COST STSM Report

Reference Code: ***ECOST-STSM-FA1103-121015-069926***

Participant: ***Dr. Michele Ferrari***

Period: ***From 09/11/2015 to 07/12/2015***

Host institution: ***Flinders University of South Australia, Adelaide, Australia***

Host: ***Prof. Christopher M.M. Franco, Flinders University***

"Actinobacteria: Isolation, identification, characterization and their possible use in biological control"

ABSTRACT

In recent years, new actinobacteria species have been isolated as endophytes from plants and are sought after for the role of bio-control inoculants for sustainable agriculture (1).

In particular, my studies focus on the isolation of some endophytic actinobacteria from tomato, with a potential antagonistic activity against the causal agent of bacterial canker of tomato: *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*).

Cmm is a plant-pathogenic bacterium belonging to the order of *Actinomycetales*.. It infects tomato plants, spreads through the xylem and causes bacterial wilt and canker which can be considered to be the most important bacterial disease of tomato causing substantial economic losses worldwide (2).

PURPOSE OF THE STSM:

Task 1: Collection of plant material:

Tomato fruits from different agro-systems (glasshouses, tunnels, open field) and cultivation methods (organic, non-organic tomatoes) were considered as a source of endophytic actinobacteria. Tomato placental tissue from fruits was used to isolate the actinobacteria.

Task 2: Establishment of an actinobacteria collection

A collection of different isolates will be established, keeping a separation among strains coming from organic cultivation, glasshouses, tunnels and open field. A comparison will be done between sets of isolates coming from different cultivation conditions. The whole collection will be assayed in order to search possible endophytes with a strong antagonistic activity against *Clavibacter michiganensis* subsp. *michiganensis in vitro* for the selection of prospective actinobacteria to be used as bio-antagonist in field experiments.

INTRODUCTION:

Clavibacter michiganensis subsp. *michiganensis* (the causal agent of bacterial canker of tomato)

Cmm is the causal agent of bacterial canker of tomato (*Lycopersicon esculentum*). Cmm is the major concern worldwide, included in the EPPO A2 quarantine pest list and regulated according the European Union Directive 2000/29/EU. The pathogen spreads through infected seeds (seed-borne pathogen) and transplants, and such propagation plant materials are responsible for long distance dissemination. Tomato is cultivated throughout Europe and conditions are conducive to disease development in open fields in southern Europe and greenhouses. The disease causes a range of symptoms on aerial parts, including fruits.

Seed-borne pathogens of tomato represent a serious threat to modern agricultural cropping systems, as they can be disseminated to many geographical regions around the world. The quality control, as seed-health testing including detection, is an important step to prevent the introduction and spread of these harmful pathogens, considering the increasing of the trend of global seed production and trade.

- **Environmental role of the actinobacteria**

Actinobacteria are a phylum of Gram positive bacteria, formerly known as actinomycetes (from Greek "*actis*" ray, beam and "*mykes*", fungus),.. Morphologically they resemble fungi because of their elongated cells that branch into filaments or hyphae. They are all gram-positive, facultatively aerobic (3) (4).

Actinobacteria is one of the dominant bacterial phyla and contains one of the largest of bacterial genera, *Streptomyces* (5). The 16S rRNA gene sequence is normally used for phylogenetic classification of actinobacteria. Actinobacteria are responsible for the peculiar odour emanating from the soil, mainly in warmer climates. *Streptomyces* is the main genus responsible for decomposition of organic matter (6). During the process of composting mainly thermophylic (adapted to high temperatures) and thermo-tolerant actinobacteria degrade natural substances such as chitin or cellulose. During the initial phase of composting the intensive increase of microbial activity leads to self heating of the organic material. High temperatures in composting help to kill viruses, pathogenic bacteria and weed seeds present.

Actinobacteria are known as secondary metabolite producers, in particular antibiotics (7), antifungals (8) and hence of high pharmacological and commercial interest.

In 1940 Selman Waksman discovered that the soil bacteria he was studying made *Streptomycin*, a discovery for which he received a Nobel Prize. Since then, hundreds of naturally occurring antibiotics have been discovered in these terrestrial microorganisms, especially from the genus *Streptomyces* (5).

Actinobacteria need oxygen for their metabolism because they live predominantly aerobically. The compost material should therefore be well aerated. Generally, actinobacteria grow on fresh substrates more slowly than other bacteria and fungi.

Actinobacteria inhabit plants (9) (10), including a few plant pathogens, such as *Clavibacter* spp., *Rhodococcus fascians, Streptomyces scabies, S. turgidscabies, S. acidiscabies, S. europaeiscabies.*

MATERIAL AND METHODS:

- Collection of plant material

Organic tomato fruits (642 g) were used as the source of plant material. Tomato fruits, called from now on “the sample”, were cut horizontally by a sterile scalpel and the placental tissues extracts by sterile spoon. Placental tissues (containing gelatinous membranes and seeds) were collected from each of the fruits, placed in sterile Petri dishes and crumbled into small pieces until a heterogeneous

reddish blight suspension was obtained. The seeds were separated from the suspension by centrifugation.

- Surface sterilisation of fruits

Before starting the collection of the material, the sample was washed thoroughly with tap water, than washed once in sterilised reverse osmosis (RO)-treated water and gently dried on a paper towel. The sample was immersed in sodium hypochlorite solution (3% available chloride, freshly prepared) for 2 minute, followed by washing in sterile water five times to remove the chemicals. The epicarps of the fruits were plated by rolling onto different media to evaluate the effectiveness of the surface sterilisation and potentials contaminations recorded.

- Isolation method

Approximately 5 -10 g of small tomato pieces and suspension were plated onto 4 isolation media in triplicate for each medium. Furthermore, different pH of the media (5,5, and 7,4) and incubation temperatures (27° and 37° C) were tested. The isolation media were:

1. Humic acid vitamin B agar (HVA) (Hayakawa MT, 1987)
2. Tap water yeast extract (TWYE)
3. VL70 gellan gum with a mixture of D-galacturonate, D-glucoronate, L-ascorbate, and D-gluconate (GGAG, 0,5 mM of each substrate)
4. VL70 gellan gum with a mixture of D-glucose, D-galactose, D-xylose, and L-arabinose (GGXA, 0,5 mM of each sugar)

The composition of VL70 medium was taken from *Joseph et al.* (11). The pH of all media was adjusted to 7,2. Each medium was supplemented with 10 mg/ml nystatin as antifungal agent. Plates were kept in small sealable plastic boxes which were lined with wet paper towels to maintain the moisture levels during the long incubation times and incubated at 27° and 37° C.

- Purification of isolates

Isolation plates are examined weekly. Emergence time of each colony was recorded, and whole colonies were removed from the isolation plates every week and purified. Colonies were purified by streaking onto half strength potato dextrose agar (HPDA, Difco) plates. Pure cultures were maintained on HPDA slants at 4° C and in 20% glycerol at -80°C, for further study.

Fig. 1,2: Collection of plant material (left) and surface sterilisation (right).

Fig. 3,4: Preparation of the sample (left) and plating on HVA and TWYA agar (right).

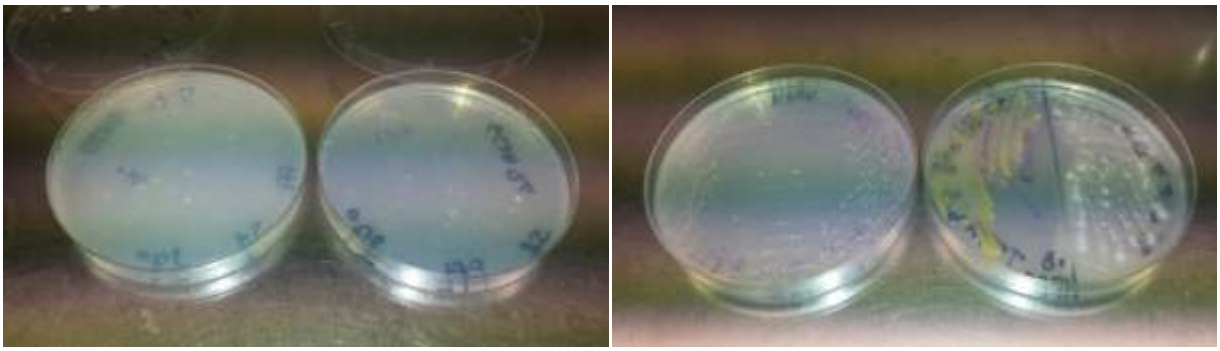

Fig.5,6: Endophytes colonies on selective media (VL70 GGAG and VL70 GGXA) on the left and their purification on HPDA (semi-selective medium) on the right.

RESULTS:

Endophytes Diversity and Isolation Media

Numbers of isolates (actinobacteria-like colonies) were collected from each isolation medium, at different pH and different incubation temperatures, are listed in table 1. During the next 2 weeks other endophytes will be isolated from plates to reach the number of 50 – 60 actinobacteria–like colonies for further analysis.

N. of Isolates	N. of weeks (after inocula 24/11/2015)	Medium	pH	Incubation T (°C)
2	1	HVA	7,4	27
5	1	TWYA	7,4	27
2	1	TWYA	5,5	27
4	1	VL70 (GGAG)	7,4	27
1	1	VL70 (GGAG)	5,5	27
3	1	VL70 (GGXA)	7,4	27
2	1	TWYA	7,4	37
1	1	TWYA	5,5	37
2	1	VL70 (GGAG)	7,4	37
1	1	VL70 (GGXA)	7,4	37
1	1	VL70 (GGXA)	5,5	37
1	2	TWYA	7,4	27
1	2	TWYA	5,5	27
1	2	VL70 (GGAG)	7,4	27
1	2	VL70 (GGXA)	7,4	27
1	2	VL70 (GGXA)	5,5	27
1	2	VL70 (GGXA)	7,4	37

Table 1: Number of endophytes emerged from different isolation plates at different times and different incubation temperatures.

pH	N. of isolates	Incubation T (°C)	N. of Isolates
5,5	7	27	22
7,4	23	37	8

Table 2: Total amount of endophytes emerged on plate after 2 weeks from the inocula related to the pH of the media and the incubation temperature.

CONCLUSION:

During the first and second weeks of incubation, a relationship between incubation time and pH of the medium was observed. As shown in Fig. 7,8,9,10, the highest number of isolates was obtained onto plates with pH 7,4 and 27°C incubation temperature was most appreciate than 37°C (first and second week).

This is an important aspect to continue the isolation of more endophytes from different parts of tomato plants (roots, stem and leaves). The high number of isolates obtained during the first and second growing week reveals the possibility to isolate novel actinobacterial strains able to colonize quickly the host plant vascular tissue. This encourages our further analysis in endophytes bio-control activity against bacterial canker of tomato caused by *Cmm*.

Further studies will be done during the next months on the isolated strains to identify actinobacteria from non-actinobacteria by morphology. Actinobacteria antagonism will be tested on *Cmm* and the most active will be characterize in order to obtain a collection for next *in-vivo* analysis.

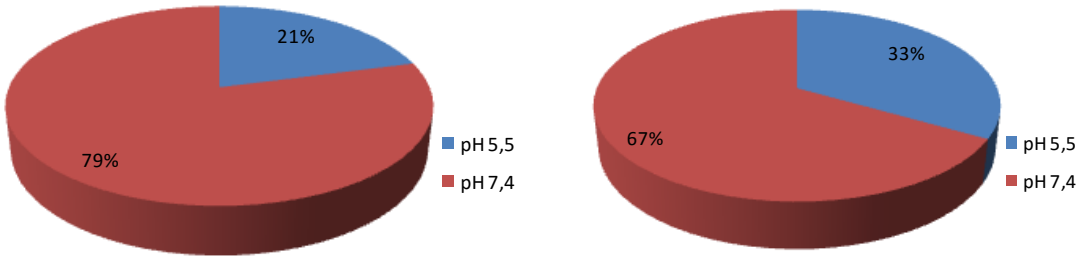

Fig. 7,8: Percentage of endophytes emerged on plate related to the pH of the media after 1 week (left) and 2 weeks (right) from tomato.

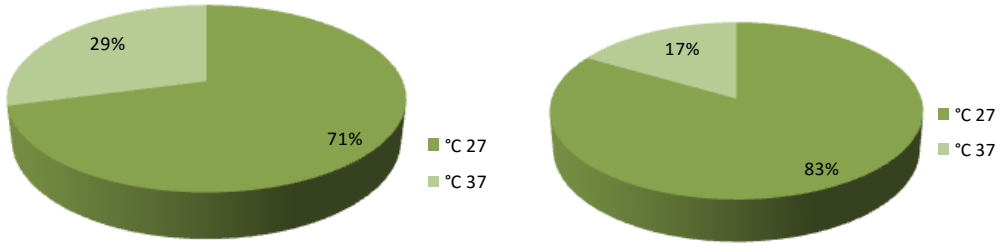

Fig. 9,10: Percentage of endophytes emerged on plate related to the incubation temperature after 1 week (left) and 2 weeks (right) from tomato.

REFERENCES:

1) *Kaewkla O., Franco C.M.M.* (2013) "Rational approaches to improving the isolation of endophytic actinobacteria from Australian native trees" Microb. Ecol. Plant Microbe Interaction. 65: 384-393
2) *Karl-Heinz Gartemann, Oliver Kirchner, Jutta Engemann, Ines Gräfen, Rudolf Eichenlaub, Annette Burger* (2003). "*Clavibacter michiganensis* subsp. *michiganensis*: first steps in the understanding of virulence of a Gram-positive phytopathogenic bacterium" Journal of Biotechnology 106(2003) 179-191.
3) *Ventura, M.; Canchaya, C.; Tauch, A.; Chandra, G.; Fitzgerald, G. F.; Chater, K. F.; van Sinderen, D. (5 September 2007).*"Genomics of actinobacteria: tracing the evolutionary history of an ancient phylum". *Microbiology and Molecular Biology Reviews 71 (3): 495–548.* doi*:*10.1128/MMBR.00005-07. PMC 2168647.PMID 17804669.
4) *Servin JA, Herbold CW, Skophammer RG, Lake JA (January 2008).*"Evidence excluding the root of the tree of life from the Actinobacteria". *Mol. Biol. Evol. 25 (1): 1–4.*doi*:*10.1093/molbev/msm249. PMID 18003601.
5) *C.Michael Hogan*. (2010). Bacteria. Encyclopedia of Earth. eds. Sidney Draggan and C.J.Cleveland, National Council for Science and the Environment, Washington DC.
6) *Ningthoujam, Debananda S.; Tamreihao, SuchitraSanasam K.; Nimaichand, Salam (2009).* "Test". Afr. J. Microbiol. Res. 3 (11): 737–742.
7) *Mahajan, GB (2012).* "Antibacterial agents from actinobacteria - a review". Frontiers in Bioscience 4: 240–53.doi:10.2741/e373.
8) *Gupte, M.; Kulkarni, P.; Ganguli, B.N. (2002).* "Antifungal Antibiotics". Appl. Microbiol. Biotechnol 58: 46–57.
9) *Bressan, W (2003).* "Biological control of maize seed pathogenic fungi by use of actinobacteria". Biocontrol 48 (2): 233–240.doi:10.1023/a:1022673226324.
10) *Atta, M.A (2009).* Austral. J. Basic and Appl. Sci. 3: 126–135.
11) *Joseph SJ, Hugenholtz P, Sangwan P, Osborne CA, Janssen PH* (2003) Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl Environ Microbiol 69:7210-7215.