COST STSM Reference Number:	STSM-FA1103-061014-048817
Period:	6/10/14 – 28/11/14
COST Action:	COST Action FA1103
STSM Applicant	Christian Huber; christian.huber@helmholtz-muenchen.de
STSM Host	Muriel Raveton; Muriel.Raveton@ujf-grenoble.fr
STSM type:	regular
STSM Topic:	Role of maize endophytes in the phytoremediation of organo-chlorines

1. Purpose of the STSM

The main purpose of the STSM was to evaluate the role of maize endophytes in the phytoremediation of organochlorines (Lindane). Due to practical reasons (construction work in some of the relevant facilities, illness of coworkers) the scientific focus had to be shifted from endophytic bacteria towards endophytic fungi on short notice. However the metabolism of organochlorines and the involvement of endophytes was followed with good success.

2. Description of the work carried out during the STSM

Maize seeds were pre-germinated on agar plates. Seedlings of the same size were collected and transferred to plastic pots containing 70 g of standard plant soil. For the treatment with Lindane, plants were watered every 3-4 days with 40 ml of water containing 7 mg of Lindane per Liter. Control plants were watered with the same amount of tap water respectively. This work was done by the host institution. Plants were harvested for the first time after one month on the first day of the STSM. There were five plants for the control and three for the Lindane treated group. Unfortunately a contamination with *Fusarium spp*. was observed on the second set of plants, which lead to the decision to discard them and to repeat the experiment.

Colonization of endophytic fungi was based on both, microscopic observation and molecular tools. Fungal root colonization was studied after staining the roots for 30 minutes with trypan blue. Root fragments were scanned for fungal structures using a light microscope. Fungal structures were counted and rated by intensity of colonization. DNA was isolated from maize (150 mg of plant material) using the FastDNA SPIN Kit and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA. Extraction of DNA from soil was performed using the FastDNA SPIN Kit for soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA). 500 mg of rhizosphere soil (attached to the plant roots) and bulk soil were used for every preparation. Quality of the DNA extraction was confirmed on an 1 % agarose gel. Content of DNA was quantified using a NanoDrop system.

For leaf endophytic fungi the focus was set on the genus *Neotyphodium* which represents a group of plant associated fungi which are known to modify relationships between diversity and ecosystem properties. The endophyte specific primers NRPS1F and NRPS1[1] were used to amplify the non-ribosomal

[1] Rasmussen et al. (2007) High nitrogen supply and carbohydrate content reduce fungal endophyte and alkaloid concentration in *Lolium perenne*. New Phytologist 173 787-797

peptide synthetase (NRPS gene). PCRs were carried out using AmpliTaq Gold DNA Polymerase; primers were synthesized by Eurogentec.
To access the foliar endophyte concentration the NRPS gene was quantified by real-time PCR on an IQ5-system (Biorad). Fungal laccase genes were chosen to evaluate the abundance and impact of fungi contributing to the degradation of Lindane in the soil and in the roots of maize plants. Amplification of laccase genes was accomplished using degenerate primers Cu1F and Cu2R[2]. For real-time PCR, the relative abundance of laccase genes was determined from the ratio of laccase gene abundance to Basidiomycetes ITS gene abundance. Basidiomycetes abundance was determined using 5,8sr and ITS4-B primers[3]. The formation of PCR product of the expected size was followed at any time on 0,1 % agarose gels.

3. Description of the results obtained

Microscopic analysis revealed the occurrence of fungal hyphae structures on any of the analyzed maize roots, although no significant differences in colonization could be observed between control and Lindane treated samples. The young age of the plants was the reason for the generally low degree of fungal root colonization since mycorrhization occurs normally on older plants.
DNA extractions from leaf, root and soil samples were successful and resulted in DNA concentrations between 120-250 ng/µl (plant samples) and 50-70 ng/µl (soil samples).
PCR analysis with primers for general fungi as well as specific primers for NRPS revealed the occurrence of *Neotyphodium* in all analyzed leaf samples.
In soil samples, PCR reactions with both primer sets (Laccase: Cu1F, Cu1R; Basidiomycetes: 5,8sr and ITS4) obtained PCR products of the expected size, which confirmed the quality of the primers and the PCR conditions. Quantification of gene abundance was done by real-time PCR. Standards of each gene were generated from pooled PCR products from all samples, serial diluted and run in triplicates together with the genomic DNA. Huge effort in time was made to set up the best conditions for real-time PCR for each set of primers. Unfortunately severe problems occurred regarding the negative, non-template control during the PCR reaction. In each of the reactions it showed a positive signal.
To rule out any sources of contamination new sets of primers for each gene were ordered twice in different qualities and aggregates, different sources of water were tested as well as different set of pipettes. Nevertheless the potential contamination remained, probably due to the high amount of fungal spores present in the laboratories (under construction).
In order to accomplish the goals of the experiment, community analysis with 454 sequencing will be done in the next experiment with a sufficient number of biological replicates. The same applies for the analysis of phytohormons which could not be realized in this experiment due to a lack of plant material and time.
Personal note: Intensive efforts in time and material were made to solve the problem with the negative control in real-time PCR. The formation of primer dimers that is frequently observed in real-time PCR could be ruled out by comparison of melt curves of the amplified products as well as checking the size of the reaction products on agarose gels. In every case the melt curves and the size of the fragments were

[2] Luis et al. (2004) Diversity of laccase genes from basidiomycetes in a forest soil. Soil Biol Biochem 36: 1025-1036
[3] Fierer et al. (2004) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbiol 71: 4117-4120

identical with the testes standard genes. Even though I could not succeed with this problem in the given period of time the systematic work on solving the issue was an intensive training in molecular biology and especially in real-time PCR, which is extremely helpful for my future research.

4. FUTURE COLLABORATION WITH THE HOST INSTITUTION

As the topic fits well into the area of research of both institutions, further cooperation is intended.
As the host institution undergoes massive construction work, it is planned to repeat the experiment in the greenhouse of the Helmholtz Zentrum München. Analysis of the samples will be performed based on the experience obtained in the preliminary experiment during this STSM in close cooperation with the host laboratory. A joint publication is envisaged.

UMR 5553

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TO WHOM IT MAY CONCERN

Dear Mr./Mrs.,

I hereby confirm that Christian Huber, Post-doctoral student at Helmholtz Zentrum München, involved in Cost Action FA1103, has successfully concluded his short research stay in my group at the dept. of Pollution-Environment-Ecology-Ecoremediation, Grenoble University, from 6.10 to 31.11.2014.

During this period, Christian acquired the total experience of endophyte DNA-extraction, -purification, -titration in view to pyrosequencing and RT-qPCR analyses. Follow-up collaboration and publications/articles are planned and foreseen.

Yours sincerely,

Grenoble, le 9/12/2014

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12/09/14

Confirmation letter

This is to confirm that Dr. Christian Huber from my working group (Plant Endophyte Phyisology) at the Helmholtz Zentrum Muenchen performed a successful Short Term Scientific Mission (STSM) within COST Action 1103.

During the period of 6/10/14 to 28/11/14 Dr. Huber visited the working group Perturbations Environnementales et Xénobiotiques (PEX), *LECA , of University of Grenoble, FR. There he had the opportunity to study the* Role of maize endophytes in the phytoremediation of organochlorines.

In this mission, Dr. Huber obtianed valuable results on the physiology and performance of fungal endophytes, and the degradation of the xenobiotic, Lindane.

We are grateful for this support of his work, as it enabled us to deepen the cooperation with Prof. Raveton´s group and will also be the basis for a joint publication in the near future.

Sincerely

Prof. Dr. Peter Schröder

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