COST Action FA0807 Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Short-term Scientific Mission (STSM) Report

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STSM Topic: Cytochemical characterisation of the phloematic tissue in *in vitro* plants of *Ca*. P. mali-resistant and –susceptible genotypes infected with *Ca*. P. mali

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Background

Apple proliferation (AP) disease is the most important graft-transmissible and vector-borne disease of apple in Europe and is caused by '*Candidatus* Phytoplasma mali' (*Ca*. P. mali). Resistance against *Ca*. P. mali was observed in the wild genotype *Malus sieboldii* (MS) and in few hybrids derived from the crossings of MS with the *Ca*. P. mali susceptible *Malus x domestica*. A study of the gene expression after *Ca*. P. mali infection in both resistant and susceptible genotypes was carried out at AlPlanta. The results led us to the hypothesis that the resistance in MS genotype involves the production of H_2O_2 induced by the phytoplasma either through direct activation of the plant defense or through modification of metabolic pathways. Our analysis suggests that a mechanism resembling the "recovery" phenomenon could be involved in the *Ca*. P. mali-resistant MS. Experiments carried out by Musetti and colleagues showed that it is possible to visualize at cellular level the modifications and the ultrastructural features characterising the phloematic tissues of recovered plants. In their study, a cytochemical analysis allowed the individuation of the peculiar changes occurring in the response associated to the "recovery" giving new insights on the phytoplasma-apple interaction.

Purpose of the STSM

The mechanisms associated to the resistance in MS still remain elusive. The cytochemical analyses would be of great interest to investigate if the modifications characterising the "recovery" phenomenon are also taking place in MS supporting our hypothesis. Therefore, the general objective of the mission was to adapt and establish an analytical procedure through cytochemical analyses for the characterisation of the phloematic tissue in *Ca.* P. mali-resistant and –susceptible *in vitro* plants infected with *Ca*. P. mali.

The specific objectives were:

- analysis through Transmission Electron Microscopy (TEM) for the presence and localization of H_2O_2 in phloematic tissues of *Ca*. P. mali infected and healthy *in vitro* plants
- quantification of Ca^{2+} levels in *Ca*. P. mali infected and healthy *in vitro* plants

Work carried out during the STSM

A selection of healthy and *Ca*. P. mali-infected *in vitro* plants of both susceptible and resistant genotypes were used in the experiment for the comparison of their phloematic tissue. The material was produced and provided by AlPlanta and transported to the host institution. In particular, the following material was analysed:

- *M x domestica* cv Golden Delicious (GD; susceptible genotype). Infected and healthy *in vitro* plants
- *Malus sieboldii* (MS; resistant genotype). Infected and healthy *in vitro* plants
- Hybrid D45 (showing resistance against *Ca*. P. mali) derived from the crossings MS x *M x domestica.* Infected and healthy *in vitro* plants

In order to gain more information the TEM analysis was carried out in two different tissues: leaves and branches. The materials were collected from plants chosen based on their phenotypical characteristics. For healthy plants, for all the genotypes, plantlets with similar size and development grade were selected. In case of infected plants, plants with characteristic AP symptoms were chosen for GD where symptoms influence the plant growth in a drastic way. For MS and D45, where the symptoms are not evident, the plants were chosen randomly but with homogeneous physiological state. Five plants were used for each genotype and state. For each plant, 4-5 leaves and 2-3 branch cuttings were collected and treated in the same tube as single sample. In total, 30 samples (5 plants x 3 genotypes x 2 states) were prepared for the TEM analysis.
For the quantification of the Ca^{2+} levels, the plants were pooled in order to obtain enough material (about 1 g) to carry out the cellular extraction for the chemical analysis. The latter was performed with the support of the Department of Chemical Sciences and Technologies (Udine University).

Main results obtained

Transmission Electron Microscopy (TEM) analysis

The first question that we wanted to address was if the *in vitro* material was a good starting material for the TEM analysis. In this regard, we are particularly satisfied by the results obtained from the preparation of the tissues chosen for the TEM analysis. This was an interesting point also for Dr. Musetti since our material is considerably different compared to that commonly used in her analyses.
During the procedure we had to slightly adapt the protocol to our material. Compared to the procedure carried out on material collected from plants grown in field particular care has to be taken in the handling of the samples due to the fragility of the *in vitro* plant tissues. However, the protocol for the preparation of the embedded samples was successfully carried out. Thus, it was possible to produce sufficient sections for the following TEM analysis. In the latter, it was possible to well distinguish and characterise the cytochemical modifications taking place in the tissues analysed. From the TEM analysis of the sections in GD infected leaf and branch we could clearly observe that the intracellular structures were strongly compromised compared to the healthy tissues. More in detail, the phloem cells were heavily occluded by callose deposits (Fig. 1C). Deposits of starch were observed in several

cells (Fig. 1B) as well as a clear disorganisation of the organelles with the impossibility to identify the intracellular structures (Fig. 1B). Differently, in MS and D45 the deposition of callose in the sieve tubes of infected plants was restricted to few cells and the cellular organisation was maintained in both leaf and branch tissues (Fig. 2B).

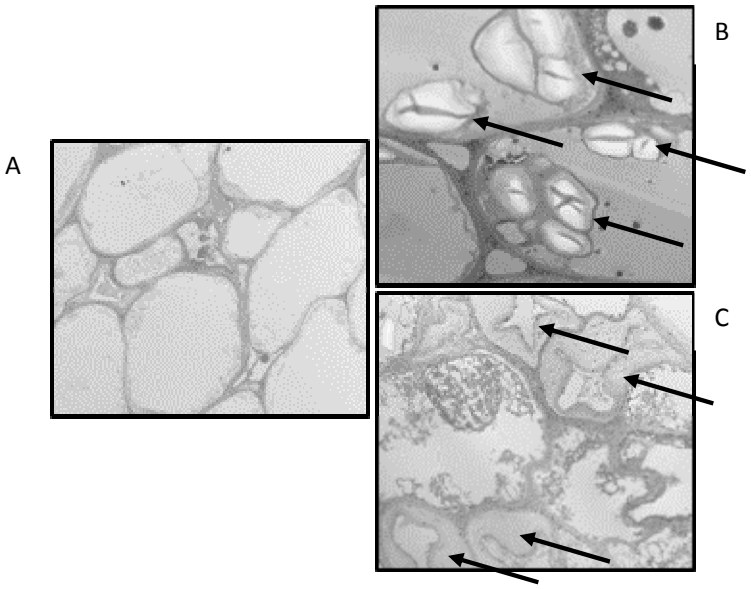

Fig. 1: sections of Golden Delicious leaf tissue. Comparison between healthy (A) and infected state (B and C). The arrows indicate starch deposits (B) and callose deposits (C)

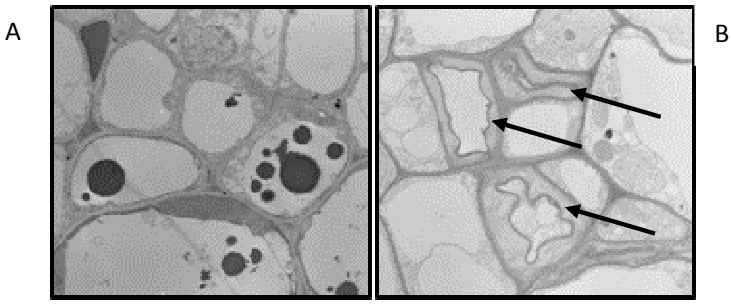

Fig. 2: sections of *Malus sieboldii* leaf tissue. Comparison between healthy (A) and infected state (B). Arrows indicate callose deposits.

A general and diffused labeling (commonly associated to the presence of H_2O_2) was observed in both healthy and infected tissues of resistant and susceptible plants. The localisation mainly in the interstitial space between the cells and in correspondence of the cell wall regions could indicate a young tissue with a highly active metabolism due to the development of the cellular structures. Thus, in contrast to the observations in recovered plants, in our resistant genotypes we could not observe a specific labelling of intracellular localised H_2O_2.

Quantification of the Ca^{2+} levels

This analysis required a considerable amount of material. Due to limitation in the number of plants available it was not possible to produce independent samples for a statistical analysis. However, the results obtained, though preliminary, were of high interest since we could observe a marked difference (about 10 times) in the Ca^{2+} level of MS healthy compared to GD healthy. In addition, the Ca^{2+} levels decreased in both cases in the infected tissues but in a higher degree in MS infected compared to GD infected. The results obtained for the tissues of D45 were not reliable therefore it was not possible to compare this genotype with the others. Further investigation is necessary to confirm these data but the preliminary indications showed a promising result.

Future collaboration with host institution

The results obtained from this preliminary study are not conclusive about the differences that can be observed between resistant and susceptible plants in regard to the presence of *Ca.* P. mali. Considering this, it is reasonable to think about further and more exhaustive experiments to conduct in collaboration between the two institutions.

Foreseen publications/articles

Presently we cannot foresee if the results will end up in a publication