REPORT
SHORT-TERM SCIENTIFIC MISSION

University of Neuchâtel, Neuchâtel (CH)
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1 Introduction

In this report an evaluation is made of my Short-Term Scientific Mission (STSM) at the University of Neuchâtel.
In chapter 1 the STSM proposal will be briefly summarised. Chapter two outlines the several activities performed in Neuchâtel including some results. In chapter three concluding remarks considering the planned and achieved goals will be given.

1.1 Description project

The title of the research project is ‘Chemical information exchange in soil; interaction between conifer roots, vine weevil larvae and entomopathogenic nematodes (EPN)’. The aim of the project is to elucidate the environmental chemistry of semiochemicals, attractive towards entomopathogenic nematodes, released by plants (*Thuja* spp.) into the soil when under attack by the vine weevil larvae (*Otiorhynchus sulcatus*). The three main research questions are the following: 1) Which semiochemicals are released by the plant, 2) How do the nematodes respond to this/these semiochemical(s) and 3) What is the fate of the semiochemical(s) in the soil.
This is a very short description of the project. In the STSM proposal a more detailed description has been given.

1.2 Goal visit University of Neuchâtel

Ted Turlings is one of the leading ecologists in the world and an expert in identifying compounds involved in behavioural ecology of insects. His research group, which is part of the University of Neuchâtel, has recently developed techniques including olfactometers combining bio-assays with chemical analysis of the infochemicals released by both insects and plants in multitrophic systems. One recently designed olfactometer is applicable for analysing the behaviour of insects/nematodes and chemicals in the soil. With this belowground six arm olfactometer system one of his PhD-students, Sergio Rasmann, has identified a semiochemical in a tritrophic system with maize, *Diabrotica* larvae and EPN. This promising result, achieved with their biological and chemical techniques, is of great interest for my research. The goal of my stay in Switzerland is seeing if their techniques are applicable to a tritrophic system which consists of *Thuja*, vine weevil larvae and the entomopathogenic nematode *Heterorhabditis megidis.* Besides, this visit will enlarge my contact network of scientists in the field of chemical ecology, and enable me to exchange information and to discuss opportunities of collaborating.

1.3 Planned Activities

1. Discuss collaboration between the University of Neuchâtel (UN) and Plant Research International (PRI) / University of Amsterdam (UVA).
2. Familiarize with the <u>equipment</u>, including the costs.
3. Testing the six arm <u>olfactometer</u> with the tritrophic system of Sergio Rasmann (maize-*Diabrotica-Heterorhabditis megidis)*.
4. Testing the six arm <u>olfactometer</u> with the following tritrophic systems:
    a. *Thuja*- vine weevil larvae- *Heterorhabditis megidis*
    b. *Taxus*- vine weevil larvae- *Heterorhabditis megidis*
5. Identifying possible semiochemicals from Thuja and *Taxus* with <u>Solid Phase Micro Extraction</u> (SPME) and GC-MS using the method used at the University of Neuchâtel. Identification screening of the compounds which are released by the roots of *Thuja* and *Taxus* is planned.
6. Discussing the <u>results</u> with Ted Turlings and Sergio Rasmann.

2 Description of the activities

All activities described in the STSM proposal were conducted. The first three days were spent on discussing the possible collaboration between PRI or the UVA and UN. Besides, an experiment with the six arm olfactometer with maize spiked with their semiochemical was performed. The semiochemical was collected with SPME and measured subsequently with GC-MS. However, I mainly focused on the tritrophic system with *Thuja* and *Taxus* and *O. sulcatus* during the stay in Switzerland. Therefore I will not outline the experiments with the maize system but only the experiments performed with *Thuja* and *Taxus.*
The following experiments were performed:

1. *Thuja* and *Taxus* experiment
    - Testing the attraction of the nematodes towards *Thuja* in the tritrophic system in the six arm olfactometer
    - Collecting the semiochemicals from the gas phase in the soil by SPME and measuring the extracts with GC-MS
    - Extracting the roots, collecting the semiochemicals using SPME and determining the extracts with GC-MS
2. SPME experiment
    - Collecting samples of the soil with SPME, in which an undamaged and damaged *Thuja* was incubated, at two distances from the roots. The compounds collected by SPME were subsequently measured with GC-MS. In this experiment the olfactometer was not used.

2.1 Thuja and Taxus experiment

This experiment is divided into three sub experiments:

1. Testing the attraction of nematodes towards *Thuja* in the olfactometer
2. Identifying semiochemicals from the gas phase of the soil
3. Identifying semiochemicals in the headspace of the roots

2.1.1 Olfactometer

The goal of this experiment was testing the attraction of EPN's in three different treatments of every plant in the olfactometer. The treatments were: 1) a healthy plant (HP), 2) a plant with 5 larvae (DP) and 3) 5 larvae (L) (Table 1). All treatments were set up in an olfactometer arm with silver sand. The remaining three arms were filled with just silver sand functioning as three blanks (B) (Figure 1).

Table 1. Treatments of the olfactometer experiment (repeated 4 times)

Treatment code	Treatment *Thuja*	Treatment *Taxus*
DP	*Thuja* + 5 larvae + silver sand	*Taxus* + 5 larvae + silver sand
HP	*Thuja* + silver sand	*Taxus* + silver sand
L	5 larvae + silver sand	5 larvae + silver sand
B	silver sand	silver sand
B	silver sand	silver sand
B	silver sand	silver sand

Since the paper with information on the six arm olfactometer has not been published yet, details of the set-up and research approach will not be provided in this report. Two olfactometers could be used at the same time, each olfactometer experiment lasting four days.
The results of the olfactometer experiments are shown in Figure 1. The damaged Thuja and *Taxus* appear to be more attractive towards the EPN's than the healthy plants, larvae and blanks. However, the differences in response of EPN's are statistically non significant. The EPN's used with Taxus show

a higher response towards all treatments compared to the EPN's used with Thuja, namely 13.5% against 2.5% of the total amount of nematodes added previous to the soil. Especially two blanks show a high response of the EPN's. This may have been caused by the position of these two blanks that may have been situated in the arms next to the damaged plant, thereby possibly receiving more EPN's than the blank opposite of the damaged plant. However, since I forgot to distinguish between the three blanks, it cannot be proven that the blanks with the higher response of EPN's indeed were surrounding the damaged plant. The position of the blanks should be checked in further research.
The total response of the nematodes varies between 2.5 % and 13.5%.

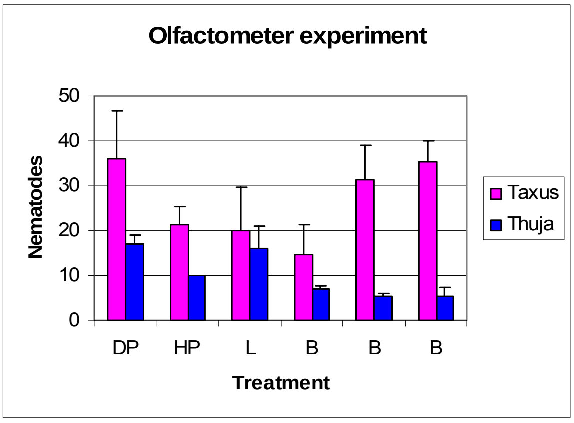

Figure 1. Response of nematodes in the olfactometer experiments with Thuja and Taxus.

2.1.2 Identifying semiochemicals from the gas phase of the soil

The goal of this experiment was to elucidate the compounds released by the damaged plant which was used in the olfactometer experiment, since the damaged plant was more attractive to the EPN's than the other treatments. One olfactometer arm with a damaged *Thuja* and damaged *Taxus* was used in this experiment. The compounds released by the plant into the soil were collected with SPME at a distance of 15 cm from the roots of the damaged plant and subsequently analysed with GC-MS. Measurements were performed during one day at time intervals of 30 minutes.
The chromatograms of the GC were very promising, since one compound, R-limonene, was identified with a certainty above 90% in both *Thuja* and *Taxus.* Since only a damaged plant was measured (i.e. no non damaged control), it cannot be said whether this is a semiochemical which is induced by herbivory.
R-limonene is released in a larger amount by *Taxus*. *Thuja* releases less R-limonene with time, until a stable level is reached, while the release of R-limonene by *Taxus* varies with time. The variation in release of R-limonene by *Taxus* could be indicating that the larvae were not continuously eating from the plant and therefore the plant is not continuously releasing the semiochemical. Whether or not such a direct relationship between chemical release and herbivory exists needs further investigation. The lack of variation in the release of R-limonene by *Thuja* could indicate that *Thuja* releases R-limonene even in a healthy state.

2.1.3 Identifying semiochemicals in the headspace of the roots

The goal of this experiment was to identify the compounds in root extracts of weevil damaged and undamaged *Thuja* and *Taxus.* For this purpose the roots were pulverised in a mortar after freezing with liquid nitrogen. The volatiles of the roots of the damaged and undamaged *Thuja* and *Taxus* were collected from the headspace of the root powder with SPME and measured with GC-MS.
The chromatograms show that *Thuja* releases more different compounds and in with a higher abundance than *Taxus*. Most of the compounds are terpenes. The compounds which are released by the roots of *Thuja* are similar compounds that were found by Nickavar *et al* (2002) in the essential oils of fruit and leaves of *Thuja*. Nickavar *et al.* found that the most dominant compounds of the leave oils

are α-pinene (21.9%), α-cedrol (20.3%), 3-carene (10.5%) and R-limonene (7.2%). These compounds were also present in the root powder of *Thuja* in enlarged amounts.
The chromatograms also showed that there were plant specific compounds that were not present in the other plant. *Thuja* specific compounds are for instance (+) - cycloisosativene, borneol, cedrol, camphor, Fenchol, ß-myrcene and thujopsene. Specific compounds released by *Taxus* are camphene, (-)-2-myteneylacetate and eugenol.
The compounds found in a damaged plant were not induced by herbivory, since the compounds were also present with an undamaged plant. However, five compounds released by a damaged *Thuja* seem to have increased compared to the undamaged plant. Especially α-humulene and R-limonene showed an increased abundance in the chromatograms. In the chromatograms of a damaged *Taxus* two compounds were induced, (-)-2-myteneylacetate, α-pinene, and one compound, (+)-3-carene, showed an increased abundance compared to the undamaged *Taxus.*
Compared to the compounds found in the soil, more compounds are identified in the roots of the plants.

2.2 SPME experiment

The goal of this experiment was to elucidate the compounds released by *Thuja* both undamaged and damaged by the larvae of the vine weevil. For this purpose the soil in which the plant was incubated is sampled by SPME and the extracted compounds are subsequently measured by GC-MS. In the previous experiments, described above, the SMPE fibre was inserted at a distance of 15 cm from the roots. In the current experiment the fibre was inserted at a distance of 15 cm from the roots and at a distance of 1 cm. To investigate the release of the compounds by the roots in time, measurements were done at regular time intervals.
From the chromatograms many observations can be made. These will be described briefly.

<u>Comparison measurement at two distances from the roots</u>
An increased amount of compounds is collected by the fibre at 1 cm distance from the roots, both with an undamaged and damaged plant.

<u>Comparison damaged and undamaged Thuja at a distance of 15 cm from the roots</u>
The identity of the volatiles collected at a distance of 15 cm from the roots in this experiment and those from the previous experiment with the soil (paragraph 2.1.2) are almost similar. In both experiments R-limonene was found in similar amounts. During the SPME experiment, (+)-3-carene was found as well. Both compounds, (+)-3-carene and R-limonene are present in a larger amount with the undamaged Thuja. This confirms the suspicion that the compound was also present in *Thuja* in a healthy state.

<u>Comparison damaged and undamaged Thuja at a distance of 1 cm from the roots</u>
As mentioned above, more compounds are identified nearer to the roots. Undamaged *Thuja* releases the compounds in greater amounts than damaged *Thuja*. However, some compounds, like (+)-4-carene, (+)-α-pinene, 2-carene, thujopsene and α-humulene, are only present with the damaged Thuja and seem to be induced. Especially thujopsene is released in large quantities upon attack by larvae.

<u>Difference in release of compounds in time</u>
Like with *Taxus* in the olfactometer experiment (paragraph 2.1.2) the release of compounds varies in time whenever the plant is under attack of larvae. This phenomenon can also be observed near the undamaged roots at a distance of 1 cm. Though, at a distance of 15 cm from the roots the compounds of the undamaged Thuja do not vary that much with time, but decrease slightly until its level is stabilising. The release of compounds at a distance of 15 cm from the roots does not show temporal differences. Within the rhizosphere one can expect a less constant release by the undamaged plant of compounds, leading to a difference in time which will be levelled out at a distance of 15 cm from the roots. Though no conclusions can be made, it seems that there is no constant release of volatiles from the plant when it is under attack of larvae, while in a healthy state the release is constant.

3 Conclusion

In this chapter I will reconsider whether I achieved the goal of my stay in Switzerland and I will focus on the planned activities (chapter 1).
In paragraph 3.1 I will focus on the collaboration, while in paragraph 3.2 I will describe how this stay will help my research.

3.1 Collaboration

The research group of Ted Turlings is interested in collaboration within the field of chemical ecology. They feel that exchanging information is useful and that the chemical knowledge of the University of Amsterdam can be helpful in putting the research within a bigger scope. However, the PhD-research is almost finished and at the moment they do not know whether they will continue the subject. If my research proposal and approach is further developed and results can be shown, the collaboration will be further discussed.

3.2 Research approach

The main goal of my stay was to verify whether the research approach and equipment of the University of Neuchâtel was applicable for my research. Many experiments have been done. From this experiment I gained many experimental skills that will help be develop my own research approach. Besides, the results obtained led to different subjects on which I can focus on within my own research.

Experimental Skills
The six arm olfactometer could be a useful instrument and will be used for my own research. The experiments showed that it is very important to make a standard protocol for rearing the nematodes and applying the larvae. The response of the nematodes in the experiments was very low and the difference in response of nematodes between the different treatments was hardly significant. Besides it is still unsure whether the larvae infested the plants enough to induce the release of semiochemicals in large quantities. Therefore I should minimize the amount of treatments and standardise the experiments with the nematodes, larvae and plants. Perhaps the equipment will be adjusted in the Netherlands, in order to combine the bio-assay with chemical measurements.

Results
- *Taxus* but especially *Thuja* releases many compounds even in healthy state. This will complicate the experiments. Maize, which is used in Switzerland, hardly releases any compounds in a healthy state.
- The amounts of compounds which are released by the plant upon attack of larvae seem to differ in time. It will be interested to focus on the cause of it. Perhaps the larvae are not continuously infesting the plant, therefore not leading to a continuous release of semiochemicals.
- Measuring the compounds at different distance from the roots or measuring the roots itself, led to the presence of different compounds and different amounts of compounds in extracts.

The results obtained showed that there are factors that can influence the response of the nematodes and the release of semiochemicals. Three factors will definitely be included in further research.
Though the data obtained are not statistically significant and therefore can not be used directly, they will help develop my further research approach. Therefore my stay was more than useful.