COST ACTION FA1103

Endophytes in Biotechnology and Agriculture

Minutes of the Working Group 3 Meeting "New concepts and strategies for longterm cultivation and conservation of competent endophytes for plant growth and plant protection“

Place: COST Meeting room 1
Avenue Louise 149, 1050 Brussels, Belgium
16th-18th November 2013

1. Welcome to participants

The participants of the inaugural meeting of COST Action FA1103 were welcomed by the working group 3 leader *Matthias Döring* and COST Chair *Carolin Schneider*.

2. Talks

Introduction to the workshop
Matthias Döring (Germany)

There are several problems correlated with fungal and bacterial cultures on artifical media during the processes of cultivation/conservation:

1. Changing of micro/macromorphology
2. Sectoring of fungal mycelia – phenotypic degeneration
3. Loss of Virulence after subculturing on artificial media
4. Loss of Production of Metabolites
5. Loss of pigments

According to these problems and the experience of each researcher, several questions arose:

a. Are my endophytic microbial isolates really monoxenic?
b. How can we improve long-term cultivation and preservation, esp. for endophytes?
c. Can the inoculation of plant material and reisolation of microbial endophytes improve ecological fitness and colonization behaviour of the endophytes?
d. Etc

According to all these above mentioned points and considering the experience of all the experts present in the meeting that work with endophytes – fungal and bacterial, several points will be discussed, from identification of endophytic species, isolation of the species, maintenance of the strain in culture medium and conservation/preservation of the strain to future propose.

Anna Maria Pirtillä (Finland):
“Studying Endophytic Fungi as a Source of New Antimicrobials: Loss of Bioactivity in Subculture”

This presentation focused on the potential of endophytic fungi to produce antibiotics and in the problems of maintain this endophytic fungi in culture in a way of use this organisms as protection of pathogens that affect several plant species.
This work was developed within a project on Finland ecosystems isolating the endophytic species that leave in association with several natural plant species, such as *Calluna vulgaris, Labrador tea, Rhododendron tomentosum, Pinus sylvestris*, etc. Several culture medium were used, such as DM, malt agar, PDA, and MEB to maintain the endophytic fungi. Besides that, the secondary metabolites isolation was made using several methods, namely:

1. HPLC-DAD-MS
2. LC–MS/ GC–MS
3. Flash Chromatography

Nevertheless when the compound was in the final step to be isolated the fungi disappeared from the natural ecosystem and because the cultivation method was not optimized the fungal strain was also lost.
To avoid this problem a new approach was tested, using Metagenomic. To test this new approach crowberry, *Empetrum nigrum* (Pace et al., 1985; Schmidt et al., 1991) as used.
From the experience of these studies, the fungal strains can be stored at -80ºC in 30% glycerol or in culture plates at 4ºC. For the reviving of the strains, for example, the strain isolated from *P. sylvestris*, the needles were used as reviving system.
It was suggested in the end of presentation, by the other participants that the cryopreservation of the fungal strains should be directly made on the cryovial with the culture medium and not in petri dishes and then transferred to the cryovials in a way to avoid disturbing the fungal strain culture. Other suggestion was to tested the use of a cryoprotector at lower concentrations, that maybe be more effective that at higher concentration – use 5% instead of 10%. It was also suggested that the secondary metabolites should not be maintained in liquid culture.

Desiree Jakobs-Schönwandt (Germany):
"Cultivation and conservation of endophytic fungi for plant protection and –growth with regard to bioengineering aspects"

This presentation focused on the production of different ways to store microorganisms in a way that they can be applied in the field. The project includes:

1. Identification of endophytes species was made by ITS amplification.
2. Secondary metabolites were identified by HPLC-MS e Maldi-Tof-MS.
3. Endophytic species were maintained in culture medium.
4. *Beauveria bassiana* was the selected fungi to be cultivate and formulate using the fermentation process.
5. *B. bassiana* produce spores easily and in short time. The conidiospores are the spores produced under stress conditions and that are more suitable to be used in the formulation process. To obtain only the conidiospores, the fungal strain were stressed with NaCl at higher concentrations.
6. 23 culture mediums were tested to select the better to maintain the fungal strain.
7. The formulated conidiospores were sprayed on the leaves.

As a process of conservation/preservation of fungal strains encapsulation method was tested on *Metarhizium anisopliae* to avoid the stress conditions. Encapsulation was made using a Ca-alginate and a bio-polymer (5% or 10% - as the percentage of the bio-polymer increase, water activity is reduced), followed by a drying step in the flow chamber at 22ºC, 35% of humidity during 22H, after another drying step at 20ªC during 72H in a desiccator.

It was also presented a new method to test the viability of bioproducts. It is a mathematical equation that can transform, for example, the 2 years of viability tests in days – **Arrhenius** relationship equation (Peleg, 2012).

Borbala Biro (Hungary):
"Importance of suitable adaptation to certain environmental factors in better tailoring the plant-microbe interactions"

This presentation focused on:

1. Beneficial microbes and functioning
2. Adaptation to specific environments
3. Potentials of the manipulation of microbial interactions for the better plant growth

The first important information that should be taking in account is the plant-microbe interactions, that should consider:

a. The effect of plant interactions;
b. Effect of microorganisms;
c. Microbial strategisms existing in the rhizosphere.

Beneficial activities of microorganisms in soil are connected with plant growth promotion (production of hormones - plant growth regulators, PGR), protection against root pathogens (biological control of diseases, soil nematodes and insects, weeds), the enhance of nutrient use efficiency, the enhance of drought tolerance of plants, as well as the improvement of soil aggregation.
Nevertheless, it should not be forget that not all the interactions on the rhizosphere are positive, and that it must be a balance between the symbiont and its effect. Not always an increase in the amount of the symbiont have a positive effect on the plant.
The concentration of symbionts depends on severel abiotic factors (nutrient levels, climatic factors, soil-use) and also of biotic factors (other microorganisms). Environmental stress is a selective factor of the variability of the available strains in the rhizosphere.
Several studies with sinergistic benificial interactions alltogether were presented - tripartite symbiosis (functioning in the rhizosphere): *Rhizobium* + AMF hyphae + *Azospirillum* bacteria. These studies showed that combined inoculations of beneficial microsymbionts results in a synergistic positive effect on the growth and plant fitness, and that nutrient availability is a key-point of the beneficial effect (Biró et al., 2000, 2006; Michael et al., 2000).
It was also presented the effects of metal on culture medium that improve the tolerance of the strain and enhanced the growth even for the host-plant.
It was also discussed that under sterilized conditions by heat, the available nutrients may be lost and that Gama irradiation can be an alternative to sterilize with loosing nutrients available.

Nabil Hegazi (Egypt):
"The consortium of endophytic rhizobacteria: The rich diversity and dense populations

nesting the xerophytic plants of semi-arid deserts"

This presentation talks about the rhizobacteria from the desert – xerophytes, specifically those that are located on the Rhizosheath. In this rhizosphere it is possible to find *Bacillus*, *Enterobacter*, *Pseudomonas* and the *Azospirillum* groups of bacteria.
The main focus on the presentation was on the use of plant juices in the culture medium for rhizobacteria - *Mesembryanthemum crystallinum,* and the amendments with SE (soil side) and YE (growth factors), and the possibilities of this juice in support the growth of RMO (rhizosphere microorganisms).
By testing it was shown that the more diluted is the juice, better is the growth of the rhizobacteria, but this will also depend on the strain.
The question that remains is that if the juice support the viability of the strains, when used in liquid and solid medium.
Besides the juice of *M. crystallinum*, 3 more juices were tested *Zygophyllum album, Carpobrotus edulis, Trifolium alexandrinum* and all tested plant juices (except *C. edulis)* can:
promiscuously support culturing RMO in the root spheres of all tested RMO host plants and juices diluted as much as 1:40 speaks well to the economical uses of plant juices for culturing RMO.
From these experiences the plant juices seems to support the growth of culturable endophytes, but what if the strain are unculturable? Would be any phenotypic or genotypic differences from the strains cultured on plant juices, compared to those cultured on chemically-synthetic medium? And, do the plant juices support the viability of the strains? These question remain to answer but for these several other participants will test the plant juices on their strains and evaluate its potential.

Colin Ingham (Netherlands):
"A Better Petri Dish: Advancing Microbiology with Miniaturized Culture Chips Fabricated from Porous Aluminium Oxide"

This presentation point to several question of strains culture maintenance and how in the future technology can improve it. The actual culture methods are limited in handling and detection, not speed up - 1 day to weeks to culture and the agar is not a good option to be used in all the culture medium because:

1. It has a short life time;
2. Several microorganisms "feed" on it;
3. It is toxic for some microorganisms.

MicroDish BV is a small company that is designing and trying to improve microbial culture methods.
The company team is developing technology to use porous (nano) materials to replace agar as the support matrix, miniaturized formats the "Dish". Microfluidics and cheap imaging add functionality and can make such "culture chips" usable and better than what we have now. They want to integrating multiple assays (molecular + culture). To that propose they are using on aluminum oxide (porous) anodic – PAO, that the only disadvantages this was detected until now is the brightness. They are subdividing PAO to construct microdish culture chip (MDCC).
To understand the viability of this technology it has to be tested by researchers that are already using the traditional method of culture and see the potential of MDCC. It will be necessary to improve the technology to the researcher needs.

Brian Murphy (Ireland):
„Effective storage protocols for fungal root endophytes"

In this presentation the Brian Murphy expose the preservation/conservation method that his lab use, but the storage of endophytic organisms is adapted to the requirements of each lab and each species.

Nevertheless, the potential of long term preservation and the maintenance of strains viability can be just evaluated across time.
Fungi have different forms and each one of them have different requirements to be preserved. Fungal spores seem to be the most suitable form of fungi to be long term preserved. It is also important to avoid the number of sub-cultivations.
For fungi that are not cultivable the better way to store them are in plant tissue. It is very important that the chemical and physical properties of the fungi are known for that the desired characteristics can be established and to avoid undesired contaminations. The technique of storage tested in its laboratory for short term preservation is the store in pure water together with periodic reinfections on seeds. After the first step of development of the endophyte, it is regrowth again on plant tissue.
Nevertheless, the growth and development of the spore is determined by many factors: nutrient availability, host interaction, other microrganisms.
According to the types of propagules is thus the procedure to prepare it to storage and each one has different storage potential.

1. Thick-walled sexual spores: >10 years
2. Conidia: 2-3 years
3. Chlamydospores: variable, but useful for some obligate biotrophs
4. Sclerotia, thick-walled hyphae: 5 years +

The production of secondary metabolites can also been induced by nutrient limitation (particularly N) or by chemical inducers in high N growth conditions.
Techniques of preservation should also take into account the degradation of the material, and so straight freezing or treating with a cryoprotectant such as glycerol or intracellular trehalose before freezing, will be an option. Fast (or in some cases, slow) freezing and thawing essential.
Each laboratory has its own experience, its one strains, its own method, which is more or less optimized to the material that they have and the available conditions with they work to.
In our case, Brian Murphy, our major goal is to produce inoculants that will be reliable and predictable in effect, so early processing for any inoculates preparation is essential, while the spores retain maximum viability. It is of course important to periodically test for the desired characteristics. Testing of inoculant effects and storage life is on-going, but what is really needed to convince potential customers is how persistent and effective inoculants are in the field.
In the future maybe the solution can be on artificial plant systems in real time. It is to known what each one of us have done and what all can do that we are all hear, to find solutions to our problems.

Lotfi Fki (Tunisia):
"Cryopreservation of endophytic bacteria living inside date palm embryogenic and caulogenic cultures"

Phoenix dactylifera (date palm) is one of the most important plants economically in Tunisia and has difficulties in propagation – slow vegetative multiplication because:

1. Seeds with high genetic heterogeneity
2. Date palm is propagated from offshoots which are limited in quantity
3. Micropropagation

Another problem from date palm cultivation is the spread of lethal diseases and the genetic erosion. Trying to avoid all this problems date palm is being cultivated under plant tissue culture. Plant tissue culture is very important to: production of plant material, plant breeding, gene banks, production of

chemical compounds and also can help in the isolation and conservation of true endophytic microorganisms.
In plant tissue cultures there are several ways of contamination: air, tissue culturist or the own plant material. In the practice the microorganisms found in a plant tissue culture are called as “contaminants”. Nevertheless, some of these “contaminants, can promote plant tissue culture. Apparently, and endophytic microorganisms live inside the plant without causing apparent disease to the plant, being beneficial for the plant. But, the endophytic bacteria detection can be difficult in tissue culture, because, for example, the disinfectant, used to sterilize primary explants, can block bacteria growth during the initiation phase and some plant growth regulators (cefotaxime) may promote the latency of bacteria. Because of that, when plant tissue cultures are preserved by cryopreservation techniques at -196ºC, together with them can be endophytic bacteria.
During plant tissue cultures – micropropagation of date palm some bacterial strains were detected within 2-4 weeks while others started their proliferation after 2–3 years. Several test showed that liquid media, culture medium supplemented with charcoal and stress can stimulate the endophytic bacteria expression in plant tissue cultures.
From all the presented studies it was shown that:

- Endophytic bacteria are present date palm tissue cultures being beneficial to the plant, because the endophytic bacteria proliferation is under plant cell control.

Hatice Özaktan (Turkey):
“The effectiveness of endophytic bacteria stored in – 80°C for biocontrol and plant growth”

Bacterial cultures can be stored freezed, at -20ºC (with glass beads) or -80ºC (in a suspension in nutrient broth or sterile glycerol). By experience the long term storage of bacterial endophytes should be done at -80°C in liquid NB with 20% glycerol (until now 5 years have past).
The main goal of their work is to determine if endophytic bacteria (EB) stored at -80ºC, continue to have plant growth-promotion potential and provide biological control against soilborne disease, seeds and young plants of cucumber (*Cucumis sativus* cv. Gordion).
Soilborne disease is caused by *Fusarium oxysporum*. Several experience were done in greenhouses and in plant tissue cultures. The experience consits on:

1. Isolation and preliminary characterization of EB - trituration of leaves and imprinting of stem and root tissues onto Triptic Soy Agar (TSA).
2. *In vitro* tests –plant growth-promotion (PGP) and biocontrol activities of selected EB (Brick et al., 1991; Lork,1948; Schwyn and Neilands, 1987; Gaur,1990; Nejad and Johnson,2000).
3. *In vivo* tests: testing the potentials of biocontrol and plant growth promotion of selected EB strains

From the tests, can be concluded that EB isolates improved seed germination, seedling length, and plant growth of cucumber but also, when used for seed/seedling treatment, significantly reduced disease symptoms caused by vascular wilt pathogen *Fusarium oxysporum*.
These indicated that endophytic bacteria should be evaluated further for efficacy as biological control agents of vascular pathogens and that some endophytes may survive, multiply, and exhibit limited movement following introduction into cucumber.

Martin Schumacher (Germany):
“Cultivation and Conservation of Competent Endophytes – May In-Vitro Culture Help”

Leibniz-Institute DSMZ have several cultures of different plant species and microorganisms, that are supposed to be axenic and that routinley are tested to evaluate their sterility. The suspicious cells cultures are tested at two different temperatures in three different comercial culture medium.

1. M92 Trypticase Soy Broth Yeast extracts
2. M215 Infusion of potato Glucose
3. DSMZ M129 Brain Heart Infusion Cysteine Na_2S

It can happen a contamination during the process of sub-culturing, which is not evident during the initiation process.
Because of the routinely verification of cultures sterility a sytematic identification of contaminations is being undertaken in order to construct a phylogenetic tree. Until now, several species of *Pseudomonas, Rhizobium, Bacillus*, *Methylobacterium* and an yeast has been identifyed. The colonization depend on host and endophyte, but the way as the colonization happen depends also in the parts of a callus.
The presence of those contaminants were detected just in a specific phase during the regeneration of an embryogenic culture . According to all this knowledge the question remain: are the isolated bacteria really endophytes or contaminations?

- *In-vitro* cultures may be used to produce substrates for endophyte cultivation and serve as substrate for endophytic cultivation. They also help in the cryopreservation process.

The specificity of the relationship between bacteria and cell cultures vary and is still under study.
In vitro cultures serve as substrate for endophytic cultivation, maintainence and propagation under laboratory conditions, but this willdepend on the specificity of cell culture/endophyte interaction.
In cell cultures, the presence of bacteria doesn´t seem to affect the plant, nevertheless after cryopreservation and regrowth of the plant culture again, the bacteria starts to growth.

3. Discussion points

Experience about endophytic cultivation and preservation:

Preservation of fungi:
PDA with mycelial plug in filter paper – dry it – conserved at -20C

Autoclave the seeds with water and dry it – inoculate with fungal mycelium – let it grow– pick up the seeds – put in -20C or -80C

Cryo-preservation (-196°C) – It preserves better morphological characteristics and viability of the strains. Besides the cryoprotector alginate should be used as perlite. For plant cells is it the onliest opinion because at -80C plant cells die.

Lyophilization with and without a conservation agent (glycerol, dextrine malt) and frozen at -80C for bacteria and fungi

Culture techniques – innovate and/or maintain - what is known:

Suitable culture media are: BSM – bacteria screening medium (Sigma)
Low nutrient medium R2A

Organ culture (eg root organ cultures used for AMF) of plant genus *Lunularia*(mosses) with endophytic isolate

½ strength Hoagland-Solution in a semi-solid medium together with the plant and the endophyte

Low temperature for cultivation – 10C,-15C,-20C for bacteria

Liquid MS-medium stimulate the proliferation of endophytic bacteria

Plant peptide

PDA for fungi – take the mycelial fragment and put in eppendorf tube and store it at -20C

Plant juice extracts are also suitable for long-term cultivation and preservation; Nabil Hegazi has a big knowledge about it

Can *in vitro* cultures affect the genomic features of the endophytes?

When we subcultivate the strains some characteristics are lost (eg by ISSR – PCR it is possible to determine genetic diffrences between the control plant and the sub sub-cultivated strains across time; flow cytometry allow to compare between the genome size of the organisms from T0 until sub-cultured time(T1))

How is the lifestyle of endophytes affected by *in vitro* cultures?

Bacteria
Genes may be lost when bacteria endophyte is separate from the plant.

Fungi
Isolating the fungi endophyte from the plant make sometimes the endophyte loose its pigmentation, the ability to sporulate in culture medium – genes can stop to be expressed.

Is co-cultivation necessary to preserve some endophytic bacteria?

Yes. But not every time these co-cultivation need to be done in the plant. It will depends on the plant-endophyte interaction.

How can we maintain the endophyte in the plant material?

Callus cultures are suitable for it.

Commercial products – how to be manipulated by consumers?
The endophytic species has to be crop specific and depending if it is a fungi or bacteria endophyte the application method if be different.
It should also be considered the location of endophytic species on plant.
It is easier to use the metabolites from endophytic to apply in cropping than the endophytic species by it self. It is also more easy accepted by the consumers.

How we define a suitable method of conservation to the endophytic organism?

Each bacteria and fungi should be studied alone and the preservation method decided according to that. Each endophyte has ist own requirements.
A **table** may be designed according to what has been already tested and published and the experience of each group.
This will be a start point to the preservation of new strains and to strains with specific problems of long term preservation.

The same table can have information about what type of medium that is used to cultivate this endophytic species and what kind of tests can be used to evaluate the interactions between the plant and endophyte. Also collect information about viability and function of each endophytic strain.
Anyway we should also considered the possibility that sometimes during the preservation process we have to put the endophyte in the plant. Of course that according to the number of strain this will to time consuming.

4. Future Workshops and ideas

The members of the Workshop want to build up a flow chart, a kind of decision key for preservation of endophytic bacteria and fungi in future:

Table – Flow chart
Fungi
- sporolate or not sporolate;
- preservation: -20C, -80C, -196C (long term preservation); 4C (medium term conservation) , sand
- sub-cultivation: alone; with plant cells – *in vitro* tissue cultures or *in vivo* cultures
- viability and function;

Bacteria
- preservation: -20C, -80C, -196C (long term preservation); 4C (medium term conservation) , soil
- sub-cultivation: alone; with plant cells – *in vitro* tissue cultures or *in vivo* cultures

1. Isolate the endophytic strain
2. Select the strain;
3. Cultivate the strain;
4. Preserve the strain – different methods of preservation;
   Test the function and the viability according to the needs of each propose

Nabil Hegazi has the offer to organize a little meeting in Cairo about research work of plant extracts/juices and endophytes, performed by former workshop participants.

5. Miscellaneous

End of January 2014:
Each participant of workshop should provide a simple protocol with the methods that are using to preserve endophytes (word document, with pictures) and to save it in Dropbox folder

Vera Valadas
Matthias Döring
January 2014

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