant growth promoting fungi Trichoderma asperellum in banana (Musa spp.): evaluation under greenhouse conditions"*. Guidance was carried out by Professor Rony Swennen (promoter, KU Leuven) and Dr. Jassmine Zorrilla (co-promoter, KU Leuven).
- On May 11th 2020, a Bachelor project carried out in the frame of the MUSA 2020 project was defended by three KUL students (Ms Larissa Debroux, Ms Clara De Meese, Mr Sam Loos) with the title: *"The role of beneficial endophytes in natural crop resistance: a focus on major banana pests and diseases"*. Guidance was carried out by Professor Hervé Vanderschuren and Dr. Jassmine Zorrilla.
- On June 4th 2020, a third Master Theses defence took place at KU Leuven where all the results presented were obtained in the frame of the MUSA 2020 project:
- Ms Heike Van der Geest presented her work entitled *"Growth promotion in banana (Musa spp.) by microorganisms: an evaluation at the phenotypic and gene expression level"*. Guidance was carried out by Professor Rony Swennen (promoter, KU Leuven) and Dr. Jassmine Zorrilla (co-promoter, KU Leuven).
- ICIPE recruited a MSc student to develop a study on the use bacterial endophytes in the management of the banana nematode *Radopholus similis*. The is based at the IITA research station in Uganda.
- Activities developed by CENSA included a visiting scientist (Ing. Vaniert Ventura) at Istituto per la Protezione Sostenibile delle Piante del CNR, Sede di Torino (Italy, supervisor Dra. Raffaella Balestrini).
- 4 PhD. students and 2 MCs students involved in MUSA by CENSA.

Courses

Giovanni Bubici (CNR) attended the Course on the Nagoya Protocol held in Rome, Italy, on 5/4/2019: *Il Protocollo di Nagoya: quali conseguenze per la Ricerca e il suo ruolo in Horizon 2020* ?

Six posters were presented at ECFG15 Congress, held in Rome in February 2020.

Several talks about the project were given by Prof. Luis Vicente Lopez Llorca in Bari (Dec. 2019) or by Dr. Federico Lopez Moya at the IAS CSIC (Navarra). Dra Nuria Escudero from Microomics Ltd. also gave a course about metagenomics sequencing technologies available in MUSA project.

IAS-CSIC personnel participated in an informative workshop held at the Escuela de Capacitación Agraria de Tacoronte, in Tenerife (Spain), titled "Fusarium en el laboratorio"

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.

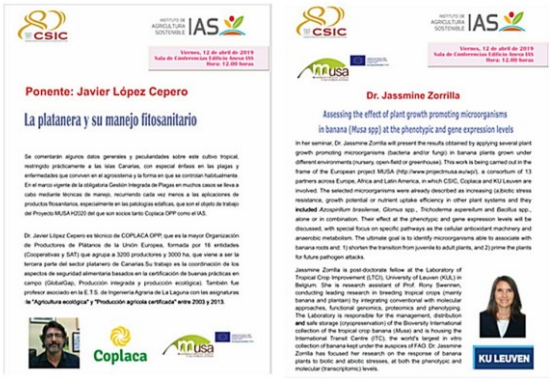
http://coplaca.es/2018/11/14/jornada-divulgativa -proyecto-musa-8-noviembre-2018/.

In April 2019 a scientific-dissemination day named *'Un paseo por la ciencia'* (A walk through science) took place in Córdoba (Spain) in which the role of banana endophytes in the biological control of Fusarium wilt was taught to the general public, mainly to children, by displaying IAS-CSIC MUSA activities in a poster and by showing live examples of *in vitro* antagonism against *Fusarium oxysporum* f.sp. *cubense.*

IAS-CSIC MUSA project's tasks have been advertised in a dissemination campaign of CSIC called "*Yo investigo' (I do research)*", aiming at promoting science to the society at large. https://www.youtube.com/watch?v=SpP0nHt-CxM&list=PLrGFH5gC-7DbBFeKLuk6XJRC423Rp1xH4&index=88&t=0s

A seminar on the biological control of plant diseases with emphasis on banana crop was held at Torrealba Secondary school in Córdoba, Spain, Freb. 26th, 2019.

In April 12th 2019, Partners Coplaca and KU LEUVEN were invited to give seminars on banana crop management and the effect of plant growth promoting microorganism in banana, respectively, in the Institute for Sustainable Agriculture (IAS-CSIC) in Córdoba, Spain.



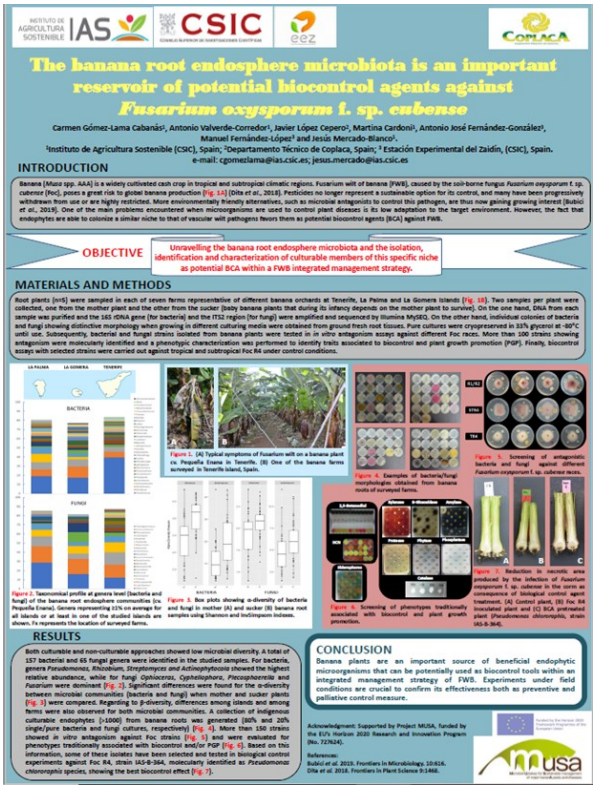
A seminar on the biological control of plant diseases with emphasis on banana crops was held at Almanzor school in Córdoba, Spain, June 6th, 2019.

UA personnel was invited to give a seminar on "Molecular mechanisms of growth and development inhibition and in fungi and plants by chitosan" at the Institute for Sustainable Agriculture (IAS-CSIC) in Córdoba, Spain, 14th October, 2019.

In September 2019, a scientific-dissemination day named "*Ciencia y música bajo las estrellas*" (Science and Music under the stars) took place during the European Night of Researchers event, in IAS-CSIC Córdoba (Spain). The role of banana endophytes in the biological control of Fusarium wilt was taught to the general public, mainly to children, by a seminar on "Biological control of plant diseases: Fusarium wilt of banana", displaying IAS-CSIC MUSA activities in a poster and showing live examples of *in vitro* antagonism against *Fusarium oxysporum* f. sp. *cubense*.

Jesús Mercado-Blanco (IAS-CSIC) was invited to present the Webinar "The Friday of innovations in agriculture: Bacterial endophytes as agro-biotechnological tools" on May 15th 2020, in which some of the activities and results from partner IAS-CSIC within the MUSA project were shown.

On December 2019, IAS-CSIC participated in Microbiotec '19 congress hold in Coimbra (Portugal) giving an oral flash presentation entitled 'The banana root endosphere microbiota is an important reservoir of potential biocontrol agents against *Fusarium oxysporum* f. sp. *cubense*'.



The International Researchers' Night is an activity aimed at popularizing science to audiences of all ages. The Plant Pathology Laboratory from UA participated in Sept. 2019 with the activity "Let's take care of the planet and the crops that keep us alive" in a successful way. In the activity, COPLACA bananas were tasted and banana crops were cropped, as well as dissemination of the MUSA project.

In these activities, dissemination on a MSc thesis project based on the effect of chitosan encapsulations on bananas was also carried out, as well as 4 BSc thesis projects, all of them involved in the activities of the MUSA project.

A VOC repellent patent was also developed during this time, as well as a book chapter at Springer (in press).

- 1 MSc thesis project
- 4 BSc thesis project
- UA Science Open Day
- 1 Paper. Int. J. Mol. Sci. 2019, 20(2), 332.
- 1 Book chapter at Springer *(in press)*
- VOC repellents patent

Int. J. Mol. Sci. **2019**, *20*(2), 332; https://doi.org/10.3390/ijms20020332 Open Access

Review

Molecular Mechanisms of Chitosan Interactions with Fungi and Plants

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Chitosan Workshop in Alicante July 2019

The Plant Pathology Laboratory of the University of Alicante participated in the European Researchers' Night promoting MUSA project

MSc and BSc studies

In July 2019 a workshop on chitosan was held at the University of Alicante, led by the Laboratory of Phytopathology. The workshop showed different members of the MUSA project how to prepare chitosan and what uses it can have for the sustainable protection of banana crops.

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

A MUSA project presentation to banana producers was held in Nueva Paz Municipality (Cuba), in March 2019. Young scientists meetings were held at CENSA during 2019.

Presentations were given at SISA Meeting related to MUSA:

Meta-analysis as strategies for decision-making in the agro-ecosystems management.

Efecto de factores abióticos seleccionados sobre la viabilidad, movilidad e infectividad de Heterorhabditis amazonensis Andalo et al. cepa HC1

La función de la capacitación en la adopción del control biológico. Estudio de caso Klamic®

Efecto in vitro del nematodo entomopatógeno Heterorhabditis amazonensis Cepa HC1 sobre adultos de Cosmopolites sordidus (Germar).

Manuals and leaflets were also printed in Spanish by CENSA.

A 2020 calendar with MUSA logo text and pics was produced by Coplaca.

Communication materials was developed by Real IPM to aid in information/knowledge transfer to farmers including banana propagators, scholars and researchers.

MUSA leaflets produced by Real IPM.

Press and media initiatives

- Banana plants' defense against deadly wilting disease may be in the soil (https://www.iita.org/news-item/banana-plants-defense-against-deadly-wilting-disease-may-be-in-the-soil/). IITA-Bulletin Issue no. 2526 (March 1, 2020).
- Unlocking the diversity of microorganisms around banana roots (http://bulletin.iita.org/index.php/2020/04/04/unlocking-the-diversity-of-microorganisms-around-banana-roots/). IITA-Bulletin Issue no. 2531 (April 4, 2020).
- "Experts study banana diseases in Tanzania" published in the newspaper *The Guardian*, on 4 April, 2020.
- 30/8/2019 - "*Il CNR di Bari Lancia la sfida al fungo killer dei banani*". La Gazzetta del Mezzogiorno, page 7.
- 20/9/2019 - "*La guerra dell'IPSP di Bari contro la Fusariosi*". L'Adriatico, page 19.

Links with other projects

SARI integrated activities of MUSA Project with research and events programmed with other cooperative Agencies and Institutes, as shown in the following banner. UNEXE carries on several project with UK Science and Technology Facilities Council Food Network+ (SFN+), ETH Zurich and Banalin, a growers association in the Dominican Republic (see WP8).

An international conference was planned in April 2020 with Coordinators of other EU funded projects, to be held at *Casa Mediterraneo*, Alicante (ES). Apart of MUSA, other Project Coordinators and representatives already agreed to participate (see the Conference draft scheme below). The meeting included various presentations and aimed at establishing links in order to integrate activities, develop collaborations and common dissemination initiatives, and present results to interested stakeholders (Coplaca, Eurobanan). Due to the Covid-19 emergency the meeting unfortunately had to be cancelled. It will be likely re-organized and scheduled for April 2021, in presence or *via internet*, depending on the evolution of the pandemic.

Scheduled activities
JORNADA EN CASA MEDITERRANEO (CM)
CM-Grupo de Fitopatología, Depto CC Mar y B. Aplicada, Universidad de Alicante (UA)
Responsable UA prof. Luis V. Lopez Llorca

Tema: "Seguridad Alimentaria en el Mediterráneo: Proyectos EU H2020. MUSA y otras Soluciones Sostenibles"
Fechas: 19, 20 y 21 de mayo de 2020

19 May

Arrival
-17.00h Welcome and Registration (Seminario Depto. Mar y B. Aplicada, Universidad de Alicante).
Visit: Grupo de Fitopatología, Project MUSA experiments, greenhouse.

20 May

9.30-11.00 Opening session, Director of CM

Conferencias Divulgativas (30 min/conferencia)
*Project **MUSA***

- Coordinador MUSA Dr. Aurelio Ciancio (CNR, Bari, Italia),
  Investigadores Participantes:
- Dr. L. Pocasangre (Earth University, Costa Rica),
- Dr. L.V. Lopez Llorca.

11.00-12.30 Coffee Break. CM Agorà: Poster Session. Photo Exibition: *Banana trade in Alicante. Historical Review* (in collaboration with Dept. de Historia, UA, Eurobanan and Coplaca). Social events: Tasting of organic bananas (Coplaca). Activities for children and young people on sustainable management of pests and diseases for food security.

12.30-14.30 Invitaded Speakers

- Dr. Altus Viljoen (Stellenbosch University, Sudafrica).
- *Project **TROPICSAFE*** Prof. Assunta Bertaccini (Univ. Bologna, Italia)

Evening social programme: visit to Castillo de Sta. Bárbara and Centro Histórico, Alicante.

21 May

9.30-11.00 Conference with other EU Project leaders: "*Seguridad Alimentaria en el Mediterráneo: Soluciones Sostenibles*".

- Dr Hans Rediers (KU Leuven, Project **C-Root Control**).
- Dr. Bruno Govin (PCS Ornamental Plant Research, Project **UNiforce**)
- Prof. Arnd Verschwele (Juluis Kühn-Institute, Germany, Project **DSS-IWM**)

11.00-12.30 Coffee Break. CM Agorà: Poster Session. Photo Exibition: *Banana trade in Alicante. Historical Review* (in collaboration with Dept. de Historia, UA, Eurobanan and Coplaca). Social events: Tasting of organic bananas (Coplaca). Activities for children and young people on sustainable management of pests and diseases for food security.

12.30-14.00

Conference of other EU projects on "*Seguridad Alimentaria en el Mediterráneo*", Round Table (to be selected) with all conference attendents.

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

Task 11.1. Management and Task 11.2 Management of innovation and results

In cooperation with all partners, the project management proceeded ensuring progress of planned activities as stated in the work plan, verifying milestones, deliverables, resources allocation and use. The required administrative and report charges were carried out, promoting internal communication and WP coordination. Evaluation procedures were applied ensuring quality and conformity to EC reporting and Consortium Agreement rules. Management of the innovation and new knowledge produced allowed the consortium to respond to the regional tasks and demand.

The Project Coordinator has been in contact with by EAB members prof. A. Bianco, prof. Rosa Manzanilla Lopez and Dr Ana Pietra Buena. Prof. Manzanilla Lopez significantly followed the project activities and updates, contributed to the work planning and communication of the work. By attending the Annual meeting she remained in direct contact with other Consortium members and Coordinator, also joined easily through email and other communication forms. Thus far no problem occurred among partners, apart of problems related to the Visa issue for some members to attend EU workshops. The information flow was efficient in most cases, and contacts occur almost on a weekly or daily basis, with different means, including cell phone, even during the Covid-19 emergency lock down.

Transfer of innovation and knowledge has been discussed with stakeholder Coplaca and an improvement is needed to promote the informatin flow from the laboratories towards farmers and field technicians. At this regard, some initiatives are under evaluation and discussion, in order to organize workshops and working days in the field in Canary Islands, to satisfy farmers' demand of knowledge and capacity building.

Task 11.3 Management of dissemination and exploitation activities

Several internal managing initiatives, aiming at an optimal development of the programmed tasks, have been promoted, trying to achieve any possible progress. The personnel involved have conducted their tasks as coordinately as possible and in good will, depending on local connectivity and in function of previous and ongoing professional links, trying to avoid overlaps, to make a rational use of time and resources. A flexible approach has often been considered as necessary and valued by beneficiaries, due to the broad range of diverse situations encountered in particular in some extra-EU areas, and to the difficulties often incurred, especially during the actual pandemic situation.

The search for interactions with specific partners seeking for synergies has been pursued, and the MUSA Coordinator has been informed in all cases. Examples of joint activities have been mentioned in previous WP summaries of this report. Attendance to requested meetings has been fulfilled, and participation in all possible dissemination actions has been pursued, when possible. IAS-CSIC has devoted time to legal aspects such as the implementation of needed MTAs and full compliance with requisites of the Nagoya protocol. Finally, in spite of difficulties, all beneficiaries contributed in due time to the deliverables produced during this second Project period:

- WP2 D2.1 Microbial collections (bacteria, fungi) and other beneficial EBCAs and selected PGPM soil microorganisms for applied IPM (by month 20).
- WP2 D2.2 Report on EBCAs host range assessment (by month 20).
- WP3 D3.1 Data on biology and effectiveness of selected EBCAs from different regions and climates, including risk analysis. (by month 24).
- WP4 D4.1 EBCAs gene expression data in controlled conditions (by month 34).
- WP4 D4.2 Metagenomic data on soil microbial consortia effects on EBCAs on susceptible /resistant banana and enset (by month 36).
- WP5 D5.1 Methods for large-scale cultivation of microbial EBCAs and EPNs, bioformulation and storage (by month 20).
- WP5 D5.2 Plant-derived products and helpers for BW management and bioformulations (by month 30).
- WP6 D6.1 Field data for integration of fungi and bacteria-based formulations with susceptible/tolerant germplasm against RKN, BW and PD (by month 26).

- WP6 D6.2 Report of phytosanitary status of propagation material and evaluation of its impact on plant growth response under different IPM regimes (by month 36).
- WP7 D7.1 Validation SWOT report on bio-formulation parameters for IPM, transfer of methodologies and management of simultaneous pests (by month 30).
- WP8 D8.1 Sampling methods and protocols for EBCAs and qualitative/quantitative epidemiologic data on changes induced in time and under various climatic change effects (by month 28).
- WP10 D10.4 Practice abstract (second series) (by month 36).

Project Meetings

The MUSA Second Yaer Annual Meeting was held at Meliá Marina, Varadero, Cuba, on 6-8 May 2019. Interview for National and regional TV. Technical tour with Nueva Paz, Mayabeque biofactory for banana vitroplant production and local farms. The meeting was attended by several MUSA partners, including stakeholders Real IPM and MS Biotech, with a presentation of the company and products. Partners also attended the subsequent III Seminario Internacional de Sanidad Agropecuaria, 2019 (SISA 2019) and the XX Congreso Latinoamericano de Fitopatología, also held at Meliá Marina, Varadero, on May 6-10, 2019.

The Third Year MUSA Annual Meeting was organized by personnel of Real IPM and ICIPE and was held at ICIPE in Nairobi (Kenya), on 24 Feb. 2020.

The Meeting was attended by 20 participants from 9 partner institutions (proceeding from all the three regions interested by the Action), and by a representative of CNIC (Centro Nacional de Investigação Cientifica de Angola, A. Tomas), who gave a talk on the crop phytosanitary situation in Angola. It also saw the participation of local students and technicians, with other Real IPM and ICIPE personnel. The report with the Meeting minutes, produced by the attending EAB representative prof. Rosa Manzanilla Lopez, has been already forwarded to the MUSA PO and is available upon request to the Coordinator.