

Scientific Report of COST STSM Reference Number: COST-STSM-ECOST-STSM-FA1103-191112-023162

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STSM Topic: Characterisation of *Mycobacteria* and *Rhodopseudomonas* species - two endophyte genera which might influence growth characteristics of *Prunus avium* shoot tip cultures.

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Period: from 19/11/2012 to 14/12/2012 **Place:** Oulu (Finland)

1. Background of the STSM

During in vitro propagation of *Prunus avium* (wild cherry), especially during rooting and acclimatisation, large-scale losses of plantlets have been observed (up to 70%) which have been related to the presence of endophytes in plantlets cultured *in vitro*. The objectives of the PhD project of Mona Quambusch are to investigate the interaction between *Prunus avium* in vitro plantlets and bacterial endophytes and to differentiate between bacteria with beneficial, neutral or negative properties for the plant. In previous studies, we were able to characterize the bacterial communities in two *Prunus avium* genotypes both by culture-independent methods and isolation of bacteria. In cooperation with the "Institut für Pflanzenkultur" (Solkau, Germany) we selected cherry genotypes with good, poor or fluctuating propagation success during in vitro propagation, rooting and acclimatisation for the studies.

A culture-independent approach was started by DNA extraction from in vitro plants and PCR on 16S rDNA. Amplified fragments were cloned and *E. coli*-colonies were analysed by ARDRA (Amplified Ribosomal DNA Restriction Analysis). The bacterial community of the well growing cherry genotype mainly consisted of *Rhodopseudomonas* sp. which made up 74% of the cloned fragments. *Rhodopseudomonas* belong to the plant growth promoting bacteria and are able to fix nitrogen and produce plant hormones (Koh and Song, 2007). Additionally, we detected *Mycobacteria* (16% of the cloned fragments) in this genotype. In the poor growing cherry genotype 58% of cloned fragments belonged to the genus *Mycobacteria*, while *Rhodopseudomonas* could not be detected.

In addition to the culture-independent approach we also isolated bacteria from shoot tip tissue culture samples. We conducted a PCR on 16S rDNA of 47 bacterial isolates and analysed them by ARDRA. 11 Groups were identified and a representative of each group

was sequenced and compared to database entries. The isolates were dominated by *Bacillus*. However, we also identified three *Rhodopseudomonas* sp. isolates and one isolate turned out to be highly related to the *Mycobacteria*. In conclusion, at this point of the project, it is of great interest to investigate the influence of *Rhodopseudomonas* and *Mycobacteria* on the plant propagation characteristics.

2. Purpose of the STSM

The results of our studies directed our interest to the genus *Mycobacteria*, which dominated the poorly growing cherry genotype, and to *Rhodopseudomonas*, which could so far only be detected in the well growing genotype. Earlier, one *Rhodopseudomonas* strain has been found to have beneficial effects on tomato (Koh and Song, 2007). *Mycobacteria* have only rarely been isolated from plants, but were shown to be highly abundant in buds of Scots pine (Pirttilä et al., 2005) suggesting a close relationship between these bacteria and plants.

Literature study on *Mycobacteria*, especially in connection with studies on endophytes, led us to the group of Anna Maria Pirttilä at the Department of Biology, University of Oulu, Finland. They had recently isolated one *Mycobacterium* sp. strain from rock plants and classified it by sequencing 16S and ITS regions of ribosomal DNA. Also, 13 unique *Mycobacterium* clones had been identified by culture-independent methods and characterized phylogenetically (Koskimäki et al., 2010).

In this STSM we wanted to further classify the identified *Rhodopseudomonas* sp. and *Mycobacterium* sp. by sequencing the complete 16S rDNA and ITS regions and to construct phylogenetic trees based on this information.

Additionally, we aimed at designing specific primers to develop a qPCR-based detection method.

If time permitted, through in-situ-hybridisation studies the third objective was to identify the plant tissues where the bacteria are abundant.

3. Description of the work carried out during the STSM

Classification of bacteria from in vitro plants

To amplify the ITS rDNA from all 11 bacterial isolates a PCR was conducted with primers FGPS 1490-72 and FGPL 132-38 (Koskimäki et al., 2010). For the sequencing reaction the BigDye Terminator Ready Reaction Premix (Applied Biosystems) was used. The sequencing data of 16S rDNA and ITS regions were evaluated by using the MEGA 5 software and the first phylogenetic trees of the isolates were constructed.

Development of specific primers for qPCR

Sequence analysis of the obtained 16S rDNA regions for *Mycobacterium* sp. and for *Rhodopseudomonas* sp. was performed by aligning sequences of all bacterial isolates of in vitro cherry (e.g. *Bacillus* sp., *Microbacterium* sp., *Pseudomonas* sp.) with ClustalW and possible specific regions were identified. Possible primer combinations were tested in silico by the program probe match at Ribosomal Database Project (<http://rdp.cme.msu.edu/probematch/search.jsp>).

In situ hybridisation

Samples of shoot-tip including apical meristems, stems and leaves of 6 cherry genotypes were embedded in paraffin blocks. Probes specific for *Eubacteria* (E11) and *Mycobacteria* (My2) were labelled with digoxigenin (Pirttilä et al., 2005). My2-sense was used as negative control. Microtome slices were hybridised with probes E11, My2 and My2-sense and stained with DIG Nucleic Acid Detection Kit (Roche Applied Science).

4. Description of the main results obtained

Classification of bacteria from in vitro plants

Sequencing of the full 16S region was successful for *Rhodopseudomonas*, *Mycobacterium* and *Bacillus* isolates. The ITS region could not be sequenced successfully due to problems with PCR. Therefore, optimisation of ITS-PCR will be continued in the home institute. Based on the 16S rDNA sequences a phylogenetic tree of the bacterial isolates was constructed using MEGA 5 and CLC software. For each isolate, three reference strains from NCBI database were used for classification.

Development of specific primers for qPCR

Based on the analysis of 16S rDNA sequences, two variable regions with 200 bp distance could be identified. For the qPCR, based on this region a primer pair was designed that covers all mycobacterial strains found in the plant material. Another primer pair was found to bind specifically to *Rhodopseudomonas* isolate N-12. Evaluation of the primer pairs in PCR and qPCR using plant material and bacterial isolates will be continued in the home institute.

In situ hybridisation

The in situ hybridisations were performed once in the host institute to learn the protocol, and a set of embedded plant tissue and microscopy slides with fixed and hybridized samples were transferred to the home institute. A preliminary evaluation of the in situ hybridisations by microscopy showed a hybridization signal with eubacterial probe E11. The mycobacterial probe My2 hybridized to some tissues of the plant samples, especially in meristematic regions of the shoot-tip. My2-sense was used as negative control and showed only a weak background staining. These results already give evidence for bacterial cells inside the plant tissue. A detailed evaluation of the complete set of

samples will be continued at the home institute. The embedded plant samples can be used for additional studies if needed.

5. Foreseen publications/ articles resulting or to result from the STSM

The results obtained in this STSM will be included in publications as part of my PhD-project.

6. Confirmation by host institution of the successful execution of the STSM

The STSM was very successful considering the time limit of the stay (four weeks) and especially the fact that I was extremely busy with teaching at the time. Luckily, my post-doc (Mysore Tejesvi) and Ph.D. students (Janne Koskimäki, Johanna Pohjanen) were active with helping the fellow to carry out all experiments planned, and even more (in situ hybridization). The fellow herself was truly independent and hard working, which can be seen in the amount of results obtained during the visit. We will be continuing the collaboration to get the work finalized and published.

- Anna Maria Pirttilä

References

Koh, R.H. and Song, H.G. (2007) Effects of application of *Rhodopseudomonas* sp. on seed germination and growth of tomato under axenic conditions. *Journal of microbiology and biotechnology* 17: 1805-1810.

Koskimäki, J.J., Nylund, S., Suorsa, M., Pirttilä, A.M. (2010) Mycobacterial endophytes are enriched during micropropagation of *Pogonatherum paniceum*. *Environmental Microbiology Reports* 2: 619-624.

Pirttilä, A.M., Pospiech, H., Laukkanen H., Myllylä R., Hohtola, A. (2005) Seasonal variation in location and population structure of endophytes in buds of Scots pine. *Tree Physiol.*25:289-297.