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: 01/10/2015 to 04/12/2015

Place: Braunschweig (Germany)

1. Introduction

Plant tissue culture is now extensively used for many purposes such as plant propagation and breeding. Tissue culture could be also used to preserve plant genetic resources and their corresponding endophytic bacteria.

Utilization of pesticides and chemical fertilizers in agriculture is increasing. This is obviously the origin of many human health problems and ecosystem misbalances.

The objectives of my mission are:

- Isolation of endophytes from date palm and some rootstocks in vitro tissue cultures.
- Characterization of such microorganisms using molecular and biochemical approaches.

2. Description of the work carried out during the mission:

2.1. Material and methods

2.1.1. Plant material

In this training, I used diverse plant material samples to isolate endophytes:

- Clean date palm vitrocultures - Contaminated date palm vitrocultures - Apparently clean but potentially contaminated date palm vitrocultures.
- Garnem vitrocultures.
- Myrobolan vitrocultures.
- Paulsen vitrocultures

2.1.2. Methods

Bacterial indexing using standard microbial media

Small pieces from vitrocultures (root, leaves, callus and stem) were cultivated on bacterial culture media. All bacteria were grown on plates in different temperature: room temperature (23°C), 28°C and 37 °C for 48 to 72 hours.

In addition, six strains of bacteria, preserved in glycerol from the last year (isolated during my last STSM), were selected and reactivated by new inoculation: Three strains from callus contaminated by the yellow bacteria and three from callus contaminated by white bacteria. For this purpose, different media have been used: Middlebrook medium, Trypticase soy broth medium, R2A medium and Potato medium.

Macroscopic and microscopic observations

Colonies 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.

Biochemical characterization

- ✓ Biochemical basic tests

Preliminary biochemical characterization of endophytic bacteria was carried out using standard methodologies:

Amino peptidase activity, KOH reaction, catalase and oxidase activities were determinate for all strains.

- ✓ Bacteria identification using API galleries and BIOLOG system

An API-20NE gallery was used for the identification of some strains. The API identification is a carbon source utilization test, but it relies on visual detection of the test bacterium. The BIOLOG system detects carbon source utilization with the reduction of tetrazolium dye in response to cellular respiration. The results were compared with a response database of Gram negative and positive bacteria, yeast and lactic bacteria. Five isolates were tested for C-source utilization pattern and identified using BIOLOG System kits. Bacterial isolates were raised on BIOLOG Universal Growth (BUG) medium and 24 hours growing cultures were then suspended in Phosphate-buffered Saline (PBS), adjusted to required optical density and inoculated to the 96 well BIOLOG plates, 95 wells contain different C substrates. Plates were incubated at 28°C and observed for color development at intervals of 24 hours. Color

development pattern was compared to the database and isolates were identified at species level.

Molecular characterization: Amplification of DNA coding for 16S rRNA and sequencing

Molecular identification of bacteria involved the extraction of bacterial genomic DNA, PCR amplification and sequencing of the 16S rRNA gene. The bacterial genomic DNA was extracted from pure bacterial cultures using traditional protocol based on cell lysis at 95 °C for 20 min. Thereafter, the 16S rRNA gene was amplified using the universal 16S rRNA primers. The thermocycler program was 94 °C for 15 s, 44 °C for 15 s and 72 °C for 30 s for 24 cycles.

The PCR products were resolved in a 1% agarose gel. The PCR products were purified using Qiagen gel purification® kit and sequenced. Finally, the 16S rRNA gene sequence of each bacterial isolate was compared with known 16S rRNA gene sequences in the GenBank database using the BLAST (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]).

3. The main results obtained

3.1. Morphological characterization

Our results showed that bacteria isolated from contaminated Date palm vitrocultures and stored in glycerol, were able to re-grow. In Table 1, we describe these endophytic bacteria.

Table1. Macroscopic characterization of Date palm endophytic bacteria

Bacterial strains reference	Optimal temperature (°C)	Medium	Colonies description
D2/26 A	23	R2A	Yellow, small, smooth, round
D2/26 B	23	R2A	Yellow, small, smooth, circular
D2/26 C	23	R2A	White, small, smooth, fast rate of growth
D2/26 D	23	R2A	White, small, fast rate of growth
D2/26 E	23	R2A	Yellow, small, slimy, round
D2/27F	23	R2A	Yellow, small, smooth, round
D2/27G	23	R2A	White, small, smooth, round
D1/31	23	Middlebrook	Big, rough
D1/31-S-SC	37	Middlebrook	White, small, circular, low rate of growth
D1/31-S-BC	37	Middlebrook	Yellow, round, low rate of growth
D1/35	37	Middlebrook	Yellow, round, low rate of growth
D1/36	37	Middlebrook	

In the same case, we tried to isolate new bacteria from new plant material. In Table 2, there is a description of the isolates.

Table2. Macroscopic characterization Date palm and Garnem endophytic bacteria

Bactrial strains reference		**Optimal temperature(°C)**	**Medium**	**Colonies description**
Date palm	1-1	37	Middlebrook	White, smooth, fast rate of growth
	1-2	37	Middlebrook	White, smooth, fast rate of growth
	2-4	23	Trypticase soy broth	Yellow, big, smooth, fast rate of growth
	2-5	37	Trypticase soy broth	White, smooth, fast rate of growth
	2-6	37	Trypticase soy broth	Yellow, smooth, round
	2-9	37	Trypticase soy broth	White, smooth, fast rate of growth
	2-11	37	Trypticase soy broth	white, big, smooth, round
	4-1	28	Middlebrook	white, small ,round
	8-1	28	Middlebrook	White, small ,rough
Garnem	5-1	37	Middlebrook	White, small, low rate of growth
	5-2	23	Middlebrook	white, round, fast rate of growth
	5-3	28	R2A	
	5-4	28	R2A	

It is important to specify that we failed to isolate endophytic bacteria from Myrobolan and Paulsen rootstock.

3.2. Biochemical characterization

Biochemical basic analyses are illustrated in table 3.

Table 3. Characterization of several bacteria by biochemical reactions

Strain reference	Amino-peptidase	KOH	Catalase	Oxidase
D2/26 A	-	-	+	-
D2/26 B	-	-	+	-
D2/26 C	-	-	+	+
D2/26 D	-	-	+	-
D2/27E	-	-	+	+
D2/27F	-	-	+	+
D2/27 G	-	-	+	-
D1/31 S-SC	+	+	+	+
D1/31 S-BC	-	-	+	+
2-4	+	+	+	+
2-5	-	-	+	+
2-11	-	-	+	+
4-1	-	-	-	-
8-1	-	-	-	-
5-1	+	+	+	+
5-2	+	+	+	+
5-3	+	+	+	+
5-4	+	+	+	+

+: positive test; -: negative test

For Gram-negative bacteria, colonies were tested by API-20NE to identify the genera of bacteria (Table 4).

Table 4. Characterization of some Gram-negative isolates based on API-20NE

	Bacterial strain references		
	5-3	**5-2**	**5-4**
NO3	+	-	-
TRP	-	-	-
GLU	-	-	-
ADH	+	+	-
URE	-	+	-
ESC	-	-	-
GEL	-	-	-
PNG	-	-	-
GLU	+	+	+
ARA	+	+	+
MNE	+	+	+
MAN	+	+	+
NAG	+	+	+
MAL	-	-	-
GNT	+	+	+
CAP	+	+	+
ADI	-	-	-
MLT	+	+	+
CIT	+	+	+
PAC	-	-	-
OX	+	+	+

Identification of five isolates was done using BIOLOG "Micro Station System" that is an automated identification system. Test result yielded a characteristic pattern of substrate utilization of each endophyte, which was compared to a current database. Table 5 gives the identity of seven isolates by API-20NE and BIOLOG.

Table 5. Identification of some isolates based on API-20NE and BIOLOG system

	Bacterial strain references	**Samples**	**Name**	**Probability (%)**
API 20 NE	5-3	Garnem	*Pseudomonas fluorescens*	99
	5-2	Garnem	*Pseudomonas fluorescens*	99
	5-4	Garnem	*Pseudomonas fluorescens*	99
	D1/31 S-SC	Date palm	*Achromobacter xylosoxidans*	99
BIOLOG system	D1/31 S-SC	Date palm	*Achromobacter grignonense*	76
	D1/31 S-BC	Date palm	*Achromobacter grignonense*	89
	D2/26 C	Date palm	*Achromobacter xylosoxidans*	88
	D2/27 F	Date palm	*Achromobacter xylosoxidans*	89
	5-2	Garnem	*Acinetobacter baumannii*	63

3.3. Molecular characterization (Sequencing):

Sequencing of about 500 bp of the 16S ribosomal DNA region was performed for 17 isolates from Date palm and Garnem vitrocultures. By comparison with the BLASTn deposited sequences, the bacteria could be classified in 7 different genera; the most important bacteria isolated from date palm (the orange one) could be classified as pathogenic bacteria like *Mycobacterium gordonae*. The second isolate from Garnem vitroplant could be classified as endophytic bacteria *Pseudomonas fluorescens*.

Table 6. Identification of some isolates by sequencing

Strain reference	Accession number	Base pairs	Genus	Identity (%)
D2/26 A	LN651153.1	550	*Brevebacterium permense*	100
D2/26 B	KF876886.1	550	*Brevebacterium avium*	100
D2/26 D	LN651153.1	560	*Brevebacterium permense*	100
D2/27E	FR799434.1	580	*Tsukomurella pseudopumae*	100
D2/27F	KT726993.1	580	*Ochrobactrum pseudogrignonense*	100
D1/36	KT347502.1	560	*Mycobacterium gordonae*	100
D1/31 S-SC	KM488321.1	550	*Achromobacter xylosoxidans*	100
D1/31 S-BC	KT005456.1	550	*Achromobacter xylosoxidans*	100
1-1	KT347502	530	*Mycobacterium gordonae*	100
1-2	KT347502	540	*Mycobacterium gordonae*	100
2-3	KT726993	540	*Ochrobactrum pseudogrignonense*	100
2-5	EF173324	530	*Paenibacillus favisporus*	100
2-11	LN890143	568	*Paenibacillus favisporus*	100
4-1	HE575950	560	*Mycobacterium paragordonae*	100
8-1	HE575950	580	*Mycobacterium paragordonae*	100
5-3	KT350501	520	*Pseudomonas fluorescens*	100
5-4	KT350501	530	*Pseudomonas fluorescens*	100

4. Future collaboration with host institution

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

5. 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.

6. Confirmation by the host institute of the successful execution of the mission

Hereby, we confirm that Mrs. Ameni Nasri carried out a Short Term Scientific Mission (STSM) in the context of EU COST Action FA 1103 “Endophytes in Biotechnology and Agriculture”.

Ameni Nasri stayed in our laboratory from the 30th of September until the 11th of December 2015 and worked on the isolation and characterization of endophytic bacteria from Date palm in-vitro plantlets.

Work to finalize the characterization of isolated bacteria will be carried on.