EUROLEGUME
2014-2017

Enhancing of legumes growing in Europe through sustainable cropping for protein supply for food and feed

FP7 Research Project № 613781

DELIVERABLE D3.1

HANDBOOK WITH PROTOCOLS FOR NODULE SAMPLING, RHIZOBIA ISOLATION, POLIPHASIC CHARACTERIZATION, EVALUATION OF BNF EFFICIENCY, COMPETIVENESS AND PERSISTENCE

LEAD BENEFICIARIES: UTAD, LLU AND AUA

M6 – JUNE 2014

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 613781

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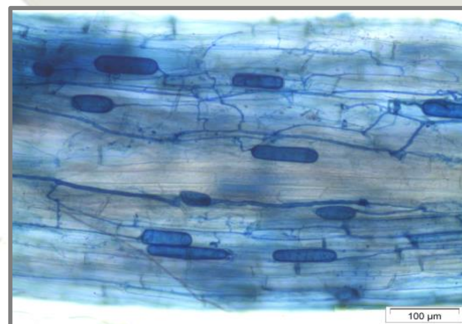
Working with Microbial Symbioses of Legumes

Handbook of Protocols



WORKING WITH MICROBIAL SYMBIOSES OF LEGUMES: HANDBOOK OF PROTOCOLS

1ST VERSION, JUNE 2014

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FP7 Research Project nº 613781

ABBREVIATIONS

AM - Arbuscular mycorrhizal

AMF - Arbuscular mycorrhizal fungi

ARA – Acetylene Reduction Assay

ARDA – Amplified rDNA Restriction Analysis

BNF – Biological Nitrogen Fixation

BTB - Bromothymol blue

CFU - Colony Forming Units

Chl - Chlorophyll

CR - Congo Red

dH_2O – destilled water

DNA- Deoxyribonucleic acid

dpi- days post inoculation

DW – Dry weight

E- Esther

EC- Electrical conductivity

EU . European Union

GPA - Grey Pea Agar

HPLC- - High-Performance Liquid Chromatography

IGS – Intergenic gene spacer

INT- Iodonitrotetrazolium chloride

LANS -Long Ashton nutrient solution

Las - Leaf area

LDWs - Leaf dry weight

MPN - Most Probable Number

OD - Optical density

PCR – Polymerase Chain Reaction

PCR-RFLP - Restriction Fragment Length Polymorphism of PCR products

PVC - Polyvinyl chloride

PVLG- Polyvinyl lactoglycerol

RCBD - Random Complete Block Design

RH- Relative Humidity

RHA - Rhizobium Agar

RHB - Rhizobium Broth

rRNA – ribosomal ribonucleic acid

rpm- revolutions per minute

SR - streptomycin resistant

SRL- specific root length

Str – streptomycin

UPGMA - Unweighted Pair Group Method with Arithmetic Mean

WBA - White Phaseolus Beans Agar

WUE- Water Use Efficiency

YM - Yeast-Mannitol

YMA -Yeast-Mannitol Agar

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1. Introduction

An increased production and consumption of legumes in the EU is highly desirable taking into account the high nutritional value, the beneficial health effects of legumes and their role in the farming systems.

Soil, as living organism, belongs to one of the most significant resources of agriculture. Climatic changes are influencing whole ecosystems including the populations of soil microorganisms. Soil microorganisms establish a framework of interactions responsible for key environmental processes, such as the geochemical cycling of nutrients and the maintenance of plant health and soil quality. The soil microbial activity is especially important for leguminous plants because the common feature for all legumes is Biological Nitrogen Fixation (BNF) in symbiosis with rhizobia. The term “rhizobia” includes a range of bacterial genera, such as *Rhizobium, Bradyrhizobium, Sinorhizobium* (*Ensifer*), *Mesorhizobium* and *Azorhizobium,* which elicit the formation of specialized organs, called nodules, on roots or stems of their hosts, in which they reduce atmospheric nitrogen and make it available to the plant. BNF is the key environmental benefit of legumes under a broad spectrum of environmental conditions and crop rotations resulting in reduced mineral N fertilizer use.

Besides their symbiotic relationship with rhizobia, legumes are mycorrhizal plants. In exchange for carbon from plant hosts, arbuscular mycorrhizal fungi (AMF) increase uptake of nutrients, resistance to diseases and drought tolerance. Although AMF are non-specific in their ability to infect a wide range of hosts, the degree of benefit to each partner in any given AMF–host plant interaction can depend on the particular species involved.

Exploiting the relation between genotype and environment is a strategy to mitigate the influence of climate change on agriculture. Using legume plants as sinks for different undesirable processes in ecosystem (soil erosion, greenhouse gas emission and carbon footprint) could contribute to the sustainable development of Europe ensuring safe environment and food for its citizens. Locally grown legumes are adding value not only to agricultural and environment resources such as improved soil properties, intensified cropping systems in sustainable way, new feed possibilities, etc., but also gain interest from agro-industries to develop innovative food products.

The FP7 EUROLEGUME project aims at the sustainable production of legumes, ensuring improved varieties, better microbial inoculants to support nitrogen fixation and plant growth, and develop innovative foods and feeds, turning EU more competitive. Multidisciplinary approaches, including investigations on

plant/rhizobia/AMF associations for different species/genotypes will be used for better understanding of ways to promote efficiency of rhizobia and AMF in biological nitrogen fixation (BNF) and nutrient foraging. Broad bean (*Vicia faba* L.) and peas (*Pisum sativum* L.) are legumes species cultivated in Europe since ancient times. Cowpeas, also known as black eye beans (*Vigna unguiculata* (L.) Walp), are to date widely cropped as grain legumes, food and vegetable crops particularly in the southern part of Europe and in developing countries. It could be used as a multipurpose crop- legume by using their leaf, grain and as forage.

This manual is intended to provide guidance on methods for working with microbial symbioses of leguminous plants, particularly rhizobia and AMF, and to standardize protocols, in order to ensure that the scientific data generated in the EUROLEGUME project is comparable between partners.

2. Working with rhizobia

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2.1 – Collection of nodulated specimens with direct sampling

- Select one representative legume for sampling. Careful excavation of the root system is required to get an accurate estimation of the nodulation status of a plant. With a spade, describe a circle with a radius of approximately 15 cm around the plant and cut out this section to a depth of at least 20 cm. Still using the spade, slowly lift out the clump.
- Carefully remove the soil from the root material with hands. Avoid detaching secondary roots from the plant as nodules may be found on the lateral roots as well as the tap root (Fig. 2.1).
- Carefully place the whole plant into a plastic bag.
-

Fig. 2.1. Sampling of plant roots from pots and from the field.

- In the laboratory, place a sieve of an appropriate size and mesh under each root sample to catch nodules than may become detached from the root. Collect healthy and complete nodules from roots.
- Determine pH and electrical conductivity of soil samples.
- For the purpose of BNF estimation, record nodule weight and/or nodule number. Collect five to ten (5-10) nodules from three (3) to ten (10) plants per cultivar. These numbers are arbitrary but are the minimal ones to get a representative sample.
- Nodules should be detached intact from the roots by cutting the root about 0.5 cm on each side of the nodule from main or lateral roots. All nodules from each plant are stored in the same vial.

- Each sample should be accompanied by a record form (Appendix 2.1). This form should provide information about: date of collection, plant host and variety, the shape, size, interior nodule pigmentation (red, pink, brown, green, greyish-green, black), location, soil type, cropping system, soil fertility conditions, soil pH, soil type and other useful information.
- Nodules are maintained at environmental temperature in plastic tubes filled with dehydrated silica gel for transportation (Fig. 2.2). Nodules can be stored in vials containing dehydrated silica gels in a refrigerator for up to 6 months. Fresh nodule samples could be stored in a refrigerator (4 ^{o}C) for up to 48 hrs. Nodules should not be frozen since freezing may kill bacteroids.

Fig. 2.2. Nodules in tubes filled with dehydrated silica gel for transportation.

2.2. Collection of nodulating bacteria with the trap method

- Soils for laboratory legume cultivation are collected from different fields. The samples are collected from acidic to neutral soils of different regions. At least, three soil samples are obtained from each field, to a depth of 10 cm, after removal of the surface litter, and the samples are homogenized to produce a single composite sample.
- The chemical properties of the soil samples should be determined before planting and at the end of the study period. The determination of mineral N in soil is useful knowledge since high concentrations of soil mineral N suppress N_2 fixation but low concentrations can promote N_2 fixation. Samples from the study site should be collected at sowing of the crop and at the end of the study period. Both samples should be collected from the same soil depth.
- Plant legume cultivars in 1 L culture pots filled with vermiculite and a 40% (v/v) N-free nutrient solution (Appendix 1.4) autoclaved at 121 ^{o}C for 20 min.
- Surface sterilize seeds by soaking in 70% ethanol for 30 s and in 1% sodium hypochlorite for 3min and then rinse with sterile distilled water for 5 times. Sown 5 sterilized seeds per pot (see also other methods in Appendix 1.5)..

- Make a suspension of soil sample (1 g soil in 99 mL dH_2O, shaken for 30 min at 100 rpm) from each of the sampling sites for the inoculation of the legume seeds sown (one soil suspension per pot). Alternatively, a soil sample (2-3 g) could be placed into the vermiculite at a depth of 2–3 cm, and the legume seeds are sown into the soil. As negative control, legume plants are grown in vermiculite without soil.
- Grow the plants for 4 weeks in controlled conditions (at 28 º C, with 16 h day, and 23 º C with 8h night).
- Isolate the nodulating bacteria as described below (2.4).

2.3 Examination of nodules and bacteroids

- Note the shape and size of the nodules recovered from the collected plants. Nodule size and shape vary depending on the rhizobia and host plant species (Fig. s 2.3 and 2.4). Characterize nodules by the shape, size, location, pigmentation.
- An active N-fixing nodule contains a protein called leghemoglobin. Its presence in the nodule can be noted by the characteristic pink, red, or brown coloration. Active nodules may also be black. Black nodules are not very common. They have been reported on *Lablab purpureus, Dolichous biflorus,* and *Vigna unguiculata* when inoculated with some strains of rhizobia.

Fig. 2.3 – Different shape of nodules. Left - Nodules on the roots of *Pisum sativum;* Middle -*Phaseolus vulgaris;* Right –*Vicia faba.*

Fig. 2.4 – Different size of nodules

- Senescent nodules are usually greyish green. When nodules on the soil surface are exposed to sunlight, they may develop a green exterior. Most ineffective rhizobia cause nodules with white interiors that lack leghemoglobin.
- Cut thin sections of nodules with a razor blade and float them on a drop of water on a microscope slide, use a cover glass and examine under low power (10×) and high power (40×) objectives.
- Gently rub the cut surface of nodule on a microscope slide to make a smear. Allow the smear to air dry and then pass the slide through a flame. Cool the slide and stain the smear with diluted carbol fuchsin for 10-20 seconds. Wash in water, blot off excess moisture, and air dry. Examine under the oil immersion objective.

2.4 Rhizobia isolation from nodules and cultivation

- Wash roots thoroughly with tap water to remove soil. Sever the nodule from the root by cutting the root about 0.5 cm on each side of the nodule. When moving the nodule, use forceps on the root appendages to reduce the risk of damaging the nodule.
- Immerse intact nodules in sterile glass or plastic containers containing 70% ethanol for 1 min. Then transfer nodules to a 3-5% (v/v) solution of sodium hypochlorite for 2-4 min. Rinse in five changes of sterile water using sterile forceps for transferring. Forceps may be sterilized quickly by dipping in alcohol and flaming.
- In cases of isolating rhizobia from desiccated nodules, a rehydration step before sterilizing is needed. Place nodules into a small beaker with clean cool water and leave in the refrigerator to imbibe overnight. A one hour soaking at room temperature is sufficient for nodules which have been desiccated for only a short time.
- Crush the surface sterilized nodule with a pair of blunt-tipped forceps or a sterile glass rod in a large drop of sterile water in a Petri dish or microtube. Streak one loopful of the nodule suspension on a Petri dish with yeast extract mannitol agar (YMA) plate (or Rhizobium agar (RHA), WBA or GPA), containing 0.0025% Congo Red (CR). (Appendix 1.1 and 1.2)
- Similarly treat one loopful of the nodule suspension on an agar plate containing 0.002% (w/v) bromothymol blue (BTB) (Appendix 1.1 and 1.2).
- Alternatively the first streaking could be done on YMA plates without indicator media, because the morphology and growth rate of rhizobia may be affected.
- Incubate Petri dishes at 25 – 30 °C in the dark.
- Daily inspect the plates for recording colony growth, possible presence of contaminants and appearance of typical colonies of rhizobia. Fast-growing rhizobia begin to appear within 3-5 days, while those of slow-growing rhizobia appear within 7-10 days.

- Typical rhizobia colonies appear white on YMA-CR plates since they do not absorb CR. Slow-growing rhizobia form blue colonies on YMA-BTB, which is indicative of alkaline reaction on BTB. Fast-growing rhizobia form yellow colonies, which is indicative of acid reaction on BTB (Fig. 2.5).
- If necessary, restreak rhizobia on YMA-BTB and/or YMA-CR plates to further purify the isolates. Check purity of culture by performing a Gram stain.

Fig. 2.5. Cultures of *Rhizobium* sp. on YAM-CR (left) and YMA-BTB (right).

Fig. 2.6 - Petri dish with colony of *Rhizobium* sp. on WBA.

- Cultures of pure isolates are maintained on YMA slants in screw-cap tubes at 4 °C for short-term storage or in YM broth supplied with 20% (w/v) glycerol at −80°C for long-term storage.

2.5 Cultural characteristics

The colony morphology of rhizobial isolates are evaluated (Appendix 2.3) as follows:

- size of the colony (diameter in mm),
- shape or form of the colony (punctiform, circular, irregular, filamentous, rhizoid, spindle),
- elevation of the colony (flat, convex, pulvinate, umbonate, crateriform),
- margin of the colony (entire, undulate, lobate, filamentous),

More characteristics of isolates may be obtained from culturing them under different "stress" conditions such as high salt concentrations, various pH and/or high temperatures.

2.6 Rhizobial enumeration

1) **Make a serial dilution and plate by one of the pour-plate, spread-plate, or drop-plate methods**

- Inoculate flasks containing 50 mL of broth media with rhizobia strain.
- Incubate the flasks at 25-30 °C on a rotary shaker at 200 rpm.
- Take the culture flask from the shaker after 4-5 days and remove 1 mL for dilution series.

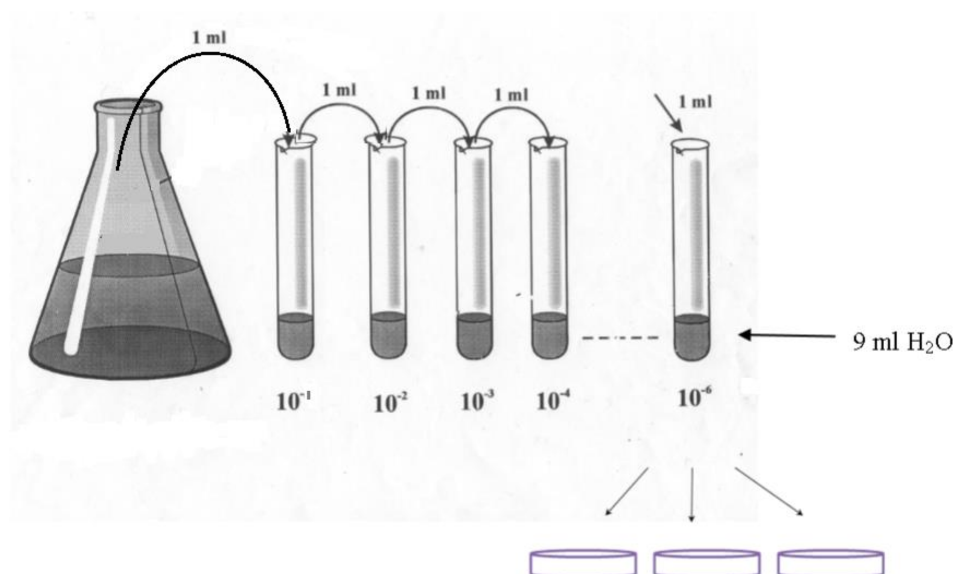

Fig. 2.7 - Preparation of dilutions.

2.6.1. Determining the number of viable cells in a culture by plating methods

A. Pour-plate method. Make serial dilution of the broth culture. Based on the total count, the number of viable cells will be approximately 1.0 x 10^9 mL^{-1}. A countable range for plate counts is 30 - 300 CFU per mL. To achieve this concentration, set out eight tubes, each containing 9 mL of sterile diluents (1/4 strength YM or RH broth, pH 6.8). One mL of the broth culture is diluted in steps, tenfold each time (10^{-1} through 10^{-8}). Use a fresh pipette for each strain. Open the Petri dish only sufficiently to allow the pipette to enter and deliver the sample. Flame pipette briefly (but do not overheat) by passing it through the Bunsen burner flame each time prior to successive removal of aliquots for replication (2 per dilution) from the same tube. Similarly with the same pipette remove 1 mL aliquots in duplicated from the 10^{-7} and 10^{-6} dilutions into more Petri dishes.

Pour 15 - 20 mL YMA or RHA (kept malted at 50 °C in water-bath) aseptically onto each of the cell suspensions in the Petri dishes. To disperse the cells evenly, gently move each Petri dish clockwise and counter clockwise. Allow the agar to set, invert the dishes and incubate at 26 - 28 °C. Read the plates after 3 - 5 days.

Multiply the average number of colonies by the dilution-factor. If the average number of colonies at 10^{-7} dilution is 50, then the original broth culture had a concentration of:

CFU per mL= (number of colonies) × (dilution factor) × (vol. of inoculum)
= 50 colonies × 10^{7} × 1.0 = 50×10^{7} cells per mL = 5.0 ×10^{8} CFU per mL

B. Spread-plate method

This method is also commonly used.

Use the same serially diluted samples. Begin with the 10^{-7} dilution and deliver 0.1 mL of the sample into each of four plates of YMA or RHA. Using the same pipette, dispense 0.1 mL samples from the 10^{-6} and 10^{-5} dilutions, in that order. Spread the sample evenly over the agar surface with a sterilized spreader. Incubate as before.

Calculate the number of viable cells as outlined for the pour-plate method, adjusting for the smaller volume that was plated (0.1 mL instead 1.0 mL).

C. Drop-plate method (Miles and Misra method)

This method is faster and consumes less material.

Use agar plates which are at least 3 days old or have been dried at 37 °C for 2 hours. Radially mark off eight equal sectors on the outside bottom of the Petri dish. Label four sectors for replications of one dilution and four for another, allowing two dilutions per plate.

Use the dilution series which had been prepared earlier. Plate dilutions of 10^{-7}, 10^{-6}, and 10^{-5}. Using a micropipette, begin with the highest dilution and deliver 20 µL to each of the appropriate four sectors of the plate. Two dilutions can be shared by one plate. Allow the drops to dry by absorption into the agar, then invert and incubate at 26 - 28 °C.

Results may not match those of the pour-plate and spread-plate methods at the first attempts. It is advisable to practice drop-plating with water before using this method for the first time. Fewer colonies per drop require more drops to be counted to provide the same statistical precision.

After 3 - 5 days of incubation, with daily observations, count the colonies formed. The preferred counting range should be 10 - 30 colonies per drop.
The following equation is used to calculate the number of colony forming units (CFU) per mL from the original aliquot /sample: CFU per mL = Average number of colonies for a dilution x 50 x dilution factor.

2.6.2. Estimating cell concentration by optical density

The optical density (OD) of bacterial suspension is generally correlated with the number of the cells. Optical density measurements are simple and convenient estimations of cell numbers as they require little manipulation.

Dilute 5-10 mL of the broth culture to 10, 20, 40, and 80% of its original concentration. Measure the light absorbance by each concentration with a spectrophotometer at a wavelength of 540 nm. Use broth to calibrate the instrument at zero. Relate the different concentrations to the actual cell count with the Petroff-Hausser chamber by plotting the optical density (OD) against the total cell number.

This method also has its limitations. It is best suited for initially clear media. Dead cells and contaminants contribute to the OD of the culture, as well as gum procedure by the rhizobia, undissolved salts or precipitates in the medium.

2.7. Development of antibiotic resistant rhizobia

Streptomycin resistant mutants are obtained by gradually adapting them to environments with higher doses of streptomycin.

- Select strains for the development of antibiotic resistant mutants. Culture the strains on WBA or GPA.
- Prepare WBA or GPA plates containing antibiotics:

Prepare stock solution of streptomycin (str) with a concentration of 1 g 10 mL^{-1} sterile water. Add 1 mL of stock solution to 100 mL of molten medium (45 - 50 °C). Each mL of medium now contains 1000 units (U) of streptomycin (str) = 0,001 mg mL^{-1} = 1 μg mL^{-1}.

Fig. 2.8 - Development of streptomycin resistant *Rhizobium* strains.

- Preparation of medium with gradually dilutions of streptomycin to obtain streptomycin resistant *Rhizobium* strains.

  Adaptation of *Rhizobium* strains to streptomycin start with a concentration of 10 units per mL (0.01 μg mL^{-1})and continues with 0.1 μg mL^{-1}, 0.25 μg mL^{-1} , 0.5 μg mL^{-1}, 0.75 μg mL^{-1} and finally 1 μg mL^{-1}.

The resulting streptomycin resistant mutants were designated SR form and stored on bean agar at +4 °C. After every 30 days storage, the resulting dressing of SR forms containing streptomycin (1 μg mL^{-1}) streak on WBA with streptomycin and in the media without streptomycin. Compare virulence of SR strains in the microvegetation experiments.

2.7.1. Identification of antibiotic-resistant marked strains of rhizobia in nodules

Antibiotic resistant marked strains of rhizobia may be identified by their ability to grow on media containing antibiotics. The antibiotic marker technique is applied in ecological studies.

- Collect nodules from experimental plants. Detach and surface sterilize nodules.
- Prepare plates for nodule typing with streptomycin and plain WBA or GPA.
- Draw a grid pattern on the bottom of each plate and number squares.
- Crush the surface sterilized nodule in a drop of sterile water with a sterile glass rod in a sterile Petri plate or Eppendorf tube. Streak one loopful of the nodule suspension on a Petri dish with WBA or GPA containing streptomycin and plain media. Incubate Petri dish at 25 - 30 °C in the dark and make daily observations.

2.8. Assessment of nodulation and BNF

Authentication of the isolates as rhizobia: the nodulation capability of each isolate is tested by inoculating seedlings of the cultivar from which the isolates were obtained (Koch's postulates). Experiments are conducted *in vitro* or in a greenhouse with plants cultured in pots. Nodulation efficiency is assessed 4-6 weeks later by determining the presence of root nodules (including number and size) and their colour (pink, white, green or pale yellow). Note: black nodules have been reported on *Vigna unguiculata* when inoculated with some strains of rhizobia.

2.8.1. *In vitro* studies

- Prepare seedling–agar using one of recipes (Appendix 1.3).
- Autoclave seedling agar at 121 ^{o}C for 15 minutes and disperse equal volume into test tubes (tube size depends on plant species). An appropriate amount of molten agar is dispensed so that after solidifying a 5-10 cm long agar face is presented for seedling growth.
- Preparation of germination plates:

  Make 0.75% (w/v) water agar. Autoclave at 121°C for 15 minutes. Pour 25 mL of melted agar into each Petri dish and allow to cool. Surface-sterilized seeds will be pre-germinated in these plates.
- Select, surface sterilize and germinate seeds.

  Batches of seeds with more than 70% viability will be suitable. Select undamaged seeds for uniformity in size and colour. Use one of method for surface sterilization of seeds (Appendix 1.5)

- Aseptically put sterilized seeds in Petri dishes and incubate Petri dishes at 20-30 ^{o}C depending of plant species.
   A. Pick up just germinated seeds with sterile forceps and place in the rhizobial suspension for 1-5 minutes. With sterile forceps transfer seeds in test tubes with seedling agar (Fig. 2.9).
   or
   B. Pick up just germinated seeds with sterile forceps and transfer into test tube with seedling agar. Cultivate till the seedling radices are 0.5-1.0 cm long and straight. Carefully clear (with a sterile glass rod) the rooting medium around the root of the plant, to the depth of 1 cm. Dispense 1 mL of the rhizobial culture into the cleared area around the root. Use fresh pipette for each new rhizobial species or strain.
- Wrap aluminium foil around lower part of tubes. It shields the roots from light and heat. Keep tubes in a growth chamber.
- Make periodical observations of nodulation.
- Periodically observe plants. Note colour and growth. Replenish tubes with sterile water as required. At the end of experiment determine plant weight and dry weight (dry for 48 h at 70 ^{o}C). Remove roots from the tubes and wash them free of rooting medium. Where nodules are present, describe nodule shape, size, pigmentation and distribution.
- Evaluation of experiment: effectiveness will be apparent from green coloration of the plant and abundant nodules that are red/ pink when sliced open.

Fig. 2.9. Cultivation of plants inoculated with *Rhizobium* sp. in large test tubes

2.8.2. Pot experiments

- Surface-sterilized legume seeds are inoculated with the rhizobial suspension and sown in pots with a substrate supplied with all nutrients except N.
- The pot experiments are performed in a completely randomized block design with six replicates.
- Uninoculated plants are used as negative control and uninoculated plants supplemented with nitrogen as control.

- After emergence five plants are left per pot.
- Three replicates per treatment are used for the determination of nodule number and dry weight, plant dry weight and nitrogen and protein content at full flowering stage.
- The other three replicates are used for seed yield.
- At the flowering stage, two plants per pot for determination of nitrogenase activity by Acetylene Reduction Assay (ARA) by gaseous phase chromatography and other three plants are used for the other measurements (Fig. 2.10).

.**Fig. 2.10.** Analysis of nodules in pot experiments.

A nodulation scoring system is suggested to be applied for assessment of nodule effectiveness (Fig. 2.11). Nodule colour, size and number are determined. The system may need to be modified for different locations and legumes. For cowpea, more than 20 nodules per plant are satisfactory after eight to ten weeks of plant growth.

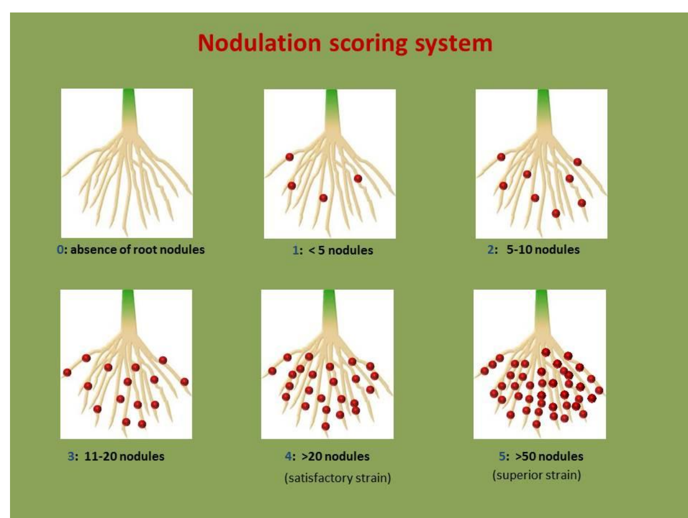

Fig. 2.10. Nodulation scoring system to be used within the Eurolegume Project based on 0 to 5 ranking.

2.8.3. Screening rhizobia for nitrogen fixation potential

The experiment is set up as a random complete block design (RCBD) with replications. There are inoculation treatments, plus- nitrogen control with no inoculation, and non- inoculated control with no nitrogen. Experiment can be organized:

- In large test tubes
- Leonard jars
- Microcosms

Fig. 2.11. Different variants of microcosms.

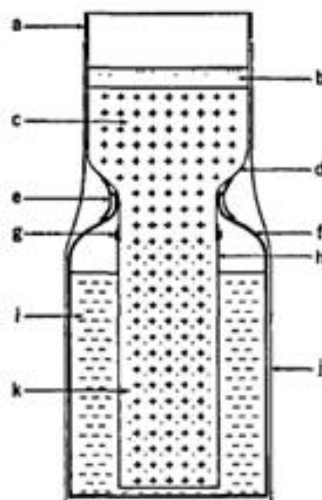

Fig. 2.12. Diagram of improved Leonard jar: a - Petri-dish; b - sterilized sand; c - vermiculite; d – inverted bottomless (growth bottle); e - heat-resistant tape; f- mayonnaise bottle (reservoir bottle); g – string (fixing growth bottle and water-prof bag); h – water-proof paper bag; i – N-free culture solution; j – aluminium foil; k – minute holes (sewing needle) (Trung and Yoshida, 1983).

Growing medias can be used the same as in previous experiments but only without agar. The plus – nitrogen control will contain 70 mg L^{-1} N applied as a 0.05% KNO_3 (w/v) solution.

Culturing the rhizobia, surface-sterilization of seeds, germination of seeds and inoculation of plants are similar as described above.

Make daily observations of the experiment. Five to ten days after planting, thin to uniform plants per jar.

Harvesting the plants

To minimize errors during harvest, the stem should be cut at the point of cotyledon attachment. This point is marked by the scar on the stem. If it is no visible, then cut at the level of growth medium. Place the shoots in labelled paper bags. Dry to constant weight at 70 ^{0}C for 2 days. Each bag should contain the plant shoots from only one jar.

Roots and adhering rooting medium are dislodged into a coarse sieve. Wash the rooting medium from the roots using gentle stream of water. Determine nodule score (Fig. 10) and describe the nodule distribution:

- Prolific tap –root nodulation,
- Occasional nodules on lateral roots
- Distant from the tap root
- Large number of small nodules
- Small number of large nodules.

Detach the nodules, count them, determine their total fresh weight.

Determine fresh and dry weight of shoots and roots for all treatments.

Determine plant pigment content in the leaves.

2.8.4. Assessment of Biological Nitrogen Fixation (BNF) by measuring nitrogenase activity of nodulated roots – acetylene reduction assay

The measurement of nitrogenase activity in the nodules is done by means of the acetylene reduction assay, which is a measure of ethylene production (see below). Nodules are detached from nodulated roots and put in a suitable air-tight vessel into which acetylene can be introduced. After a specified incubation period (30-45 minutes), samples are withdrawn and analyzed for ethylene produced with a gas chromatograph.

Note: Do not wash the roots to clean them as wetting decreases the nitrogenase activity significantly. A wet nodule probably traps the acetylene on the surface of the nodule by slight solution in water, thus making less acetylene available to the nitrogenase in the nodule. If the root system becomes wet, the nodules should be dried by blotting prior to being placed in the bottle.

Remove the nodules from the incubation vessels after gas samples have been removed for analysis. Obtain the fresh weight of nodules after blotting dry, and finally, oven dry the nodules at 70 ^{o}C.

Acetylene reduction assay

Nitrogenase reduces acetylene (C_2H_2) to ethylene (C_2H_4), and the ethylene formed can be very sensitively detected by gas chromatography. The acetylene reduction assay (ARA) is the most sensitive method to elucidate biological N_2 fixation activity. It could be used to compare nitrogenase activities among treatments in laboratory experiments as well as in field experiments. However, the ARA cannot be quantitatively converted to the amount of N_2 fixed. It should be noted that the ARA does not relate directly to N_2 fixation, but instead measures electron flux through nitrogenase. Total electron allocation for both N_2 fixation and H_2 evolution is measured by ARA. In addition, ARA is difficult to be applied in field experiment mainly due to difficulty of full recovery of nodulated root system.

The standard ARA method involves enclosed detached nodules or nodulated root systems in air tight containers and exposing them to an atmosphere containing C_2H_2 (Peoples and Herridge 1990). However, detached nodules are ready to desiccate and decrease N_2 fixation activity.

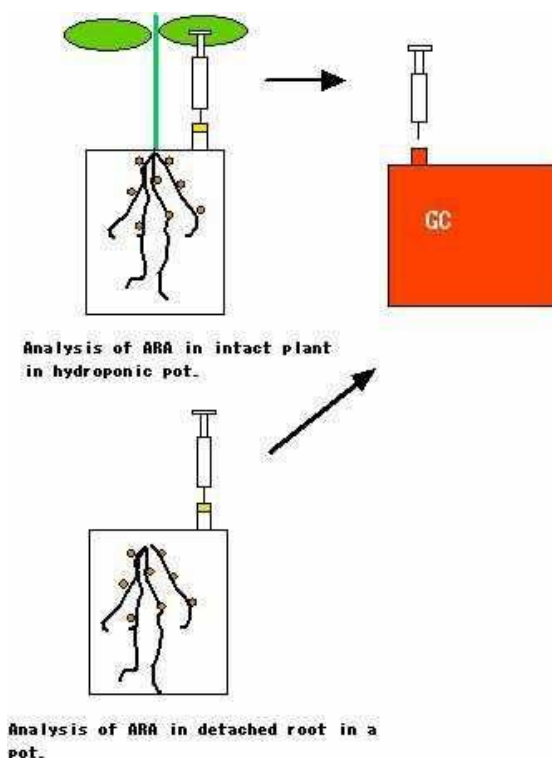

Fig. 2.13. ARA measurement of nodulated legumes in intact plants and detached roots

For hydroponically cultivated legume plants, the ARA can be measured in intact plants in a sealed pot (Fig. 2.13). About 10% of the air volume inside is replaced by pure acetylene. After incubation in a short period for

15 min or 30 min, 0.5 mL of the gas inside is sampled using a hypodermic syringe, and the gas is analyzed by GC (gas chromatography) equipped with FID detector. The column is Porapack N (GL Sciences). About 99.5% standard ethylene is diluted to 1000 times and 0.5 mL is injected.

Detached nodulated roots are most frequently used for ARA analysis (Fig. 2.14). Detached root system is placed in a glass jar (700 mL) and 10 % of the gas inside is replaced by acetylene. After 20 min incubation at 25°C, 0.5 mL of gas inside the jar is taken and concentration of ethylene is analyzed. After ARA measurement, roots are dried and nodule DW is measured. ARA is expressed as µmole ethylene formed per hour per plant or µmole ethylene formed per hour per g DW of nodules (specific ARA).

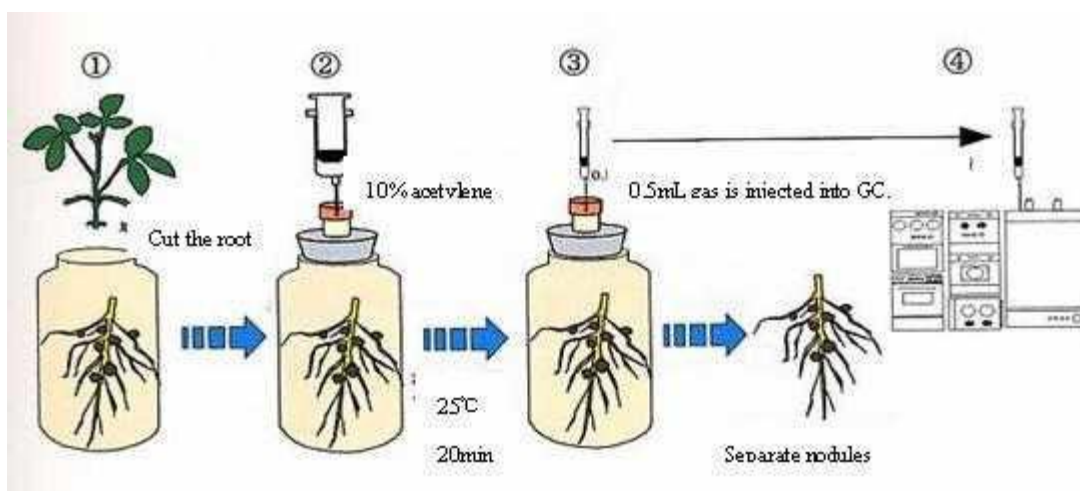

Fig. 2.14. ARA measurement of nodulated legumes in intact plants and detached roots.

2.8.5. Selecting effective strains of rhizobia in potted field soil

The effectiveness of mixed and single strain inocula are compared. Infective native rhizobial population in field soil are determined. The experimental design is a randomized complete block with as minimum three replicates.

Besides tested number of rhizobia strains, each experiment contains 3 controls, plus N control without inoculation; and two sets of non- inoculated controls. At 2 weeks the extra set of non- inoculated control is removed for inspection for nodulation by native rhizobia. If nodulation is observed in the non- inoculated controls, initiate MPN counts of native population using soil set aside for this purpose.

Preparing inoculum

Use antibiotic resistant strain from collection, propagate in the broth and dilute with water to reach turbidity 1×10^9 cells mL^{-1}.

Choosing the site for collecting soil

The ideal site for soil collection is one where the field experiment (which follows the pot experiment) is to be conducted. The site soil should be low in nitrogen. The native rhizobia population should be less than 10^9 rhizobia per g soil; no previous history of inoculation and cultivation with the intended legume.

Collecting, preparing and potting field soil

With steel spade or other suitable implement, obtain field soil from the depth of 10-15 cm. Soil samples should be taken randomly within a soil type. Collect and transport the soil (approximately 150 kg) in strong plastic bag to a clean area covered with plastic film. Mix the soil thoroughly and remove debris (e.g. stones, roots, leaves, etc.). Sift the soil using a 5 mm mesh screen. Take sample to determine the soil pH. If the soil is acid, add lime to bring the pH to 6.0-6.5. Mix the soil and lime thoroughly and allow to equilibrate for at least 7 days. During the equilibration period, cover the soil with plastic sheet.

Pots with diameter larger than 15 cm, higher than 18 cm, with a capacity over 3 L and, at least, with one hole on the bottom, are suitable for potting. Pots should be clean. Weigh soil in each plastic pot. Gently tamp the pots on the floor to compact the soil. Soil in all pots must be tamped down to occupy nearly the same volume to achieve similar bulk density.

Set aside 250 g of soil in a refrigerator (4 ^{o}C) for MPN count of the native rhizobial population.

Applying fertilizers

The fertility of the soil must be adjusted to optimal levels to obtain good growth of the plants (Appendix 1.4). Prepare the fertilizers (except insoluble triple superphosphate) in the form of solutions and pipette them on to the soil surface and allow to dry. Add the triple superphosphate. Mix the soil in each pot thoroughly to ensure uniform distribution of the nutrients (mixing is easily achieved by removing bag of soil from the pot and massaging).

Planting and inoculating the seeds

Surface sterilize seeds are treated with inoculum of *Rhizobium* and allow to imbibe water and bacteria for 10-60 minutes, avoid direct sunlight. Plant seeds at the depth of 2 cm. Each variant can be prepared separately. Label the treatments. Water the soil in the pots to field capacity.

Randomize the pots on the greenhouse bench.

Fig. 2.15. Vegetation pot experiments.

Periodical observations, care and harvest of plants

When the plants are 5-10 days old, thin to uniform plants per pot.

Inspect non- inoculated control plants for nodulation by native rhizobia.

Irrigate the pots and make periodic observations.

Harvest the half of plants at the flowering stage. Determine fresh and dry weight of shoots and roots, number, shape, size, pigmentation and location of nodules for all treatments, nitrogen content in the plants.

The rest of plants are harvest at the end of vegetation. Determine dry weight of shoots and roots, number of pods, number and weight of seeds, nitrogen content in the seeds.

2.8.6. Verification of the nitrogen – fixing potential of rhizobia in the field conditions

Strains of rhizobia, previously selected in potted field soil, are evaluated in the field environment so as to further identify the most effective strains for inoculants' production.

Select rhizobial strains and prepare the inoculants

Choose the inoculants strains and/or mixture. Inoculants can be prepared advance of the experiment and kept in the room temperature up to 2 weeks or stored up to 4 weeks in a fridge.

Prepare the field and apply fertilizers

Make calculations about treatments and plot size. Set up treatments with rhizobia strains, control without rhizobia and control without bacteria but with nitrogen fertilizer in four replications. Each treatment plot is franked by inoculated 0.5-1 m border. Treatments should be randomised.

Plough the field, remove rocks, plant roots and other debris. Till the soil to break up lumps and prepare smooth, firm seed bed. Alternatively, the sowing may be done without ploughing. This will minimize disturbance to the soil and release of soil nitrogen. Mark the plots and designate treatments for the different plots.

Cross contamination from rain wash may be controlled by the preparation of elevated seed beds.

Sprinkler and drip irrigation methods may be used if these are available.

Fertilize soil to optimal conditions (recommended fertilisers doses see in Appendix 1.4). Lime soil to pH 6.0-6.5. Apply the lime 2 weeks before applications of other fertilisers.

Inoculate the seeds and plant

Planting distance depended from plant species. Calculate seed number needed for each variant. Inoculate the seeds just before planting. Keep the inoculated seeds in their plastic bags, Petri dishes or flasks and in a cool place away from direct sunlight.

Set aside 20 seeds of each inoculated treatment and with minimum delay determine the number of rhizobia per seed.

Plant the controls and guard rows first and cover the seeds on completion of each row.

To prevent contamination of seeds, sterilize hands when handling each bath of seeds inoculated with different strain. Hands are easily sterilized by thorough washing with soap and water followed by swabbing with alcohol after the hands are dry.

Inspect the field and weed as necessary.

Inspect the field frequently for plant damage by diseases and insect pests. Take appropriate measures to control these pests. Weed the plots whenever necessary.

Make frequent observations of plant growth and colour.

Harvest at 50% flowering, examine nodulation.

Record when 50% of plant population to initiate flowering. Make early harvest at that time.

Harvest plants for dry matter yield. Observe nodule size, pigmentation, and distribution on the roots. Obtain fresh and dry weight of nodules.

Harvest plants

Record time for the plants reach maturity. Process the plants determining grain yield (dried to storage moisture).

2.8.7. ^{15}N natural abundance

The ^{15}N natural abundance method has been successfully applied to the study of N_2-fixation by legumes. ^{15}N Natural abundance technique is based on the principle that the proportion of N derived from the air (%Ndfa) via biological N_2-fixation (BNF) is proportional to the difference in ^{15}N abundance between the ''N_2-fixing'' legume and that of a suitable reference plant which only obtains nitrogen from the soil.

According to Unkovich et al., (2008), several methods are available for the determination of %Ndfa by field-grown legumes, each with their own advantages and limitations. The ^{15}N natural abundance isotope-based method is the most commonly employed, because it provides a time-integrated measure of the total amount of nitrogen fixed over the entire growth period. Moreover, this ^{15}N-based technique has the potential to separately evaluate the inputs of BNF- and soil-derived nitrogen to the legume based on a single harvest of the legume crop at maturity.

The strategy applied to exploit this technique is to assume that reference plants which are known to be unable to obtain nitrogen from N_2-fixation accumulate nitrogen only from the soil. Thus, if N_2-fixing legumes (eg. such as soybean (*Glycine max*), French/common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*) have significantly lower ^{15}N abundance than the reference plants, then the difference can be interpreted quantitatively to assess the contribution of biological nitrogen fixation.

The calculation of the proportion of N in the legume plant derived from the air (%Ndfa) is calculated using the equation of Shearer and Kohl (1986):

$$\%Ndfa = (\delta^{15}N(reference) - \delta^{15}N(N\text{-}fixing) / \delta^{15}N(reference) - B) \times 100$$

Procedure:

The ^{15}N natural abundance method can be applied both in greenhouse and field experiments. It allows the estimation of N_2 fixation in locations, where legumes and non N- fixing plants (usually non legumes) are present.

- Choose **a reference plant (a non N- fixing plant)** and include it in your experiment. More than one reference plant is recommended. You must have samples of reference plants from each experimental treatment and preferably from each replicate plots. In greenhouse trials very often specific reference treatments are included in the experimental design, whereas in farmers' field weeds can also be used

as reference plant. The choice of reference plant must be very careful, since the reference plant must have almost the same biological cycle as the legume crop that you are using for your experiment. Another point relates the choice of the reference plant is that it has to follow the same N pattern as the legume crop. The reference plant must exploit the same N soil as your main legume crop.

- Reference plant must be sampled at the same growth stage as the legume crop.

In case you want to use your 'B' value ('B' is the $\delta^{15}N$ of shoots of legumes that are fully dependent upon N_2 fixation and sampled at the same growth stage as the field plants)

- To estimate the 'B' value you must establish a greenhouse trial where plant must be grown in an inert substrate without N fertilization. Usually it is used sterilized sand for pots experiments, or if you are familiar with hydroponics, you can also use hydroponic substrates such as pumice or perlite. **The substrate must not contain any N source.** The plants must be inoculated with the same strain that you are testing in the field. The greenhouse trial must provide a reasonable 'B' value to be used in the field experiment.
- Otherwise, it can be used 'B' value from literature.

Sampling

- Whole shoots should be sampled. Do not split your plant material into (leaves, stems, pods)
- Legumes and reference plants must be sampled at the same time before reaching its maturity, preferably at the stage of early pod- filling.
- If roots from pots can be harvested a whole plant can be determined for ^{15}N.
- Nodules can also be harvested and analyzed for ^{15}N.

Standard Operating Procedure for Total-N and ^{15}N determinations

Milling and weight plant and soil samples

1. Divide plant material in to shoots, roots, nodules contained with large brown paper bags.
2. Dry in an oven at 70 ^{o}C until the plant material reaches its stable weight
3. Milled each sample to a fine powder: not very fine (i.e. not like bread flour), but resembling ground black pepper.
4. Nodule samples need ground in a mortar and pestle or RetchTM (ball) mill.
5. Store each ground sample in a sealed (air-tight), glass or plastic container

Weighing for mass spectrometry (**Total-N and ^{15}N determinations)**

1. Into tin capsules (#SC0010 (x500) SerCon Ltd, Crewe UK; www.swercongroup.com), weigh 0.3 - 0.4 (MAX) mg of powdered plant material (on at least 4 Fig. balance).

For *soil samples*: use no more than 5 - 6 (MAX.) mg per capsule.

2. Record the weight of each sample.

3. First crimp the capsule by clasping the top together and fold-over towards the base, re-shaping the capsule into a tubular roll. Take one end of the roll and fold again, this time along the length of the role to form a tight-coil. Ensure there are no sharp edges (these may catch on the delivery mechanism and stop the sample processing).

4. Place each sample for processing into a labelled (you may have more than 1), 96 well micro-titre (ELISA) plate, and note the orientation - recording the position (row and column coordinates), and weight of each sample in a sample record sheet (see example in Appendix 2.3). Use a lidded ELISA plate and record your name, date and plate descriptor on the lid.

5. Send for processing; indicating specifically that these are for "combined ^{15}N AND Total-N determinations".

Additional information can be found in Unkovich et al., 2008.

2.9. Molecular characterization

The genotypic characterization of nodulating bacterial isolates is performed by applying different molecular methods:

1) PCR-restriction fragment length polymorphism (PCR-RFLP) of 16S rRNA genes (also known as "amplified 16S rDNA restriction analysis (ARDRA)"). Primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') are used to amplify the 16S rRNA genes (Weisburg et al. 1991). The amplicon sizes are expected to be ~1500 bp.

2) PCR-RFLP of 16S-23S rRNA intergenic gene spacer (IGS). This method has high discriminating power and is useful to identify genomic groups at the intraspecific level. Primers IGS-F1 (5'-GTCGTAACAAGGTAGCCGT-3') and IGS-R1 are used to amplify the IGS regions (5'-TGCCCAAGGCATCCACC-3') (Sarr et al. 2011). PCR is performed as described by Laguerre et al. 1996. The amplicon sizes could range between 800-1800 bp.

3) Sequencing of 16S rDNA, IGS, *nifH* and *nodC* from selected isolates.

4) If necessary, genomic fingerprinting of the isolates would be made by rep-PCR using the primers for the ERIC elements (Versalovic *et al*., 1994).

2.9.1. PCR amplification of 16S rDNA

PCR reactions are performed in 50 μL reactions containing 100 ng genomic DNA; 200 μM each dNTP; 1.5mM MgCl2; 2.5 units GoTaq FlexiDNA polymerase (Promega) or other equivalent polymerase, 5 μL 5 X GoTaq Flexi Buffer (Promega), and 0.2 μM of both forward and reverse primers. PCR cycling conditions consist of a single cycle of initial denaturation at 95◦C for 5min, followed by 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), extension (72°C for 1 min) and final extension at 72°C for 7 min.

2.9.2. General procedure for PCR-RFLP analysis

- Aliquots (10 µL) of PCR products are digested with 5 U of restriction endonuclease in 20-µl reaction volumes by using the manufacturer's recommended buffer and incubation conditions. At least four tetracutter endonucleases (*Hha*I, *Hinf*I, *Msp*I and *Rsa*I) are used for fingerprinting analysis. The following restriction enzymes are also recommended: *Alu*I, *Cfu*I, *Dde*I, *Hae*III, *Hha*I, *Hinf*I, *Msp*I, *Nde*II, *Rsa*I, *Sau*3A1 and *Taq*I.
- Digested PCR amplicons are analyzed by horizontal electrophoresis in agarose gels (1-3%).
- The gels are stained and photographed by a digital camera. The digitized images are stored in a computer for pattern analysis with suitable softwares for the analysis of DNA fingerprints (e.g. Gel ComparII, Phoretic 1D, GelQuest or others).
- The obtained patterns are grouped into categories and presented in a Table.
- A dendrogram is constructed based on the UPGMA clustering algorithm by analyzing the similarity between the restriction fragments with the software used.

2.9.3. Sequencing of PCR amplicons from selected rhizobial isolates

Isolates belonging in different 16S PCR-RFLP and IGS PCR-RFLP types are chosen for sequencing analysis of selected rhizobial genes.

Procedure:

1. Isolates from the glycerol stocks are streaked on YMA plates and are incubated at 28 ◦C to allow colony development.

2. Total DNA from isolates are extracted from an YM liquid cultures grown to the exponential phase (0.4 < OD600nm < 0.6), using commercially available kits for isolation of bacterial genomic DNA or manual protocols.
3. Genomic DNA is used as PCR template for further molecular characterization. Genes for identification and phylogenetic characterization involve 16S, IGS, *nifH* and *nodC*. Primers for 16S rDNA and IGS are reported above while degenerate primers for *nodC* and *nifH* are used (Laguerre et al., 2001; Sarita et al., 2005).
4. PCR products are purified using commercial PCR clean-up kits.
5. Purified PCR products are cloned in TA vector to be subjected for sequencing analysis.
6. Standard reference strains belonging to several different genera are included in the study.
7. Sequences are used for phylogenetic analysis and identification of the isolates.

An useful resource is the “ICSP Subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* - diversity, phylogeny and systematics“ (edzna.ccg.unam.mx/rhizobial-taxonomy/), devoted to different issues related to the molecular systematics, taxonomy, genomics, ecology and evolution of rhizobia. Besides up-to-date links to all validly described rhizobial/agrobacterial taxa, the site provides tutorials in phylogenetic analysis, key papers, etc..

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3. Working with arbuscular mycorrhizal fungi

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3.1- Introduction

Arbuscular mycorrhizal fungi (AMF) live in intimate mutualistic symbiosis with almost of 80% of terrestrial plants, which they provide with water and inorganic nutrients, mainly phosphorus (P). The fungal mycelium acts for plants as an extension of their root systems, allowing them to optimize the use of soil minerals and water from a much larger volume of soil.

Mycorrhizal symbioses improve plant growth under low-fertility conditions, improve tolerance towards environmental stresses such as drought and pollution, and increase resistance to root pathogens. AMF are a major component of soil fertility and have an important role in the soil biological activity because of their abundance as well in the soil aggregation and stability, reducing soil erosion through better plant rooting capacity.

Mycorrhizal symbiosis stimulates plant growth while substantially reduce fertilizer requirements. Because most crop plants are mycorrhizal, AMF play an essential role in sustainable agriculture and should be more integrated in the agricultural production systems.

All AMF are grouped in the phylum *Glomeromycota*, with almost 220 species actually described with the majority of representatives belonging to the order Glomerales.

Many of the following protocols are available at the website of International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) (http://invam.caf.wvu.edu), a valuable resource for those working in mycorrhizal research, where additional protocols can be found.

3.2 - Extraction of spores from soils or pots and their observation

Spores isolated from root-rhizosphere soil mixtures enable to reveal the diversity and spore abundance of the arbuscular fungi sporulating in the pots or in the field. The determination of the species diversity of arbuscular fungi occurring in a given ecological site needs to conduct long-term and regular sampling of this area, as well as to induce sporulation of some fungal species in trap cultures.

Spores of AM fungi in soil can be collected by the **wet sieving and decanting method** (Gerdemann and Nicholson, 1963) to remove soil particles while retaining the spores on sieves of various sizes. Since the spores are lighter than that of soil particles, successive decantation of soil suspension followed by sieving can concentrate the spores from soil.

For collecting spores from pots, remove a pie-shaped slice of substrate from top to close to the bottom of the pot. It is critical (also in field soils) include roots in the sample because some species in all genera except *Gigaspora* produce intraradical spores.

Procedure:

1. Collect a mixture of roots and rhizosphere soil from a depth of 10-30 cm, using a shovel. Place in plastic bags. In the laboratory, remove the coarse materials with a 2-mm sieve, air dry and weigh the samples (this allow spore number to be expressed relative to soil weight). Store the samples in a refrigerator at 4 ^{o}C until processing.

2. Put the soil sample (10-50 g) in a waring blender, add 500 mL to 1 L tap water and blend at high speed for approximately 5 seconds (Fig. 3.1). Longer blending times do not affect spores, but break up roots to the extent that more organic detritus is associated with spores in the soil suspension.

3. After 10-30 seconds of settling down of soil particles, the blended material is decanted through a sieves with various mesh size - at least 1 mm, 100 µm and 50 µm, arranged in descending order of mesh size (Fig. 3.2). Usually AM fungal spores are collected on 100 µm sieves. Some small spores are on 50 µm sieves. The procedure should be repeated until the upper layer of soil suspension is transparent. Use stainless steel sieves commercially available or make a plastic sieve with PVP tubes and nylon mesh.

Fig. 3.1. Soil sample in a waring blender.

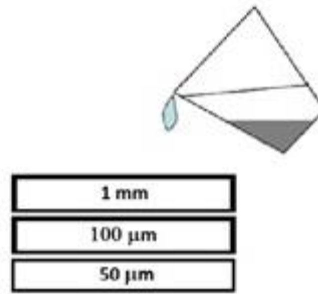

Fig. 3.2 – Sieving of soil suspension (Adapted from Morton et al., 1993)

1. The top sieve (1 mm or 500 µm) captures roots, debris, large spores, spore clusters and sporocarps. Despite the amount of organic material, these spores and frutifications are large enough to be easily detected and collected.

The material in the top sieve is washed, transferred to a large Petri dish, and viewed through a stereomicroscope in order to verify that no spores are present. The sievings on the fine mesh is collected into a small beaker and dispersed with weak ultra-sonication (ex: 30W for 30s). Strong sonication may destroy fungal spores. The dispersed sample is again passed through the sieve or layered onto a sucrose solution gradient.

The **sucrose density centrifugation** technique is often used to separate spores from the organic debris when is hard to find the spores hidden by the debris.

Procedure:

- The contents of the 50 µm sieve is collected in a 50 mL beaker and then transferred into 50 mL tubes containing a sucrose solution (20/60% [wt/vol]) gradient and centrifuged at 2000 rpm for 2-3 min. The resulting supernatant is passed through the 50 µm sieve, washed with tap water to remove sugar, and transferred to Petri dishes.
- Under a stereomicroscope collect manually the spores using fine Pasteur pipettes, sharpened with the flame of gas burner, to separate from organic material.
- Spores can be stored at 4 ^{o}C for up to 30 days (checking weekly for parasitized spores which then are immediately removed).
- The final (and most laborious) step is to separate spores of each morphotype (to mount on slides to make vouchers, inoculate plants to establish monospecific species, extract DNA, perform germination assays, etc.). Some taxonomic expertise is needed, but if the spores are in good

condition, anyone can initiate monospecific cultures. Identification to species can be made by molecular techniques (see below).

Throughout this procedure, record the **origin information of the isolated fungi** in detail as much as possible (i.e. site description: latitude, elevation, vegetation, soil type, cropping history etc.) and prepare and store **voucher specimen of spores**.

3.3 - Enumeration of Spores

The method for count the number of spores in a sample depends on the spore density (from invam.wvu.edu/methods/spores/enumeration-of-spores):

High density - Ocular field

1. Using a fine ruler, determine the diameter of the ocular field of the stereomicroscope at a magnification where spores can be easily distinguished from mineral particles and organic debris. Then calculate the area of the ocular field at that magnification.
2. Use plastic Petri dishes to count spores because the base of the plate is flat. The dish also is hydrophobic, thus, enough water must be added to have complete coverage of the base. Calculate the area of the base of the plate.
3. Calculate the number of fields in the dish = Area of the base of the plate/area of ocular field (**X**)
4. Add the spore suspension to a Petri dish and then randomly rotate the dish to spread out spores as evenly as possible. Note: This method will not work for extracted spores that have been stored more than 24 h due the formation of aggregates.

If there are less than 30-40 spores per field, count them in 40 fields randomly chosen over the area of the dish. Calculate average number of spores per field and multiply this number by **X** (number of fields/dish).
If there are more than 30-40 spores per field, then place spore extract in a test tube, dilute 1:1 with water. Vortex and remove a specific volume (10 mL works well), transfer to Petri dish, and recount. Make sure to keep track of each dilution to calculate spores in total sample.

Example:

Spores are extracted from a 50 cm^3 soil sample and suspended in 15 mL water.
In Petri dish, too many spores are observed in each field to be counted. This volume then is diluted 1:1 to 30 mL. Spores are counted from a 10 ml aliquot of this dilution. X=289. An average of 28 spores were found in 40 fields of view.

28×289 = 8092 spores in the 10 mL sample.

8092×3 = 24,276 spores in the total sample (30 mL and by extension, the original 50 cm^3 soil from which spores were extracted)

24276/50 = 486 spores/cm^3

Low density - Direct counts

When there are less than 3-4 spores in some fields of view and no spores in others (25-50% of fields examined), then the method above cannot be used because it will overestimate the total number of spores in the sample. In this case, spores are few enough to be counted directly.

1. Transfer spore suspension to a test tube, vortex, and transfer 1 ml to a watch glass. Perform this step three more times to count spores in four replicates.
2. Swirl water in watchglass (clockwise or counterclockwise) to concentrate spores in the center. Average the counts from four watchglasses and multiply result by dilution factor (1/x total mL in test tube).
3. If number of spores in watchglass is too many to count, increase the dilution and recount.

3.4 - Determination of the spore viability

Collect randomly thirty spores from a sample. Add the vital stain iodonitrotetrazolium chloride (INT) solution (1 mg mL^{-1}) and left at room temperature for 48h (Walley and Germida, 1995).

Count the spores at the stereomicroscope (40x). Spores were considered viable when turned red after reacting with iodonitrotetrazolium chloride (INT) and non-viable when they maintained the original color.

3.5 - Morphological observation of spores for identification

Morphology of spores is a basis for identification of AM fungi, because the hyphae and the organs such as arbuscules and vesicles are not specific to species. Spores collected from soil often deteriorate so that they may be used only for tentative identification at genus level. For detailed observation, culturing the AM fungus is required, mixing the soil samples with sterilized sand or vermiculite (1:1, v:v) for **trap cultures** (see below) and the spores from pot culture should be used.

Observation of intact spores under dissecting microscope

Spores collected from soil are put in a watchglass or a small Petri dish, and their shape, color and the attachment to spores are observed. Spores should be classified into each spore morphotype based upon detailed observation. At least 30-50 spores from the same morphotype should be observed.

Observation of spores mounted on slide glass under a compound microscope

Spores are mounted with polyvinyl lactoglycerol (PVLG) on a slide glass. Several slides should be made: intact spores mounted with PVLG; crushed spores mounted with PVLG; spores mounted with PVLG containing Melzer's reagent (1:1; v/v).
For best results, mounted specimens with PVLG should be studied only 2-3 days after they were mounted to give time for spore contents to clear.

The morphological characteristics of spores (shape, size, colour, surface ornamentation, wall structures and presence of sporiferous saccule) should be recorded because they may be helpful to identify genus of the target fungus. However, some genera need also molecular data. For species identification, the characteristics should be compared with those in the species description in the original reference or using reference cultures (Morton *et al.*, 1993). Many species description and pictures are available in INVAM website (http://invam.wvu.edu/the-fungi/species-descriptions) and in the website maintained by Janusz Blaszkowski (http://www.zor.zut.edu.pl/Glomeromycota/index.html) (Fig. 3.3).
For spore size, at least 40-50 spores should be examined.

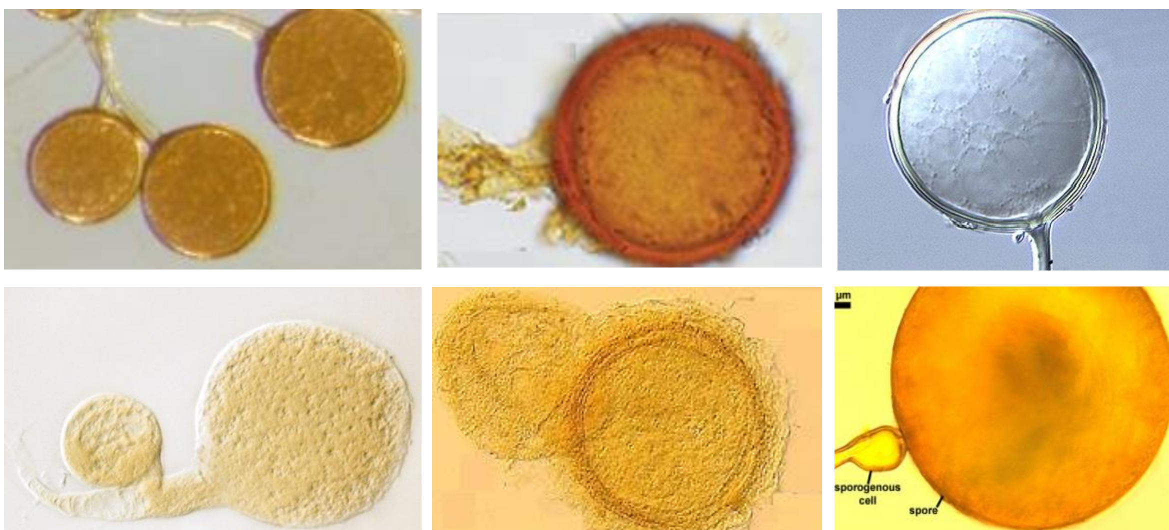

Fig. 3.3 - Morphology of representative genera of AM fungi: a) *Glomus,* b) *Diversispora*, c) *Paraglomus*, d) *Acaulospora*; e) *Entrosphospora; f) Gigaspora* (photos of J. Blaszkowski, with permission).

3.6 - Culturing AM fungi – Trap cultures

As AM fungi are obligate symbionts they cannot be cultivated without their association with plants. Traditionally they are propagated in pot cultures - *in vivo* cultures. These cultures are maintained for taxonomic, biochemical, and agronomical investigations. The production on inoculum using *in vivo* propagation systems necessitates greenhouses and growth chambers and intensive work for the maintenance and evaluation of cultures as well as constant monitoring to prevent cross-contamination between cultures and to maintain their monospecificity.

AMF can also be propagated under axenic culture conditions on excised roots - *in vitro* cultures. The Glomeromycetes in vitro Collection (GINCO) (www.agr.gc.ca/eng/science-and-innovation/research-centres/ontario/eastern-cereal-and-oilseed-research-centre/the-glomeromycetes-in-vitro-collection/?id=1236786816381) is an international collection which objective is to provide the scientific community and industry with a source of high quality starting inoculum of AM fungi, free of contaminants, produced under in vitro conditions.

Soil Trap Culture and Plant Trap Culture

Since the AM fungi need the symbiotic association with plants for survival and growth, is necessary to grow and inoculate the host plant.

For the AM fungal inoculum production or species detection, spores collected from soil can be used. However, spores in soil are not always active in colonizing plants or lack informative taxonomic characteristics due to alteration and deterioration by soil organisms. Therefore, trapping culture is often employed. Establishment of trap cultures using bulk soil or by mixing rhizosphere soil and root pieces with sterilized diluents and growing with suitable hosts (**Soil Trap Culture**), represents a strategy to yield a large number of healthy spores which can be readily identifiable. Trapping is also necessary to obtain abundant healthy spores for use as inoculum to establish monospecific cultures.

To isolate AM fungi colonizing roots, mycorrhizal plants collected from field, with their roots washed free of soil, can be transplanted to potting medium as **Plant Trap Culture** (Morton et al., 1993).

Trap cultures are important in the following situations:

- When mycorrhizal colonization is high in roots of a plant community, but little or no sporulation occurs (a common condition in many arid and hydric soil conditions).
- When the objective is to obtain abundant healthy spores of different species and establish monospecific cultures for specific purposes.

For preparing trap cultures collect samples (soil or mycorrhized plants) from the upper 20 cm of the soil profile.

Pots and potting medium: Pot size is critical, with the volume not less than one liter to optimize root occupation and sporulation. Sterile soil or soil-sand mixture is usually used. Various potting materials for horticulture can be also used. However, the materials should be low in available phosphate and preferably not rich in organic matter.

Host plant: Various host plants can be used: leguminous species (i.e. *Trifolium pratense*, *Medicago* spp., *Vigna unguiculata*) and grass species (i.e. *Sorghum bicolor*). Onion and leek (*Allium* spp.) are also good hosts. AM fungi generally do not show host specificity but some species show host preference. Therefore, the plant species from which the target AM fungus is isolated can be used as a host plant. The choice of host also can differ with objective.

Seeds should be surface-sterilized by soaking in 50 % household bleach for 10 min and rinsed three times with autoclaved distilled water.

Growth conditions: Use conditions which support good growth of host plants. To avoid contamination, a growth chamber is preferable (with a 16h photoperiod at 24 °C day, 20 °C night). If greenhouse is used, it should be kept clean. However, cross-contamination or contamination from dust is inevitable under open-air conditions, even in growth chamber. To prevent cross-contamination from other pot culture in the same chamber the pots could be covered with plastic bags (SUNBAG, Sigma Co.).

In general, pot cultures are used for three purposes:

1. trapping as many fungal organisms as possible that are indigenous to a field soil
2. establishing each organism of a species as unique accession (or culture)
3. increasing inoculum of each organism for use internally or for distribution to users.

After three multiplication cycles, the spores are extracted from the soil, separated according to their morphology and mounted on slides with PVLG (polivinyl-lactoglycerol alcohol) and with Melzer + PVLG (1:1; v/v) (Morton et al., 1993).

Single spore isolation: To purify an isolated fungus, single spore isolation is needed. Even if the spores are morphologically identical, it often contains contaminants whose morphology is very similar. For genetic studies or population genetics, the purification through single spore isolation is essential.

Procedure:

- Collect rhizosphere soil with a shovel or some other tool. Cut shoots at the crown and chop the roots with the associated soil using a sharp chopper.
- Mix the chopped roots and soil with autoclaved coarse sand (1:1; v/v) (use a plastic bag for a good homogenization of the mixture). Transfer for a 15 cm plastic pot.
- Seed pots with a suitable host plant with abundant root systems (eg. *Sorghum bicolor* or *Trifolium* sp.).
- Grow the cultures in a greenhouse or growth room for at least four months. Plants are maintained for 30-60 days after cessation of shoot (and root) growth to optimize spore yields. Keep fertilization to a minimum, to encourage AMF proliferation. Fertilizer should be applied only when plants show signs of phosphorus deficiencies (purpling of leaf sheaths), or nitrogen deficiency (chlorosis of young leaves). At 3-4 months, check for sporulation. By the fourth month sporulation may be at the peak.

Fertilization:

Apply to each pot 100 mL of modified Long Ashton nutrient solution (LANS, Hewitt, 1966) (Appendix 1.4).

- Let the pots to dry in a shaded room with a stable temperature so that the drying period is not too rapid (1-2 weeks).
- Spores should be extracted **before** pot contents are dry to obtain the brightest and cleanest specimens. If the spores are for establishment of monospecific cultures only dry long enough to eliminate free moisture. Harvest the spores using the sieving and decanting techniques or the density-gradient centrifugation technique.
- Store trap cultures in gallon zip-loc plastic bags for at least 30 days before spores are extracted for inoculation onto seedlings, because spores of some species (particularly those in *Acaulospora* and *Entrophospora*) appear to require a dormancy period before they are infective. The 30-day rest period appears to reduce or eliminate this variable.
- When colonization and/or sporulation is low after one culture cycle, then additional cycles often will result in recovery of additional species in greater abundance (Stutz and Morton, 1996). The bagged material is used as inoculum (undiluted) and set up within 30 days of harvest. If sporulation is extremely low, the pots aren’t harvested, but shoots removed and the pot contents reseeded.

- To obtain spores and hyphae free of soil particulates, the plant host in soil of interest can be compartmentalized in nylon mesh (40 mm openings) and surrounded with coarse sand (into which hyphae and spores will grow).
- The dried soil containing spores can be stored for a year at 4-5°C. It is advisable that the isolated fungi are re-cultured every year.

3.7 - Establishment of Monospecific Cultures

Monospecific cultures must be started from carefully selected spores. These spores can originate from a field soil if they are healthy and infective, more consistent results are obtained using spores collected from pot cultures: 1) many spores are newly formed and therefore usually healthy and more similar in age and 2) morphological distinctions between species are easier to detect (minimizing contamination).

Spores are extracted from field or culture 2-3 days before inoculation onto plant seedlings using wet sieving method or sucrose density-gradient centrifugation. The spores of each target species are collected manually with a Pasteur pipette and stored in a watch glass (sealed in Petri dish) at 4°C. Spores are examined daily until the day of inoculation. Any spores with changed morphology (loss of contents, collapse, colour change) or parasitism are removed.

Before inoculation, water is added to the spores, the watch glass agitated, and any particles, hyphal fragments or atypical spores are removed.

Containers (sterilized in 10% bleach) are first filled to the top with sterile sand: soil mix and 6-7 cm deep holes made in centers with a sterile glass rod. Label the containers with the inoculated morphotype/species.

An alcohol lamp and a beaker of 95% ethanol is set up for flaming the glass rod after each transplant operation.

Preparation of the plants

Approximately 12 days before a monospecific culture is planned on being set up, approximately 40-50 sorghum or clover seeds are evenly spaced in a 15-cm diameter pot using a sand-soil mix.

By 12 days, pot contents can be removed intact. This mass is placed in a large glass bowl filled with water so that it is completely immersed. Roots are gently teased from the growth medium and from each other and then left undisturbed for at least 30 minutes.

Inoculation

Transplant 3 seedlings into the center hole (made with a glass rod) of a container. Apply spores (in 200 µl water) in the root zone. The number of spores placed along roots varies with fungal species. Those that germinate readily and with high frequency (e.g. most *Gigaspora*, some *Scutellospora* and *Glomus* species) number 50-75. For all others, numbers range from 100-120 spores. *Acaulospora* species are the most unpredictable and so at least 100 spores are applied.

The soil is topped with a sterile growth medium, watered gently and the containers placed in a room with indirect lighting for 24h. Then transfer to the greenhouse or growth room.

Containers are not fertilized for at least 14 days after transplantation to optimize initial mycorrhizal development. The exception is when the potting medium consists of sand or some other soilless medium.

Watering is done manually each morning with care and attention to pot size, so as to minimize the volume of drainage into trays and also to prevent splashing.

After 4 months cut the shoots and check the presence of the required morphotype/species. After 3-12 months of storage, the soil, which consists of spores, infected roots and hyphae can be used as inoculum.

Flow of isolation and culture of AM fungi is summarized in Fig. 3.4.

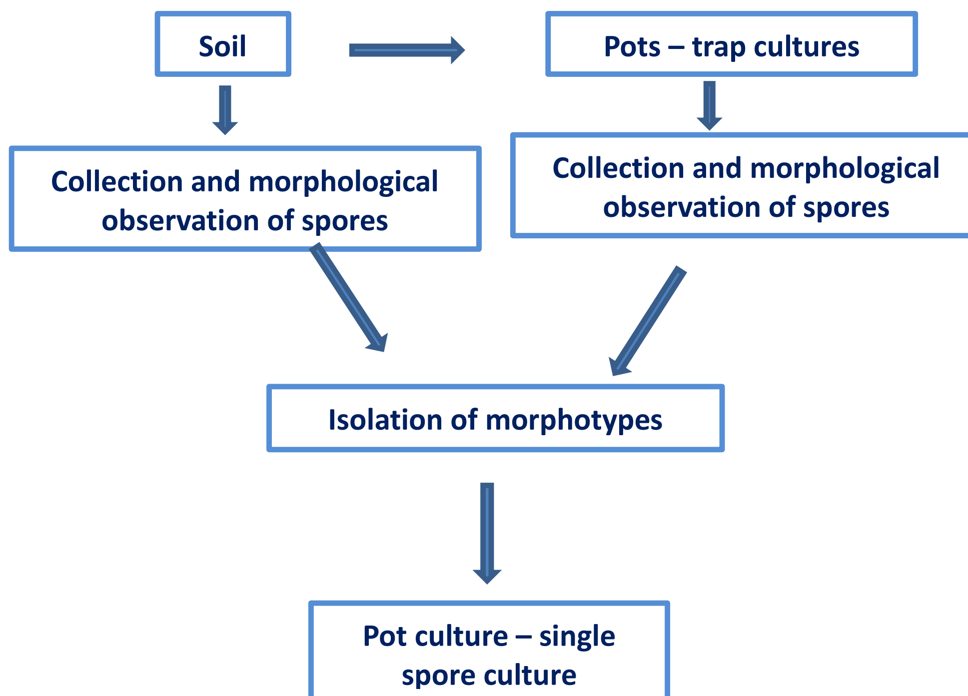

Fig. 3.4. Flow of isolation and culture of monospecific cultures of AM fungi.

3.8 - Staining endomycorrhizal roots

Method of Koske and Gemma, 1989:

- Wash freshly collected roots with tap water on a sieve (250 or 500 μm) to remove soil. Remove a subsample to determine dry weight.

NOTE: Roots may be stored in water at 4°C like this for a week or so before staining. (If they need to be stored for a longer period, place the roots in a 1:1:1 lactic acid: glycerol: water solution at 4°C)

- Remove another subsample and place in tissue cassettes, vials or test tubes for staining.
- Clear roots by soaking them in 2.5 % KOH overnight, or by soaking them for 15-20 minutes in a water bath or Bunsen burner by heating the KOH solution at 90°C.

NOTE: Different root types will require different clearing times and intensities. Check for color change to light brown then remove from heat. Do not over-clear because cortex of roots will disintegrate.

- Pour off KOH in a waste bottle, and rinse cassettes in destilled water 2-3 times.
- Acidify roots for 5-10 minutes (or overnight) in 1% HCl at room temperature. This is necessary for Trypan Blue stain to bind to fungal structures.
- Pour off HCl in a waste bottle, and remove cassettes from HCl and place in beaker with 0.05 % Trypan Blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) for 5-20 minutes in a hot bath or burner. The cassettes can be added to the boiling solution and then the burner shut off thereafter.

- Pour off Trypan Blue solution in a waste bottle (or recycle after filtration), and place cassettes in de-stain solution (50 ml glycerol: 48 ml water: 2 ml lactic acid) or lactoglycerol (1:1:1 lactic acid: glycerol: water) for a few days.

NOTE: If stained roots need to be stored for a longer period, place the roots in lactoglycerol or water-glycerol mix (2:1 v/v) with 1-2 drops of sodium azide, at 4°C in air tight containers. Stain is retained in fungal tissue for more than one year. Monitor for fungal contamination and if roots become to destained, repeat steps 5-7.

Method of Vierheilig et al., 1998 (ink in vinegar):

A more recently developed staining method uses ink and vinegar (Vierheilig et al. 1998, 2005). This staining solution consists of a 5% ink diluted in vinegar. Good staining results are obtained with black writing inks (Shaeffer Jet Black; Cross Black; Pelikan Black) and some blue inks (Pelikan Blue) (Brundrett et al. 1996).

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In this method, after the clearing the roots in KOH and rising in destilled water, roots are stained by boiling for 3 min in a 5% ink–vinegar (5% acetic acid) solution.

3.9 - Quantification of mycorrhizal colonization (modified line intersection method of McGonigle et al. 1990)

After clearing and staining them, root length can be measured simultaneously with mycorrhizal colonization by a gridline intersection procedure (Giovannetti and Mosse 1980), or separately by viewing slides with a compound microscope (McGonigle et al. 1990).

In the modified line intersection method of McGonigle et al. 1990 a compound microscope with an eye piece cross-hair moved to randomly selected positions is used to measure the mycorrhizal colonization as shown below.

- Mount the root fragments in parallel on glass slides. For each treatment prepare 3 slides (containing 4-5 root fragments, approximately 3 cm long). Calculate the occurrence of AMF structures recording at least 70 line intersections per slide (Fig. 3.5).

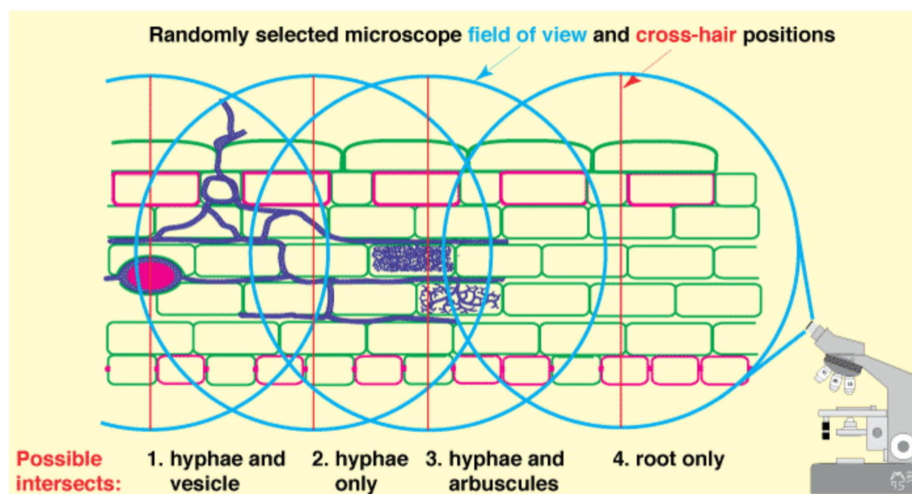

Fig. 3.5 – Microscopic observation of root segments to quantify arbuscular mycorrhizas (Brundrett et al. 1996).

The parameters of mycorrhizal colonization - frequency of mycorrhization (F%); percentage of mycorrhization (M%); percentage of arbuscules (a%) and presence of arbuscules in the root system (A%) - are determined according to microscopical observation.

- Frequency of mycorrhization (F%) - percentage of segments showing internal colonization
- Intensity of mycorrhization (M%) - average per cent colonization of root segments
- Percentage of arbuscules (a%) - average presence of arbuscules within the infected areas
- Presence of arbuscules in the root system (A%) - average presence of arbuscules in the whole root system.

3.10 - Infectivity assays

AMF infectivity in the soil is assessed using a bioassay with bait plants. This allows to measure the ability of the different target plant species to enrich the soil rhizosphere with infective AMF propagules, building up the native AMF inoculum (Brundrett and Abbott, 1995). Bait plants in the bioassays can be the same as the plants used in the greenhouse. Pre-germinated seeds are sown into the soil immediately after the harvest of the plants, and are left to grow for four weeks.
Mycorrhizal colonization is estimated as described above. In the bioassay approach, percent root colonization of the bait plant is assumed to reflect the number of total infective propagules (spores, mycelium and colonized roots from other plants).

Infectivity assays in greenhouse: if the aim is to determine infectivity of fungal propagules in a particular experimental soil which the fungus was collected, then the source field soil should be used (mixed with sand) after sterilization twice, at two consecutive days, at a minimum temperature of 80 ^{o}C.

- In pot experiments it is important to add a microbial wash from soil inoculum, reintroducing bacteria and fungi, to mycorrhizal treatments (Koide and Li, 1989).
- To prepare the soil filtrate, suspend 200 g of soil in 1000 mL sterile water for 60 min. After shaking and decanting, filter the suspension twice (Whatman nº 1).
- Install a drainage system to avoid contact among running waters from different AMF treatments
- The pots with different inoculation treatments should be periodically rotated to different bench positions to minimize differences due to their location in the greenhouse.
- Every pot from the non-mycorrhizal treatments should receive the same amount of AMF inoculum autoclaved twice (121 ^{o}C, 25 min.) in consecutive days.

Biomass measurements: At each harvest, four replicates of each treatment are selected randomly. The plants are separated into leaves, stems, roots and nodules, and oven-dried at 70ºC for 48h. A root sub-sample from each treatment was taken before oven drying for estimation of AMF infection of the root tissue. The fresh mass of the sub-sample was recorded so that the dry mass of the sub-sample could be added to the total root dry mass.

3.11 – Molecular analysis

Molecular analysis is essential for identification of AM fungi. Sequence data for conserved genes such as rRNA is obtained by PCR amplification followed by sequencing with DNA extracted from spores.

DNA extraction

Many protocols for DNA extraction are reported for several authors, for example the protocols of Morton et al., 1993, described below. DNA can be extracted from spores or from roots colonized by AMF.

Cleaning of spores

Healthy-appearing spores from an extract are transferred to a watch-glass in tap water and then checked under a stereomicroscope to remove any hyphae or debris that may be present. The watch-glass is placed in a sealed petri dish (to reduce evaporation) or in a capped vial and stored at 4 ºC for at least 48 h.

Examine spores again and remove all that have any variation to normal appearance (spore discoloration, spotting, disorganization of contents, excessive opaqueness, signs of bacterial or hyphal growth on spore surfaces).

Collect clean spores with tweezers or fine Pasture pipette. Wash spores several times with sterile water with sonication for 10-20 seconds. If spores show water repellency, surfactant such as Tween 80 can be used. Microplate with 6 or 12 wells is convenient for successive washing.

Crushing spores

A cap of Eppendorf tube can be used. The cap is cut from the tube and placed upside down on the stage of a dissecting microscope. Apply 20 mL of InstaGene (Bio-Rad) to the cap. This resin binds to

PCR inhibitors rather than DNA. A spore or spores are put into the cap and crushed thoroughly with a micro-pestle or any fine rod. Put the tube on the cap with crushed spores. Centrifuge the tube for spinning down the reagent with crushed spores from the cap into the tube. DNA in the supernatant could be ready for PCR but further purification is sometimes needed to remove inhibitors for PCR amplification.

Alternatively, spores are crushed in PCR buffer and the boiled for 15 s. After centrifugation at 13000 rpm, 2 μL of supernatant is used as template for PCR.

PCR amplification

Conditions of PCR amplification depend on the primers used.

The primers NS1 (5'-GA GTC ATA TGC TTG TCT C-3') and NS4 (5'-CTT CCG TCA ATT CCT TTA AG-3') universal primers are used in the first amplification (White et al., 1990) followed by nested amplification with the primers pair AML1 (5'-ATC AAC TTT CGA TGG TAGGAT AGA-3') and AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3') (Lee et al., 2008). PCR amplifications are performed using one polymerase following the thermal profile described for each couple of primers. PCR products of the expected size (~800 bp) were then checked by gel electrophoresis and purified from agarose gel using commercial gel extraction kits.

Cloning

The resulting product is cloned into vector system I and transformed into *Escherichia coli*. To obtain PCR products for restriction fragment length polymorphism (RFLP) analysis, putative positive transformants are selected by picking the bacterial colonies with a toothpick into a 10 ml H_2O and then 15ml of the PCR mix was added to each sample tube. Amplification is performed with the vector primers.

Restriction analysis

The positive clones are digested with restrictions enzymes (ex: *Hinf* I and *Mbo*I). Mix 5μL of each PCR product with 10 μL of digestion mix for 3 h at 37°C. Analyze fragment patterns on agarose gel containing 1 % regular agarose and 1% high resolution agarose.

Sequencing

Select clones for sequencing on the basis of the RFLP typing. Sequence one clone from each RFLP type found.

Phylogenetic analysis

A phylogenetic analysis is performed by comparing the sequences of the analysed samples with the corresponding publish genomes of other mycorrhizal fungi. The sequences are aligned by using the CLUSTAL W program, and neighbor-joining tree constructed by using MEGA software.

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4. Plant phenotyping protocols

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4.1 Introduction

Above- and belowground phenotypic traits are important parameters reflecting plant performances; this description is designed to outline a manual phenotyping approach to select better pea and bean varieties for increased commercial use or future breeding efforts within the EUROLEGUME project. For example, information on photosynthesis rates and number and arrangements of leaves is important because this (partially) determines carbon assimilation rates and subsequently growth and yield - while transpiration rates partially determine the water use efficiency of a plant. Root architectural differences not only determine the plants' ability for water and nutrients uptake and its transport efficiency but entail also certain carbon costs below ground. The correlation between root traits and shoots traits and crop yield, should be studied in bean and pea plants in order to determine high yielding varieties adapted to suboptimal or stressful environmental conditions. The capability of pea and bean plants to form symbiosis with rhizobia for nitrogen fixation and arbuscular mycorrhizal fungi (AMF) for improved nutrient uptake cannot be ignored either; however, information about root system development and functioning with different symbionts is scarce. Thus, in the third and fourth year of the EUROLEGUM project, the influence of different rhizobia and AMF strains on the legume phenotypes and plant functioning under suboptimal environments will be central.

4.2 Greenhouse experiment

The phenotyping facility at BOKU is a 27m long, 7m wide and 3.5 m high foil house orientated in North-South direction and located in Tulln, Austria (48.33° N, 16.05° E). However, any kind of greenhouse could be used for growing plants for phenotyping purposes. Photosynthetic active radiation (PAR), mean daily maximum and minimum temperature and humidity are recorded during the experimental period in 30 min intervals. In addition, soil properties (e.g. grain size distribution, water holding capacity) as well as the amount of water for irrigation and the amount and quality of applied fertilizers (see below) are recorded. Some columns will be equipped with soil moisture and temperature sensors to exemplarily determine the water and temperature distribution.

4.2.1. Experimental set up at BOKU

Plants will be grown in 1 m high, 20 cm wide plastic columns filled with 0.7 to 1.2 mm-sized quartz sand – allowing for unrestricted root growth, regulation of water and nutrient supply and easy sterilization for controlled rhizobia and AMF application. Columns are closed at the bottom with a perforated lid; a layer of mineral wool facilitates water drainage and prevents leakage of sand.

Columns are arranged in 8 blocks with North to South direction and up to 50 columns per block (each variety/treatment occurs in one replicate per block; Fig. 4.1). Different varieties are randomly distributed within each block.

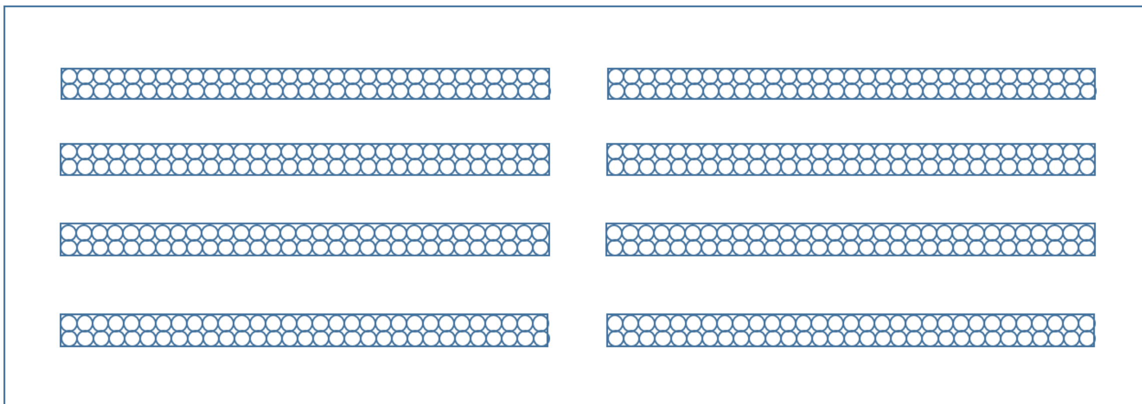

Fig. 4.1 Arrangement of soil columns (1m height, 20 cm diameter) in eight blocks at BOKU phenotyping facility, Tulln, Austria. For each variety (and treatments in subsequent years) replicates are partitioned between blocks (n =8).

4.2.2 Germination, rhizobia and AM inoculation and transplanting

Visually intact seeds of similar size are weighed, surface sterilized and germinated in darkness at 22±0.1°C in 0.7mm to 1.2mm-sized sand in a growth chamber. The sand will be enriched with 2.5% of mycorrhizal inoculant provided by Symbiom s.r.o., Czech Republic (Ales Latr). For surface sterilization, seeds are shook in 1% sodium hypochlorite for 1 min before rinsing under tap water. Subsequently, seeds are placed for 10 minutes in a strain-specific Rhizobia mixture – the mixture is prepared by diluting the content of one normal-sized Agar plate with Rhizobia in 100 ml of water. Rhizobia strains are kindly provided by Ina Alsina (Latvia). The seeds are planted within 1 hour after being coated with rhizobia. Germinated plants will be grown the first days under full irrigation and low N fertilization; 400µmol m^{-2} light intensity and a photoperiod of 14 h. Three to four days after germination, seedlings with similar size are transferred within their germination sand to the columns; one plant individual is planted in each column. About 2 g of additional AMF inoculant are placed in the planting pit before planting.

4.2.3. Fertigation

A modified Long Ashton nutrient solution is used to supply the non-nitrogen micro- and macro-nutrients (Apendix 1.4). A lower quantity of KNO_3 will be added to allow for full rhizobia symbiosis. Different KNO_3 levels will not be used for basic phenotyping but to determine plant-environmental interactions in the second year. As standard, concentration of nitrate-N is 0.71 mM KNO_3 (10 ppm N). The pH of the nutrient solutions is adjusted to pH 7 and the solution is filled up to 1000 mL with distilled water before being applied to plants via drip fertigation (Jia et al., 2004). Frequency and amount of surface drip irrigation is determined in practice; however, for basic screening, excessive amount of water are applied to avoid nutrient accumulation. Starting in the second year of EUROLEGUME, columns are modified to serve as weighing lysimeters; drainage water, evaporation from empty columns and water content of sand will be determined to measure the water use and the water use efficiency of varieties under different environment and/or different symbionts.

4.2. Nondestructive measurements above ground

1. Leaf number

Beans have compound leaves divided into three oval or heart-shaped leaflets that are dark green and rough in texture. The leaves are arranged alternately on the stem. Peas have pinnately compound leaves with some of the leaflets modified into tendrils. Leaf number is determined biweekly by counting.

2. Stem diameter

Stem diameter is measured biweekly at the first node of the stem with Vernier caliper (Ramirez-Vallejo and Kelly, 1998).

3. Plant height and growth habit

The height of plant is measured from soil surface to the top of erect plant. If it is a vine like shoot, then shoot length (mm) might be measured by stretching the longest tendril. Reference starting points along the shoots are marked with tape, resulting in all shoot tips bearing exactly two leaves from the reference point on day 0 (Cramer et al., 2007).

The growth habit is evaluated in the 6th week after sowing; the following categories are used:

1. Acute erect (branches form acute angles with main stem)
2. Erect (branching angle less acute than above)
3. Semi-erect (branches perpendicular to main stem, but do not touch the ground)

4. Intermediate (lower branches touch the ground)
5. Semi-prostrate (main stem reaches 20 or more centimeters)
6. Prostrate (plants flat on ground; branches spread several meters)
7. Climbing

4. Photosynthesis, stomatal conductance, intercellular CO_2 concentration, and transpiration

Photosynthesis measurements are carried out in the morning form 9:00am to 11:00am in order to assess the stomatal conductance, net CO_2 assimilation, intercellular CO_2, transpiration and water use efficiency (WUE) under greenhouse growth conditions. For these measurements, the most recent mature leaf is enclosed in the IRGA Li-6400 (Licor Inc.), under a light intensity of 700 PPFD [CO_2] of 400 µmol/mol, leaf temperature at 25°C, and relative humidity (RH) between 40 and 55%: readings of photosynthetic parameters are taken after stable values are reached.

5. Chlorophyll (Chl) a fluorescence

Measurements on the most recent mature leaf are performed from 9:00 until 11:00 in the morning with a portable MINIPAM fluorometer (Walz, Effeltrich, Germany). DLC-8 leaf clips (Walz) are used for 10 min dark adaptation before recordings.

6. Date of anthesis of the first flower

Time of first flowering is defined as the day when the flowers on the first flowering node on the main stem are fully open (Stoddard, 1993, Khan et al., 2010).

4.4 Destructive measurements above ground

4.4.1 Harvest

Harvest will take place 4 to 8 weeks after first flowering (to be determined according to plant development, same for all varieties). In doing so, shoots are cut 1 cm above the soil surface and then divide it into stem and leaves. If not needed for other analysis, leaves are collected, stored in labeled paper bags and dried in the oven (48h, 70°C) and then weighed with fine scale for dry biomass. The total leaf biomass includes samples used for EC, chlorophyll content, specific leaf area, and venation (see below). Stem biomass is stored in labeled paper bags and dried in the oven (48h, 70°C) for dry biomass (fine scale).

4.4.2 Electrical conductivity (EC)

Sample two healthy leaflets randomly, record fresh weight and cut them into 2 cm discs, rinsed with ddH_2O and then put into tubes with 20 ml dd H_2O. Then the samples are put on a shaker. The EC value is taken after 24 hrs. EC value is taken again after samples are autoclaved. The samples are oven dried (48h, 70°C) and weighed with fine scale.

4.4.3 Chlorophyll content

Two healthy mature leaves are harvested and the fresh weight (18–30 mg) (fine scale,) of each sample is recorded. Leaves are then frozen in liquid nitrogen and stored at −80°C in a deep freezer until further analysis. Chlorophyll a+b and Carotenoid contents are measured after 8 h extraction in ethanol (65°C) with a microplate reader (Waren, 2008).

4.4.4 Specific leaf area (SLA) and leaf dry matter content (LDMC)

Select six fully expanded and illuminated leaves, without serious herbivore or pathogen damage (Garnier et al., 2001) (2 from the top, middle and bottom of the plant if possible). The fresh weight of leaf samples are recorded immediately (fine scale) and then stored in plastic bags preventing water loss. The sampled leaves are scanned with flatbed table scanner (Epson XL-10000) within 3 days. Leaf area (LAs) is analyzed by WinFolia (Régent, Quebec, Canada). Then the sampled leaves are dried in the oven (48h, 70°C). Leaf dry weight is measured with a fine scale.

4.4.5 Leaf and stem anatomy

Select three leaflets randomly, record the fresh weight (fine scale) and keep them in tubes with 75% ethanol. Leaf anatomy such as vascular bundles and e.g. epidermis, mesophyll thickness is possibly analyzed later. A one cm-long stem segment is sampled from the first internode above the primary leaves, placed in storage tubes filled with 75% ethanol and stored in cooling room for anatomical analysis. Digital pictures of cross sections will be analyzed for xylem vessel frequency and diameter with Image J (NIH, USA). The theoretical hydraulic conductivity will be determined and related to leaf area (Huber value).

4.5 Belowground measurements

The soil is pushed out of the column onto a 2 mm mesh. After determining the maximum root system depth, the soil column is separated into depth classes 0-20 cm and 20-100 cm; each of them is rinsed carefully with a soft water jet. Roots crowns are excavated from the 0-20 cm soil section and placed in a container with soap for washing. Detergent is added to the water, helping to separate roots from soil particles, without significantly damaging the root system or causing the loss of a large number of fine roots. Then, root samples obtained from root crowns are washed and rinsed in clean tap water (Miguel, 2012).

1. Possible adventitious roots from the stem (some varieties might not have)

Diameter of a representative adventitious root is taken 1 cm from the point of attachment. The fresh weight of all the adventitious root samples is determined with a fine scale immediately after sampling. Keep all of them in tubes filled with tap water and stored in cooling room. Further analysis of all the adventitious roots is done through scanning.

2. Basal root (some varieties might not have)

Basal root angle is measured on a large protractor. The 1 cm stem left on the root system is used as a reference, i.e. set at 90 degree. The number and angles of all basal roots are recorded. Diameter of basal roots are measured at 1 cm distance from the tap root. The roots are separated from the tap root and roots are plotted dry paper towel; basal root fresh weight is taken immediately. Facultative: One representative basal root is used for root respiration measurement. It is set in a chamber, and the increase in CO_2 concentration is measured by infrared gas analyzer in a closed system (Dannoura et al., 2006). The dry weight and length of are also recorded for later normalization. Place all basal root samples in tubes filled with tap water for later scanning.

3. Tap root and laterals on tap root

Tap root diameter is taken 1 cm down from the boundary between the tap root and hypocotyl for the tap root measurement (Barlow, 2011).

The length is measured with a ruler and fresh weight (fine scale) is taken immediately after sample is dried with paper towel. Dry biomass (oven-dried, 65°C, 48h) is also taken with a fine scale. Facultative: One representative tap root segment (5 cm from the soil surface) / tap root lateral (5-7 cm from the soil surface along the tap root) can be used for root respiration measurements (see above). The whole tap root is scanned separately from all her laterals for further analysis.

4. Nodule density

The number of nodules per root length is counted on representative samples of basal root, tap root and lateral roots, then up scaled to determine the total nodule number. In addition, the color (pink, white, green or pale yellow) and size of nodules can be measured by analyzing the digital scans with Image J (NIH, USA).

5. Mycorrhization

After clearing and staining roots, root length is measured simultaneously with mycorrhizal colonization by a gridline intersection procedure (Giovannetti and Mosse 1980), or separately by viewing slides with a compound microscope (McGonigle et al. 1990). In the modified line intersection method of McGonigle et al. (1990) a compound microscope with an eye piece cross-hair moved to randomly selected positions is used to measure the mycorrhizal colonization. Endomycorrhiza will be stained with ink before colonization is determined; if necessary, roots will be bleached before staining.

6. Elemental and N_2 fixation analysis

All the samples will be oven dried (48h, 70°C). Dry samples of leaf, stem and root may be used for element analysis (ICP-MS) and sent for N_2 fixation analysis. A 'weighted' ^{15}N composition based on the ^{15}N abundance and proportional amounts of N in each organ can be used to calculate total ^{15}N and total N content of the whole plant (Denton et al., 2013).

7. Biomass

The biomass of the different root types will be determined and related to surface area to determine morphological parameters such as specific root length (SRL) and specific root area (SRA).

Table 1: Frequency of phenotyping measurements

Biweekly	End of the experiment
Leaf number	EC analysis
Stem diameter	Chlorophyll content
Plant height	Specific leaf area
Photosynthesis	Leaf anatomy
Stomatal conductance	Stem anatomy (theoretical hydraulic conductivity)
Intercellular CO2 concentration	Fresh and dry weight
Transpiration	Adventitious root diameter, length, fresh and dry weight

	Basal root whorl number, root angle,
	Basal root diameter, length, fresh and dry weight
	Tap root diameter, length, root fresh and dry weight
	Maximum rooting depth, root fractions in the top soil (0-20 cm) and deep soil
	Nodule number
	Elemental and N_2 fixation analysis
	Degree of mycorrhization

4.6 - Field measurements below ground

While no field experiments are performed at BOKU within the EUROLEGUME project, the following outlines the principles of a short belowground phenotyping program other partners could perform along or in collaboration with BOKU.

4.6.1 Root analysis

During growth or very soon after aboveground harvest 8 roots systems per variety/treatment (minimum 4-5!!) will be excavated (with a spade). Shake the soil off carefully and place in 20-liter containers with soap-water for rinsing the remaining soil off. Detergent is added to the water, helping to separate roots from soil particles without significantly damaging the root system or causing the loss of a large number of fine roots. Then, root systems are washed and rinsed in clean tap water (Miguel, 2012). The direction of plants within rows can be marked before excavation by pinching a hole in the remaining stem with a needle.

Three classes of roots are usually distinguished in bean roots: Adventitious, basal and tap roots. Basal and tap roots are larger diameter roots and will have lateral (side) roots (Fig. 4.2).

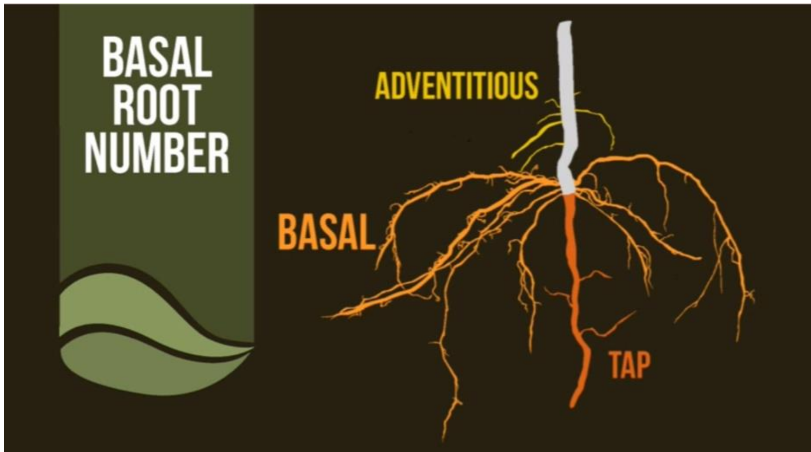

Fig. 4.2: Structure of Common bean root system (Jimmy Burridge, Shovelomics, Youtube video)

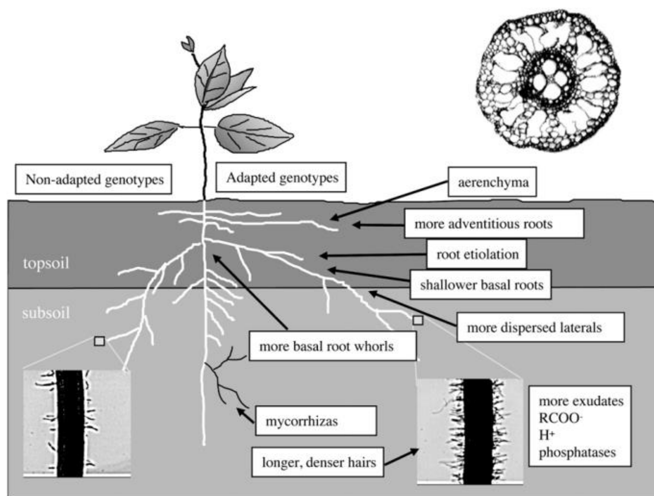

Fig. 4.3: Root systems of bean genotypes varying in P uptake efficiency, left: non-adapted to low P, right: adapted to low P conditions (Lynch, 2011)

1. Adventitious roots

Count the number and determine fresh and dry weight (48h, 70°C) of adventitious roots (Ramirez-Vallejo and Kelly, 1998).

Advanced: Scan a representative adventitious root in a petri dish filled with water on a flatbed scanner, grayscale, 300dpi WITH A TRANSPARENCY UNIT (not too expensive e.g. http://www.imaging-resource.com/SCAN/V700/V700.HTM) !! BOKU will analyzed pictures for you with WinRhizo Pro (http://www.regentinstruments.com/assets/winrhizo_about.html), e.g. for surface area, volume, length – results (together with dry weight): tissue density, specific root area, specific root length, total root length, total root length)

2. Basal root

Basal root whorl number (some varieties might not have)

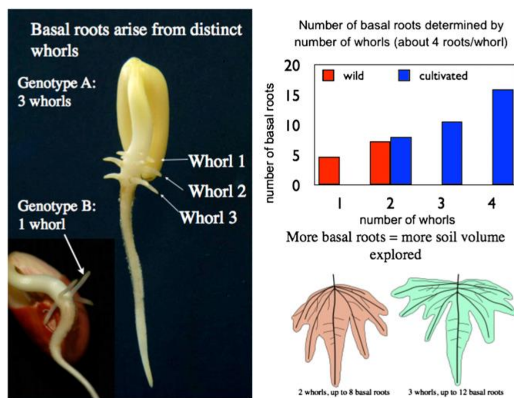

Fig.4.4: Whorls are defined as distinct tiers of basal roots that emerge in a tetrarch fashion along the base of the hypocotyl (Lynch, 2011).

Count the number of whorls.

Basal root angle, number and diameter

The angle of all basal roots is measured on a large protractor. Diameter can be taken with a caliper 1cm from the point of attachment.

Basal root fresh and dry weight

Cut basal roots from tap root. Determine fresh and dry weight (48h, 70°C) of basal roots.

Basal root morphology

Advanced: Scan a representative basal root in a petri dish filled with water on a flatbed scanner, grayscale, 300dpi WITH A TRANSPARENCY UNIT (not too expensive e.g. http://www.imaging-resource.com/SCAN/V700/V700.HTM) !! BOKU will analyzed pictures for you with WinRhizo Pro (http://www.regentinstruments.com/assets/winrhizo_about.html), e.g. for surface area, volume, length – results (together with dry weight): tissue density, specific root area, specific root length, total root length, total root length). Very very advanced: Separate the lateral roots from the basal root before scanning and scan, dry and weigh separately.

Basal root anatomy

Very very advanced: Store 10-12x 2 cm long segments per variety/treatment (taken approx. 5 cm from the beginning of the basal root at tap root) for anatomical analysis in 70%EtOH and send to BOKU.

3. Tap root

Tap root diameter, rooting depth tap root, fresh and dry weight

Determine fresh and dry weight (48h, 70°C) of tap roots. Measure diameter 1 cm down from the boundary between the tap root and hypocotyl (Barlow, 2011). For length measurements, measure the actual depth of tap root tip in soil (don't pull straight!) from hypocotyl/tap root boundary.

Tap root morphology

Advanced: Scan the tap root in a petri dish filled with water on a flatbed scanner, grayscale, 300dpi WITH A TRANSPARENCY UNIT (not too expensive e.g. http://www.imaging-resource.com/SCAN/V700/V700.HTM) !! BOKU will analyzed pictures for you with WinRhizo Pro (http://www.regentinstruments.com/assets/winrhizo_about.html), e.g. for surface area, volume, length – results (together with dry weight): tissue density, specific root area, specific root length, total root length, total root length). Very very advanced: Separate the lateral roots from the tap root before scanning and scan, dry and weigh separately.

Tap root anatomy

Very very advanced: Store 8x 2 cm long segments per variety/treatment (taken approx. 10 cm down from the beginning of the tap root) for anatomical analysis in 70%EtOH and send to BOKU.

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APPENDIXES

1 - MEDIA AND SOLUTIONS

1.1- Culture Media

Yeast Mannitol Agar (YMA)

Yeast Mannitol Broth 1 liter

Agar 15 g

Preparation:

- Prepare YMB
- Add agar, shake to suspend evenly, autoclave.
- After autoclaving, shake flask to ensure even mixing of melted agar with medium.

White bean agar (WBA)

Distilled water 1000 mL

White bean 50 g

Sucrose 10 g

K_2HPO_4 1.0 g

Agar 15.0 g

Preparation: Slowly boil the beans for 1.5 hours allowing them to dissolve. Then filter them through cotton wool. Take 500 mL of filtrate and add other components and water till 1L. Check pH 7.2 and autoclave 20 min at 1.0 atm.

Grey peas agar (GPA)

Distilled water 1000 mL

Grey peas 100 g

Sucrose 20 g

K_2HPO_4 1.0 g

Agar 15.0 g

Preparation: Slowly boil the peas 1.5 - 2 hours till peas are soft. Then filter through cotton wool. Take 500 mL of filtrate and add other components and water till 1L. Check pH 7.2 and autoclave 20 min at 1.0 atm.

YMA with streptomycin

Stock solution: 400 mg str / 100 mL water (4 mg str/mL).

Add 5 mL str stock / 500 mL YMA to make plates containing 40 µg str / mL.

10 mL str stock / 500 mL YMA for plates containing 80 µg str / mL.

Autoclave YMA together with magnetic stirring bar in an Erlenmeyer flask. Add filter sterilized antibiotics after the agar has cooled below 80 °C. Mix well and pour after bubbles resulting from mixing have dispersed.

1.2 - Dyes and reagents for media and microscopy

Bromothymol Blue (BTB)

Stock solution: 0.5 g / 100 mL ethanol

Add 5 mL stock / liter YMA

Final concentration of BTB: 25 ppm.

Congo Red (CR)

Stock solution: 0.25 g / 100 mL

Add 10 mL stock / liter YMA

Final concentration of CR: 25 ppm

Carbol Fuchsin Stain

Basic fuchsin 1g

Ethanol 10 mL

5% phenol solution 100 mL

The fuchsin stain should be diluted 5 - 10 times with distilled water before use.

Polyvinyl-Lacto-Glycerol (PVLG)

Polyvinyl alcohol (PVA)* 16,6 g

Lactic acid 100 mL

Glycerol 10 mL

Destilled water 100 mL

*polymerization 1000-1500

Dissolve the PVA in destilled water for several hours in a hot water bath at 80 ºC (complete dissolution may need 6 h). Mix the solution with glycerol and lactic acid.

Store at room temperature for at least 24h before using. This solution stores well in **dark bottles** for one year.

Melzer's Reagent

Dissolve 100 g of chloral hydrate, 1.5 g of Iodine and 5 g of Potassium iodide in 10 mL of destilled water.

For permanent mounts mix Melzer's reagent with PVLG in a volume ratio of 1:1 and store the mixture in a dark bottle. Iodine staining reactions will vary from **pale pink** (weak reaction) to **dark red-brown** (moderate reaction) to **dark reddish-purple** (intense dextrinoid reaction).

1.3 - Seedling agar recipes

Component	Somasegaran, 1994	Tepper, 1993	Grodzinskij, 1973
	g	g	g
$CaHPO_4$	1.0	2.0	0.172
K_2HPO_4	0.2	0.2	
KH_2PO_4		0.2	
$MgSO_4*7H_2O$	0.2	0.2	0.123
$CaSO_4*2H_2O$			0.344
NaCl	0.2	0.1	
KCl			0.160
$FeCl_3*6H_2O$	0.1	0.01	0.025
$FePO_4$		1.0	
$NaNO_3$		0.05	
$Na_2B_4O_7$		0.005	
$MnSO_4*4H_2O$		0.005	
Microelements	*1.0 mL		**10 mL
Agar	15	8	10
Water	1L	1L	1L
pH		6.8 – 7.2	

*From stock containing 0.5% B; 0.05%Mn; 0.005% Zn;0 .005% Mo and 0.002% Cu.

**-Na_2 MoO_4*2H_2O - 25 mg L^{-1}; $CuSO_4*5H_2O$ – 2.5 mg L^{-1}; $CoCl_2*6H_2O$ - 2.5 mg L^{-1}; H_3BO_3- 620 mg L^{-1} ; $MnSO_4*4H_2O$ -2230 mg L^{-1} ; $ZnSO_4*4H_2O$ -860 mg L^{-1}

Autoclave seedling agar at 121 °C for 15 minutes and disperse equal volume into test tubes (tube size depends on plant species). An appropriate amount of molten agar is dispensed so that after solidifying a 5-10 cm long agar face is presented for seedling growth.

1.4 – Nutrient solutions

Long Ashton Nutrient Solution (LANS)

100 × stock Micro nutrients	100 × stock Macronutrients
NaH_2PO_4 20·8 g L^{-1},	$CaCl_2$ 50·0 g L^{-1},
$MgSO_4{\cdot}7H_2O$ 36·9 g L^{-1},	K_2SO_4 21·75 g L^{-1},
$MnSO_4{\cdot}H_2O$ 0·223 g L^{-1},	Fe EDTA 3 g L^{-1}.
$CuSO_4{\cdot}5H_2O$ 0·024 g L^{-1},	
$ZnSO_4{\cdot}7H_2O$ 0·029 g L^{-1},	
H_3BO_3 0186 g L^{-1},	
$(NH_4)_6Mo_7{\cdot}O_{24}4H_2O$ 0·004 g L^{-1},	
$CoSO_4{\cdot}7H_2O$ 0·003 g L^{-1},	
NaCl 0·585 g L^{-1}.	

Recommended doses of mineral fertilisers

		Somasegaran, 1994		Villija	Prjanishnikov
Element	Applied as	kg ha^{-1}	mg per 1 kg of soil	mg per 1 kg of soil	mg per 1 L of soil
Phosphorus, P		100			
	triple super-phosphate	500	220	500	
	or KH_2PO_4		195		
	$CaHPO_4$				172
Potassium-K		200			

		Somasegaran, 1994		Villija	Prjanishnikov
Element	Applied as	kg ha^{-1}	mg per 1 kg of soil	mg per 1 kg of soil	mg per 1 L of soil
	KCl	382	168	250	160
	K_2SO_4				
Calcium-Ca					
	$CaSO_4*2H_2O$				344
Magnesium, Mg		5			
	$MgSO_4*7H_2O$	50	22		123
Zink, Zn		10			
	$ZnSO_4*7H_2O$	46.8	21		
Molibdenum, Mo		1			
	$(NH_4)_6Mo_7O_{24}*H_2O$	1.76	0.8		
Nitrogen , N (for control pots)		100			
	$CO(NH_2)_2$*	222	91		
	$NaNO_3$			167	
	NH_4NO_3				240

- 25% N is applied at planting and the remaining 75% at 3 weeks.

Recommended fertilisers rates g per m^2.

	Somasegaran, 1994	Villija
triple superphosphate	50.0	P- 3.06
KCl	38.2	K- 6.63
$MgSO_4$ x $7H_2O$	5.0	
$ZnSO_4$ x $7H_2O$	4.6	
$(NH_4)_6Mo_7O_{24}$ xH_2O	0.17	
$CO(NH_2)_2$	0.64+1.93*	
NH_4NO_3		

*Apply only for plus- nitrogen control. 25% at sowing time, 75% at 4 weeks

1.5 – Seed sterilization methods

Somasegaran, 1994		Tepper, 1993
	1 minute in 70% ethanol solution. Drain off.	2 minutes in concentrated H_2SO_4 (ρ=1.34) Rinse with sterile till pH of water is neutral. First rise should be done quickly to avoid heat damage of seeds
3% sodium hypochlorite solution for 3-5 minutes	3-5 minutes in commercial bleach	
Rinse seeds with five till eight changes of sterile water	Rinse seeds with five till eight changes of sterile water	

2- RECORD FORMS

2.1 - Sample record form

Sample ID	Collector's name	Collection date	Location	Agricultural-ecological climatic region	Cropping system	Legume host and variety	Nodule parameters (shape, size, pigmentation)	Soil type	Soil pH	Soil fertility	Other informations

2.2. Colony morphology of isolates on bromothymol blue (BTB) plates after 7-10 days incubation at 30 ◦C.

Isolate's name	Location	Species name	Shape	Size (mm)

Species name is referred only to the sequenced isolates.

2.3. Sample Record Sheet for combined ^{15}N and Total-N determinations

Position	Code	Description	Weight		Position	Code	Description	Weight
A1					E1			
A2					E2			
A3					E3			
A4					E4			
A5					E5			
A6					E6			
A7					E7			
A8					E8			
A9					E9			
A10					E10			
A11					E11			
A12					E12			
B1					F1			
B2					F2			
B3					F3			
B4					F4			
B5					F5			
B6					F6			
B7					F7			
B8					F8			
B9					F9			

B10					F10			
B11					F11			
B12					F12			
C1					G1			
C2					G2			
C3					G3			
C4					G4			
C5					G5			
C6					G6			
C7					G7			
C8					G8			
C9					G9			
C10					G10			
C11					G11			
C12					G12			
D1					H1			
D2					H2			
D3					H3			
D4					H4			
D5					H5			
D6					H6			
D7					H7			
D8					H8			
D9					H9			
D10					H10			
D11					H11			
D12					H12			

3 - QUANTIFICATION AND EVALUATION OF QUALITY OF DNA

Spectrophotometry:

The nucleic acids absorb UV light at a wavelength of 250 to 280 nm with a maximum at 260 nm . The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. The ratio between the 260 and 280nm reading allows estimation of nucleic acid sample purity.

1 . Connect the spectrophotometer at 260 nm, set the zero.

2. Add the sample to quantify, read absorbance at 260 and 280nm.

3 . Calculate the ratio of the two readings (A260/A280) to have idea of DNA purity.

4. Calculation of DNA concentration

[DNA] ng / Dilution ratio = A260 x 50

(A260nm = 1 = 50 ng/µL)

Alternatively, use Nanodrop® in laboratories with this equipment.

Agarose gels

The integrity and concentration of DNA can be evaluated and quantified in agarose gels at 0.7% by comparison with DNA standards of known concentration.

Preparation of agarose gels

Weigh 0.7 g of agarose to 100 mL of 1x TBE, buffer mix, place in the microwave until it is transparent. Apply 2 µL of ethidium bromide solution (1mg/mL). Allow to cool and polymerize and put it in the electrophoresis apparatus submersed in TBE buffer.

Electrophoresis

Mix 5 µL of loading buffer with 5 µL of DNA sample. Besides samples to quantify, include a DNA ladder of known concentration. Proceed with electrophoresis at a constant voltage of about 3V/cm distance between electrodes.

Visualization

Place the gel on UV light and capture the image