Short-Term scientific Mission Report

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STSM topic:	Identification of endophtic bacteria from healthy and phytoplasma-infected apple trees.

Background

Apple proliferation (AP) is one of the most serious apple tree diseases in several European countries. The causal agent of AP is '*Candidatus* Phytoplasma mali' (16SrX group) and it determines different symptoms such as proliferation of secondary shoots (witches'-brooms), reduced flowering, phyllody, enlarged stipules, leaf rosettes, chlorosis, yellowing and early leaf reddening. Symptoms of economic relevance are small fruit size, lower fruit quality and overall yield decreases.

Different control measures have been used to manage phytoplasma diseases, such as use of the resistance rootstock (Seemuller et al., 2008), antibiotic, chemical treatments against the insect vector (*Cacopsylla picta* (Förster) (syn. C. costalis) and *C. melanoneura* (Förster)) and thermotherapy. However, this measures are not curative and they can cause negative effects on plant productivity and viability. So, there is a need to find new environmental friendly strategies to control AP.

Several studies demonstrated that bacterial endophytes, beside growth promotion, can produce beneficial effects on host plants by different mechanisms. To date, cultivation as well culture-independent approaches have been applied for the analysis of endophytic bacterial diversity in different plant species, such as citrus, poplar, rice, eucalyptus, and grapevine.

Purpose of the visit

This STSM experience aims to isolate and identify endophytic bacteria associated with healthy and phytoplasma-infected apple trees by the use of cultivation-dependent and cultivation-independent methods.

Work carried out

Apple roots collection, samples preparation and DNA extraction

Apple root samples were collected from each of ten plants in Lombardy region (north Italy). Plants were chosen among asymptomatic (five plants) and symptomatic (roots with witches'-brooms) (five plants) for apple proliferation (AP) (Table 1). On the basis of previous work carried out on grapevine plants, apple roots were washed with tap water and sterilized by treating with ethanol 70% for 3 min, sodium hypochlorite 2% for 5 min, and ethanol 70% for 30 sec, and fivefold washing with sterile water. Total DNA was extracted from 5 g of roots with the method describe by Doyle and Doyle (1990) with some modifications. Total DNA was quantified by spectrophotometer (NanoDrop) and it was used as template for endophytic bacteria characterization by cultivation-independent methods.

Table 1. Asymptomatic and symptomatic apple samples

	NO	Sample name	Location	Date
Asymptomatic	1	F7P18	Lombardy	06.04.2011
	2	F16P30	Lombardy	06.04.2011
	3	F7P48	Lombardy	06.04.2011
	4	F9P30	Lombardy	06.04.2011
	5	F13P7	Lombardy	06.04.2011
Symptomatic	6	F15P3	Lombardy	06.04.2011
	7	F15P2	Lombardy	06.04.2011
	8	F15P47	Lombardy	06.04.2011
	9	F16P49	Lombardy	06.04.2011
	10	F16P3	Lombardy	06.04.2011

'*Ca*. Phytoplasma mali' detection in apple root samples

Root samples were tested for AP phytoplasmas by PCR on total DNA extracted as previously described. In detail, '*Ca*. Phytoplasma mali' was detected by the use of primer pairs fAT/rAS specific for 16SrX phytoplasmal group. The direct PCR was performed in 25 µl-reaction containing 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.25 µM primer fAT, 0.25 µM primer rAS, 1.25U *Taq* polymerase, 14.625 µl H_2O and 1 µl DNA template. The samples were amplified in thermocycling and consisted of an initial denaturation at 95°C for 5 minutes followed by 35 cycles of 30 s at 95°C, 56°C for 1.15 min, 72 °C for 1.30 min and finally 7 min at 72°C. PCR products were separated on 1% agarose gel and visualized by UV transilluminator. This analysis confirmed the presence of '*Ca*. Phytoplasma mali' in the symptomatic plants. Moreover, the phytoplasma was detected in three of five asymptomatic plants. Thus, only the two plants resulted healthy were used for endophytic bacteria identification by cultivation-independent methods (Table 2).

Endophytic bacteria identification by cultivation-independent methods

Endophytic bacterial community associated with healthy and AP-infected apple roots was carried out by 16S rRNA gene libraries and sequencing. The bacterial 16S rDNA was amplified by the use of bacterial universal primer pairs 799f/1492r. PCR reaction mixture (25 µl) containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM of each primer, 1.25 U *Taq* polymerase, 15,375 µl H_2O and 1 µl template DNA. The samples were amplified in thermocycling and consisted of an initial denaturation at 94°C for 5 minutes followed by 35 cycles of 1 min at 94°C, 45 s at 52°C, 1 min at 72 °C and finally 8 min at 72°C. PCR products were separated on 1% agarose gel and visualized by UV transilluminator. The bands specific for bacterial 16S rDNA were excised and purified from the gel with the QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer's instructions. Purified products were cloned in the plasmid vector pCRII TOPO (Invitrogen) and propagated in *Escherichia coli* as described (Shuman, 1994). The plasmid DNA was extracted from *E. coli* colonies with the QIAGEN Plasmid Mini kit (QIAGEN), and will be sequenced with an ABI 3730 sequencer (Primm, Italy).

Isolation of endophytic bacteria from roots with cultivation-dependent methods

Endophytic bacteria associated with healthy and diseased roots were also described by cultivation-dependent methods. Apple roots (10 samples, table 1) were sterilized as previously describe. After sterilization, roots samples (2 g) were ground in mortar and mixed

with a 'Ringer solution (10 ml). Partial volume of homogenates (100 µl), serially diluted, were spread on the TSA (Tryptic Soy Agar) and LB (Luria-Bertani) agar medium. The samples were incubated at 30ºC for 5 days. After growth, bacterial colonies were selected on the basis of phenotypic characters (shape, color etc.). Bacterial density in the roots was calculated as CFU ml^{-1}. In healthy plants bacteria CFU ml^{-1} were 10^5, while in diseased plants were 10^3- 10^4 (Table 2). In order to compare endophytic bacterial community living in healthy roots with those present in infected ones we decided to identify only the isolates from samples n. 1, n. 4, n. 8 and n. 10

Table 2. CFU/ml of endophytic bacteria isolated from apple roots.

	NO	Sample name	fAT/rAS	**Number of the colony**	
				LB Agar	**TSA**
Asymptomatic	1	F7P18	-	**$1{,}8 \times 10^5$**	**$2{,}4 \times 10^5$**
	2	F16P30	+	**$2{,}8 \times 10^4$**	**$3{,}6 \times 10^4$**
	3	F7P48	+	**$8{,}3 \times 10^3$**	**$1{,}5 \times 10^3$**
	4	F9P30	-	**$1{,}1 \times 10^5$**	**$1{,}6 \times 10^5$**
	5	F13P7	+	**7×10^4**	**$1{,}1 \times 10^4$**
Symptomatic	6	F15P3	+	**3×10^3**	**$7{,}5 \times 10^3$**
	7	F15P2	+	**6×10^3**	**7×10^3**
	8	F15P47	+	**9×10^3**	**$1{,}1 \times 10^4$**
	9	F16P49	+	**$1{,}2 \times 10^4$**	**$1{,}3 \times 10^4$**
	10	F16P3	+	**1×10^4**	**$8{,}6 \times 10^3$**

DNA extraction from bacterial cells

Bacterial isolates from sample n. 1, n. 4, n. 8 and n. 10 were grown in LB broth overnight at 30ºC. Bacterial DNA was extracted from the cells with a MicroLysis kit (Microzone). Liquid cultures were centrifuged at 13.000 rpm for 5 minutes and the obtained pellets were suspended in 100 µl NaCl (5M). 1µl bacterial suspension was added to 19 µl microlysis extraction buffer and it was incubated at 2 min. at 65ºC, 2 min. at 96ºC, 4 min. at 65ºC, 1 min. at 96ºC, 1 min. 65ºC and 30 sec. at 96ºC. The bacterial DNA extracted was used as template for PCR. PCR was carried out by the use of universal primer pairs 27F/1495R. PCR was performed in 50 µl-reaction containing 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM

dNTP, 0.25 µM of each primer, 1.25 U *Taq* polymerase, 15,375 µl H_2O and 1 µl template DNA. The samples were amplified in thermocycling and consisted of an initial denaturation at 95°C for 2 minutes followed by 35 cycles of 1 min. at 95°C, 50°C for 2 min, 72 °C for 3 min and finally 7 min at 72°C. PCR results were analyzed on 1% agarose gel and visualized under UV. PCR products will be cloned and they will be sequenced for bacteria identification.

Future perspectives

This STSM led to start the description of endophytic bacterial community associated with healthy and diseased apple roots. We plan to complete the screening and the sequencing of the libraries carried out on total DNA extracted from healthy and AP-diseased roots. The prosecution of the work implies to carry out experiments for identification of the bacterial species from apple roots. Such experiments will continue basically in the lab of Milano (prof. Bianco and cooworkers) with exchange of information concerning the next steps of the work.