FA COST Action FA1103 Endophytes in Biotechnology and Agriculture

EU COST FA1103 Training School 2014: „ Isolation and characterization of fungal root endophytes “

Locality: Leibniz-Institute of Vegetable and Ornamental Crops (IGZ)
Theodor-Echtermeyer-Weg 1, D-14979 Großbeeren (Close to Berlin), Germany

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Surface sterilization of root fragments

1. Wash with a brush under running tap water the root system or root samples to remove soil particles.
2. Keep the root under running tap water (time depends on soil particles attached to the roots).
3. Select sections of the root for disinfection.
4. Immerse the roots for disinfection in 70% ethanol for 1 min. From this step onwards, work at sterile conditions.
5. Transfer root fragments to 2.5% NaOCl and keep for 5-10 min. (Time and hypochlorite concentration depend of the diameter and constitution of the root.)
6. Wash 3 to 6 times (each 1 min) with sterile distilled water in a different container (e.g. Falcon tube).
7. Take out the samples with sterile pincers and dry them on a sterile serviette or filter paper. (Time depends on the size of the root).
8. Divide the samples into three parts for DNA extraction, endophyte isolation and microscopy. Place root fragments for microscopy into 70% EtOH.

Isolation of endophytes

Placing root fragments on Petri dishes

1. Cut fragments of approximately 1 cm on a sterile paper using sterile pincers and sterile scalpel.
2. Verify the effectiveness of surface sterilization using the imprint technique (Schulz et al. 1999). For this place the fragment for 1 min on potato dextrose agar (PDA). Incubate these dishes at 25°C and control regularly for any growth of epiphytes. If epiphytes grow, discard the fragments on the dishes used for isolation.
3. Cut each fragment on new sterile paper into three pieces and transfer each piece to Petri dishes containing e.g. PDA, Sabouraud dextrose agar, 2% malt extract agar and water agar (WA). It is recommended to use also a root extract as medium. For fungal endophytes the media should be supplemented with antibiotics e.g. chloramphenicol (200 mg/L).
4. Incubate all dishes at 25°C in the dark.
5. Check each fragment every 2 to 3 days for mycelia emerging from the cut ends.

Colony transfer

1. Transfer mycelia emerging from the cut ends to new Petri dishes.
2. Incubate at 25°C in the dark to achieve pure fungal colonies.
3. Conserve one sample of the pure colony in sterile 0.3 % $NaCl_2$ at 4°C or in glycerol at -20^{o}C.
4. The rest of the colonies can be kept on Petri dishes at 4°C for further analyses.

Visualization of endophytes

Trypan blue staining

(Phillips and Hayman 1970)

1. Wash out fixative (two general fixatives: 70% EtOH or FEA formaldehyde : 70% EtOH : concentrated acetic acid, 5:90:5 (v/v)) in H_2O (2-3 times for some min).
2. Place roots in closed vials and add 10% KOH.
3. Heat the roots in a water bath at 95°C for 5 to 30 min. Time depends on the degree of lignification and thickness of the roots (*Medicago truncatula* ca. 5-10 min, *Hedera helix* and *Panax ginseng* 15 min, *Solanum lycopersicum* >15 min)
4. Take the vials out of the water bath and exchange KOH by 5% HCl (154 mL 32% HCl in 1000 mL H_2O). Roots stay in the solution for 3 min at room temperature. (Do not put them back to the water bath!!!)
5. Remove the HCl and add trypan blue solution to the roots. Heat for 3 to 7 min in the water bath.
6. Remove the dye, add 96% EtOH and heat it for 2 min at 95° in the water bath.
7. Replace EtOH by lacto-solution.

Trypan blue solution: 5 drops stock-solution (1% trypan blue in H_2O) in 5 mL H_2O

Lacto-solution: lactic acid, 87% Glycerin, H_2O (1:2:1)

Alternative 1 (Vierheilig et al. 1998):

4. Stain roots in 5% ink/acetic acid at 80°C in water bath.
5. Wash roots with tap water and de-stain in 5% (v/v) acetic acid for 30 min.
6. Remove acetic acid and fill with tap water, store in fridge.

Alternative 2 (optimized for tomato roots; Pelican black worked best for tomato)

1. Wash roots in dist.water (change 3 times)
2. Put roots into 5% acetic acid (5-10 min)
3. Stain roots in 5% ink*/acetic acid at 90°C (8 min)
4. Wash out the stain from roots in 5% acetic acid
5. Store in lactic acid on room temperature

Cover samples in lactic acid (or in PVLG (polyvinyl alcohol/lactic acid/glycerol (PVLG; Omar et al. 1979), if permanent slides are needed) preferably using 24x32 mm (or larger) cover slips.

- Typical fungal colonization patterns will be checked.
- Methods for quantification of colonization (estimation, segment-based, intersection-magnification method) will be discussed.

Molecular identification

DNA extraction

with DNeasy Plant Mini kit from Qiagen

1. Grind a fresh or frozen sample of plant tissue (~ 200 mg) by hand in liquid nitrogen. Dry samples can be ground in a mill (e.g. Retsch mill).
2. Put 1/3 for further processing into a 1.5 mL tube, freeze 2/3 for other studies.
3. Add 400 µL AP1 solution and 4 µL RNase (100 mg/mL) to the sample (max. 100 mg fresh sample or max. 10 mg dry sample) and vortex.
4. Incubate for 10 min at 65°C.
5. Turn tube upside down 2 to 3 times.
6. Add 130 µL P3 solution and vortex.
7. Cool 5 min on ice.
8. Transfer the lysate on a QIAshredder Mini Spin Column (purple color).
9. Centrifuge for 2 min with 14,000 rpm.
10. Transfer flow through without pellet to a new tube.
11. Add 1.5 times the volume of buffer AW1 to the lysate and mix with a pipet.
12. Transfer 650 µL to a Dneasy Mini Spin Colum (white color).
13. Centrifuge for 1 min with 8,000 rpm.
14. Discard flow through.
15. Centrifuge for 1 min with 8,000 rpm.
16. Add 500 µL AW2 buffer.
17. Centrifuge for 2 min with 14,000 rpm.
18. Transfer Dneasy Mini Spin Colum to a new tube.
19. Place 100 µL AE buffer directly on the membrane.
20. Incubate 5 min at RT.
21. Centrifuge for 1 min with 8,000 rpm.
22. Take the flow through and determine DNA concentration by optical density measurement at 260 nm and 280 nm.

(Optical density ratio of 260 nm to 280 nm should be 2:1; dsDNA OD=20 means 1 mg/mL; ssDNA OD=33 means 1 mg/mL; RNA OD=25 means 1 mg/mL)

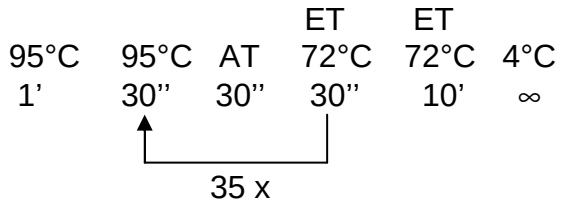
Alternative DNA extraction method

According to Dellaporta et al. 1983 (DNA will not be very pure, but of sufficient quality for PCR.)

1. Grind 1 g tissue in liquid N2.
2. Add to a centrifuge tube with 15 mL extraction buffer (0.1 M Tris/Cl pH 8; 0.05 M EDTA; 0.5 M NaCl, 10 mM β-mercaptoethanol) .
3. Add 1 mL 20% SDS.
4. Incubate 10 min at 65°C.
5. Add 5 mL 5 M potassium acetate.
6. Incubate 20 min on ice.
7. Spin 20 min at 8,000 rpm.
8. Filter supernatant through gauze into a new centrifuge tube.
9. Add 10 mL isopropanol.
10. Incubate 30 min at –20°C.
11. Spin 15 min at 8,000 rpm.
12. Discard supernatant.
13. Dissolve pellet in 0.7 mL TE buffer (10 mM Tris/Cl, pH 8.0; 1 mM EDTA).
14. Transfer to a 1.5 mL tube.
15. Spin 10 min at 12,000 rpm.
16. Add 75 µL 3 M sodium acetate pH 4.8 and 0.5 mL isopropanol.
17. Spin 30 min at 12,000 rpm.
18. Wash pellet with 80% EtOH.
19. Spin 5 min at 12,000 rpm.
20. Dissolve pellet in 100 µL TE.

Standard PCR

	Volume [µL]	final concentration
dH_2O	12.9	
Polymerase buffer (includes $MgCl_2$ and dNTPs)	4.0	200 µM dNTPs
forward Primer (10 µM)	1.0	0.5 µM
reverse Primer (10 µM)	1.0	0.5 µM
Taq-Polymerase (100 U MyTaq HS)	0.1	0.5 U
Template (genomic DNA)	1.0	10 – 30 ng
Σ	20.0	



Annealing temperature (AT) depends on the primer sequences. Elongation time (ET) depends on the expected PCR fragment size.
If PCR fragments will be cloned, add a 10 min step at 72°C after the last circle. This will lead to an attachment of one nucleotide desoxy adenosine triphosphate (dATP) at the end of the fragment which you need for cloning. If you do the cloning not immediately, you can do this step before the ligation, by incubating the PCR product with dATP and Taq-Polymerase at 72°C for 10 minutes.

Primer	sequence	AT [°C]	product size [bp]
ITS1	TCC GTA GGT GAA CCT GCG G	57	700 – 800
ITS4	TCC TCC GCT TAT TGA TAT GC	57	
ITS1-F	CTT GGT CAT TTA GAG GAA GTA A	57	600 – 750
ITS4-A	CGC CGT TAC TGG GGC AAT CCC TG	57	
FLR3	TTG AAA GGG AAA CGA TTG AAG T	54	373
FLR4	TAC GTC AAC ATC CTT AAC GAA	54	

Agarose gel electrophoresis

1. Dissolve agarose in TAE buffer (40 mM Tris/Cl, pH 8; 20 mM acetic acid, 1 mM EDTA) by heating in the microwave (attention!). Concentration (0.8% to 3%) depends on the expected DNA fragment size.
2. Pure the agarose gel (Do not forget the comb!) and let cool down.
3. Remove the comb and place agarose gel into the electrophoresis chamber.
4. Mix 7 µL of the PCR product with a drop of loading dye (50% glycerol, 75 mM EDTA pH 8, 0.2% bromophenol blue) and pipet into slots.
5. Run at 100 V until blue marker has entered 4/5 of the gel.
6. Stain the gel in an ethidium bromide solution (0.2-0.5µg/mL in TAE buffer).
7. Wash the gel in TAE buffer and visualize the nucleic acids under UV light.

Ligation

2 x Ligase-Puffer (includes dATP)	5 µL
pGEM T-easy vector	1 µL
PCR-product	3 µL
Ligase (3 Weiss U/µL)	1 µL
Σ	10 µL

Ligate the PCR product with the vector at least 1 hour at RT (better over night at 4°C).

Transformation

1. Add 5 µL of the ligation reaction solution to 50 µL of chemo-competent cells (DH5α) and incubate for 30 min in an ice bath.
2. Incubate for 45 sec at 42°C (heat shock).
3. Add 450 µL SOC medium.
4. Shake horizontally for 1.5 hours at 37°C.
5. Distribute 50 µL and 100 µL on Petri dishes (LB agar with ampicillin, IPTG and X-GAL.
6. Incubate dishes over night at 37°C.

LB (for 1 L):
10 g peptone
5 g yeast extract
10 g NaCL
15 g Bactoagar (for Petri dishes)

1. Autoclave.
2. Add 150 mg ampicillin for plasmid selection.
3. Pour Petri dishes.
4. Before distributing the bacteria on the agar, add 40 µl 2% X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 20 mg/ml DMF: dimethylformamide) and 10 µl IPTG (isopropyl β-D-1-thiogalactopyranoside; 200 mg/ml H_2O) for insert selection.

SOC (for 1 L):
20 g tryptone
5 g yeast extract
0.5 g NaCl
3.6 g glucose
10 mL KCl (250 mM)
5 mL $MgCl_2$ (2 M)

Colony PCR

1. Take white colonies with sterile toothpicks and suspend in 50 µL LB in a 0.2 mL tube.
2. Vortex.
3. Add 1 µL of the bacterial suspension to a standard PCR reaction with M13for and M13rev primers (see above) and check result by gel electrophoresis.
4. Based on gel electrophoresis results select clones with inserts and add the remaining 5 µL to 3 mL LB medium with ampicillin.
5. Incubate on a rotator over night at 37°C.

Primer	sequence	AT [°C]	product size [bp]
M13for	GTTTTCCCAGTCACGACGT	56°C	depends on
M13rev	CAGGAAACAGCTATGAC	56°C	insert

Plasmid preparation

1. Cool down Sol 1 and Sol 3 in an ice bath and keep Sol 2 at RT.
2. Transfer 1.5 mL of the ON culture to a 1.5 mL tube.
3. Spin down for 5 min at 8,000 rpm and discard supernatant.
4. Suspend pellet in 100 µL Sol 1 with a pipet.
5. Add 5 µL RNase (10 mg/mL) and mix by shaking.
6. Incubate for 5 min at RT.
7. Add 200 µL Sol 2 and mix by shaking.
8. Incubate for 5 min in an ice bath.
9. Add 200 µL Sol 3 and mix by shaking.
10. Incubate for 5 min in an ice bath.
11. Spin down for 10 min at 13,000 rpm.
12. Transfer supernatant to a new 1.5 mL tube.
13. Add 800 µL 96% EtOH and mix by shaking.
14. Incubate for 30 min at –20°C.
15. Spin down for 5 min at 13,000 rpm and discard supernatant.
16. Add 70 % ETOH and vortex.
17. Spin down for 5 min at 13,000 rpm and discard supernatant.
18. Air-dry the pellet for 5 min.
19. Dissolve pellet in 40 µL TE buffer.
20. Check DNA concentration by OD measurement.

Sol 1: 25 mM Tris/Cl pH 8; 10 mM EDTA ; 50 mM glucose; 5 mg/mL lysozyme

Sol 2: 0.2 M NaOH; 1% SDS

Sol 3: 3 M potassium acetate (pH 4.8 with acetic acid)

Restriction enzyme digestion

H_2O	? µL
appropriate 10x buffer	1 µL
ca. 1 µg plasmid DNA	? µL
Eco RI (? U/µL)	1 µL
Σ	10 µL

Incubate for at least 2 hours at 37°C.

Check result by agarose gel electrophoresis.

Outline of the molecular taxonomy practice

(example sequences and datasets will be distributed on the practice)

1. *Checking electrophoregrams of Sanger sequencing using Staden Package
2. Overview of main points of dataset establishment, GenBank, blastn, choosing outgroup etc.
3. Multiple alignments
    a. PRANK online and/or PRANKSTER implementation of PRANK
    b. mafft – online
4. Checking/editing alignments
    a. Different file formats
    b. (one possible program: ProSeq – for Win)
5. *Problem of ambiguous sites – Gblocks
6. *Coding indels – GapCoder
7. *Nucleotide models
    a. *jModeltest
8. Phylogenetic analyses
    a. pilot MEGA
    b. RAxML – by raxmlGUI
    c. MrBayes
        i. Checking convergence: AWTY online
9. Editing trees
    a. MEGA
    b. FigTree

* Because of time limitations, most probably demo

Programs:

• PRANKSTER: http://www.ebi.ac.uk/goldman-srv/prank/prankster/
• (rec) ProSeq: http://dps.plants.ox.ac.uk/sequencing/proseq.htm
• MEGA: http://www.megasoftware.net/
• raxmlGUI: https://sites.google.com/site/raxmlgui/
Phyton is needed, 2.5 or higher but Phyton 3. is not supported.
• MrBayes: http://mrbayes.sourceforge.net/
Might be problems with 3.2.x versions on x64Win, 3.1.2 is perhaps better.

Online tools:

• PRANK: http://www.ebi.ac.uk/goldman-srv/webPRANK/
• mafft: http://mafft.cbrc.jp/alignment/server/
• AWTY: http://king2.scs.fsu.edu/CEBProjects/awty/awty_start.php

Additional media

Water agar

15 g pure agar/L

Plant nutrition medium

(Shahollari et al 2007)

Amounts per 1 litre:

(1) 5 mL 1 M KNO_3

(2) 2 mL 1 M $MgSO_4$

(3) 2 mL 1 M $Ca(NO_3)_2$

(4) 1 mL 10 µM Fe-EDTA

(5) 1 mL micronutrient mix

(6) 10.5 g Agar

pH 5.6

Autoclave

Add through sterile filter (0.22 µm) 2.5 mL 1M KH_2PO_4 (pH 5.6)

Micronutrient mix:

Per 100 mL

(a) 70.0 mM H_3BO_3	add 432 mg
(b) 14.0 mM $MnCl_2$	add 276 mg
(c) 0.5 mM $CuSO_4$	add 125 mg
(d) 1.0 mM $ZnSO_4$	add 28.8 mg
(e) 0.2 mM Na2MoO_4	add 4.8 mg
(f) 10.0 mM NaCl	add 58.4 mg
(g) 0.01 mM $CoCl_2$	add 0.23 mg

M medium

(Bécard & Fortin)

	final concentration [mg/L]	stock solutions	for 1 L from stock solution
KNO_3	80	3.2 g/L	
$MgSO_4$	731	29.2 g/L	
KCl	65	2.6 g/L	Add 25 mL
Na-Fe-EDTA	8	3.2 g/L	Add 5 mL
$MnCl_2$	6	6 g/L	
$H2BO_4$	1.5	1.5 g/L	
$ZnSO_4$ x 7 H_2O	2.65	2.65 g/L	
KI	0.75	750 mg/L	
Na_2MoO_4 x 2 H_2O	0.0024	2.4 mg/L	
$CuSO_4$ x 5 H_2O	0.13	130 mg/L	Add 1 mL
$Ca(NO_3)_2$x4 H_2O	288	115.2 g/L	Add 5 mL
KH_2PO_4	4.8	9.6 g/L	Add 1 mL
Glycin	0.3	0.3 g/L	
Myo-Inositol	50	5 g/L	
Nikotinsäure	0.5	50 mg/L	
Pyridoxine HCl	0.1	10 mg/L	
Thiamin HCl	0.1	10 mg/L	Add 10 mL
sucrose	10 g		10 g
phytagel	3-4 g		3.5 g

after adding sucrose adjust the pH to 5.5 with KOH

make aliquots of vitamin solutions and store at –20 °C

pour plates and let dry under the sterile hood; condensation water is the source for contamination