Scientific report of a Cost STSM

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STSM topic: Application of in vitro tissue culture to the propagation of some rootstocks and isolation of corresponding endophyte consortia

Host: Dr. Martin Schumacher (mas@dsmz.de) in The DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures), Braunschweig (Germany)

Period: 1/05/2014 to 30/06/2014

Place: Braunschweig (Germany)

Problematic and objectives of the STSM:

In recent decades a new area of research has opened up in the field of microbiology, as the scientific community cottoned on the importance of endophytic microorganisms in plants life cycle. Endophytes are organisms which find their ecological niche in the plants. They do not cause damages to the colonized vegetable and, in many cases; they may even bring them numerous benefits.

Plant tissue culture technology is now extensively used for many purposes such as large scale propagation, improvement, conservation and bioactive molecules production. Tissue culture today plays also an important role in the study and the preservation of plant genetic resources and their corresponding endophyte consortia.

Utilization of pesticides and chemical fertilizers in agriculture is increasing. This is obviously the origin of many problems related to human health, in particular, and to ecosystems misbalance in general. There are currently many resistance systems that are explained by endophytes, starting with understanding the details.

The objectives of my mission were:

- Isolation of bacteria from *in vitro* tissue cultures of date palm and some rootstocks that have the ability to resist to biotic and abiotic stress.
- Characterization of such microorganisms using molecular and biochemical approaches.

Description of the work carried out during the mission and the main results obtained

Endophytic bacteria were isolated from *in vitro* tissue cultures of date palm, Garnem, Myrobolan and Paulsen.

Different types of media have been used to isolate and later to identify some bacteria strains:

- ✓ Middlebrook medium
- ✓ R2A medium
- ✓ 535 medium
- ✓ Potato medium
- ✓ Trypticase soy broth agar
- ✓ BSM medium

Bacterial indexing of tissue cultures and Electronic microscope observation:

We used one explants for each specimen to inoculate the different media (liquid and solid for each medium):

- ✓ Liquid media: The explant were cutting in small pieces and incubated in100 ml of liquid medium in the shaker® during 3 days.
- ✓ Solid media: containing solidifying agent agar (18 g/l). Small pieces were used to inoculate the culture media.

All bacteria were grown on plates at 22, 28 and 37 °C for 48 to 72 h. Liquid cultures were grown for 24 h at 28 °C.

Diverse isolated bacteria strains from date palm and the rootstock Garnem were observed by the electronic microscope.

Colonies of isolated bacterial were characterized for the following traits: color, form, elevation, margin, diameter, surface, opacity, and texture. Motility, morphology, size, and division mode were also evaluated by performing electronic microscopy at a magnification of 1,000 as described previously.

Endophytic bacteria colonizing date palm in *in vitro* cultures were isolated in samples from all varieties. They could be growing up in middlebrook and R2A media especially at 28 °C. These orange-yellow bacteria had been observed by electronic microscope. It could be one isolate as bacillus bacteria.

The second isolate from date palm could be proliferating in potato liquid medium and middlebrook solid medium at 28 °C. These white bacteria had been observed also in electronic microscope.

Any isolation from healthy samples of date palm has been detected in the different media but the fourth variety of date palm (probably disinfected) had been showing high bacterial density

in the different media especially at 28 °C. This mixture of bacteria has been observed by electronic microscope and different forms of bacteria have been detected as some motile one.

Some bacteria have been identified from the rootstock Garnem in 535 medium. The microscope observation showed high density of bacteria (different form, size...) and some motile bacteria have been observed.

No endophytic contamination from rootstock Myrobolan and Paulsen have been detected by inoculation in liquid and solid media.

Molecular identification:

Genomic DNA was extracted using standard methods with a cell lysis performed at 95 °C for 20 min. PCR was performed using the universal 16S rRNA primers. The thermocycler program was 94 °C for 15 s, 44 °C for 15 s, 72 °C for 30 s and it was repeated for 24 cycles.

Bacterial DNA was used also as template in polymerase chain reaction (PCR) with specific primers allowing amplification of 18S RNA gene to verify the presence of fungus in the bacterial mixture.

Samples were subjected to electrophoresis in a 1% agarose gel. The PCR products were purified using Qiagen gel purification® kit and sequenced. Nucleotide sequence identities were determined by the BLAST and seq matches programs.

Sequencing of about 500 bp (from 480 to 615 bp) of the 16S rRNA region was performed for 3 isolates from date palm (the white and the orange-yellow isolates) and the rootstock Garnem. By comparison with the BLASTn deposited sequences, the bacteria isolated from date palm (the orange one) could be classified as pathogenic bacteria intercellurar mycobacterium. The second isolate from Garnem rootstock could be classified as endophytic bacteria Herbaspirillium. It is noteworthy that this bacterium was present in all date palm vitroplants and we considered that as preliminary results.

Extraction of genomic DNA and cloning:

DNA molecules were extracted from leaves of different vitroplants following the protocol Kit “Invisarb Spin Plant Mini Kit”. This extraction was followed by electrophoresis in agarose gel (1%) was run for 1 hour at 120 V to check the quantity of DNA.

DNA was amplified by PCR using specific primers to amplify bacterial genome. Samples were subjected to electrophoresis in a 0.4% agarose gel. The positive bands were cutting and purified by Qiagen gel extraction kit® and DNA is ready to cloning.

Specific protocol using invitrogen Kit® was used, based at heating shock of E.coli to insert the vector. Positive clones of E. coli were growing in LB medium containing an antibiotic ampicillin (50mg/1000ml) at 37 °C overnight. Positive strains were used to extract bacterial DNA.

The Bacterial DNA was used also as template in polymerase chain reaction (PCR) with specific primers (M13) allowing amplification of insert gene. The positive results were purified using Qiagen purification kit® and it will be sequenced.

Future collaboration with host institution

The interaction with the host institution will continue to find results that can be published**.**

Foreseen publications/articles resulting or to result from the STSM (if applicable)

We expect to publish a paper with the collaboration of the host institute.

Confirmation of the successful execution of the STSM

Hereby we confirm that Miss. Ameni Nasri carried out a "short term scientific mission" in our laboratory. The results obtained were described in her report. We could successfully isolate several endophytic bacteria from in vitro date plant materiel by cultivation as well as by molecular methods. Most of the bacteria isolated still have to be characterized. Two bacteria could be characterized as Herbaspirillium spec. and Mycobacterium spec. The isolated Mycobacterium belongs to the Mycobacterium avium complex and a further characterization of both bacteria cannot be based only on 16S sequence data.

A further cooperation for the final characterization of the isolated bacteria is intended.