Aspects of Applied Biology ***120****, 2013*
Positive Plant Microbial Interactions:
Their role in maintaining sustainable and natural ecosystems

Studying the microbiome of AMF cultivated *in vitro*

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Summary

Numerous methods for the large-scale production of the beneficial arbuscular mycorrhizal fungi (AMF) have been developed in the last decades. All these methods have specific advantages and constraints regarding their design, commercialisation, and domain of application. Production of *in vitro* AMF and improvement of this method is being carried out as part of the VEGEDURABLE II project. Due to the sporadic appearance of microorganisms that may take over the root organ culture and mycorrhizal fungi, a number of experiments were undertaken to research and attempt to understand this aspect. Putative endophytic bacteria were isolated from mycorrhizal fungi using tryptic soya medium. Amplification of the resulting 16S rDNA with universal bacterial primers followed by sequencing and analysis in GenBank suggested that the isolated bacteria does not have close matches, therefore could belong to the new unidentified group. Transmission electron microscope observation of spores suggests that these bacteria-like organisms are endophytic and embedded inside of the spore. Fluorescence microscopy showed the presence of stained DNA that was likely to be of bacterial origin in contrast to larger stained fungal DNA also present.

Key words: Arbuscular mycorrhiza, spore associated bacteria bacteria

Introduction

Mycorrhiza is the most common underground symbiosis and is present in 92 % of plant families studied (80% of species), with arbuscular mycorrhizas being the ancestral and predominant form. Beneficial associations of microorganisms with roots contribute to sustainable horticulture and agriculture. Most agricultural crops will perform better and are more productive when well-colonised by mycorrhizal fungi. However, due to heavy fertilisation, tillage and application of pesticides such beneficial microorganisms are not available or not active in all agro-ecosystems, thus application of mycorrhizal inoculum could be critical for sustainable crop production.

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs and cannot live in the absence of their association with plants. Colonisation by AMF supplies a benefit to the plant by contributing to their mineral nutrition by the capture and the transport of mineral nutrients, essentially of phosphorus (P), from soil to plant (Koide & Schreiner, 1992). In return the plant provides the fungus with photosynthetically derived carbon.

Different methods of mass-scale production have been developed, that generally fall into two main groups – *in vivo* where substrate based cultivation or aeroponics are used with living plants and *in vitro* where only the transformed roots grow under aseptic conditions (IJdo *et al*.,

2011), however both methods have their advantages and constrains. The *in vitro* method allows production of a wide range of AMF species in fairly natural conditions, but does not guarantee total absence of unwanted microorganisms, whereas the *in vitro* method offers the potential for completely sterile production. The diversity of AMF that have been grown *in vitro* and produced many thousands propagules is lower than in pot cultivation, and could be seen as a disadvantage (IJdo *et al*., 2011). However, plant diversity on agricultural fields is not always present, so the use of the most effective monostrain of mycorrhizal fungi would be acceptable. The form of the final product using *in vitro* cultivation potentially makes it more attractive as it could be applied in liquid or mixed with many different carriers.

This paper presents results from VEDERURABLE II project that is currently running within the Interreg VI, A program for which experiments for improving *in vitro* method of production were conducted. One of the objectives was to determine the cause of a repetitive occurrence of putative bacterial 'contamination' in the in vitro culture and the methods to control it.

Bacteria are often associated with eukaryotic cells and can establish endocellular symbioses (Bianciotto & Bonfante, 2002; Moran *et al*., 2008). Bacteria associated with AMF spores have been found to stimulate the germination of spores (Xavier & Germida, 2003; Lumini *et al*., 2007); stimulate the colonisation of roots (Lumini *et al*., 2007); stimulate the growth of AMF up to the formation of fertile spores in the absence of host (Hildebrandt *et al*., 2002, 2006) and inhibit different plant fungal pathogens (Budi *et al*., 1999).

Materials and Methods

Isolation of putative bacteria from spores

In order to isolate bacteria sterile 10 mL Falcon tubes filled with 3% tryptone soya (TS) broth were inoculated with potential sources of putative bacteria: spores (500 in 1 cm^3 agar), transformed chicory roots colonised with arbuscular mycorrhizal fungi *Rhizophagus irregularis* (approx. 200 µg) and uncolonised roots. All samples were cultivated at 250 rpm and 25°C. Culture density was determined at 600 nm using spectrophotometry UV; (1800 Shimadzu). Samples that had the highest OD600 value, aseptically harvested whole spores (20 per Petri dish) and crushed spores were inoculated onto TS agar plates and incubated at 25°C for 2 weeks.

Molecular studies

In order to determine the presence of the bacteria within the AMF and investigate the phylogeny of the isolated bacteria, universal bacterial primers were used for PCR amplification of the 16S rDNA. Bacterial colonies grown on TS agar media were selected based on their morphology; to obtain a representative range of bacteria colonies presented on a straight line across Petri dish were also picked. Using DNeasy Plant Mini Kit (Qiagen) DNA was extracted from aseptically harvested spores (approx. 500), transformed chicory roots alone and colonised with AMF following manufactures instructions. Obtained DNA samples alongside with bacteria isolated on nutrient media were used in PCR reactions with 27F and 1525R universal bacterial primers (Relman *et al*., 1992; Warwick *et al*., 2005). The presence and the size of amplicons were checked by gel electrophoresis. The samples with positive amplification were purified and sequenced. General Bioinformatics analysis was performed using the BioEdit sequence editor and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo). DNA sequences were analysed using the basic local alignment search tool (BLASTed) on the dataset of the National Centre for Biotechnology Inormation (http://www.ncbi.nlm.nih.gov).

Microscopy studies

Using a dissecting microscope two Petri dishes containing spores of *Rh. irregularis* were selected for a transmission electron microscopy (TEM): one with the presence of bacteria, one without. Phytagel was dissolved using 10 mM sodium citrate solution and from each Petri dish

approximately 2,000 spores were aseptically harvested (Doner & Becard, 1991). Spores were fixed in 2.5% (v/v) glutaraldehyde in phosphate buffered saline (PBS) for 2 h with and postfixed in 1% (v/v) osmium tetroxide in PBS for 2 h, and vacuum was periodically applied during fixation and postfixation to encourage penetration. The spores were dried through a graded ethanol series, then placed in propylene oxide-resin (1:1) for 30 min and in LV resin for 2 h twice and polymerised for 24 h at 60°C. Ultrathin sections were cut with diamond knife, mounted on the carbon-coated gold grids, stained for 30 min at 60°C in uranyl acetate followed by 10 min in lead citrate. The samples were dried before examination at them using a JEOL1230 transmission electron microscope.

For fluorescence microscopy spores and hyphae of *in vitro* culture were harvested aseptically, placed on a slide (10–20 spores per slide), crushed with a cover slip and heat fixed. About 300 µL of stain 500 nM propidium iodide (PI) nucleic acid stain prepared according Invitrogen protocol was used for one cover slip preparation. The preparation was incubated for 1–5 min and immediately examined under a Nikon Labophot microscope with an episcope fluorescence attachment at excitation wavelength of approximately λ_{535} nm.

Results

Isolation of microorganisms using nutrient media

TS agar plates inoculated with liquid culture, obtained from the inoculation of TS broth with AM spores in the experiment described above, showed growth of putative bacteria after 2 weeks. Three morphologically different bacterial colonies were observed: very small colonies of < 1 mm in diameter, medium sized colonies with a dense white core and slightly clear edges and large, approximately 10 mm diameter, white colonies.

Plated spores on TS agar had bacteria encircling them however did not spread throughout the whole plate (Fig. 1). The crushing of spores was no more efficient at releasing bacteria as only 20% of inoculated plates showed positive bacterial growth.

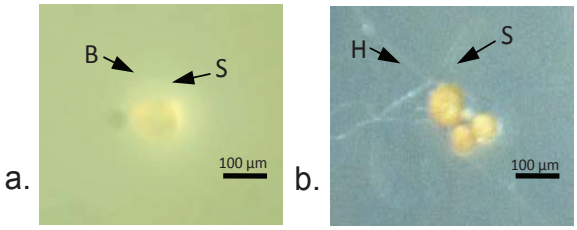

Fig. 1. AMF spores plated on TSA and incubated for 2 weeks. a. spore (S) surrounded by putative spore associated bacteria (B); b. germinating spores with associated hyphae (H) in the absence of putative bacteria. Bars correspond to 100 µm.

DNA amplification and sequencing

Not all prepared samples showed positive bacterial DNA amplification (Fig. 2). Samples with extracted DNA from roots (colonised and uncolonised) and spores showed no discernible amplification (Fig. 1, G, H). There was a positive amplification in chicory roots samples, but when the DNA sequence was analysed using the basic local alignment search tool (BLASTed) in GenBank database it appeared that it had 99 % maximum identity over 100 % query cover to an environmental contaminant. Positive amplification was achieved for samples taken from isolated colonies as well as for bacteria surrounding the plated spores.

Microscopy studies

Electron microscopy of the AMF spores confirmed the presence of rod-shaped bacteria in the fungal cytoplasm (Fig. 3). They were sparse throughout the preparation and were shown to be 0.3 – 0.5 µm in size.

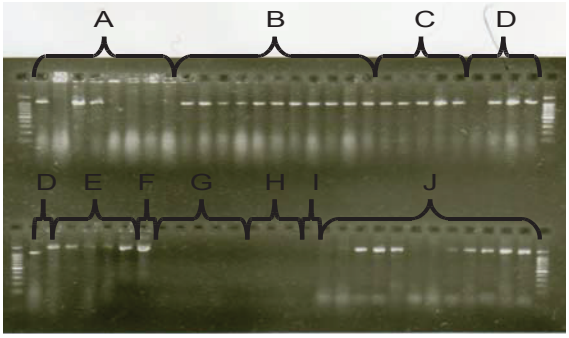

Fig. 2. Electrophoresis of DNA obtained from different sources: crushed (A) and whole spores plated on TS agar with the arising colony used for PCR; A type of bacteria (C); B type of bacteria (D); C type of bacteria (E); chicory roots (F); chicory roots colonised with AMF (G); *in vitro* spores (H); negative control (I); bacterial colonies that were situated across the line (J).

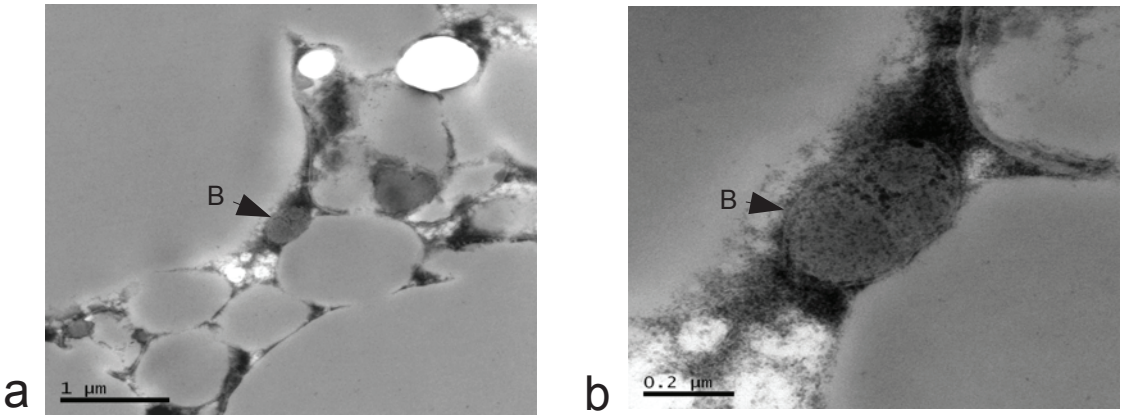

Fig. 3. Electron micrographs of putative bacterium (B) inside of *Rh. irregularis* spore (a, b).

It was not possible to discriminate bacterial DNA from fungal nuclei in cytoplasm released from crushed spores. However, as shown in Fig. 4(a), there was a clear difference between large fungal nuclei DNA and small bacterial nucleoid DNA. Fig. 3(b) shows the absence of bacterial nucleoids in hyphae.

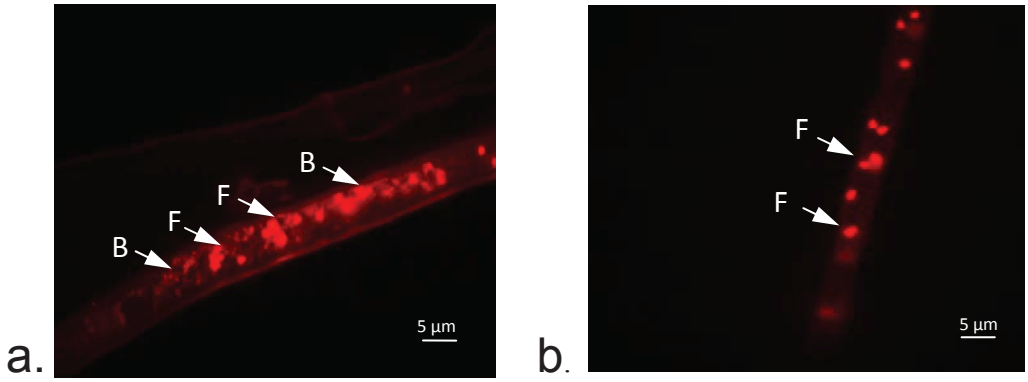

Fig. 4. Hyphal fragments stained with propidium iodide (PI) from samples, where contamination was observed (a), possess fungal DNA (F) and bacterial DNA (B); and hyphae from sample, where contamination was not detected under dissecting microscope, possess only fungal DNA.

Discussion

Despite the high nutrient content in TS agar, bacteria grew only slowly and the first observation of colonies was only made 1 week after inoculation. This clearly indicated that the growth conditions

were not favourable for these microorganisms. A possible reason could be the (preferred) requirement of the presence of host organism, in this case AMF. Bharadwaj *et al*. (2008) suggested that low strength media can help in isolating a greater number of bacteria, 1% TS agar was tested here, however no growth of any bacteria was stimulated. When spores were spread onto TS agar plates, this did not elicit high growth of microorganisms on the plate either, although the presence of putative bacteria was observed around the spores themselves. The possible explanations of this phenomenon are: these microorganisms are associated with spores and require specific conditions such as the presence of AMF spores, they require particular spore-derived exudates, and/or they are present inside of spores and because they were not released from the spores the microorganisms were not spread onto the plates. In spite of slow growth (first observation of the putative bacteria was possible only after 2 weeks of incubation), it was possible to cultivate putative bacteria outside of spores. This may indicate that sporadic appearance of microorganisms was triggered by stress encountered by the spores (different substrate, absence of host roots, treatment with antibiotics, etc.). This hypothesis was also supported by the experiment, where spores were crushed to release the bacteria, but the absence of whole spores inhibited growth or did not support it at all. The plates, where few colonies were observed, contained less damaged spores and this allowed the putative bacteria to grow.

Microorganisms, isolated from spores, were used in PCR amplification using universal bacterial primers alongside with DNA extracted from spores and roots. However, DNA electrophoresis showed that there was no amplification of bacterial DNA in latter samples. A possible reason of the PCR failure was very low concentration of bacteria within the fungal structures. During an optimisation procedure it was shown that samples with DNA concentrations less than 20 pg μL^{-1} will give a negative amplification in standard PCR. In addition a rich background of endogenous fungal and/or plant DNA also made the reaction less effective.

However, we had several positive amplifications using the universal 16S rDNA primers to conclude that the microorganisms associated with spores were of bacterial origin confirmed by sequence analysis. Phylogenetic analysis (not presented in this work) showed all bacteria to be divided in three distinctive groups. Two groups were clustered together with uncultured bacteria from an environmental sample registered by Taubel *et al*. (2009) and *Staphylococcus*, commonly found on skin (Heylen *et al*., 2006); therefore the bacteria from these groups were assumed to be common contaminants. However, the third group did not have any close matches in the GenBank database and could belong to bacteria that are very specific and have been not yet identified. This group comprised of bacteria that were isolated from different sources such as bacteria from TS broth inoculated with spores; bacteria that grew around spores plated on TSB and bacteria grown from crushed spores inoculated on TS agar plates. These bacteria seemed to appear at varying time points through the fungal lifecycle, thus is likely that these bacteria have either a symbiotic or a neutral relationship with mycorrhizal spores.

The examination of spore ultrastructure gave us an indication of the location the endosymbiotic bacteria. These bacteria were observed growing inside the cytoplasm, unlike the bacteria described by Walley & Germida (1996) that were shown to be embedded in the spore wall. In this case there were no bacteria observed within the spore wall structure and TEM observations showed very low numbers of bacteria within the spore. This observation supports our assumption about the failure of the PCR experiments, i.e. insufficient bacterial DNA extracted from AMF spores. There was no clear cell wall observed in bacteria located inside the spores, however, Gram staining showed that the isolated and cultivated bacteria are Gram negative.

Fluorescence microscopy also proved the presence of bacteria within *Rh. irregularis*. It was not possible to determine the bacterial nucleoid within the spores due to rich fungal nuclei background, however, examination of hyphae showed the presence not only large fungal nuclei, but much smaller bacterial nucleoid. This finding confirms that bacteria are not only present in spores, but also in hyphae.

Acknowledgements

VEGEDURABLE II – this project is sponsored by selected under the European Cross-border Cooperatio–n Programme INTERREG IV A France (Channel) – England, co-funded by the European regional development fund (ERDF). European Union. Investing in your future.

We would like to thank Ian Brown for assistance with the sample preparation for TEM.

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