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

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Methods for large scale cultivation of microbial EBCAs and EPNs, bioformulation and storage

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History of changes	Changes introduced	page
1	Five initial lines moved to page 2, five sentences added with 1 figure	2
2	Added: eight sentences	3-5
3	Added: Table 1 and five sentences	6
4	Added: three sentences and paragraph on phenotypic studies	7
5	Added: *Biochemical characterization* paragraph with two figures	8
6	Added: text on *in vitro* mass rearing, EPN sterilization and production	9
7	Added: six sentences, Table 2 and a graphic plot	10-11
8	Added: Fig. 10 and two sentences	12
9	Added: 7 text pages, 9 figures and 3 tables	14-20
10	Added: 12 text pages, 12 figures, 10 tables	21-34
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12	Added: *Novel elements in mass production generated by the Project*	38

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1. Methods for large-scale cultivation of EBCAs

1.1. Fungi

A different liquid-solid production method for production of the fungus *Pochonia chlamydosporia* var. *chlamydosporia* was developed by SacomLab/MSBIO, divided into the following steps (Fig. 1 A-D):

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

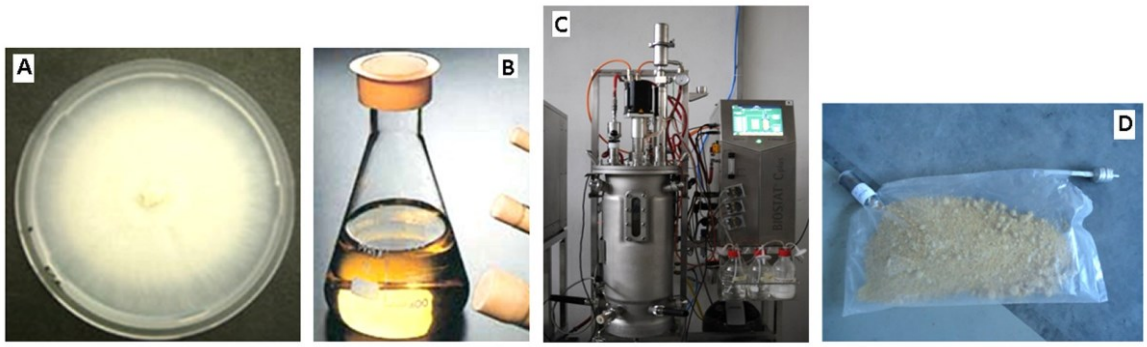

Figure 1. Initial steps followed for the liquid production of the *P. chlamydosporia* inoculum, starting from a petri dish culture (A) used for liquid inoculum preparation (B) and subsequent massal production in liquid (C). Finally, the inoculum is introduction on rice grains-based solid medium in plastic bags, for mass production (D).

The production begins in the laboratory, with *in vitro* cultures used as starter (Fig. 1 A) to obtain the fermenter pre-inoculum in a liquid growth medium (Fig. 1 B). The volume of the microbial biomass in liquid is then increased in fermenters (Fig. 1 C). The biomass produced in liquid provides the final inoculum for subsequent fermentation on a solid medium (Fig. 1D).

Figure 2. Downstream mass production of *P. chlamydosporia* on solid medium.

Immediately after, the downstream process follows on a solid medium, in controlled environmental, aerobic conditions (Fig. 2). Once the sporulation is completed, the biomass is subjected to an extraction processes for recovery of the chlamydospores and mycelial matters on a solid substratum. This represents the inoculum used for the preparation of the

commercial products, suitably formulated and integrated with other ingredients, in order to preserve their stability, efficiency and shelf-life.

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

CENSA set up a continued mass production of referenced strains *P. chlamydosporia* var. *catenulata* (IMI SD187) and *Trichoderma asperellum* (Ta.13), performed using a SSF Technology developed for two commercial products, KlamiC® and SevetriC, respectively. The isolates have been used in MUSA in experimental assays to evaluate new applications and use as Endophytes and Biological Control Agents (EBCAs) of banana.

Both isolates showed endophytic activity in banana roots. As a previous, background knowledge, IMI SD187 was known for a nematocidal activity against *Meloidogyne* spp. eggs and eggs-masses, and other nematodes (Hidalgo-Díaz *et al*., 2017). KlamiC® , based on IMI SD187, is a bionematocide registered in Cuba.

Ta.13, although had a lower activity *vs Meloidogyne incognita* (Hernández-Ochandía *et al*., 2015), was used as an active ingredient of Trichomax®, a product registered in Nicaragua. This product was tested for activity against *Radopholus similis* in experiments carried out in banana fields by Partner EARTH, in MUSA. Ta.13 had been tested, before MUSA project, in toxicological and ecotoxicological studies, and the fungus resulted as a safe organism for crop applications (background knowledge provided by CENSA).

For all these properties both products were evaluated for use on banana in the present project. Their mass rearing methodologies were investigated to obtain and improve the quality of the material for experiments. The continued mass SSF of referenced strains IMI SD187 and Ta13 was performed with quality control systems by personnel with a technical preparation for these activities. The work team used these technologies to produce, in laboratories, batches for experimental assays to evaluate new applications as EBCAs (Fig. 3).

Figure 3. Images for SSF mass reproduction of *P. chlamydosporia* var. *catenulata* (strain IMI 187SD) and *T. asperellum* (Ta13) using liquid pre-inoculum and solid reproduction over substrates. The final products are stored in bags.

1.2. Entomopathogenic Nematodes (EPN)

In the EPN of genera *Steinernema* and *Heterorhabditis*, the invasive stages (infective juveniles, IJ) carry cells of symbiotic bacteria belonging to genera *Xenorhabdus* and *Photorhabdus*, respectively. The nematodes penetrate the insect hosts through their natural openings or soft zones. The bacterial cells are then released within the insect body, in which they proliferate. Due to the characteristic of the symbiosis complex, relative to the function of bacteria, the insect fluids and tissues are transformed into a soft substrate allowing the nematode development. The resulting septicemia in fact kills the host allowing the feeding and reproduction of the bacteriovorous nematodes inside the insect cadaver. The phenotypic phase of the symbiotic bacteria (for *in vivo* production) and their release in media (for *in vitro* production) are important steps in mass production technologies.

The EPN efficacy as biocontrol agents of insect pests in different agricultural productive conditions has been an incentive to develop many *cottage laboratories* and companies to produce (*in vivo* or *in vitro*) and formulate several species/populations (Nagesh *et al.,* 2017; Saleh *et al*., 2020). EPN may be reproduced using *in vivo* and *in vitro* (solid and liquid) technologies and some characters are highly required in EPN strains: high virulence against the target insect(s) and ease-of-culture. Also desirable are a superior stability (i.e. shelf-life) and versatility (i.e. effectiveness against multiple insect pests) (Gaugler and Han, 2002).

Background knowledge: in Cuba, EPNs have been produced since the '90s, in *in vivo* conditions, using *Galleria mellonella* as host (Rodríguez, 2015). Sanchez *et al*. (2001), and Sánchez (2002) developed at CENSA the *in vivo* methodology and selecedt *Heterorhabditis amazonenesis* HC1 strain (native) for mass rearing, based on high virulence *vs* several insect pests and high reproduction capacity in *G. mellonella*, with first studies on solid growth media (Castellanos and Sánchez, 2000; Sánchez *et al*., 2006). The EPN do not need being registered in Cuba (Ceballos and Monter de Oca, 2016).

In the frame of MUSA, CENSA evaluated HC1 against the banana weevil (BW) either alone and in comparison with new *Heterorhabditis* isolates found in banana/plantain (*in vitro*). The pathogenicity of HC1 on BW *in vitro* was superior (Deliverable 2.3). Considering these results, the teamwork decided to perform studies to improve the *in vitro* liquid mass rearing method, using HC1 strain. According to Ehlers (1996), *Heterorhabditis* spp. liquid culture was the standard of different successful companies. These nematodes may achieve a significant control efficiency for biocontrol purposes, in the field.

1.2.1. *In vivo* EPN mass production

This procedure represents a low input technology and a labour-intensive process, used in more than 30 Cuban cottage laboratories for EPN production, since the '90s (background knowledge). The methodology has two main processes. The first one aims at the production of healthy late-instar larvae of the wax moth *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). The second step aims at the production of infective EPN.

The method for *in vivo* EPN production developed by Dutky *et al*. (1964) was modified by CENSA research team for massive use in Cuba, including some quality control steps (background knowledge, Rodríguez-Hernández, 2015). The document with the protocol modifications produced by Sánchez *et al*. (2006) is deposited in the Cuban Copyright Center (Centro Nacional de Derecho de Autor, Cuba, n. 09613-2002) and represents the reference for production of EPN in cottage laboratories.

Production of late-instar larvae of the greater wax moth

Adults of the greater wax moth are placed in glass or plastic containers. Their eggs are then collected starting 2-3 days after mating. The eggs are then introduced in selected substrates (previously sterilized) with honey. After 10 days, the small wax moth larvae are transferred to a large container with the same substrate and kept more than 25 days. The larvae are then manually separated from the substrate and classified, using the more developed for EPN production (Fig. 4, A-D). The smaller larvae can be introduced in the substrate again.

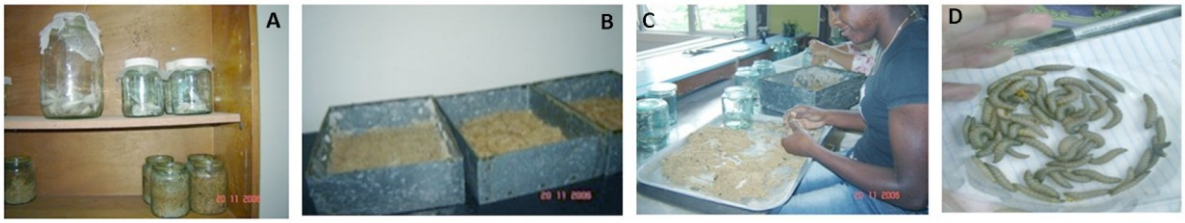

Figure 4. Initial steps in EPN hosts production. Mating of adult moths (A), larvae development in alternative substrates (B), wax moth larvae extraction (C) and collection of healthy late-instars.

Production of entomopathogenic nematodes

A water-nematode suspension is added to to containers with healthy *G. mellonella* larvae (weigh 0.2 g) followed by incubation for 10-12 days at 25-27 ºC. the *G. mellonella* dead body, with characteristic colour at 72 hours after inoculation, indicate that the bacteria has developed the phenotypic phase I and good yields of infective juveniles must be expected. The infective juveniles are then harvested using the White's Trap and a vacuum pump. The suspension is then cleaned of drebris and residues and the juveniles are formulated in nylon bags, using clean sponges (Fig. 5, A-E).

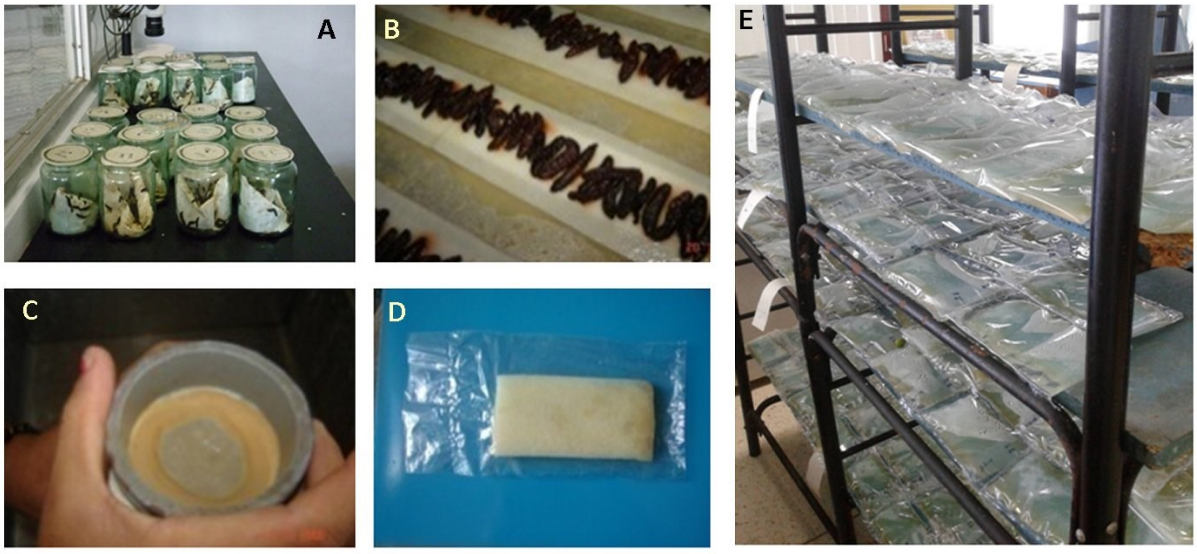

Figure 5. Steps followed in EPN production on *G. mellonella* moth larvae. Incubation of larvae inoculated with EPNs (A). Harvest of nematodes by White's trap (White, 1927), using trays and glass pieces (B). Cleaning and concentration of nematode suspensions using sieves and vacuum (C). Simple formulation of nematodes suspension in distilled water in sponges (D). Storage of bags with EPNs at room conditions (25ºC) for 2.5 months (E).

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

This methodology for mass rearing EPN is used in Cuba in several cottage laboratories (Rodríguez, 2015). However the amounts produced do not cover the national demand. For this reason, it is necessary to develop an *in vitro* mass rearing method for EPN. MUSA has provided the platform to develop studies to approach of objective declared in the project: to obtain an initial low scale (5-10 liters) methodology for *in vitro* liquid culture of EPNs in Cuba, for subsequent scale-up of production.

1.2.2. *In vitro* mass production

In vitro production of EPN demands a higher technology level based on suitable inputs and capital investment. In Cuba some facilities are available to obtain EPN in *in vitro* liquid fermentation technologies (Fernández-Larrea, 2012). CENSA aimed at obtaining some basic elements to improve the methodology of *in vitro* production, increasing the research data needed. Final goal is to develop, on the medium term, a final technology of *in vitro* liquid reproduction of EPN for transfer to LABIOFAM-group industry, that in Cuba produces and commercializes biopesticides (Fernández-Larrea, 2012).

Using the flow chart of EPN production described by Ehlers (2001) as a general guide for work in MUSA, CENSA personnel approached the flask culture phase and studies were performed on the EPN life-cycle, the isolation of EPN-associated bacteria, the characterization of phase I, the HC1 conservation and growth in selected media. Further studies aimed at IJ sanitation and development of nematode-bacteria complexes, in alternative media.

The life-cycle of *Heterorhabditis amazonensis* HC1 in *G. mellonella*

The CENSA team studied the HC1 life-cycle in *G. mellonella* to get baseline information to set up *in vitro* production. Previous studies on the life-cycle of *Heterorhabditis* spp. *in vivo* (on *G. mellonella*) and *in vitro* (in culture media) showed in fact some close similarities (Zioni *et al*., 1992). The life-cycle of HC1 was investigated in the Nematology Lab. at CENSA, following the protocols described by Andaló *et al*. (2009). The *G. mellonella* larvae were obtained in the laboratory using the methodology described before. The experimental unit were Petri dishes (9 mm) with a double layer of filter paper (humid chamber) with one larva per plate, inoculated with 10 IJ each. The Petri plates were incubated for 48 hrs in darkness, at 28°C. At 72 hrs, 10 *G. mellonella* larvae were dissected every 24 hrs under a stereoscope in Ringer solution. The EPN numbers and stages were determined until the nematodes had completed their life-cycle and depleted all nutrients available from the host. The nematodes recovered were fixed using hot TAF for countings and to allocate the specimens in each phase, following the criteria of Wouts (1979) and Zioni *et al*. (1992).

HC1 completed a long life-cycle (Table 1), from the initial IJ population inoculated (10 IJ / *G. mellonella* larva). The nematodes of this genus have long and short life cycles, depending on the number of IJ that enter the insect body (Wouts, 1979).

In the 1st generation, the J4 or IJ occur at 192 hours, but the highest numbers of IJ must be obtained at second generation, that required 288 hours (~12 days), similar to results reached in *in vivo* reproduction. The final 3rd generation cycle lasted 423 hours (~18 days), suggesting

that HC1 has a long life-cycle. The accumulated yields at 12-14 days, were 2.6 - 5.5 · 10^5 IJ per *G. mellonella* larva, with a medium value of 3.6 · 10^5 IJ. These results were higher than those reported by Sánchez (2002), that used a higher inoculum level per *G. mellonella* infected larva (20-40 IJ).

Table 1. Extension of life cycle of each *H. amazonensis* HC1 stage.

Developmental stages	Time (hrs)*		
	1st generation	2nd generation	3rd generation
J4	48-72		
Hermaphrodites	96		
Hermaphrodites + eggs/ J_1	120		
Hermaphrodites +J_2	144		
J_3/J_4	192		
Males and females		216	336
females + eggs (J_1)		240	360
females + eggs and J_2		264	384-408
J_3 - J_4		288	432
J_4		312	

*10 IJ per *G. mellonella* were used as inoculum. Data include the time spent for dissection.

The IJ numbers at the life-cycle completion was linked to the strain characteristics, or the stages used as inoculum. The period of 192 hours for 1rst generation was reported by Muthulakshmi *et al*. (2012) for *H. indica*. For *H. amazonensis* RSC5, Andaló *et al*. (2009) reported that the highest value of J3/J4 was obtained, at the second generation, at 240 hours, using 5 IJ as initial inoculum.

This study indicated that the highest yield of HC1 *in vitro* can last up to 12 or more days. according to some specialists the long period necessary to obtain high yields of EPN in fermenters could represent a critical point to discuss with industry. As a comparative term, the production of *Bacillus thuringiensis* takes hours (Fernández-Larrea *et al*., 2009).

Due to the characteristic of the symbiotic complex, the bacteria must transform the media before nematodes are introduced in the system. For *in vitro* mass production, the bacteria must be first introduced in the media (i.e. in plates, Erlenmeyer's flasks or fermenters), followed by the nematode introduction. For these reasons the symbiotic bacteria had to be isolated, characterized and reproduced before being used for HC1 *in vitro* mass rearing.

Production of the bacterial inoculum

Two methods, for isolation of the HC1 endosymbiotic bacterium were evaluated on:

1) IJ = the nematodes were obtained from *G. mellonella* using White traps, modified (Sanchez *et al*., 2001). At least 10^3 IJ were concentrated in 1 ml of water in Eppendorfs and surface sterilized using Na hypochlorite (0.1%), then washed two times in sterile distilled water. Working in a flow cabinet, a drop with the IJ were placed in plates with Nutrient Agar (NA) and MacConkey Agar (MA).

Some phase I colonies of *Photorhabdus luminescens* (symbiotic in HC1) were obtained in both media, However presence of contaminants, likely present in the IJ cuticle required a longer time for washing and decanting.

2) Dead inoculated *G. mellonella* = larvae were incubated at room temperature during 24-48 hours. The larvae showing the characteristic color suggesting they reached the phase I, (Sánchez 2002; Rodríguez, 2015) were selected for bacteria extraction. In flow cabinet, the *G. mellonella* cadavers were disinfected with ethanol (90%) followed by a brief heating (ignition). The cadavers were then opened using a sterile needle in their ventral area, near the head. The hemolymph was taken with the needle and used for producing bacterial colonies in MA media, incubated for 24-48 hours.

The use of ethanol and brief ignition of the dead body was satisfactory, yielding near 80% of pure colonies in NA and MA. On this last medium the neutral red stain (Fig. 6) was indicative of the Phase I development.

This second method resulted easier than others using *G. mellonella* cadaver hemolymph, to isolate the bacteria in phase I (Yoo *et al*., 2000; Singh *et al*., 2012). It yielded pure colonies of *P. luminescens* from HC1 and was used in the following tasks.

Summary of the method. Dead inoculated *G. mellonella* larvae are incubated at room temperature during 24-48 hours, disinfected with ethanol (90 %) with a brief ignition. The dead bodies are flamed, open with a sterile needle in their ventral area near the head. The haemolymph is then taken with a needle and is used for producing bacterial colonies in McConkey agar media, incubated for 24-48 hours. On this medium the neutral red stain (Fig. 6) is indicative of the Phase I development.

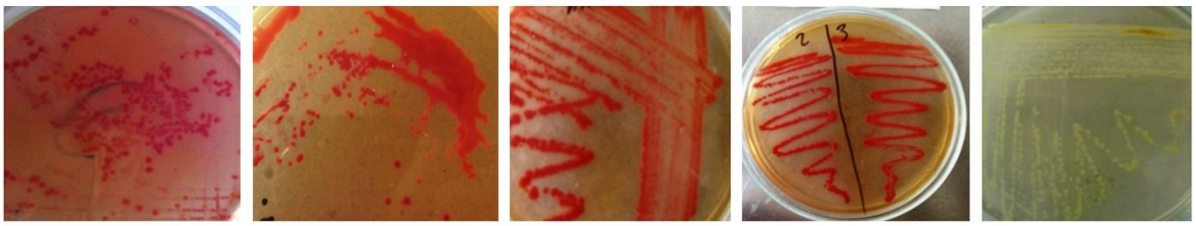

Figure 6. Colonies of *Photorhabdus luminescen*s growing on McConkey (red) and nutrient agar (irregular borders and yellow color).

Phenotypic studies of *Photorhabdus luminescens* strain HC1

Species from *Photorhabdus* genera have been associated with *Heterorhabditis* species. This work aimed at characterizing the symbiotic bacteria of strain HC1 in different culture media.

The method described before was used for bacterium isolation from cadavers hemolymph inoculated in MacConkey (incubated 24 - 48 hours, 28°C). Other six media were used.

No	media
1	NA
2	Potato Agar (300 g/L) + NaCl, K_2HPO_4, pH 7.3 ± 0.2
3	Soybean tryptone agar, pH 7.3 ± 0,2
4	Blood Agar
5	Tryptase media, soybean peptone, glucose, NaCl, K_2HPO_4, pH 7.3 ± 0.2
6	Tryptase media, Yeast extract, pH 7.3 ± 0.2

The colonies morphology was observed and their consistence checked. Their bioluminescence was observed in 48 hours cultures, during 10 min in a dark room. The growth curves in media N. 2 and 5 were produced in 200 ml Erlenmeyers inoculated with

10% of final vol. and incubated (10 hrs, 28°C, 90 rpm) in an orbital shaker. Samples were taken for 8 hrs, at intervals of 2 hrs and readings of the culture optical density (OD) were made at 600 nm, using an UV/Vis spectrophotometer.

Biochemical characterization

The API 20 E (Enterobacteria) identification system was used. One colony of *P. luminescens*, grown in MacConkey agar during 48 hrs, was re-suspended in 5 ml of NaCl (0.85%) and drops were laid in the API system, incubated at 28°C and read at 24 and 48 hrs. The bacteria were inoculated in sheep blood agar (10%) at 25°C to evaluate their hemolytic activity (Boemare *et al.* 1997). Catalase activity was determined adding drops of H_2O_2 (30%) on a glass slide with 24-hour old colonies.

The bacteria appear as short bacilli with rod-shaped extremes, and a Gram negative reaction (Fig. 7). The colonies do not dissolve in water. In API 20E test, they produced positive reactions to: glucose, gelatin and urease, and were negative to indole.

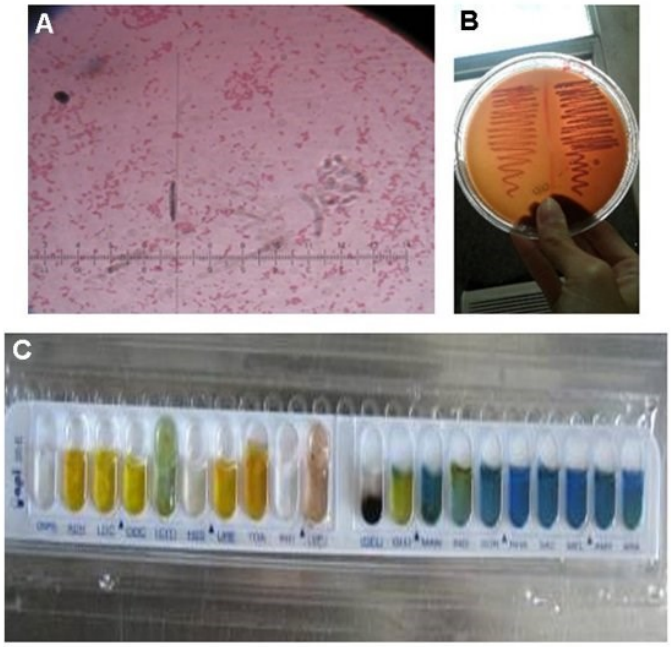

Figure 7. Morphology and color of *P. luminescens* in Gram test (A). Red colonies growing in MacConkey agar (B). Example of API test results of *P. luminescens* HC1 strain (C).

The isolate obtained from HC1 is catalase positive and produce hemolysis in sheep blood agar. Martin (2007) proposed that symbiotic bacteria of HC1 belong to *P. luminescens* species, but its identification until sub-species requires molecular tools.

The growth kinetic in two media showed that the adaptation phase (*lag*) requires 4 hrs, in which the bacterium starts to adapt to the growth conditions. Later on it starts the exponential phase, increasing the bacterial cell yield (expressed in optical densities, Fig. 8). In both media, the behavior was similar, with high values in medium N. 5. The pH in both media increased up to 7.8 in the growing phase, suggesting the production of secondary metabolites, an aspect that will be studied in future assays. The growth rate in media N. 5 was higher.

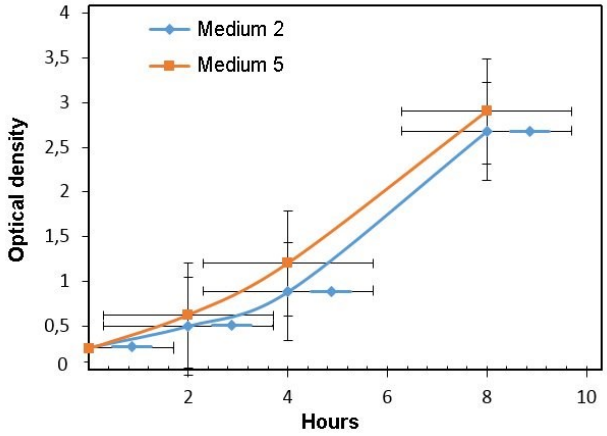

Figure 8. Growth curves of *Photorhabdus luminescens* in two media until 8 hours.

***In vitro* mass rearing of *H. amazonensis* HC1**

Standard for conservation of *P. luminescens* HC1 strain stock cultures

Best method for preservation of of *P. luminescens* from HC1 was investigated using two media and two temperatures, to keep the symbiotic phase I bacteria in good conditions as stock cultures. For this purpose, Nutrient broth with a 24 hrs *P. luminescens* colony was used, centrifugated (1000 rpm, 10 min), washing the pellet with saline solution folowed by centrifugation again. The biomass was re-suspended in two growth media with skim milk:

- skim milk (10%), yeast 0.5% and glucose 1%
- skim milk (10%), yeast 0.5% and glucose 1% + Nutrient broth (v/v)

For conservation the colonies were stored at -20 and -18 °C. The viability of *P. luminescens* was evaluated by growth in MacConkey media at 7, 15 and 30 days storage, expressed as negative or positive growth, by observation of colonies. All sample stored 7 and 15 days were positive, with colonies normally growing in the media. However, after 30 days of storage the bacterium did not grow. Lyophilization (when available) and new methods (changing media and temperatures) are under evaluation.

Sterilization of infective juveniles

The nematodes must be sterilized before their introduction in the culture medium for *in vitro* reproduction. Two reagents have been evaluated: thimerosal and sodium hypochlorite (0.1 and 0.6 %). For *in vitro* culture of *Heterorhabditis*, eggs from females (Gil *et al*., 2002) of surface sanitized juveniles (Surrey and Davies, 1996) have been used. The IJ of HC1 were produced on *G. mellonella* (as described) and collected using a White Trap modified (Sánchez, 2002). In a 100 ml beaker the IJ were rinsed three times with sterile distilled water and the suspension was passed through a 20 µm sieve. The IJ concentrated in the third rinse were divided in three groups, using a laboratory spoon, and each group was placed in one assay tube. When the nematodes decanted at the bottom of each tube, three treatments were performed: thimerosal (0.2%) and Na hypochlorite (0.1 and 6%) for 1 hr. Then each IJ group was rinsed several times with sterile distilled water. A few drops from each treatment were placed in a slide and the IJ viability was measured under a microscope. The IJ alive from the treatments were laid in MacConkey and Nutrient Agar and incubated at room temperature for 48 hrs. The IJ treated with thimerosal (0.2%) and Na hypochlorite (6%) did not survive and showed several body disruptions and deformations. In the plates laid with IJ treated with

Na hypochlorite (6%), only the symbiotic bacterium grew, and was selected for sanitation IJ for *in vitro* mass rearing. The best results were obtained using Na hypochlorite at 0.1 % concentration.Other substances were also tested for sanitation of IJ such as formalin (0.1%) (Surrey and Davies, 1996) and benzethonium chloride (0.1%) (Yoo *et al*., 2000).

In vitro EPNs production

According to Ehlers (1996), the liquid culture has obvious advantages because with increasing bioreactor scales, the proportion of labour and capital decrease although the operating costs increase. Operating costs include the expense of culture medium (Gaugler and Han, 2002). This is hence one of the most promising targets for research and development efforts concerning production costs. For this reason, several media have been developed using components such as animal liver or kidneys, vegetable oils, milk, yeast, chicken offal and egg yolk (Surrey and Davies, 1996; Wang and Bedding, 1998), or other alternative components (Islas-López *et al*., 2005; San-Blas *et al*., 2015).

Using the composition of solid culture media described by Sánchez *et al*. (2006, Patent OCPI 882/2006) as a reference, ten further media were evaluated as liquid substrates (including co-product from animal industries and botanical products), in three trials, using 150 ml Erlenmeyer flasks on orbital shakers. Six media did not yield juveniles. One medium was selected, composed by animal and vegetal co-products, that yielded 16896 IJ/ml (Fig. 9). The yields was still lower, however, compared to those reported by other authors.

The IJ from *G. mellonella* were reproduced as described before. The IJ were sanitized with Na hypochlorite (0.1%, 1 hr) and rinsed three times with sterilized distilled water.
Media composition: three trials were performed. The substrates used in the 2nd and 3rd trials are described in Table 2. Erlenmeyers with a 150 ml capacity were used. The bacteria were inoculated in the media (50 ml of nutrient broth), incubated 72 hrs in orbital shaker (24ºC). At 72 hours, the nematode inoculum (1000 IJ / ml) was added in the Erlenmeyers that were kept in an orbital shaker until final evaluation (Fig. 9). Nine sub-samples of one ml each were taken at 5 and 12 days and placed in a counting plate, and the stages of live and dead specimens were counted with an optical microscope, and pictures were taken.

Table 2. General composition of substrates evaluated for bacterium-nematode production.

Trial	Medium	Media composition
2	1	Sweet potato + yeats + cholesterol + salts
2	2	Soybean triptone
2	3	Animal protein (co-products from fishering industry) + yeast + cholesterol + salts
2	4	Lactose + yeast + peptone + glucose
2		*G. mellonella* maceraste (Control)
2	5	Potato + cholesterol + saltes
2	6	Potato + animal protein (co-products from fish industry) + salts and + vegetable oil
2	7	Buttermilk + honeybee + salts + vegetable oil + NaCl
3	9	Potato + animal protein (co-products from fish industry) + salts and + vegetable oil
3	10	Buttermilk + honeybee + salts + vegetable oil

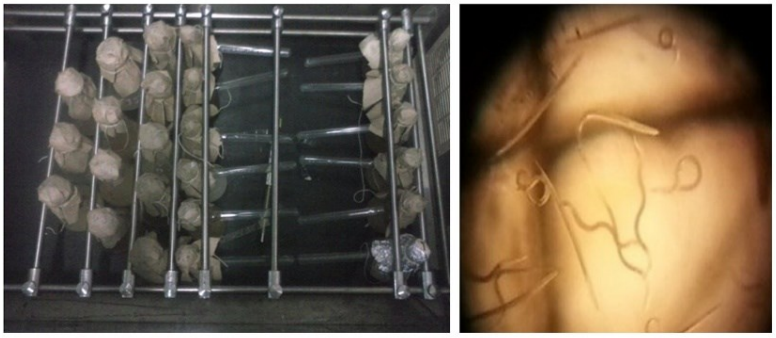

Figure 9. EPN production *in vitro*.

2. EBCAs formulations and storage methods

MSBIO

Three products have been developed in the Microbiological Lab. of partner MSBIO:

- a formulation based on *Beauveria bassiana*, with insecticidal activity;
- a formulation based on *P. chlamydosporia*, with nematocidal activity;
- a formulation based on different *Bacillus* spp. isolates, with fungicidal activity.

For the shelf-life study, different formulations were evaluated:

B. bassiana

Treatment / Oil		%	Emulsifier (10 %)
A-Em1	A	100	Em1
A-Em2			Em2
B-Em1	B	100	Em1
B-Em2			Em2
C-Em1	C	100	Em1
C-Em2			Em2
AB-Em1	AB	50+50	Em1
AB-Em2			Em2
AC-Em1	AC	50+50	Em1
AC-Em2			Em2
BC-Em1	BC	50+50	Em1
BC-Em2			Em2

P. chlamydosporia

Treatment / Oil		%	Emulsifier (10 %)
A-Em1	A	100	Em1
A-Em2			Em2
B-Em1	B	100	Em1
B-Em2			Em2
C-Em1	C	100	Em1
C-Em2			Em2
AB-Em1	AB	50+50	Em1
AB-Em2			Em2
AC-Em1	AC	50+50	Em1
AC-Em2			Em2
BC-Em1	BC	50+50	Em1
BC-Em2			Em2

Bacillus sp.

Treatment	Plant Extract	Molasses (%)
EV	100	0
M	0	100
M-EV	10	90

For formulations based on *B. bassiana* and *P. chlamydosporia*, formulations based on vegetable oil and emulsifier were considered. Three different vegetable oils have been tested, singly or mixed together (Oil A 100%, Oil B 100%, Oil C 100%, Oil A 50% + Oil B 50%, Oil A 50% + Oil C 50%, Oil B 50% + Oil C 50%), with 2 emulsifiers (Em1, Em2) at 10%. For all prototypes hypothesised, the shelf-life at 3 different temperatures (4, 16 and 25 °C), was evaluated.

For the formulations based on *Bacillus* spp., various combinations based on molasses and plant extracts were considered, in various ratios: 100% molasses, 90% molasses + 10% vegetable extracts, 100% plant extracts. For all of the prototypes hypothesised, the shelf-life at 3 different temperatures (4, 16 and 25 °C) was evaluated. The best prototype additives are shown in Fig. 10 (see also Deliverable D5.2).

The *B. bassiana* and *P. chlamydosporia* isolates after six months maintained the initial concentration in Oil A with Emulsifier 1 at 10%, at each temperature considered. Also *Bacillus* sp. kept the initial concentration values constant, over the time considered, in the formulation with molasses and plant extract.

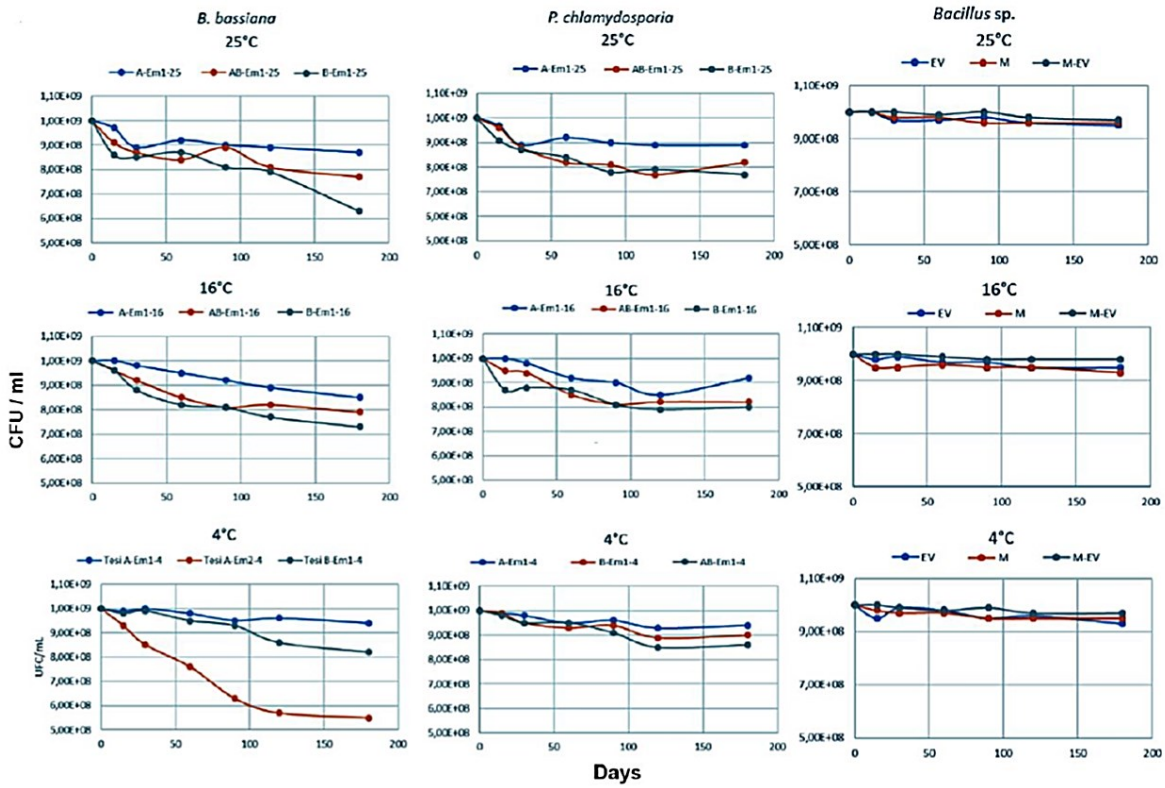

Figure 10. Shelf-life of *Beauveria bassiana*, *Pochonia chlamydosporia* and *Bacillus* sp., at different temperaures, in presence of oils and emulsifiers (*B. bassiana*, *P. chlamydosporia*), or additives (*Bacillus* sp.).

2.1. Fungi

CENSA

Since ’90s CENSA developed BCAs in more than 100 cottage laboratories disseminated all over the country. There is a need to improve the process in the phases of recovery and formulation of BCAs, in cottage and industrial productions (Fernández-Larrea, 2012).
KlamiC is a bionematicide formulated with *P. chlamydosporia*, a facultative parasite of nematode eggs (mainly root knot nematodes), which colonizes the soil and plant roots gradually, reducing nematode populations below the critical level. It is used in organic and conventional production systems. The fungus is also a root endophyte and a plant growth promoter. *Active ingredient*: chlamydospores of *P. chlamydosporia var. catenulata* IMI SD 187. The product also has the following specifications for quality control: humidity: < 8 %; shelf life: 3 months. Formulation: colonized substrate in tightly sealed nylon bags.

SevetriC is a fungicide formulated with *Trichoderma asperellum*, a fungal antagonist of many phytopathogens. It is used in organic and conventional crop production systems. *Active ingredient*: spores of *T. asperellum* Ta. 13. The product also has the following specifications

for quality control: humidity: < 10-15%. Microbial limit: < 10^5 CFU; shelf life: 6 months. *Formulation*: colonized substrate in tightly sealed nylon bags.

In MUSA, the effect of five tensioactives as candidates for fungal formulation and the solubilization of essential oils (with a biopesticide action) were evaluated on *in vitro* growth of *P. chlamydosporia* and *T. asperellum* were evaluated by CENSA. The tensioactives tested were: Tween-80, Tween-20, DMSO, TritonX-100 and D-octil, from Chemical Ecology Laboratory at National Centre for Animal and Plant Health (CENSA). They were added independently to the fungi culture media (Clark *et al*. 1982).

2.1.1. *Pochonia chlamydosporia*

Two concentrations (1.5 and 3 %) were evaluated for each tensioactive, using the natural medium Potato agar-50 (natural potato: 50 g L^{-1}; Bacteriological agar, BioCen: 15 g L^{-1}). Each treatment was prepared in an Erlenmeyer flask with 100 mL of culture medium autoclaved (121ºC for 15 min). The tensioactives were added to the fresh, sterilized medium into sterile Petri dishes. Each tensioactive was considered a treatment with five replicates using Potato agar-50 alone as control. Each dish was inoculated using a 5 mm diam. disc proceeding from a 15-day colony of *P. chlamydosporia* var. *catenulata* IMI SD 187 (a.i. of KlamiC® bionematicide). The dishes were incubated in dark at 25 ± 1ºC during 13 days.

The diameter of the colonies micelial growth was measured and chlamydospore production was quantified in a Neubauer chamber. The chlamydospore suspensions were obtained by adding 5 mL of sterile distilled water to each dishes, then sweeping the colony with a blazed Drigalsky rod. The chlamydospore sporulation on each colony was calculated and the percent of inhibition was estimated, in relation to control. Finally, the compatibility of the tensioactive with the fungus was calculated through toxicity, using the formula by Alves *et al*. (1998):

$$T = [20\ (CV) + 80\ (ESP)] / 100$$

where: T = toxicity; CV = mycelium growth in relation to control (%); ESP = chlamydospore production in relation to control (%). The tensioactives were classified according to the next limits: 0 – 30 = highly toxic; 31 – 45 = toxic; 46 – 60 = moderately toxic; > 60 compatible.

Differences in cultural characteristics of *P. chlamydosporia* IMI SD 187 were observed in colonies grown on media with different tensioactives (Fig. 11). The treatment with D-octil showed no fungal growth at all concentrations evaluated.

The calculated toxicity showed that DMSO was very toxic for the fungus at the two concentrations applied. Even when no effect on growth was observed, the sporulation was significant lower. The compatibility could not be assessed on Triton-X 100 (3%) because the medium did not solidify. D-octil was the highest toxic substance, as when it was added no fungal growth was observed. The other tensioactives were compatible with IMI SD 187. It is recommended using tensioactives and concentrations promoting the sporulation without significantly affecting the vegetative growth, i.e. Tween-20 (1.5 %) and Tween 80 (3 %).

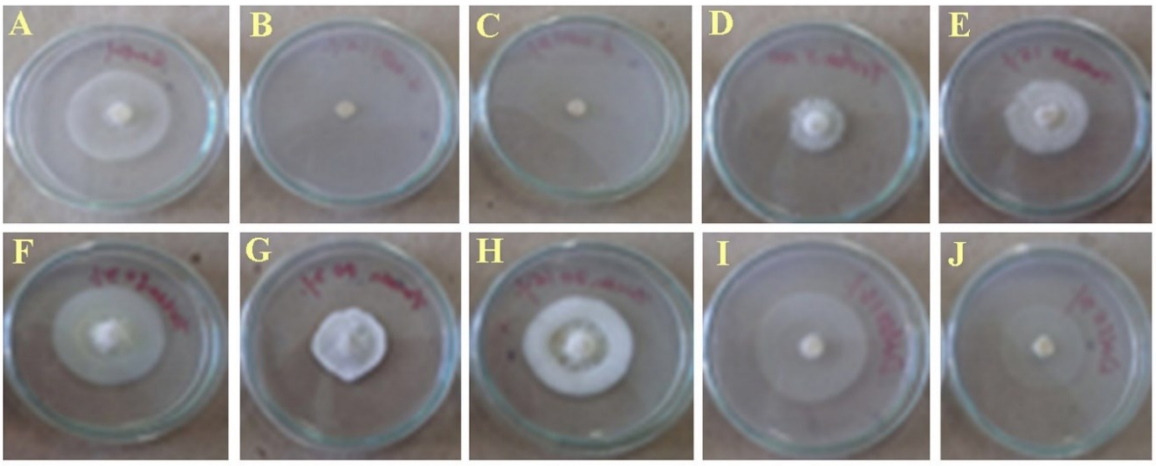

Figure 11. Colonies of *P. chlamydosporia* var. *catenulata* (IMI SD 187) on Potato agar-50 medium with different tensioactives applied. A: Control; B: D-octil (1.5%); C: D-octil (3.0%); D: Triton X-100 (1.5%); E: Tween-80 (1.5%); F: Tween-80 (3.0%); G: Tween-20 (1.5%); H: Tween-20 (3.0%); I: DMSO (1.5%); J: DMSO (3.0%)

The colony diameter of IMI SD 187 was more affected by the highest concentration (3%) of all tensoactives, except Tween-80, when the greatest colony growth was observed at the higher concentration (Fig. 12).

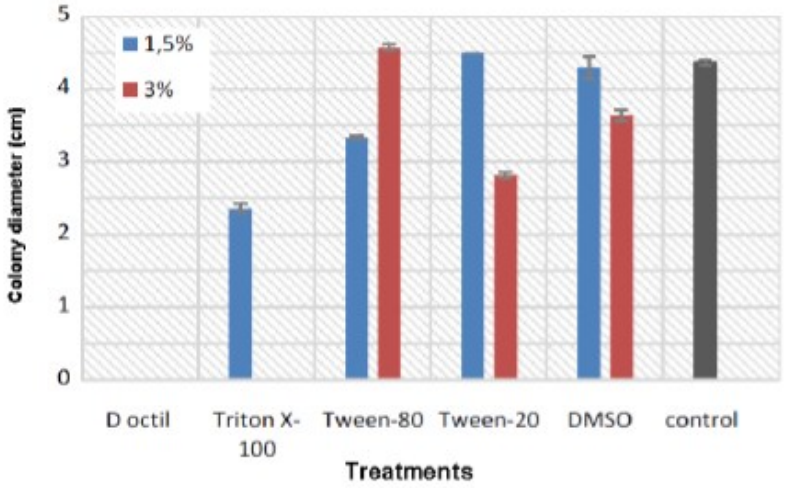

Figure 12. Colony diameters of *P. chlamydosporia* var. *catenulata* IMI SD 187 on Potato Agar-50 medium, with different tensoactives.

The chlamydospores produced by the fungus in all media with Tween-20 and Tween-80 (at two evaluated concentrations), were significantly higher than control (2.2 - 3.4 · 10^5 chlamydospores/colony) (Fig. 13). The sporulation of the fungus on the media with Triton X-100 (1.5%) did not differ from the control treatment and reached 1.6 · 10^5 and 1.5 · 10^5 chlamidospores/colony, respectively. The sporulation of the fungus on DMSO was significantly lower, with 8.8 · 10^3 and 3.8 · 10^3 chlamidospores/colony, at 1.5 and 3%, respectively.

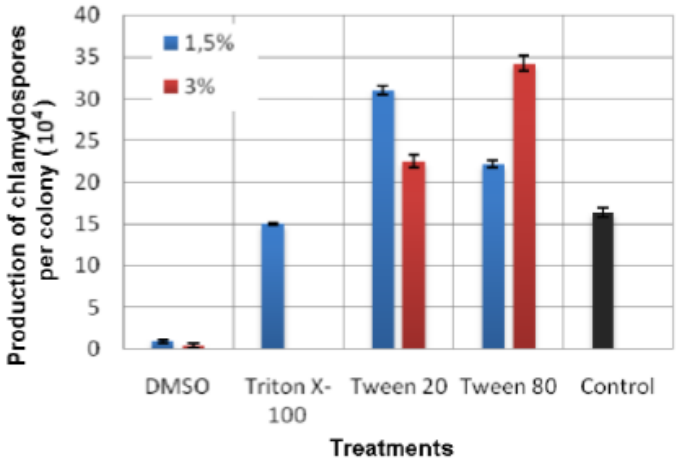

Figure 13. Chlamydospores production of *P. chlamydosporia* var. *catenulata* IMI SD 187 on Potato Agar-50 medium with different tensoactives.

The toxicity test indicated that DMSO at the two evaluated concentrations was very toxic to the fungus, even though on the fungal growth was not affected, the sporulation was significant lower (Table 3). Compatibility with Triton-X 100 (3%) could not be determined because the medium did not solidified. D-octil was the highest toxic substance: no fungal growth was observed when this substance was added to the media. The other tensoactive substances were compatible with IMI SD 187. However, those tensoactive substances and concentrations that promoted the sporulation without affecting the vegetative growth significantly are recommended, for example Tween-20 [1.5%] and Tween 80 [3%].

Table 3. Results of the compatibility assays for tensioactive substances with *P. chlamydosporia* var. *catenulata* IMI SD 187 on Potato agar-50 medium.

Treatment	Vegetative growth (%)	Sporulation (%)	Toxicity (T)	Campatibility
Control	100.00	100.00	-	-
DMSO (1.5%)	98.40	5.32	23.93	Highly toxic
DMSO (3.0%)	83.30	2.28	18.48	Highly toxic
Tween 20 (1.5%)	102.97	188.35	171.28	Compatible
Tween 20 (3.0%)	64.53	136.71	122.27	Compatible
Tween 80 (1.5%)	76.43	134.89	123.19	Compatible
Tween 80 (3.0%)	104.81	208.10	187.44	Compatible
Triton-X (1.5%)	54.00	91.59	84.08	Compatible
Triton-X (3.0%)	Medium not solid	-	-	-
D-octil (1.5%)	No growth	-	0.00	Highly toxic
D-octil (3.0%)	No growth	-	0.00	Highly toxic

Characterization of *P. chlamydosporia* spore powder and candidate clays as excipients for pre-formulation

To determine the physicochemical characteristics of spore dust and clay particles from *P. chlamydosporia* var. *catenulata* formulation, a number of analytical assays were performed during WP5 on the bioformulations produced, in view of their suitability for field

applications. 200 g of spores were sampled from three production batches of IMI SD 187, with a particle size less than 300 μm, using a vibrating sieve. The physical properties established for wettable powders (WP) were determined, according to the Manual on development and use from FAO and WHO specifications for pesticides (2004). These properties include: appearance, density, fluidity, wettability, humidity, pH, porosity index, persistence of the foam and granulometry. The methods described in USP 35:2012 were used.

Characteristics of the spore powder (SP) of *P. chlamydosporia* IMI SD 187 were:

- Moderately fine particle size (<300 μm)
- Maintains coloration (ocher: 5 YR 5/8)
- Does not form lumps.
- It has good fluidity: the flow rate of 6.34 g / cm^2s^{-1} (≥ 7g / cm^2s^{-1} indicate good fluidity) and the angle of repose was 29.57 (< 300 = excellent flows).
- The Carr index indicated a compressibility of 15.73% (11-15 = good), which can be used in packaging or to be included in a system that requires external actions for grouping.
- The pH behaved in the stable range for the fungus (5-7).
- The SP particles have an irregular surface, only 19.19% correspond to a smooth surface. This is an important value, if a formulation involving the complete coating of particles such as granules, is developed.
- SP showed limitations in wettability (does not wet) and persistence of foam.

The main limitations of the SP were wettability and foam persistence. The SP does not get wet, which is an element to consider if the product has to be incorporated in water for application. Furthermore, SP remains on the water surface, because it is less dense than water, which is a problem with dispersion in this medium. For a liquid formulation in water, it is necessary to use tensioactive agents, to achieve a homogeneous suspension of the particles in the medium.

The clays have densities greater or equal than water, which indicates that they sediment at the bottom of the system. In addition, the settlement density and the real density is greater than water, which allows a rapid wettability (Fig. 14).

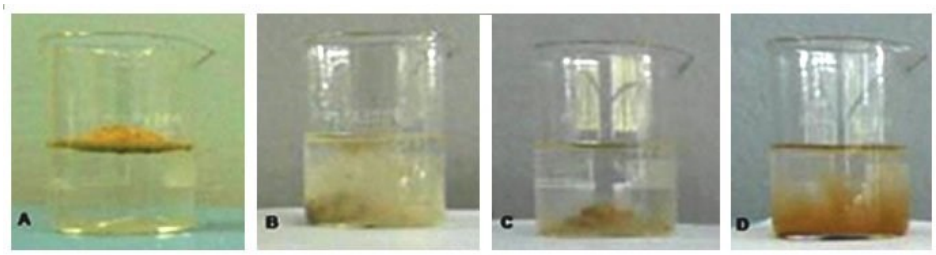

Figure 14. Wettability *for P. chlamydosporia* spore powder (SP) and clays: SP (A), Clays 1, 2 and 3 (B, C and D).

Regarding humidity, Clay 3 absorbed little water. However, Clays 1 and 2, due to their absorbent characteristics, retained high levels of water. This characteristic is convenient to avoid contamination and degradation of the active ingredient (AI). In formulations, humidity is one of most important parameters for stability and quality of the product. It is necessary a balance between humidity to keep the AI without interference by contamints. In case of

formulating with SP, the spore most remain in its state, so it is essential to eliminate water from its environment. The evaluated clays can be used for this purpose.

SP foams at 2 cm height was persistent, which induces problems in stirring when the vehicle is aqueous. Clays do not form foam, so their incorporation into a system that requires stirring is convenient (Fig. 15).

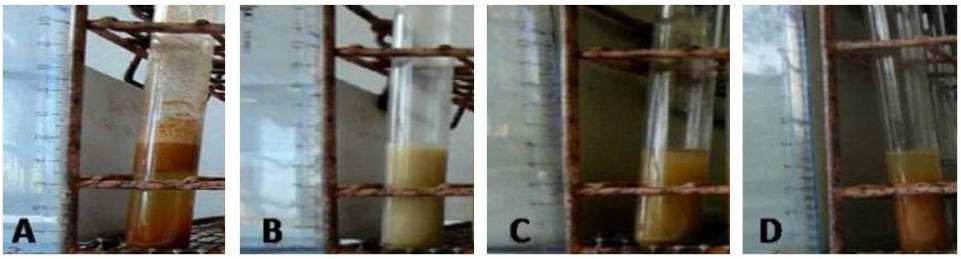

Figure 15. *P. chlamydosporia* SP produce foam, but clays do not. SP (A), Clays 1, 2 and 3 (B-D).

In vitro compatibility of *P. chlamydosporia* with clays

The *in vitro* compatibility of *P. chlamydosporia* var. *catenulata* IMI SD 187 and the three characterized clays was evaluated by two methods. In a first test, the clays were incorporated into the Potato Dextrose Agar (PDA, BioCen, Cuba) culture medium in Petri dishes. The clays were sterilized in an autoclave (121ºC, 30 min.) and incorporated into the sterile medium, in five replicates. A disc of the fungus proceeding from the edge of a pure colony (21 days old, kept at 25ºC), was inoculated in the center of each plate. Subsequently, the plates were incubated for 21 days (25 ± 1ºC, dark).

Radial growth was measured in each colony at 21 days. After incubation, the sporulation in the clays was determined in each colony. The number of chlamydospores per colony was determined in a Neubauer chamber with a Zeiss optical microscope (20×). Compatibility was calculated using the formula proposed by Alves (1998):

$$T = \frac{20\,(CV)+(ESP)}{100}$$

where: T = corrected value of vegetative growth and sporulation for product classification. CV = % of vegetative growth in relation to control. ESP = % sporulation in relation to control. The clays were classified according to the scale: 0 - 30 = very toxic, 31 - 45 = toxic, 46 - 60 = moderately toxic, > 60 compatible.

In a second trial, the Soil Receptivity method (Monfort *et al*., 2006) was used, with modifications, to evaluated the ability of the product to colonize different substrates. The clays were used as substrate and PDA media was used as control. Each treatment consisted of five Petri dishes, with 20 g clay per plate. The clays, once distributed on the sterile plates, were moistened, avoiding saturation, with sterile water. A sterile 45 µm cellulose acetate membrane was placed on top of the clays with tweezers, to prevent the formation of bubbles. A disc (Ø = 5 mm), selected from the youngest zone of a 21-day-old pure colony of the fungus on PDA, was placed on the membrane in each plate. The plates were kept in incubators for 15 days (25±1ºC, 100 % R.H., dark). This test was carried out with sterilized and not sterilized clays.

At 15 days the disc was extracted with forceps, without damaging the mycelium. The membrane was rinsed in sterile water to remove substrate debris, placed in non-sterile Petri dishes and stained with 3 ml of lactophenol blue, under a fume hood. They were incubated for 12h at 25ºC. Subsequently, the membranes were rinsed in distilled water. The colony size and diameter were determined and the presence of chlamydospores in the treatments was evaluated by optical microscopy (10×).

The growth of IMI SD 187 on clays showed differences. The highest growth was found in the PDA control medium and, among clays, the number 3 had the best values. The highest percent of sporulation was obtained in the clays. IMI SD 187 strain is able to take and use some elements present in clays for their own growth (Fig. 16).

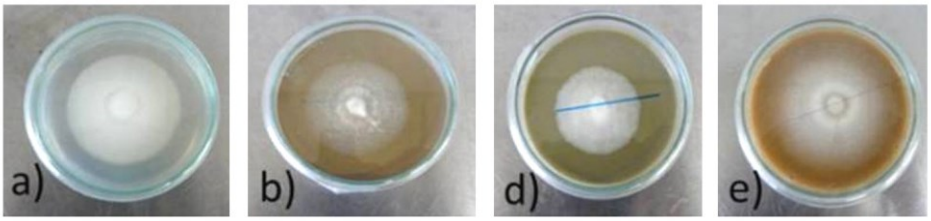

Figure 16. *P. chlamydosporia* IMI SD 187 strain mycelia growth at 21 days in control PDA media (a), clays 1, 2 and 3 (b, d, e).

The three evaluated clays were compatible with IMI SD 187, so they could be used as fillers or excipients in future formulations (Table 4).

Table 4. Compatibility assay between *P. chlamydosporia* and clays**.**

Treatment	Growth (%)	Sporulation (%)	Compatibility (%)	Result
Control	100	100	-	-
Clay 1	86.8	363.69	139.178	Compatible
Clay 2	90.28	225	117.224	Compatible
Clay 3	91.67	105.88	94.512	Compatible

Fungal growth was observed in all treatments, although it was significantly higher in the sterile treatment. The clays did not cause a toxic effect on the fungus, indicating that they can be used as excipients in formulations. The colony diameter in clay 3 was higher than the other treatments and lower than the control (Fig. 17).

The use of *P. chlamydosporia* spore powder, as an active ingredient, in formulations is recommended to improve its density, wettability, viability and stability. Results suggest the use of the three clays as vehicles for *P. chlamydosporia* formulations.

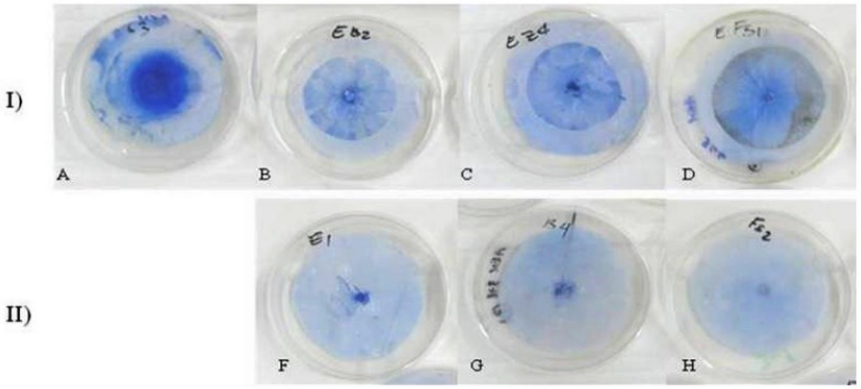

Figure 17. *P. chlamydosporia* IMI SD 187 strain mycelia growth at 15 days on cellulose acetate membrane: (I) sterile substrates: A PDA control, B = clay 1, C = clay 2, D = clay 3. (II) Non-sterile substrates: F = clay 1, G = clay 2, H = clay 3.

Evaluation of *P. chlamydosporia* pre-formulation powders

The stability of 9 pre-formulations in wettable powder form was evaluated, using as fillers 2 clays (1 and 2, from previous trials). Although clay 3 offered the best results in the previous experiment, clays 1 and 2 were selected for further studies due to their higher availability and good results in compatibility tests. The active ingredient (AI) used was the spore powder (SP) of *P. chlamydosporia* var. *catenulata* from the product KlamiC, obtained by separation in a Mycoharvester (Mod. MH-1), with a particle size < 300 µm.

The clays were previously sieved to homogenize the particles size of formulations (< 300 µm), as well as the SP used, and were sterilized in an oven for 1 hour at 180 ºC, afterwards residual humidity (%) was measured, as for the SP. The components of each formulation were then weighed. Formulations were made using different proportions of IA (fungus spores): 10%, 30%, 50%, and a formulation with 100% SP was used as a control. The clays were mixed in the proportions shown in Table 5 and distributed in 1g transparent polyethylene sachets.

Table 5. Components of pre-formulations.

	Components (%)		
N.	AI	Clay 1	Clay 2
1	100	-	-
2	10	3	87
3	10	30	60
4	10	-	90
5	30	10	60
6	30	30	40
7	30	-	70
8	50	15	35
9	50	30	20
10	50	-	50

The pre-formulations obtained in this first phase were not stable over time with less than $1 \cdot 10^6$ CFU $\cdot$ g^{-1} in 3 months. However, it was observed that the formulation that presented the

lowest viability at 90 days was formulation 1 (control) that contained 100% AI. This suggested that the clays used as fillers in the formulations influence the stability of the spore powder (Fig. 18).

The percentage of moisture allowed for current KlamiC formulation is 8% (colonized substrate), with a stability of 3 months. With the formulation we analyzed in MUSA, we aimed at decreasing the AI in the formulation and increasing its viability over time, with the addition to the current formulation of clays used as fillers.

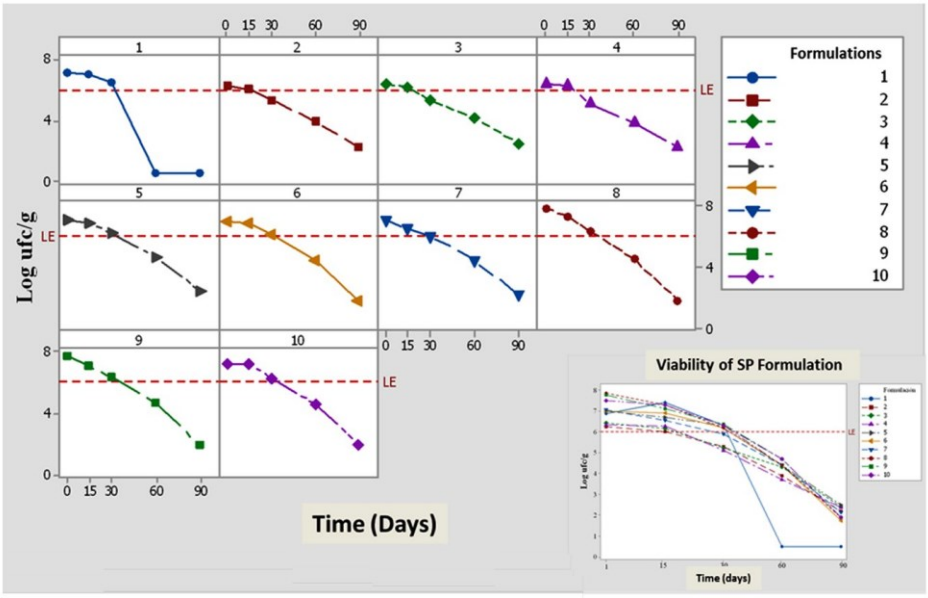

Figure 18. *Pochonia chlamydosporia* (IMI SD 187) spores viability.

The results of these first tests showed that, in the first 15 days, there was a decrease in residual humidity, caused by the absorbent capacity of the clays used as fillers. However, subsequently there was an increase in residual moisture in all pre-formulations (Fig. 19). None of the formulations fill the proposed specification limit for the KlamiC® product (< 8%RH).

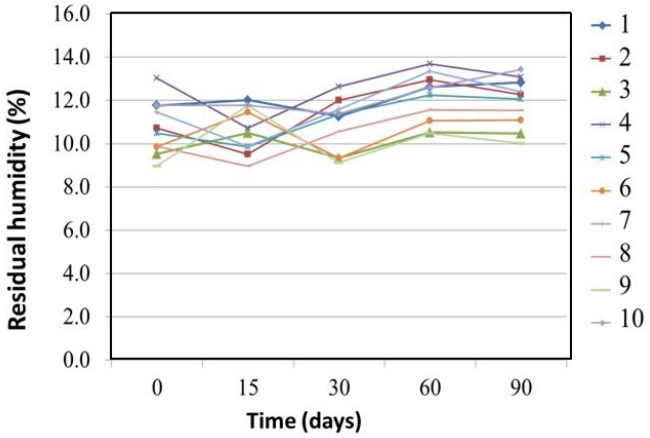

Figure 19. Residual humidity in a time course experiment.

The results obtained after three months were analyzed in the same graph, with the aim of integrating the behavior of the formulations in the proposed parameters. It was observed that the RH ranged between 10-15% while the CFU/ml values were very low with respect to the quality standards of the product (> 10^6 CFU/g). Formulation 3, for this time and at the tested conditions (25ºC), had the highest levels of CFU with the lowest humidity (Fig. 20).

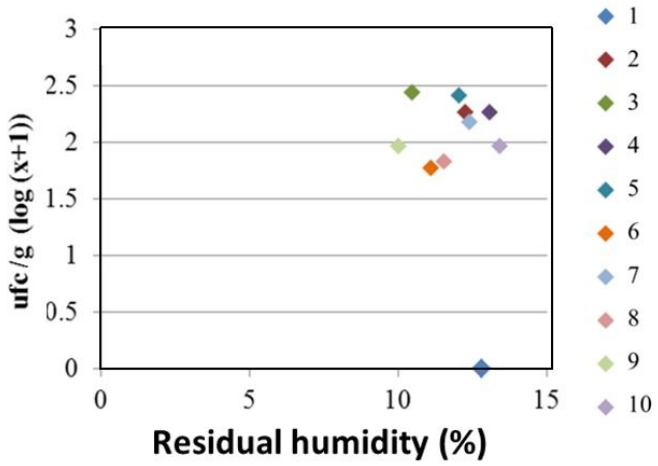

Figure 20. Evaluation of residual humidity and CFU at three months for all pre-formulations.

2.1.2. *Trichoderma asperellum*

The tensioactive added to the culture media as described before at 3% concentration and malt agar were tested on four strains of *T. asperellum* (Ta.13, Ta.78, Ta.85 and Ta.90) in three replicates per tensioactive. Malt agar alone was used as control. Two 6 mm diam. mycelium discs of *T. asperellum* from the edge of a 5 days old colony grown at 28 ± 2 ºC were used for each strain in Petri dishes, at 70 mm of distance on opposite sides, followed by dark incubation at 28 ± 2 ºC.

The colony diameters were measured at 24, 48 and 72 hrs. The radial growth inhibition percentage (PICR) was calculated using the formula of Samaniego *et al*. (1989),

$$PICR=(C-T/C) *100$$

where C = growth of fungal colony in the control (no tensioactive); T = growth of colony in the medium with the tensioactive.

All strains showed different cultural characteristics in the media with different tensioactives, in comparison to the control (Fig. 21 A-E). The culture of *T. asperellum* was not affected by Tween 80. However, in presence of D-octil there was no fungal growth. In DMSO and Triton X-100, the fungal growth was significantly lower, compared to the control. In general, there were significant growth differences among the *T. asperellum* strains in the media with different tensioactives (Fig. 22 A-D). In presence of Tween 80 the growth of all strains was stimulated, and at 48 hrs the colonies covered the whole dish. The results were corroborated by the analysis of proportions, as the strains showed no growth inhibition in presence of Tween 80 (Fig.23 A-D).

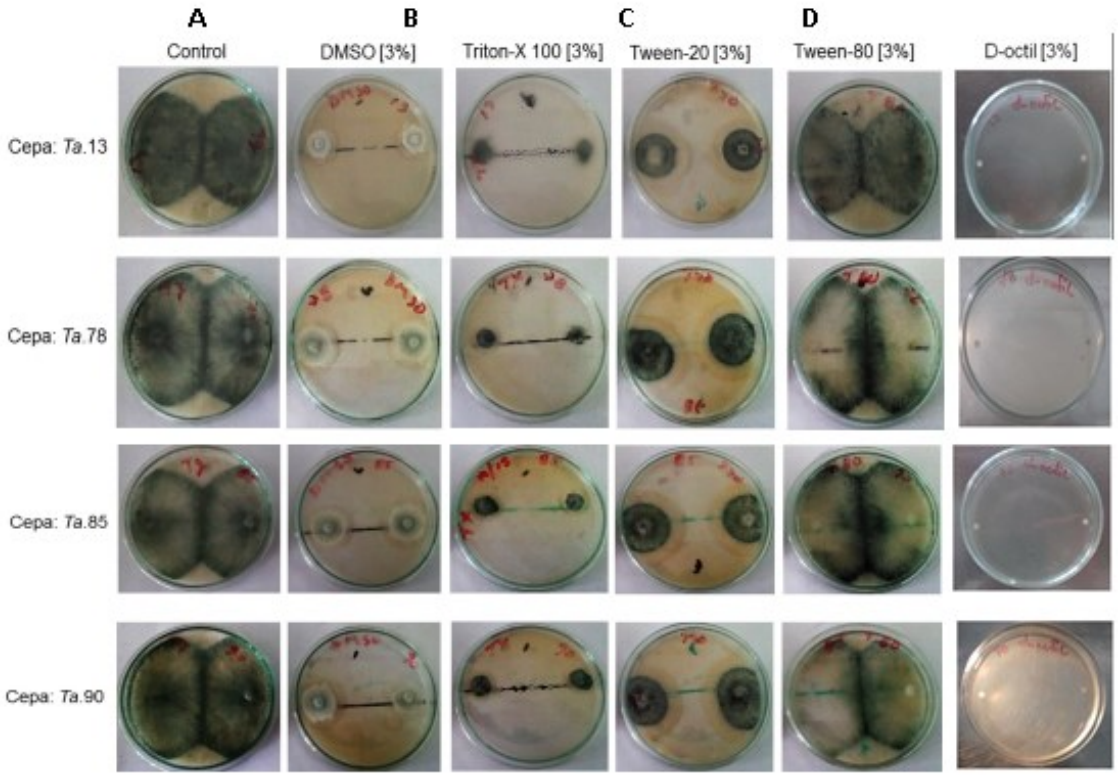

Figure 21. Colonies of *T. asperellum* grown on malt agar with different tensioactives.

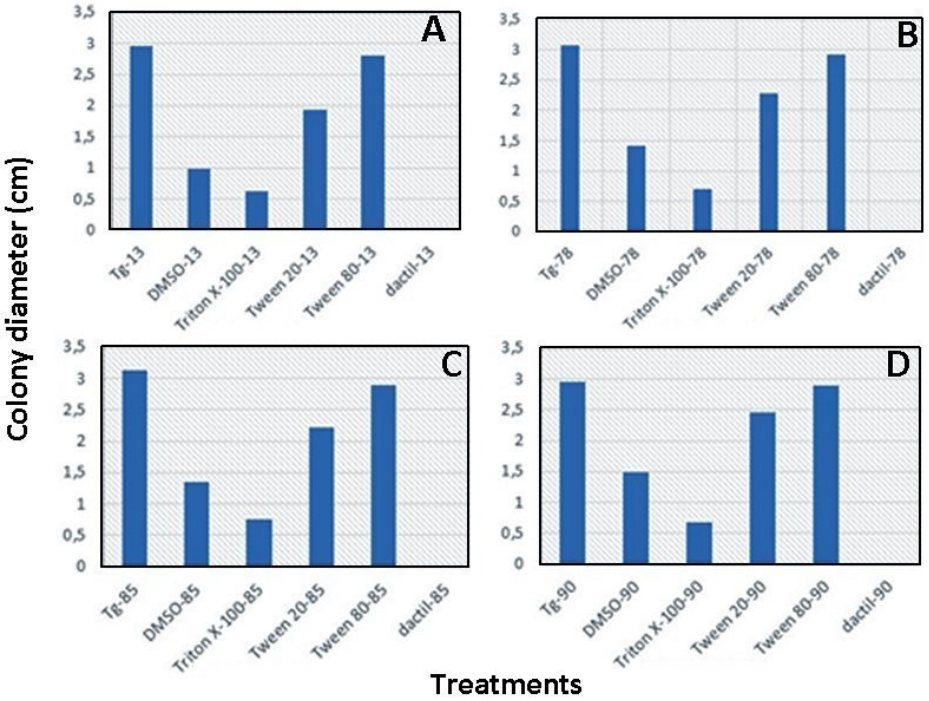

Figure 22. Colony diameter of different *T. asperellum* strains grown on malt agar with different tensioactives (A: Ta.13, B: Ta.78, C: Ta.85, D: Ta.90).

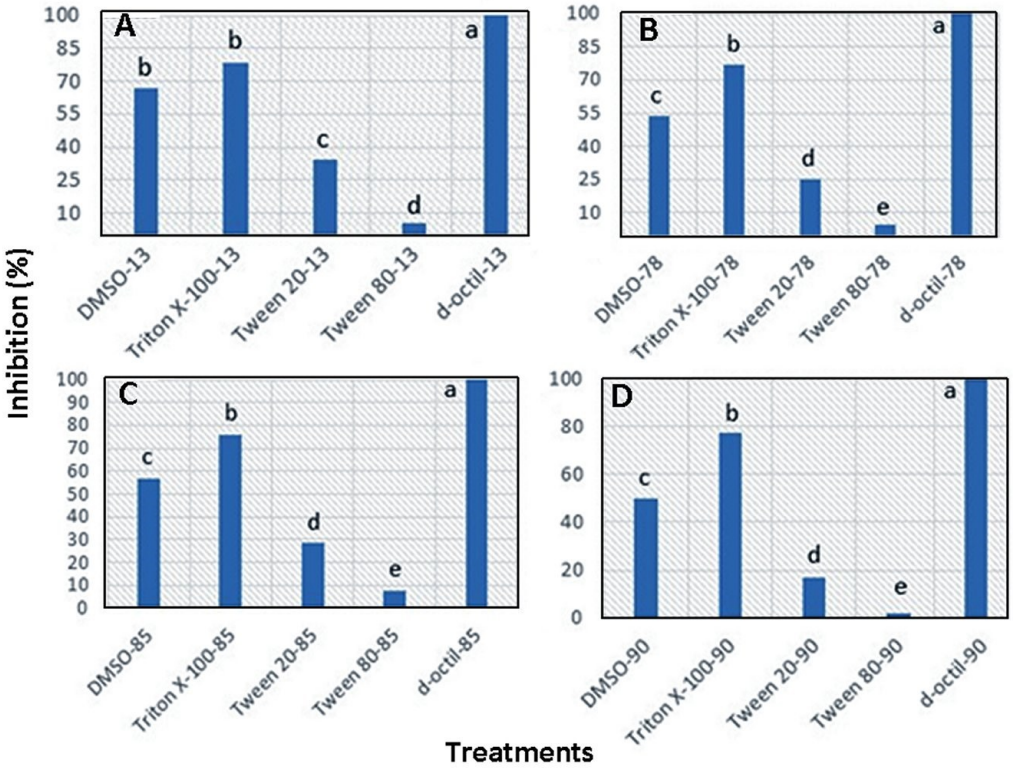

Figure 23. Colony growth inhibition of *T. asperellum* with different tensioactives (A: Ta.13, B: Ta.78, C: Ta.85, D: Ta.90).

T. asperellum excipients assay

The development of a liquid formulation was considered of interest by CENSA for applicatioins on banana in MUSA, as well as for other crops. Before testing the excipients to develop a formulation, the quality indicators listed by Chirino *et al*. (2011) were:

physico-chemical properties:

- physical state = solid
- density = acceptable. Spill or apparent density = 0.366. Slump or runoff density = 0.448.
- pH = 5.5 to 5.8
- Suspensibility = one g of product (spores with substrate), up to 100 ml deionized water in a 250 ml cylinder and with slight manual stirring, evaluated after five minutes at three depths, is distributed at: surface (6.35 - 8.05 · 10^6 spores · ml^{-1}), medium (4.7 - 8.75 · 10^6 spores · ml^{-1}) and background (6.55 - 8.10^6 spores · ml^{-1}), which shows an almost homogeneous distribution.
- Particle size = 0.1 to 5 mm as formulated with substrate, based on the AI at approx. 10 µm.
- Humidity = around 10-15% (in the granulate).

Active agent concentration: the concentration > 10^9 conidia · g^{-1}

Nature and quantity of the other components: residue from rice production, rice head (840 g) and rice husk (160 g) per kg.

Purity = $\geq$ 95%

Subsequently, seven excipients were tested and, in order to select the most suitable one, their effect on the conidia germination was evaluated. For this, 10 µg of pure conidia of *T. asperellum* T.a.13 were placed on a slide containing two drops of the product to be analyzed. Three observations (visual fields) were made on each slide, in six replicates for each excipient. The slides of each treatment were placed in 22 cm ø Petri dishes, which contained sterile filter paper moistened with water and 10% glycerin at the bottom, then incubated at 28 °C, in dark. The control was sterile distilled water. The evaluation was carried out at 16 hrs of incubation. In each visual field, the number of germinated and non-germinated conidia were counted, and the percent of germination was calculated for each excipient.

The best variants were xanthan gum 0.5% and polyethylene glycol 8.5%, both with more than 90% germination of the conidia, compared to the rest of the excipients. Both excipients are suitable for use in a liquid formulation (Fig. 24).

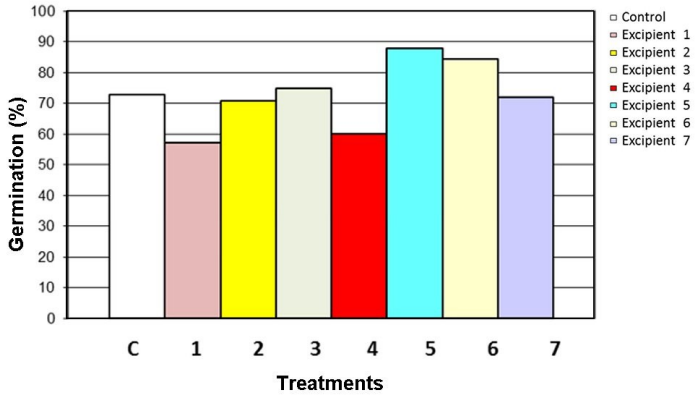

Figure 24. Effect of different excipients on germination of *T. asperellum* conidia.

2.2. EPN

Formulations include one or more ingredients as additives or co-formulates. These components have special characteristics favoring the establishment and maintenance of the product efficacy (Ravensberg, 2011). For biopesticides they have the function of preserving the active ingredient at a low metabolic activity, however alive and virulent. Several conditions must be matched to achieve a useful formulation such as pH, temperature, water quality and preservatives, among others.

2.2.1. Evaluation of abiotic factors on EPN

To evaluate the effect of water quality, pH and temperature over *H. amazonensis* HC1), the effects of four water qualities, three pH values and three temperatures over two EPN concentrations, were evaluated on viability, motility, search capacity and infectivity of *H. amzonensis* HC1 in laboratory conditions during 42 days, as part of a formulation study. The trials were replicated three times, using *G. mellonella* as target. EPN viability was determined by observing the infective juveniles with a stereomicroscope. A one-on-one procedure was used to measure infectivity of individual nematodes. For seach capacity, a two cameras olfactometer and an infectivity test were used (courtesy of Dr.s Gabriela Lankin and Erwin Aballay, Univ. de Chile).

Effect of abiotic factors

Three trials were developed, with IJ kept under the effect of each factor for 6 weeks. The assays were repeated twice, between October - December 2017 (1rst set of trials) and April - July 2018 (2nd trials). All trials were developed in the CENSA Nematology Lab. The IJ of *H. amazonensis* HC1 (GenBank: BankIt1899363 Hamaz_HC1 KU870321) were obtained in *G. mellonella* using the methodology described before. The IJ were collected using Traps (White 1927) as modified by Sánchez *et al*. (2001), with 100% of viability. Healthy last instar larvae of *G. mellonella* (≥ 200 mg) were used. The IJ concentrations were calculated as described by Glazer and Lewis (2000). The experiments were developed using complete random designs.

Effect of water characteristics

In the first test, the effect of water characteristics (tap, boiled, distilled and deionized) on JI was evaluated at the concentrations of $5 \cdot 10^4$ and 10^5 IJ per plate (15 cm diam). The effect on IJ viability was determined in Petri dishes with 100 ml of each water with IJ concentration. For each treatment pH and electric conductivity were determined (Table 6) to know is they fit the standards (USP35-NF30; NC 27:1999; NC 827:2010).

Table 6. Average pH values and electrical conductivity of water used in the study.

Water type	Used in this study		Standards (USP35-NF30, NC 27:1999, NC 827:2010)	
	pH	conductivity (µS/cm)	pH	conductivity (µS/cm)
Tap	7.5	560	6 - 9	< 4000
Boiled	8.6	47.2	No reference	
Distilled	7.4	0.3	5 - 7	< 1.1
Deionized	7.2	0.1	5 - 7	< 1.1

The plates were maintained in laboratory conditions (22 ± 1°C) during 49 days and the evaluation were made weekly (each 7 days). Analyses considered data taken at 7 and 35 days. At each evaluation time, the pH and electrical conductivity, the number of live IJ and the presence of IJ aggregates in water (Ishibashi and Kondo, 1990) were determined. The data on viability of IJ were processed through the two-factor analysis of variance and Duncan´s multiple range test (95 % significance level).

The IJ viability, showed significant differences at 7 and 35 days. The highest IJ viabilities were obtained with distilled and boiled water at $5 \cdot 10^4$ IJ per plate (> 90%). The lowest viability (67.6%) was observed in deionized water. In water the IJ formed aggregates. According Ishibashi and Kondo (1990) this phenomenon is common in several EPN species and sustains survival of nematodes, against desiccation or solar light. In distilled water, the IJ aggregates showed a normal behavior with nematodes in good conditions. Distilled water was selected for pH and temperature trials.

Effect of different pH and temperatures

Using distillated water, three solutions at pH 5, 7, and 9 were prepared and evaluated at the 22 ± 1, 25 ± 2, and 27 °C. For analysis, the data were collected at 7 and 42 days. For trials, 24 holes plates (Costar) were used, using one plate per treatments (three pH values and three values for temperature). In each hole, 2 ml of solution (for pH tests) or distilled water (pH

7,4 with 0,3 µS/cm electric conductivity, for temperature assays) with 10 IJ were used. In pH assay, the plates were maintained at 22 ± 1 °C. For temperature evaluation, the IJ were kept in distilled water at 22 ± 1°C (acclimation room), 25 ± 2°C (room temperature in laboratory) and 27°C (incubator). The effects of treatments (different values of pH and temperatures) were checked at 7 days intervals until 42 days, observing 40 IJ per treatment, testing mobility, and the living IJ were used in infectivity tests (Glazer and Lewis, 2000). The viability and infectivity of IJ at the temperatures and pH tested were determined by using the one-in-one test with larvae of *G. mellonella* (Glazer and Lewis, 2000). The color of *G. mellonella* cadavers was observed and compared with the color described for this strain (Rodriguez, 2015). The cadavers were dissected under a stereoscope to verify EPN presence, as pathogenicity indicator (Lortkipanidze *et al*., 2019). Data were processed by Wald's test.

At 7 and 42 days, highest IJ viability and infectivity in *G. mellonella* were obtained at pH 7. The lowest viability was observed at pH 9 (Fig. 25). According to Kung *et al*. (1990), pH between 4-8 guarantee the superviral of EPN.

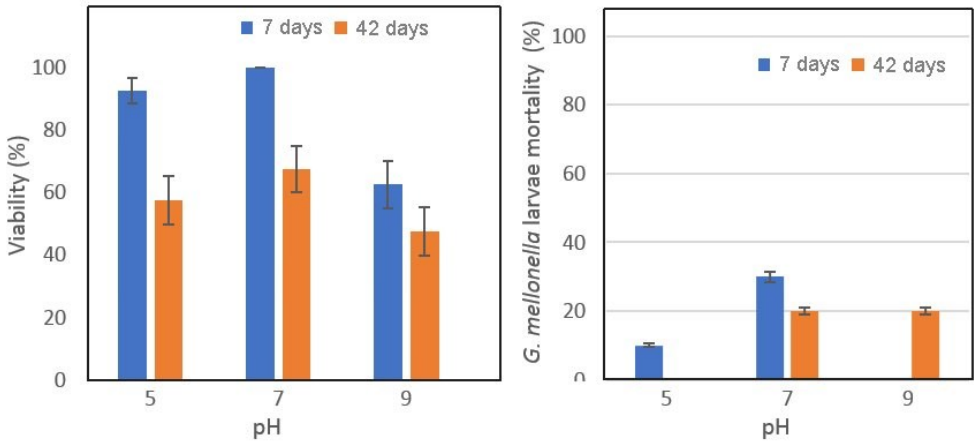

Figure 25. Viability and mortality of *G. mellonella* in the one-on-one (infectivity test, made according to Glazer and Lewis methodology) trial at pH 5, 7, and 9 ($p<0.05$).

Temperature affected the IJ viability (Fig. 26) more than pH, as the IJ did not reach 100% viability at the three temperatures tested. Additionally, the infectivity test showed lower rates of *G. mellonella* mortality.

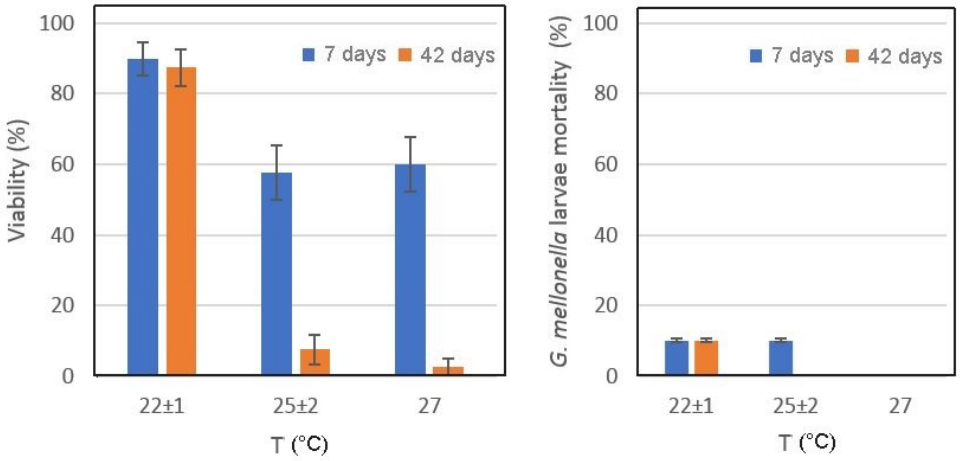

Figure 26. IJ viability of *H. amazonenesis* HC1 and mortality of *G. mellonella* in one-on-one trial at 22 ± 1, 25 ± 2 and 27 ºC.

The highest viability was observed in treatments with distilled and boiled water, that were statistically different from tap and deionized water. The pH value most favourable for nematodes was 7, with 22 ± 2 ºC as optimal temperature. The results of these trials allowed selection of best temperature and pH values, and of the type of water to be used for formulation and stored nematode solutions.

HC1 behavior tests

Different equipments (cottage or industrial manufactures) can be used to evaluate the EPN search and parasitic capabilities, in different conditions. They are useful mainly to evaluate the behavior of IJ that were reproduced and formulated in laboratories.

A six-arm olfactometer, as described by Rasman *et al*. (2005), (courtesy of G. Lankin, E. Aballay *et al*., Fac. de Ciencias Agronómicas, Univ. de Chile), made with PVC pipes of different diameters (Fig. 26) was used by the CENSA Nematology Lab. The olfactometer, to which a glass cylinder and a plastic net were introduced to avoid displacement of the target insect larvae, allowed determining displacement and infection capacity of IJ in laboratory trials.

For the assays, the assembly of a two branches device, parts of a six arms olfactometer, with glass cylinders and plastic meshes (3000 µm) (Fig. 27) were used to avoid *G. mellonella* displacement. As substrate, sterile silica sand with 10 % humidity was used.

Recently emerged IJ with 100% viability of HC1 were used. The last instar larvae of *G. mellonella* used as target proceeded from a laboratory rearing and weighed about 200 mg. A 2000 IJ suspension was applied to the substrate on the end opposit to the PVC tube in which the *G. mellonella* larvae were kept in a glass cylinder protected with a plastic net, to avoid their exit. Three replicates were used at 27°C. Larval mortality was recorded at 24, 48, and 72 hours after nematodes application.

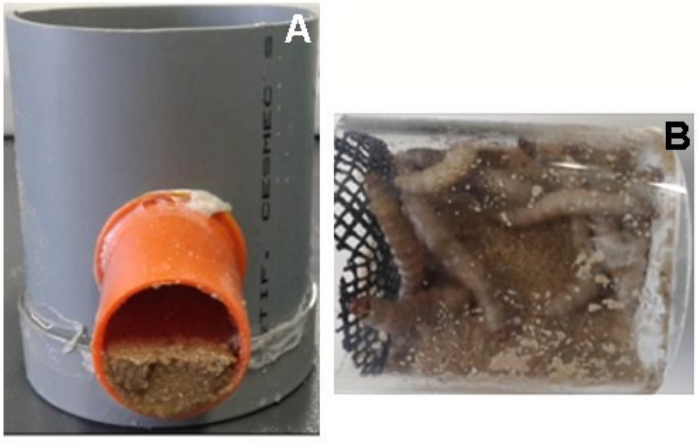

Figure 27. Fixed arm of one of the chambers containing sterile silica sand (A). Larvae of *G. mellonella* contained within the glass column filled with sterile silica sand with 10 % humidity (B).

IJ mobility was checked in the olfactometer assay. The nematodes moved along 19.5 cm in 60 hrs. Mortality of *G. mellonella* larvae reached 84.7% (Fig. 28). The two-branches olfactometer, with the introduction of a glass cylinder and a plastic net, was useful for laboratory studies testing the effect of abiotic factors on EPN.

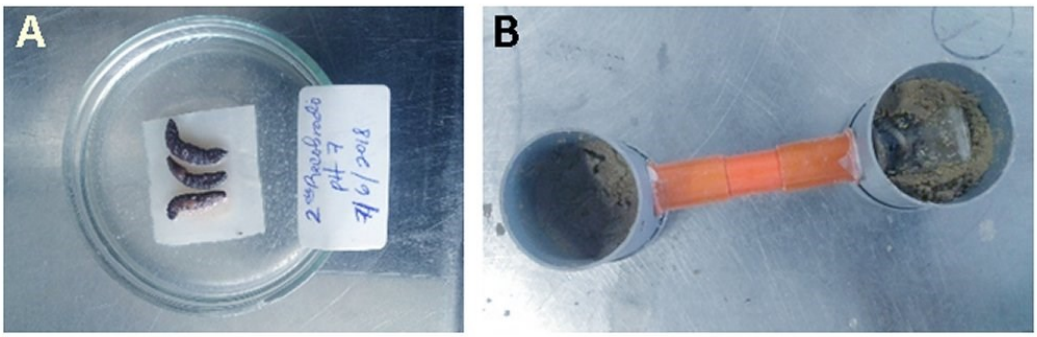

Figure 28. Late-instar larvae of *G. mellonella* dead by EPN maintained in pH 7 solution more than a month (A). Mobility (section of a six-arms olfactometer) and pathogenicity test with EPN treated with a pH 7 solution for one month (B).

EPN formulation database

A data base was built with more than 400 papers edited relatives to EPN formulations, and 53 papers presented measures of statistical dispersion for each treatment (preserves, carriers, others) for metanalysis (in progress). The prior analysis of papers showed that 45% of pepers are related to solid formulations, 35% to encapsulation and 20% to liquid formulations, in relations with nematode species/strain and crops/productive systems, among others elements.

Adjuvants evaluation

The adjuvants evaluated were a mix of methyl paraben (0.18 %) and propyl paraben (0.02 %), streptomycin sulphate (0.05 %) and ascorbic acid (100 ppm) in Ringer solution (9 g NaCl, 0.49 g KCl, 0.4 g $CaCl_2 2H_2O$, 0.2 g $NaHCO_3$ in 1 L of distilled water). Twenty four holes plastic plates were used with 10 IJ in 2 ml of adjuvant tested, at 21-23 °C. The observations were made at 7, 14, 28 and 42 days and viability and infectivity were evaluated.

Best results were obtained when the infective juveniles were preserved in a mix of methyl paraben (0.18 %) propyl paraben (0.02 %) and streptomycin sulphate (0.05 %). Ascorbic acid affected the viability and infectivity of juveniles (Table 7).

The viability of IJ *H. amazonensis* HC1 decreased abruptly in treatments with ascorbic acid at 14 days, and all nematodes died at 21 days (Table 7). This reaction differs from the positive effects of this product reported on juveniles of *H. indica* by Strauch *et al*. (2000), who found 80% of viability at 28 days.

Botanic products can be used in formulations to increase the efficacy of biological control agent or their shelf life. The lethal toxic effects of essential oils and chitosan were evaluated measuring the viability of *H. amazonenesis* HC1 IJ. The essential oils were obtain by hydro-distillation with Clevenger equipment, following ISO 65-71:84 instructions. Each oil was dried over $NaSO_4$ and maintained at 4 °C. Chitosan (N-acetil-D-glucosamineacetate, 2.5 % inerts, 4 % excipients, acetic acid, phosphoric acid) was obtained from VenAgro (Venezuela). Tensioactives and organic dissolvers were evaluated (Table 8).

Table 7. Effect of adjuvants on viability (V, %) and infectivity (I, %) of *H. amazonenesis* HC1 JI*.

Days	Treatments							
	methyl paraben (0.18%) + propyl paraben (0.02%)		streptomycin sulphate (0.05%)		ascorbic acid (100 ppm)		Ringer solution	
	V	I	V	I	V	I	V	I
0	100	50	100	50	100	50	100	50
7	62.6	20	100	20	96.7	0	94.7	40
14	96.3	30	96.7	50	53	60	100	11.1
21	89.1	30	64.9	40	0	0	87.4	70
28	96.3	50	88.6	40	-	-	91.0	30
35	83.8	50	92.6	40	-	-	60.8	20
42	70.1	30	65.6	60	-	-	46.9	10
49	100	30	73.8	30	-	-	9.5	50
56	22.9	16.7	60.4	70	-	-	0	0

* Assays carried out at 21-23°C.

Table 8. Tensioactives and organic dissolvers using for solubilization of essential oils.

Treatment	Final concentrations (%)	Supplier
H_2O		
Acetone	2	LPV 13-03-2013 (99.5 %)
Acetone	1	
Ethanol	2	Merck (absolute for analysis)
Ethanol	1	
Methanol	2	Burdick Jackson (HPLC)
Methanol	1	
DMSO	2	Merck
DMSO	1	
Tween 80	0.5	Riedel de Haën
Tween 80	0.25	
Tween 20	0.5	Riedel de Haën
Tween 20	0.25	
Triton X-100	0.5 (3)	Scharlau (98 %)
Triton X-100	0.25	
Kitosana	0.5	VenAgro (Venezuela)

Tritón X-100 (0.5 %) showed low toxic effects to IJ and was selected to increase the bio-availability of the essential oils evaluated. The oils number 34, 48, 99 and 114 were mixed with Triton X-100 (1%) and were evaluated at 0.5 % of final concentration, in 16 replicates. *Heterorhabditis amazonensis* HC1 was compatible with essential oils numbers 34, 48, 99, 114 and chitosan, considering the low toxicity shown by these products (Table 9).

Table 9. Effect of essential oils n. 34, 48, 99 and 114 on *H. amazonensis* HC1.

Treatments (final concentration)	Mortality (%)*
Water	15.15 a
Triton X-100 (0.5 %)	12.68 ab
n. 34 (0.5 %)	9.79 abc
n. 48 (0.5 %)	7.95 bc
n. 99 (0.5 %)	5.23 c
n. 114 (0.5 %)	5.71 c

* Same letters show treatments not differing for P ≤0.05 (Wald test).

For biological evaluation of substances that are not very soluble in water, such as EOs and their components, the use of compounds that increase their bioavailability in the test medium is required. In general, organic solvents and surfactants are used for this purpose. However, the susceptibility of the target organisms can vary depending on the species and even the strain, and it is therefore of great importance to adapt the tests to each specific organism.

The lethal toxic effect assay for organic solvents and surfactants on *H. amazonensis* HC1 showed that IJ are more susceptible to organic solvents. Of the surfactants evaluated, Triton X-100 did not cause a significant mortality of IJ at the two concentrations used and was selected for solubilization of the samples in the compatibility bioassays of essential oils (EOS) and their components with the EPN studied (Tables 10-13).

Table 10. Effect of organic solvents and surfactants on *H. amazonensis* HC1.

Treatments (final conc. in wells)	**Mortality***
Control Water	5.7 fg
Acetone (1%)	23.3 bcd
Acetone (2%)	16.6 cde
Methanol (1%)	22.8 bcd
Methanol (2%)	24.0 bcd
Ethanol (1%)	18.7 cde
Ethanol (2%)	23.2 bcd
DMSO (1%)	31.3 ab
DMSO (2%)	40.0 a
Tween 20 (0,25%)	13.3 def
Tween 20 (0,50%)	17.5 cde
Tween 80 (0,25%)	25.5 bc
Tween 80 (0,50%)	23.8 bcd
Triton X-100 (0,25%)	10.0 efg
Triton X-100 (0,50%)	6.9 fg

* Equal letters show no difference for $p \leq 0.05$.

Table 11. Lethal toxic effect of essential oils n. 34, 48, 99 and 114 on *H. amazonensis* HC1.

Treatment	JI Mortality (%)	
Water	3.8	cd
Tritón X-100	2.9	d
Essential oil 34	8.8	ab
Essential oil 48	13.7	a
Essential oil 99	7.5	bc
Essential oil 114	2.5	d

[a] Final concentration in well = 0.5%.
* Same letters show treatments not differing for p ≤0.05 (Wald Test).

The mortality rates of *H. amazonensis* produced by the applied oils were in all cases lower than 14%. Oils n. 99 and n. 114 did not produce a significant toxic effect on IJ, while n. 48 and n. 34 caused mortalities that did differ statistically from controls (Table 10). Oils n. 114 and n. 99, did not cause significant levels of mortality or reduction in infectivity, which suggests that they may be used in conjunction with the EPNs in IPM programs (Table 11).

Table 12. Sub-lethal, toxic effect of different essential oils on *H. amazonensis* HC1 induced mortality in *G. mellonella* larvae.

Treatment[a]	*G. mellonella* mortality (%)	
Water	63.6	a
Tritón X-100	91.6	a
Essential oil 34	83.3	a
Essential oil 48	63.6	a
Essential oil 99	91.6	a
Essential oil 114	91.6	a

[a] Final concentration in well = 0.5%.
* Same letters show treatments not differing for p ≤0.05 (Wald test).

The assessment of the lethal toxic effect of the selected EOS components evidenced that the IJ of *H. amazonensis* exposed to camphene, p-cimene and piperitone were not significantly affected by these substances. The 1,8-cineole, limonene, camphor and methyl chavicol caused mortality below 20%, but differed statistically from the control (Table 12). It is recommended to evaluate the effect of these terpenes and terpenoids at shorter exposure times. Carvacrol, eugenol, linalool and thymol were highly toxic to the EPN studied and produced mortalities > 50% in ascending order, up to 100% death of the IJ treated (Table 12).

Table 13. Lethal toxic effect of essential oils components on *H. amazonensis* HC1.

Treatment	Mortality (%)	
Water	0.7	i
Triton X100	0.3	i
Camphene	0.9	hi
p-cimene	1.6	ghi
Piperitone	2.0	ghi
1,8-cineole*	3.6	fgh
Limonene*	4.2	fg
Camphor	6.0	f
Methylchavicol	16.8	e
Carvacrol	52.7	d
Eugenol	67.6	c
Linalool	97.4	b
Thymol	100	a

[a] Final concentration in well = 0.5%.
* Same letters show treatments not differing for $p \leq 0.05$ (Wald test).

Microscopic observation of dead larvae, after exposure to these treatments, evidenced a disorder in the disposition of the internal body content, with formation of vacuoles or large granules that were observed in all cases. In the cephalic region the contraction of the body and the detachment of the cuticle was evident. In the case of thymol, the vacuolization and internal disorder were greater and collapsed internally in some regions (Fig. 29).

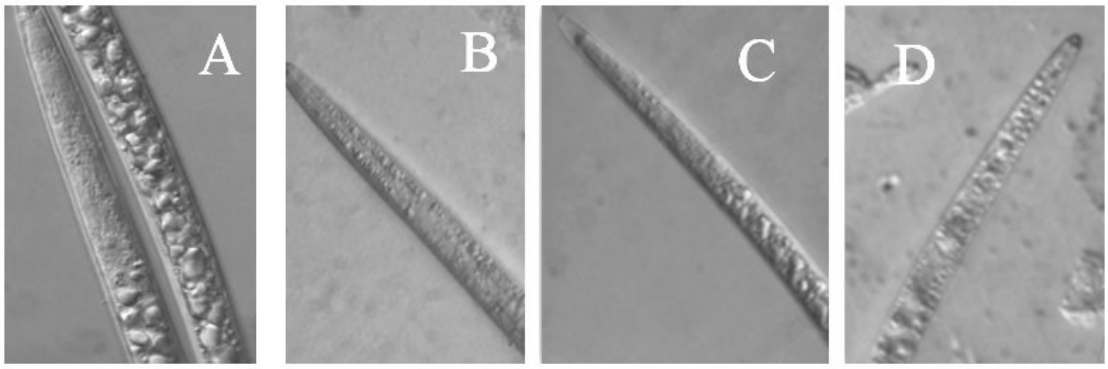

Figure 29. Alterations caused by essential oil components in *Heterorhabditis amazonensis* by thymol (A), linalool (B), eugenol (C) and carvacrol (D).

Formulations include one or more ingredients and additives or co-formulates. These components have special characteristics favoring the establishment and maintenance of the product efficacy (Ravensberg, 2011). For biopesticides they have the function of preserving the active ingredient at a low metabolic activity, alive and virulent.

Effect of different carriers

In this study, newly emerged IJ of *H. amazonensis* HC1 were used, obtained by *in vivo* reproduction as describe before, with a 100% viability. To calculate the amounts of IJ and

the nematode concentrations established for the test, the dilutions method (Kaya and Stock, 1997) was used.

As part of pre-formulation studies, the effect of three national products (carriers 1, 2 and 3), were evaluated on the viability of HC1. Transparent polyethylene bags with 10, 20 and 30 g of each carrier were prepared and sterilized. Three replicates of each treatment were compared with the control (polyurethane sponge in bags) each with $1 \cdot 10^6$ IJ. The experiment was maintained in the laboratory at 27 ± 1 °C during 124 days, with evaluations at 31, 62 and 124 days. The IJ extraction was carried out by sieves of 124, 68, 46 and 38 μm. At each evaluation, live and dead IJ were observed under a stereoscope at 2.5 × and counted. The normality of the viability variable was tested using the Shapiro-Wilks test and the homogeneity of variance by F test. Data were compared with analysis of variance and Duncan multiple range test (significance level = 95%).

High mortality of IJ occurred with the three carriers evaluated (Table 14), without greatly impacting the weight of the packaging. This study is continued to determine how to bring the IJ to the state of anhydrobiosis, which allows them to be formulated in solid media without large mortality rates.

Table 14. Evaluation of carriers for EPN formulation.

Treatments (g used)	**IJ viability (%)**		
	31 days	62 days	124 days
Carrier 1 (10)	0.3 a	0.1 a	0.08 ab
Carrier 1 (20)	1.4 a	1.1 a	0.3 cd
Carrier 1 (30)	1.0 a	0.7 a	0.3 d
Carrier 2 (10)	0.6 a	0.06 a	0.3 abcd
Carrier 2 (20)	0.5 a	0.08 a	0.1 abc
Carrier 2 (30)	0.2 a	0.2 a	0.2 abcd
Carrier 3 (10)	1.3 a	0.2 a	0.02 a
Carrier 3 (20)	0.6 a	0 a	0.1 ab
Carrier 3 (30)	0.4 a	0.01 a	0 a
polyethylene bags (control)	64.3 b	59.2 b	0.1 ab
EE	2.69	1.7	0.08

Excipients evaluation

In this test carboxymethyl cellulose (CMC) and glycerin were evaluated in seven treatments Ttable 15). The 24 holes plastic plates were used, adding in each hole 1 ml of each sample and 10 IJ, with 9 replicates per treatment. The experiment (27 ± 1ºC) was repeated three times. The evaluations were made at 24, 48 and 72h using a stereomicroscope.

Table 15. Treatments applied using CMC and glycerin.

Treatment n.	CMC (%)	glycerin (%)
1	0.1	1
2	0.5	1
3	1	1
4	0.1	-
5	0.5	-
6	1	-
7	-	1

At 24 hrs, the IJ viability were around 70% or more. At 72 hrs, the highest IJ viability was obtained with treatment 3, followed by treatment 1 (Fig. 30).

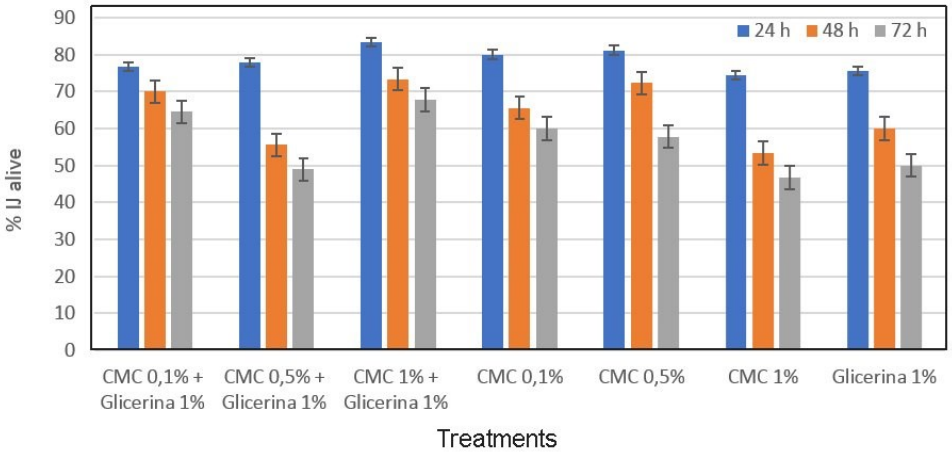

Figure 30. Viability of *H. amazonensis* HC1 IJ in treatments with excipients.

Evaluation of spray drying for IJ formulation

A preliminary study using a spray drier equipment available at CENSA was performed (Fig. 31). Parameters evaluated innclude 140ºC as entry temperature and 80ºC for exit temperature, and 15% and 100% pump flows. The IJ concentration used was 10^6, previously treated in vacuum for water extraction, obtained from 0.66 g of IJ paste.

Figure 31. Spray drier equipment available at CENSA and used for a preliminary study on IJ formulation.

The formulation was prepared by: IJ (0.66g) + maltodextrin (9.6g) + Arabic glue (2.7g) + distilled water (85 ml). at the end of process, 0.27 g were taken and suspended in 15ml of distilled water and observed in stereo- microscope Zeiss® (STEMI DV 4).
More than 80% of IJ dead. The team will continue develop trials changing temperature and flew velocity, among other parameters.

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3. Summary of novel elements in mass production generated by the Project

General novelty aspects for bioformulation production (CENSA)

The following general innovation aspects were identified by partner CENSA in relation to the mass production of bioformulations:

- New isolates of *Pochonia*, *Trichoderma* and *Heterorhabditis* spp. were found in Cuba soils, increasing the knowledge on their diversity and availability in collections in the country, and for their potential use in mass productions;
- *Pochonia chlamydosporia* var. *catenulata* IMI-SD 187 (KlamiC® bionematicide) was compatible with FitoMas®, urea, Bayfolan® and Dimetoate and was incompatible with Cuproflow. This knowledge is novel, and has relevance for the application of commercial products based on this EBCA on banana *vitro*-plants, during the *ex vitro* adaptation phase.
- Studies *in vitro* showed that *T. asperellum* T.a.13 grows faster than *P. chlamydosporia* var. *catenulata* IMI-SD 187, but there was no direct hyphal contact between them. The *P. chlamydosporia* isolate colonized the root system of banana cv. FHIA-03 and the rhizosphere of plantain *vitro*-plants when the fungus was applied alone or in combination with *T. asperellum*. Both fungi promoted the growth of the *vitro*-plants.
- Methods developed to introduce *P. chlamydosporia* var. *catenulata* IMI-SD 187 and *T. asperellum* T.a.13 in banana/plantain seed production (*in vitro* plants) represent a new achievement.

Innovation related to plant derived products

- Several essential oils were studied for BW management, and some of them showed insecticide, repellent or attractive activities. This new knowledge opens novel opportunities to include plants or essential oils in IPM of BW.
- Studies with essential oils and components showed the possibilities of use products derived from selected plants, integrated with the EBCAs (*P. chlamydosporia* var. *catenulata* IMI-SD 187, *T. asperellum* T.a. 13 and *H. amazonensis* HC1). All these applications in banana crops are new.

Specific aspects of mass production methods and/or formulation of EBCAs

Innovation related to P. chlamydosporia var. *catenulata* IMI-SD 187 and *Trichoderma asperellum* T.a.13

- KlamiC® and SevetriC are commercial products formulated as rice colonized by the fungi; studies carried out in MUSA showed compatibility between *P. chlamydosporia* var. *catenulata* IMI-SD 187, *T. asperellum* T.a.13 and tensioactives, clays and other components and their suitability to develop new pre-formulations, with a longer shelf life.

Innovation related to EPN mass production

In the frame of MUSA, some aspects of a liquid, *in vitro* mass production method and elements to develop new EPN formulations were studied:

- using local available components, liquid media were developed at Erlenmeyer's level, achieving an EPN infective juveniles (IJ) yield of 16896 IJ / ml.

– the formulations include one or more ingredients and additives or co-formulates. Parameters such as temperature, pH and water characteristics, adjuvants, tensioactives, clays, organic dissolvers and essential oils were evaluated in MUSA as a base to develop new formulations and improve efficacy of those already in use.

Innovations in mass production of fungi and bacteria for management of nematodes and Fusarium oxysporum (MSBIO)

General aspects

The innovation aspects identified by partner MSBIO for mass production regard: 1) new isolates of fungi *Pochonia*, *Beauveria* spp. and bacteria such as *Bacillus* spp. that have been found in Italian soils and 2) their use in mass production.

Innovations in isolation and related procedures

1) Use of alternative methods for the isolation of facultative microaerophilic bacteria with nematocidal action, therefore able to work in depth in soil conditions of poor aeration, which can be mass produced in high aerobic conditions with high yields.

2) Demonstration of the antagonistic behavior of *Bacillus licheniformis* isolate SGB413. From an *in vitro* test, this isolate proved to be effective against root-knot nematodes as well as indigenous *Fusarium oxysporum* strains.

3) For *B. licheniformis* SGB413, *B. amyloliquefaciens* SGB0013 and *B. velezensis* SGB100Z strains, *in vitro* tolerance tests were performed for abiotic stresses such as temperature, salinity and extreme pHs. This knowledge allowed to determine in which environmental conditions the microorganisms can be produced, applied and result effective.

Innovation in mass production

1) For the mass production of fungi (i.e., *P. chlamydosporia*) the previous system used, with active air flow inside the bioreactors containing a solid medium, has been changed by using static bioreactors without continuous air flow (Fig. 32). This allowed to reduce fermentation times and increase yields. In addition, a semi-automatic method for inoculating the bioreactors has been implemented.

2) For development of best substrate for mass production of fungi such as *Pochonia* and *Beauveria*, substances such as yeast extract, soy peptone and vitamins have been evaluated as additions, in various ratios, to the classic natural substrates used in solid fermentation (corn and rice). The best substrate for production was the one with the addition of vitamins.

Figure 32. Production of *P. chlamydosporia* on static bioreactors with intermittent air flow.

3) Different media were tested for the liquid production of bacteria such as *Bacillus* spp. The classic composition of the *Bacillus* spp. broth has been changed with the addition of different protein sources, such as yeast extract and soy peptone, and various mineral salts. Furthermore, the ratios among them have been varied.

4) The individual parameters for liquid mass production were varied in order to obtain the maximum yield for *Bacillus* spp., once the best substrates were identified. The parameters investigated were: temperature, pH and oxygen consumption. For the latter, even if some strains such as *B. licheniformis* are microaerophilic, it was experimentally found that increasing the oxygen dissolved in the substrate during fermentation to levels $\geq$ 50%, allowed a consistent and satisfactory production.

Specific innovations in products bioformulation

1) Products based on *Pochonia* and *Beauveria* have been formulated with vegetable oils and emulsifiers. Assays demonstrated their stability in the mixture with various oils and emulsions and their suitability for development as new bioformulations, with a longer shelf life.

2) Bacteria-based products have been formulated in molasses and plant extracts in various ratios, giving rise to new bioformulations with longer shelf-life.