IPM-4-CITRUS
H2020-MSCA-RISE-2016
Project n°734921
April 2017-Jan 2023

FINAL MEETING & IDB 2022
– 19th & 20th December 2022 -

AGENDA

19th December 2022 @MEDIS (Nabeul, Tunisia)

8h30	Arrival & Registration
9h00	**Welcome forewords** by MEDIS and IPM-4-Citrus coordinators Introduction of official representatives
9h05	IPM-4-Citrus project: Aims & Context Les Laboratoires MEDIS, business corporate presentation
9h15	**Overview and state point** about our achievement and perspectives WP1 Management (main indicators, history, funding and human resources, actions, reporting: deliverables overview), WP5 Networking & Outreach activities (events, consortium meeting, training, workshop and Round table) and WP6 Dissemination & Exploitation (scientific valorization, social and economic impact)
10h00	**Focus on Scientific and technical Work Packages**: WP2 Bioprocess, WP3 Formulation & Biocontrol activity and WP4 Transfer & economic maturation (3x15min).
	Coffee break (15min)
11h00	**Exchanges with officials and audience**: Question/Answer
11h15	Presentation of "MEDIS Santé végétale", from concept to production capacity
11h30	**Visit of production unit**
	Lunch (buffet on site)
14h00	**Inauguration of production unit** (media, civil society and stakeholder: project overview, production unit and local impact
14h15	**Poster session**: scientific and technical exchanges with audience **Press briefing** (for media with consortium scientists)
	Coffee break (15min)
15h15	**Round table**s (2 x 20min) Theme 1: Technology transfer as a driver for the development of the Tunisian economy (scientific point of view, industrial point of view) Theme 2: Perception of biopesticides by the civil society (synthesis of the "survey". What about your opinion?

20th December 2022 @IDB2022 (Hammamet, Tunisia)

9h30	**Satellite session IPM-4-Citrus** – (open session / 2h30+coffee break)
	Lunch (IDB2022)
14h00	**Consortium time for auto-evaluation** (restricted to consortium members) – 1h30

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IDB 2022 – 20th December 2022
Satellite session IPM-4-Citrus
9h30 – 12h30

IDB 2022 Satellite session IPM-4-Citrus - 9h30 – 12h30 / 20th December 2022	
WP2	**Proof of concept, from *B. thuringiensis kurstaki* cultivation to δ-endotoxin production (bioperformances, bioprocess and scale-up)**
WP2-1	**Physical limitations induced by a wheat bran-based medium for the production of biopesticides by *Bacillus thuringiensis* serovar *kurstaki*.** Rita BARSSOUM, Karim Mohamed CHALBI, Rayan NASSERDINNE, Julien CESCUT, Mireille KALLASSY, César ACEVES-LARA, Luc FILLAUDEAU
WP2-2	**Modelling and Dynamic Optimization of protein and spore production by *Bacillus thuringiensis*.** Tatiana Segura MONROY, Nouha ABDELMALEK, Souad ROUIS, Mireille KALLASSY, Joanna ABDOUD, Julien CESCUT, Nadia BEN SAÏD, Luc FILLAUDEAU, César Arturo ACEVES-LARA
WP2-3	**In-depth genomic analysis of *Bacillus thuringiensis* serovar *kurstaki* strains: Genome dynamics in play.** Nancy FAYAD, Rita BARSSOUM, Nathalie MARSAUD, Julien CESCUT, Luc FILLAUDEAU, Mireille KALLASSY
WP3	**Optimisation of the new biocontrol activity (formulation, efficacy at lab and field scales, risk assessment and ecotoxicology)**
WP3-1	**Study of ecotoxicological effects and efficacy tests of biopesticides based on *Bacillus thuringiensis* "Lip" and "BLB1" against *Phyllocnistis citrella* and *Prays citri*.** Rayan NASSEREDDINE, Zhanerke AMANGELDI, Gül AYYILDIZ, Fatma IDRIS5, Souad ROUIS, Mireille KALLASY AWAD, Asime Filiz CALISKAN KEÇE, Stephan DIETRICH.
WP3-2	**Novel biopesticides *Bacillus thuringiensis kurstaki* : Toxicity Studies on Non-Target aquatic Organisms.** Rim EL JENI, Sayda DHAOUADI, Hazar KRAÏEM, Gül AYYILDIZ, Zakaria BENLASFAR, Zeynep YURTKURAN CETEREZ and Balkiss BOUHAOUALA-ZAHAR
WP3-3	**Toxicity assays on Rabbit, Rat and Guinea pig: Biosafety assessment of new BLB1 and LIP *Btk*-based formulated products.** Balkiss BOUHAOUALA-ZAHAR, Gül AYYILDIZ, Hazar KRAÏEM, Rim EL JENI, Sayda. DHAOUADI, Zeynep Yurtkuran-CETERE & Zakaria BENLASFAR
WP4	**Transfer & economic maturation (benchmarking, business plan)**
WP4-1	**Economic maturation & business plan development for new Bacillus thuringiensis based biopesticide.** Souad ROUIS, Nadia BEN SAID, Tayssir HAMMAR, Sara FALVO

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Physical limitations induced by a wheat bran-based medium for production of biopesticides by *Bacillus thuringiensis* serovar *kurstaki* (WP2-1)

Rita BARSSOUM[1-2-3], Karim Mohamed CHALBI[2], Rayan NASSERDINNE[1], Julien CESCUT[3], Mireille KALLASSY[1], César ACEVES-LARA[2], Luc FILLAUDEAU[2]

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Background and aim: Biopesticides are an alternative to chemical pesticides that respond to the development of the bio-economy and are respectful to humans and the environment. *Bacillus thuringiensis* is a gram-positive, spore-forming bacterium that forms the basis of most biopesticides. This insecticidal activity is mainly due to the production, during the sporulation phase, of δ-endotoxins constituted by an assembly of crystalline proteins called Cry. The European project IPM-4-Citrus (MSCA RISE, No. 734921, 2017-2023) aims to optimise this bioproduction during the culture of 3 strains of *Bacillus thuringiensis serovar kurstaki* (Lip, BLB1 and HD1) in a wheat bran based complex medium. The biochemical composition and nutritional limitations induced by the medium were identified. This study aims to explore the evolution of the physical properties of wheat bran suspension (morpho-granulometry, solid-liquid separation by settling and rheological behaviour) as a function of grain size and concentration.

Methods: Morphological characterisation was carried out *ex-situ* using a morphogranulometer (Mastersizer G3S, Malvern Instrument). Solid-liquid separation by decantation (Falcon 50mL) was used to determine the settling velocity and the volume fraction of supernatant. The water retention capacity (WRC) is also measured. Rotational viscometry was achieved with a rheometer (Mars III, Thermo Scientific) equipped with a helical ribbon ensuring the homogeneity of the wheat bran suspensions (0-150 ghm/L).

Results: The solid-liquid separation mechanisms determines the initial operating conditions (suspension homogeneity, pumping rate) and the downstream processing. The suspension properties result from their technological pathway (with/without heat treatment and after culture) and their composition (starch and lignocellulosic particles). The main trends observed are: (i) an increase of WRC regardless of the granulometry, (ii) an increase in the pellet volume fraction with the technological pathway and (iii) a decrease in the limiting fall velocities. The apparent viscosity of the initial suspensions (after heat treatment) is reported as a function of shear rate, particle size and concentration. The suspensions evolve from a Newtonian behaviour (diluted regime, [WB]<<1 gmh/L) to a non-Newtonian behaviour (shear-thinning) in a semi-diluted ([WB] > 10 gmh/L) then concentrated ([WB] > 80-150 gmh/L) regime. A model of the rheological behaviour considering the equilibrium between the "fines" and the "coarse" can thus be proposed.

Keywords: biopesticides, *Bacillus thuringiensis*, wheat bran, rheometry, morpho-granulometry, decantation.

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Modelling and Dynamic Optimization of protein and spore production by *Bacillus thuringiensis* (WP2-2)

Tatiana Segura MONROY [1], Nouha ABDELMALEK [2], Souad ROUIS [3], Mireille KALLASSY [4], Joanna ABDOUD [5], Julien CESCUT [5], Nadia BEN SAÏD [2], Luc FILLAUDEAU [1], César Arturo ACEVES-LARA [1]

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Background and aim: Dynamic optimization of proteins and spore production by B. thuringiensis needs to use robust models coupled to control strategies. In this work, two models were proposed to describe proteins and spores production using *B. thuringiensis*. Models were calibrated and the parameters obtained were in the literature range. The best model was selected to implement two control strategies, for a Fed-batch culture and a Sequential Batch culture.

Methods: Two dynamic model were used to optimize proteins and spores production using three *B. thuringiensis strains*.

Results: The experimental simulations developed in the present study, on B. thuringiensis culture made it possible to analyse their behaviour and to adjust two models to the experimental data sets. The calibration of both models allows calculate the kinetic parameters of the culture and the experimental data presented a good fit. Additionally, although the BLB strain showed the highest maximum specific growth rate (μmax), the HD strain presented the highest yield Biomass/substrate yield coefficient values (Y1). The LIP strain presented the lowest values for this yield. As for the production of proteins, which are mainly used for insecticidal effects, the BLB strain presented the highest concentration and the proteins/substrate yield coefficient (Y2) was the highest of all the strains, while the LIP strain showed the lowest values for this yield.

In order to study the performance of the control strategies, three different cases were considered, batch without optimization and with initial Substrate equal to 25g/L, fed-batch strategy and sequential batch. It was found that the optimization of proteins and spores productions depicted an increasing profile for glucose feeding until the upper limit of volume was achieved. Fed-batch strategy had the best proteins and spores' productivity with high biomass productivity.

Conclusion: Two models were proposed in this work. One model was chosen to compare two approaches of dynamical optimization. The results demonstrated that the model based on control strategy allowed maximizing proteins and spores productivity. An experimental validation of the control law will be performed to prove the correct proteins and spore productivity.

Keywords: Model optimization, Proteins and Spores optimization, Fed-batch fermentation, Sequential batch fermentation.

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In-depth genomic analysis of *Bacillus thuringiensis* serovar *kurstaki* strains: Genome dynamics in play (WP2-3)

Nancy FAYAD[1,2]*, Rita BARSSOUM[1,3], Nathalie MARSAUD[3], Julien CESCUT[4], Luc FILLAUDEAU[3], Mireille KALLASSY[1]*

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Background and aim: Biopesticides are an eco-friendly alternative to harmful chemical pesticides. A major player in the biopesticides market are *Bacillus thuringiensis*-based products. The latter is a Gram-positive sporulating soil bacteria that owes its entomopathogenic properties to a parasporal crystal constituted of an ensemble of pore-forming δ-endotoxins. Genes encoding the latter are carried by toxin-carrying plasmids, a pillar of *Bacillus thuringiensis'* genetic makeup. *Bacillus thuringiensis* serovar *kurstaki (Btk)* is known for its activity against lepidopteran larvae. *Btk* Lip and BLB1, isolated from Lebanese and Tunisian soils respectively, exhibited a higher efficiency than the reference HD1 against lepidopteran larvae *Ephestia Kuehniella.* IPM-4-Citrus (MSCA RISE, No. 734921, 2017-2023) is a project aiming to optimize the culture of *Btk* Lip, HD1 and BLB1 on a wheat bran based medium. In this study we aimed to elucidate several in-depth genomic aspects of the three strains in question. This analysis included toxin-coding genes, mobile genetic elements, and potential virulence and human health related factors.

Methods: Lip and BLB1 entire genomes were subject to Pacific Biosciences sequencing approach with Illumina HiSeq polishing. Several bioinformatic analysis then followed including complete genome annotation by RAST and Prokka, the detection of toxin-coding genes and the mining of inter- and intra-cellular mobile genetic elements. A maximum likelihood phylogenetic analysis was also conducted.

Results: Based on the sequencing results, we identified 13 plasmids carried by HD1, 12 plasmids for Lip and 11 plasmids for BLB1. The size of the plasmids ranged between 2 and 400 Kb. Some plasmids (7) were common between the three strains, whereas other were specific for each strain. Despite belonging to the same serovar *kurstaki*, the differences in their plasmid content and detected single nucleotide polymorphisms demonstrate the strains' high genomic variability. Results pertaining to the remaining analyses are on-going. All in all, genomic data is indispensable in improving strains' bioperformances and host selection. Moreover, any microorganism which can be disseminated in the environment should be extremely verified for potential virulence factors, prior to its formulation and use as a biopesticide.

Keywords: *Bacillus thuringiensis, cry* genes, plasmids, mobile genetic elements, phylogeny

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Study of ecotoxicological effects and efficacy tests of biopesticides based on *Bacillus thuringiensis* "Lip" and "BLB1" against *Phyllocnistis citrella* and *Prays citri* (WP3-1)

Rayan NASSEREDDINE[1], Zhanerke AMANGELDI[2], Gül AYYILDIZ[3], Fatma IDRIS[5], Souad ROUIS[5], Mireille KALLASY AWAD[1], Asime Filiz CALISKAN KEÇE[2], Stephan DIETRICH[4].

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Background and aims: This study shows the results obtained in the use of an alternative biopesticide based on *Bacillus thuringiensis* tested at lab scale in the department of plant protection, Çukurova university, and in the citrus fields of Mersin targeting two main Lepidoptera: *Phyllocnistis citrella* and *Prays citri*, with the objective of discovering an alternative to chemical pesticides that lead to severe ecological issues.

Methods*:* *Bacillus thuringiensis* strain "*Lip*" was identified from Lebanese soil, and *Bacillus thuringiensis* strain "*BLB1*" was recovered from Tunisian soil. The primary goal of our research is to evaluate the efficiency of our two designed products against the citrus flower moth (*Prays citri*) and the citrus leaf miner (*Phyllocnistis citrella*), as well as to estimate the lethal dosage concentration (LD50) and verify the spore persistency through the assay.

Results: At the field scale, the results showed that the efficacy of the two applied products on both insects reached 70% within 3 days of spraying and increased to between 80-85% by 14 days. At the laboratory scale, the fatal quantity was found to be between 50 and 70 ug/ml. The spores persistence tests verified our findings. The amount of spores dropped with time, indicating that the insects digested them.

Conclusion: Overall, our findings indicate that in order to improve and confirm the efficacy of the products, it is required to first determine the lethal dosage concentration at lab scale before evaluating it at field scale.

Keywords: *Bacillus thuringiensis, Prays citri, Phyllocnistis citrella,* Ecotoxicological test, Lethal concentration, Citrus field.

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Novel biopesticides *Bacillus thuringiensis kurstaki* : Toxicity Studies on Non-Target aquatic Organisms (WP3-2)

Rim EL JENI[1], Sayda DHAOUADI[1], Hazar KRAÏEM[1], Gül AYYILDIZ[2], Zakaria BENLASFAR[1], Zeynep YURTKURAN CETEREZ[2] and Balkiss BOUHAOUALA-ZAHAR[1,3].

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Background and aim: Citrus fruits are among some of the most popular agricultural products contributing in the economic and social progress of nations. Integrated Pest Management (IPM) approach based on controlling citrus insect pests via the use of biopesticides could largely boost yield production. In this optic, newly *Bacillus thuringiensis* (*Bt*) formulations-based bio pesticides (BLB1and LIP), active against citrus pests were identified and in order to scale them up from lab to market, eco-toxicological risk assessment has to be monitored. Therefore, the acute toxicity of these new formulations on non-target living aquatic organism freshwater microalgae (*Pseudokirchneriella subcapitata*) as well as the crustaceans water flea Daphnia magna Straus, was assessed.

Methods: Toxicity assays were conducted in accordance with the ISO and OECD guidelines. The effects of various BLB1 and LIP concentrations on the sensitivity response of *Daphnia magna* and on the *microalgae* growth, were evaluated after different exposure point times. The assays were realized in comparative manner with *Bt*-standard formulation (Delfin WG).

Results: As results, realized investigations based on experiments endorsed by regulatory guidelines led us to define the impacts of the new formulations BLB1 and Lip on the environment, accurately aquatic microorganisms. The test provides the fact that *Btk* Lip and Btk BLB1 are far less toxic than the reference product Delfin.

Conclusion: The generated results reveal the no harmful effects of the new *Btk* BLB1 and Lip formulations on aquatic non-target organisms. These results prove their possible commercial exploitation counteracting the health risk factors related to citrus pests treatment by chemical pesticides.

Keywords: *Bacillus thuringiensis*, BLB1, LIP, risk assessment, non-target organisms.

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Toxicity assays on Rabbit, Rat and Guinea pig: Biosafety assessment of new BLB1 and LIP *Btk*-based formulated products (WP3-3)

Balkiss BOUHAOUALA-ZAHAR[1,2], Gül AYYILDIZ[3], Hazar KRAÏEM[1], Rim EL JENI[1], Sayda. DHAOUADI[1], Zeynep Yurtkuran-CETERE[3] & Zakaria BENLASFAR[1]

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Background and aim: Two new *Bacillus thuringiensis*-based biopests, namely *Bt kurstaki* BLB1 and LIP, efficiently targeting lepidopteron pests, were recently formulated as dried products. To assess their biosafety, toxicity assays were carried out on lab animals (i.e. rabbits, rats and guinea pigs), according to the European ISO and OECD guidelines. The ultimate dual objective was to evaluate the risk for workers/exposed populations and the impact on Health.

Methods: According to a well-established Action Plan (AP), all the experiments were conducted in comparative manners with similar reference products already on the market and a blank formulation of additives without active ingredient, in respect to animal welfares. The eye irritation/corrosion tests were carried out on cohorts of New Zealand rabbits (2-3 kg/rabbit) for acute (one instillation) and sub-acute (single day instillation, 5 days) assays, and the maximum mean total scores (MMTS) were calculated, respectively. The dermal (skin) irritation assays were carried out on three rabbits/product (single exposure) and the Primary Irritation Index (PII) determined. The acute (one gavage and 14 days observations) and subacute (daily gavage during 28 days) oral toxicity assays were carried out on rats (200-250g/rat) in collaboration with Kobay lab animal facilities. The sensitization test was performed on guinea pigs by intradermal administration.

Results: First, all the protocols received an early approval from the Ethical Committee of Institut Pasteur Tunis. The effects of BLB1 and LIP Bt-based formulations were compared to the equivalent DELFIN reference product. As results, no mortality and abnormal clinical findings were observed following external and gavage toxicity tests. No significant weight loss and reduction in feed and water consumption were observed. Barely the skin sensitization test has to be redone because of few mortality observed in negative control group. Collected data will be shown in details. Biochemical parameters and histological observations will be illustrated to support the conclusion.

Conclusion: The first evaluation of the new *Btk*-based biopests risk on human is promising. The active ingredients as well as the formulated products are non-irritating eye/skin and non-toxic on rat by gavage.

Keywords: *Bacillus thuringiensis*, formulation, toxicity, irritation, mortality

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Economic maturation & business plan development for new *Bacillus thuringiensis* based biopesticide (WP4-1)

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Background and aim: Within the IPM-4-Citrus project, different results are acquired and can be exploitable from scientific and commercial point of view. The exploitation of the project results is at the heart of its implementation. Therefore, different activities are devoted for the economic maturation and business plan development for future spin-off and/or for the creation of new production lines. The aim of this work, is to summarize different exploitation activities developed with the help of the EC Booster experts in order to bring the results closer to the market.

Methods: Key Exploitable Results (KER) are defined. Based on these KERs, Business Plan Canvas (BPC) is developed. The methodology used for BPC development includes: market analysis, legal, regulatory and Intellectual Propriety Rights aspects, clear action plan and roles, milestones, financial costs and revenues, and the impact of the project in three years.

Results: KERs defined are new innovative, cheaper, user friendly biopesticides and simplified production process. Despite the strict regulatory aspects for product homologation, the market analysis shows that biopesticide market is strongly emerging and that 90% of this growing market is based on *Bacillus thuringiensis* strains which increase the competitively. To overcome competitivity, IPM-4-Citrus product will be 25% less expensive than commercial products and will target 100% of local market and 10% of regional market. Intellectual property rights of the products formulations and process will be protected via patents. The exploitation roadmaps main actions are: finalization of the construction and installation of the biopesticide unit, technology transfer and homologation application. Revenues are estimated to increase during the first three years. Also, the project is expected to increase biological crops and to save public health and environment from chemicals effects.

Conclusion: In summary, KERs generated during the IPM-4-Citrus project are value added results that makes the economic maturation possible and the commercialization of the new biopesticides cost effective despite the high competitivity and all regulatory aspects.

Keywords: Integrated Pest Management, Biopesticides, *Bacillus thuringiensis,* Key Exploitable Results, Exploitation, Market, Economic maturation, Business Plan

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