Scientific Report – Short Term Scientific Mission (STSM)
Dr. Gennaro Roberto Abbamondi

Reference: COST Action FA1103

Host institution: Hasselt University - Centre for Environmental Sciences

Period: 09/09/2014 to 24/10/2014

Reference code: COST-STSM-ECOST-STSM-FA1103-090914-049031

- **STSM Title**

The role for plant-associated endophytes in improving production and quality of tomato

- **Abstract**

The continuing increase of the world population, and therefore the need to provide sufficient food to a growing number of people, is one of the major challenges of the twenty-first century (Berg 2009, Adesemoye and Kloepper 2009). Common strategies to enhance crop productivity are the use of chemical fertilizers, manures and pesticides. However, several studies have shown that these techniques have often a negative impact on the environment: leaching of nitrate into groundwater, surface run-off of phosphorus and nitrogen, and eutrophication of aquatic ecosystems. The growing interest in environmental sustainability has led to considerable efforts **to minimize the use of chemical fertilizers and pesticides, replacing (or integrating) these conventional techniques with more eco-friendly methods, such as microbial inoculation.** Tomato represents one of the most important agricultural products. It can be consumed as raw fruit, but also transformed by industrial processes (pulped, canned, sauce production) (Tommonaro *et al.* 2013). This vegetable is known to be rich of several bioactive compounds (folate, ascorbate, polyphenols, carotenoids). **The characterization of cultivable bacteria associated with different tomato cultivars,** and a better knowledge of **plant-growth promotion** and **plant disease control** by means microbiological techniques is an **important contribution to environmental sustainability in agriculture**.

- **Purpose of the STSM**

  - The **main objective** of this research was the genotypical and phenotypical characterization of bacteria associated with tomato plants, with the aim to improve both plant health and nutritional quality of the fruit.

Plants are well known to collaborate with microorganisms to form mutually beneficial associations (Gunatilaka 2006). These associations play essential roles in agricultural and food safety, and contribute to the environmental equilibrium (Montesinos 2003). A clear distinction should be drawn between bacteria residing in the rhizosphere or phyllosphere and **endophytes**. Endophytic bacteria reside in specific tissues of the plant (such as root cortex or xylem) and develop a very close association with the plant, with mutual

exchange of nutrients, enzymes (lipase, catalase, oxidase, etc.), functional agents (siderophores, biosurfactants, etc.), but also "signals". Endophytes deeply colonize plant hosts tissues in which they persist at high rates, without developing the negative effects of a pathogen (disruption of respiration, photosynthesis, translocation of nutrients, transpiration, etc.). On the contrary, the presence of endophytic bacteria in the host plant leads to beneficial effects on its health and/or growth.

- Therefore, as a **first objective** of this study, I isolated **endophytic bacteria** associated with different tomato cultivars.

Eleven bacterial strains, colonizing the **rhizosphere** of tomato plants, were isolated in 2012. Samples were collected in an experimental field situated in the Campania Region (southern Italy). Twelve strains were isolated from tomato plant roots in 2013. Sampling was performed in the previously described experimental field, but a specific protocol was used to isolate **endophytic bacteria**.

- As **second objective**, the genotypic characterization was performed by amplified rDNA restriction analysis (ARDRA) of their 16S rRNA gene and identification by 16S rRNA gene sequencing.

Bacteria can enhance host health, producing plant-growth regulators (auxins, cytokinins, gibberellins), but also with the suppression of stress ethylene production by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, nitrogen fixation and the mobilization of unavailable nutrients such as phosphorus and other mineral nutrients (Weyens *et al.* 2009).

- Therefore, the **third objective** of this study was to select the more promising strains on the basis of their ability to promote plant health.

Specific assays were performed to detect plant growth promotion and plant disease control characteristics of the isolates.

- **Description of the work carried out during the STSM**

For this research, a characterization of bacteria associated with tomato plants was performed, with the aim to identify strains that can improve both plant health and nutritional quality of the fruit.

On the basis of this main objective, the work was divided in three work packages:

- **Work package 1**: Isolation of bacteria associated with different tomato cultivars
- **Work package 2**: Genotypical analysis of the isolates
- **Work package 3**: Screening of the isolates on the basis of their ability to promote plant growth promotion

Work package 1: Isolation of bacteria associated with different tomato cultivars

Part of the preliminary isolations were performed at the National Research Council of Italy - Institute of Biomolecular Chemistry of Pozzuoli, Napoli (Italy). In this research institute, knowledge is gathered about the isolation and chemical characterization of secondary metabolites from different natural sources (Abbamondi *et al.* 2013, Tommonaro *et al.* 2012a). Moreover, the study of nutritional quality of tomato

(and “new tomato hybrids”) of industrial interest is explored (Tommonaro *et al.* 2012b, Tommonaro *et al.* 2013, Tommonaro *et al.* 2014).

Eleven bacterial strains, colonizing the rhizosphere of tomato plants, were isolated in 2012. Samples were collected in an experimental field situated in the Campania Region (southern Italy). Tomato roots were harvested at the late stage of maturation, placed in 50 mL tube with sterile phosphate-buffered saline (PBS; $NaH_2PO_4 \cdot H_2O$, 6.33 g L^{-1}; $Na_2HPO_4 \cdot 7H_2O$, 16.5 g L^{-1}), and stored at 4°C until the isolation step. Samples were rinsed in PBS several times to remove adhering soil particles. Roots cuttings were rubbed against a sterile 200 µm nylon mesh to retain roots particles and large cell debris. A sucrose solution (9 ml; 4% w/v) was added to the filtrates obtained from 1 g of roots. Serial 10-fold dilutions of the suspensions were prepared, and 100 µl aliquots were streaked on modified Luria-Bertani (LB) plates containing 4 g L^{-1} of NaCl instead of 10 g L^{-1} (agar 1,8% w/v). After 7 days incubation at 30°C, single colonies were selected and further purified on the basis of morphological differences. Subsequently, all strains were grown aerobically in modified Luria-Bertani (LB) medium (NaCl 0.4% w/v).

Twelve strains were isolated from tomato plant roots in 2013. Sampling was performed in the previously described experimental field, but a specific protocol was used to isolate endophytic bacteria. Fresh roots were collected in sterile 50 ml falcon tubes with 25 ml P-buffer (per L: 6,33 g of $NaH_2PO_4.H_2O$; 16,5 g of $Na_2HPO_4.7H_2O$; 200 µl Tween 40). Samples were washed several times with P-buffer until the roots were free of all attached soil particles, then distilled H_2O was used to remove foam (Tween 40). Sterilization was performed using a 5% sodium hypochlorite solution for 5-10 minutes (depending on plant material), and then roots were washed 4-5 times in sufficient volumes of new sterile water. After washing, samples were cut in smaller fragments with a sterile razor blade and crushed with sterile mortar and pestle in sterile 10 mM $MgSO_4$-solution (1 gram roots in 10 ml). Serial 10-fold dilutions of the suspensions were prepared, and 100 µl aliquots were streaked on modified Luria-Bertani (LB) plates. To check surface sterility of the roots, 100 µl of the last rinsing water was plated LB medium.

Work package 2: Genotypical analysis of the isolates

Total genomic DNA was extracted using the DNeasy® Blood and Tissue kit (Qiagen). Two primers, the universal 1392R (5’-ACGGGCGGTGTGTGTRC-3’) and the bacteria-specific 26F (5’-AGAGTTTGATCCTGGCTCAG-3’), were used for the amplification. Genotypic characterization was performed by amplified rDNA restriction analysis (ARDRA) of their 16S rRNA gene and identification by 16S rRNA gene sequencing.

Work package 3: Screening of the isolates on the basis of their ability to promote plant-growth

Plant-associated microbes can enhance plant health through different mechanisms. Growth-promoting strains can be classified as biofertilizers, phytostimulators or biocontrol agents (Bloemberg G and Lugtenberg 2001). Biofertilizing strains can fix nitrogen or increase availability of phosphorous and iron. Phytostimulators enhance plant growth in a direct way, usually by the production of phytohormones (auxins, cytokinins, gibberellins). Biocontrol agents protect plants from infections by phytogens (through competition for nutrients, induced systemic resistance, production of antimicrobial secondary metabolites). In order to perform a screening of Plant Growth Promoting (PGP) traits of the isolates, specific bioassays were carried out to detect ACC deaminase (Belimov *et al.* 2005), organic acids (OA) (Cunningham and Kuiack, 1992), Indole acetic acid (IAA) (Patten and Glick, 2002) and siderophore (SID) (Schwyn and Neilands, 2007) production.

- **Description of the main results obtained**

  - **Work package 1**: Isolation of bacteria associated with different tomato cultivars

Eleven bacterial strains, colonizing the rhizosphere of tomato plants, were isolated in 2012. Samples were collected in an experimental field situated in the Campania Region (southern Italy).

Twelve strains were isolated from tomato plant roots in 2013. Sampling was performed in the previously described experimental field, but a specific protocol was used to isolate endophytic bacteria. Results are presented in Tab. 1.

Work package 2: Genotypical analysis of the isolates

Total genomic DNA was extracted using the DNeasy® Blood and Tissue kit (Qiagen). Two primers, the universal 1392R (5'-ACGGGCGGTGTGTGTRC-3') and the bacteria-specific 26F (5'-AGAGTTTGATCCTGGCTCAG-3'), were used for the amplification. Genotypic characterization was performed by amplified rDNA restriction analysis (ARDRA) of their 16S rRNA gene and identification by 16S rRNA gene sequencing. Results are shown in a Tab. 1.

2012		***2013***	
Sample name	***Genotypic characterization***	**Sample name**	***Genotypic characterization***
[1] LIC 2 RA/1	*Ensifer* sp.	[2] SUPER-13-2	*Pseudomonas* sp.
[3] SM GIALLO RB/1	*Microbacterium* sp.	[3] SUPER-13-3	n.c.
[4] LIC 2 RB/2	*Chryseobacterium* sp.	[4] SUPER-13-4	*Rhizobium* sp.
[5] INDIGO PERÚ SA/1	*Bacillus* sp.	[5] SUPER-13-5	*Rhodococcus* sp.
[7] SUPER SM RA/1	n.c.	[6] SUPER-13-6	*Agrobacterium* sp.
[8] INDIGO PERÚ RB/1	n.c.	[7] BLACK-13-1	*Agrobacterium* sp.
[10] LIC 2 RB/1	*Bacillus* sp.	[9] BLACK-13-3	*Agrobacterium* sp.
[11] SM GIALLO RB/2	*Microbacterium* sp.	[10] BLACK-13-5	*Rhizobium* sp.
		[11] SM-13-1	*Agrobacterium* sp.
		[12] SM-13-2	*Agrobacterium* sp.

Tab.1 Isolation and genotypical analysis of bacteria associated with different tomato cultivars

  - **Work package 3**: Screening of the isolates on the basis of their ability to promote plant-growth

Plant-growth-promoting bacteria have the potential to improve plant health via three mechanisms: phytostimulation, biofertilization and biocontrol. The production of plant hormones such as indole-

3-acetic acid (IAA) is classified as “direct” promotion. The synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase can be included in the same group: ACC deaminase sequester and cleave ACC, the immediate precursor of ethylene, and thereby inhibit its biosynthesis; ethylene inhibit roots and shoots growth, therefore lower levels of this plant hormone lead plant-growth promotion. Siderophores production is also considered an indirect biocontrol trait; iron chelation limits in fact the amount of trace metals available to potential plant pathogens (Gaiero *et al.* 2013). Therefore, all isolates were tested to assess plant growth promotion and plant disease control activities. Specific bioassays were carried out to detect ACC deaminase, organic acids (OA), Indole acetic acid (IAA) and siderophore (SID) production. Results are displayed in a Tab. 2 and Tab.3.

	Sample name	***Genotypic characterization***	**No Fe(III) citrate**	**0.25 µM Fe(III) citrate**	**3 µM Fe(III) citrate**
2012	[1] LIC 2 RA/1	*Ensifer* sp.	++	++	+/-
	[3] SM GIALLO RB/1	*Microbacterium* sp.	-	-	-
	[4] LIC 2 RB/2	*Chryseobacterium* sp.	+	+	-
	[5] INDIGO PERÚ SA/1	*Bacillus* sp.	+	+	+/-
	[10] LIC 2 RB/1	*Bacillus* sp.	+	+	+/-
	[11] SM GIALLO RB/2	*Microbacterium* sp.	-	-	-
2013	[2] SUPER-13-2	*Pseudomonas* sp.	+++	++	+
	[4] SUPER-13-4	*Rhizobium* sp.	+++	+++	++
	[5] SUPER-13-5	*Rhodococcus* sp.	+++	+++	+
	[6] SUPER-13-6	*Agrobacterium* sp.	+++	+++	++
	[7] BLACK-13-1	*Agrobacterium* sp.	+++	+++	++
	[9] BLACK-13-3	*Agrobacterium* sp.	+++	+++	++
	[10] BLACK-13-5	*Rhizobium* sp.	+++	+++	+
	[11] SM-13-1	*Agrobacterium* sp.	+++	+++	++
	[12] SM-13-2	*Agrobacterium* sp.	+++	+++	++

Tab.2 Evaluation of siderophore production ability of selected bacterial isolates

	Sample name	*Genotypic characterization*	OA	IAA	ACC deaminase
2012	[1] LIC 2 RA/1	*Ensifer* sp.	+	++	-
	[3] SM GIALLO RB/1	*Microbacterium* sp.	+	+	+/-
	[4] LIC 2 RB/2	*Chryseobacterium* sp.	-	+	-
	[5] INDIGO PERÚ SA/1	*Bacillus* sp.	-	-	+/-
	[7] SUPER SM RA/1	n.c.	n.c.	++	++
	[8] INDIGO PERÚ RB/1	n.c.	n.c.	++	++
	[10] LIC 2 RB/1	*Bacillus* sp.	+++	-	++
	[11] SM GIALLO RB/2	*Microbacterium* sp.	++	+	-
2013	[2] SUPER-13-2	*Pseudomonas* sp.	-	+	+++
	[3] SUPER-13-3	n.c.	n.c.	++	++
	[4] SUPER-13-4	*Rhizobium* sp.	+++	++	++
	[5] SUPER-13-5	*Rhodococcus* sp.	-	+	++
	[6] SUPER-13-6	*Agrobacterium* sp.	+++	+++	++
	[7] BLACK-13-1	*Agrobacterium* sp.	++	+++	++
	[9] BLACK-13-3	*Agrobacterium* sp.	+	+++	++
	[10] BLACK-13-5	*Rhizobium* sp.	+++	+++	+
	[11] SM-13-1	*Agrobacterium* sp.	+++	+++	+++
	[12] SM-13-2	*Agrobacterium* sp.	+++	+++	+++

Tab.3 Plant growth promoting (PGP) traits of selected bacterial isolates: ACC deaminase, organic acids (OA) and Indole acetic acid (IAA) production

- Conclusions

In total 23 strains associated with tomato roots were isolated from different tomato cultivars and new tomato hybrids. The isolates were obtained with two different protocols; respectively, in 2012 eleven bacterial strains were isolated from the **rhizosphere** of tomato plants, while in 2013 twelve **endophytic** strains were isolated from tomato plant roots. Samples were collected in an experimental field situated in the Campania Region (southern Italy). After the isolation, only the 65.2% of the strains showed cultivable, therefore we proceeded with the genotypical analysis of 15 bacteria (6 isolated in 2012 and 9 in 2013). In a second experimental session, it was possible to restart the growth after the isolation step of other 3 strains, for a total of 18 cultivable bacteria (78.3%, 8 isolated in 2012, 10 in 2013). It was not possible to perform

genotypical analysis and evaluate organic acids (OA) and siderophore production ability of these 3 strains, therefore we have only partial results for them. On the 15 strains that were genotypically analyzed, 5 are belonging to genus *Agrobacterium*, 2 to genera *Microbacterium*, *Bacillus* and *Rhizobium*, 1 to genera *Ensifer*, *Chryseobacterium*, *Pseudomonas* and *Rhodococcus*. 73.3% were able to produce organic acids (OA), 88.9% Indole acetic acid (IAA), 83.3% ACC deaminase and 86.7% siderophores. It's interesting to underline that bacteria belonging to genus *Agrobacterium* were positive for all tested traits.

- **Future collaboration with host institution**

My visit in one of the leading research group in the field of soil-plant-microbe interactions, like Environmental Biology - Centre for Environmental Sciences of Hasselt University, will result in strengthening of the connection between groups working in similar field, hope, receiving of good scientific results. Moreover, further analysis need to be performed in order to complete the research that is subject of this STSM report. Part of the work will be carried out at the CNR - National Research Council of Italy - Institute of Bimolecular Chemistry, in particular the most promising strains will be selected for preliminary assays to detect QS activity. Signal molecules will be isolated and purified by means chromatographic techniques, and the isolated compounds will be chemically characterized by LC-MS analysis. In order to verify the effects of microbial inoculation on root development, a Vertical Agar Plate (VAP) assay will be performed at Hasselt University - Centre for Environmental Sciences in near future. The assay will be carried out at first inoculating *Arabidopsis thaliana* seedlings, then a new protocol will be designed to perform the same assay using tomato seedlings.

- **Foreseen publications/articles resulting or to result from the STSM**

The results obtained during the STMS will be presented at national and international conferences. Further analysis need to be carried out at the CNR - National Research Council of Italy - Institute of Biomolecular Chemistry, but I expect to write one or two paper(s) in collaboration with the host institute. The paper(s) will be submitted to *Microbial Ecology* or to another good impact factor journal in the field of plant-microbe interactions.

- **Confirmation by the host institution of the successful execution of the STSM**

Prof. Jaco Vangronsveld certifies that Dr. Gennaro Roberto Abbamondi successfully executed his work in UHasselt laboratories on the role for plant-associated endophytes in improving production and quality of tomato (in the frame of COST-action COST-STSM-ECOST-STSM-FA1103-090914-049031 grant) from 09/09/2014 to 24/10/2014. He certifies that Dr. Abbamondi did a very good job and his work will lead to the submission of a manuscript in near future. This grant also strengthened the collaboration with Dr. Giuseppina Tommonaro from the CNR - National Research Council of Italy - Institute of Biomolecular Chemistry.

- **References**

Abbamondi GR, De Rosa S, Iodice C and Tommonaro G. (**2014**) Cyclic Dipeptides Produced by Marine Sponge-Associated Bacteria as Quorum Sensing Signals. *Natural Product Communications*, 9(2), 229-232.

Adesemoye AO & Kloepper JW. (**2009**) Plant–microbes interactions in enhanced fertilizer-use efficiency. *Appl Microbiol Biotechnol*, 85, 1–12.

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR. (**2005**) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea L. Czern.*). *Soil Biology & Biochemistry*, 37, 241-250.

Berg G. (**2009**) Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol*, 84, 11–18.

Bloemberg G and Lugtenberg BJJ. (**2001**) Molecular basis of plant growth promotion and biocontrol by *rhizobacteria*. *Curr Opin Plant Biol*, 4, 343–350.

Cunningham JE and Kuiack C. (**1992**) Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. *Applied Environmental Microbiology*, 58, 1451-1458.

Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, Dunfield KE. (**2013**) Inside the root microbiome: bacterial root endophytes and plant growth promotion. *Am J Bot*, 100(9):1738-50.

Gunatilaka, AAL. (**2006**) Natural Products from Plant-associated Microorganisms: Distribution, Structural Diversity, Bioactivity, and Implications of Their Occurrence. *J Nat Prod*, 69(3), 509-526.

Montesinos E. (**2003**) Plant-associated microorganisms: a view from the scope of microbiology. *Internat Microbiol*, 6(4), 221-223.

Patten C and Glick B. (**2002**) Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology*, 68, 3795-3801.

Schwyn B and Neilands J. (**1987**) Universal chemical assay for the detection and determination of siderophores. *Analitical Biochemistry*, 160, 47-56.

Tommonaro G, Abbamondi GR, Iodice C, Tait K, De Rosa S. (**2012a**) Diketopiperazines Produced by the Halophilic *Archaeon*, *Haloterrigena hispanica*, Activate AHL Bioreporters. *Microb Ecol*, 63(3):490-495.

Tommonaro G, Caporale A, De Martino L, Popolo A, De Prisco R, Nicolaus B, Abbamondi GR and Saturnino C. (**2014**) Antioxidant and cytotoxic activities investigation of tomato seed extracts. *Nat Prod Res*, 28(10), 764-768.

Tommonaro G, De Prisco R, Abbamondi GR and Nicolaus B. (**2013**) Bioactivity of Tomato Hybrid Powder: Antioxidant Compounds and Their Biological Activities. *J Med Food*, 16(4), 351-356.

Tommonaro G, de Prisco R, Abbamondi GR, Marzocco S, Saturnino C, Poli A and Nicolaus B. (**2012b**) Evaluation of Antioxidant Properties, Total Phenolic Content, and Biological Activities of New Tomato Hybrids of Industrial Interest. *J Med Food*, 15(5), 483–489.

Weyens N, van der Lelie D, Taghavi S and Vangronsveld J. (**2009**) Phytoremediation: plant–endophyte partnerships take the challenge. *Curr Opin Biotechnol*, 20(2), 248-54.