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M3 - The Mycorrhiza Producing Methods

FUE-UJI

GROWTHCOOP

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INTRODUCTION

- The mycorrhizae depend on the plant for the supply of carbon, energy and an ecological niche, and in return the volume of soil is increased by means of an additional absorption system.
- However, most of the plants in modern cultivation are produced in nurseries and cannot be naturally inoculated with mycorrhizae.
- Mycorrhizae are obligate symbionts, so they need the plant to grow and reproduce.
- Several techniques of cultivation have been developed:
  - substrate-based production
  - substrate-free production
  - in-vitro production

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SUBSTRATE-BASED. Introduction

- The most widely used method.
- It consists of cultivating a plant inoculated with the fungus of interest and waiting for its roots to grow.
- It is only necessary to collect the spores or use part of the roots of the host plant as a new inoculum.
- It is recommended to carry out the multiplication of the microorganisms under a protected environment, such as a greenhouse.

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SUBSTRATE-BASED. STEP 1 & 2

1. Select the organism of interest, will be the basis of our colony and can be a single organism (pure culture) or a consortium of beneficial organisms.

   Obtained from public or private collections, commercially or by isolating the beneficial microorganism from previously inoculated cultivated or wild plants.

   - The isolation of wild plants is not recommended, they can be contaminated by other microorganisms

2. Select the host plant. The desirable characteristics are: good development of the root system, a good level of colonization, a short life cycle, easy agronomic management, and resistant to low levels of phosphorus.

   - Plants such as onion (Allium cepa), corn (Zea mays L.), sorghum (Sorghum bicolor) or clover (Trifolium spp) have all these characteristics and have been successfully used for the production of AM fungi

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SUBSTRATE-BASED. STEP 3

3. To select the appropriate substrate:
    - from nutrient-rich substrates to inert substrates such as perlite, coco peat, cork, expanded clay.
    - Normal soil or sandy soil, as well as peat, compost in combination with other inert substrates such as sand, vermiculite or perlite.
    - Preferably a mixture of nutrient rich (compost) and inert (perlite and vermiculite) substrate to achieve the correct level of nutrients in peat-based substrates (reduce) or sandy soils (increase).

The substrate must be clean to avoid cross-contamination with the soil mixture, so the only microorganism associated with our host plant and soil is AM.

- The substrate can be sterilized by heat, steam (autoclave), irradiation when we are using small volumes, or by chemical treatments for large volumes.

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SUBSTRATE-BASED. Substrate propagation detailed protocol

- Obtain a starter either from a germplasm collection or form field soil with native AM.
- Autoclave the substrate. A low nutrient content mixture such as peat:vermiculite; Vermiculite/perlite, or Vermiculite:sepiolite at a relation 1:1 can be used.
- Mix the substrate with a 10% of starter.
- Sow a host plant. Sorgum, clover, maize can be used.
- Water with Hoagland or Long Asthon solution or provide any other fertilizer with low content of phosphorus.
- The propagule will be ready once the plant roots are fully developed (6 months aprox).

Use of the inoculum

- The substrate mixed with roots can be used directly as inoculum for new substrate at a proportion 1:10. The plants sowed on this substrate will be naturally inoculated.
- The substrate can be mixed with water and filtered through a filter to obtain a suspension of AM spores. This suspension can be used to inoculate plants.

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SUBSTRATE-BASED. Examples of different nutrient solution

Nutrient	Hoagland & Arnon	Long Asthon	Cooper
N	210	168	200-236
P*	7.75	10.25	15
K	234	156	300
Ca	160	160	170-185
Mg	34	36	50
S	64	48	68
Fe	2.5	2.8	12
Cu	0.02	0.064	0.1
Zn	0.05	0.065	0.1
Mn	0.5	0.54	2
B	0.5	0.54	0.3
Mo	0.01	0.04	0.2

Concentration of P was adjusted to 25% of original in order to improve the mycorrhization.

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SUBSTRATE-BASED. Nutrient

- Nutrient level has a strong effect on AM propagation production either directly or by influencing plant growth. The optimal level will depend on the plant used.
- The use of optimal nutrition for our host plant in the first stage will increase the correct growth, promote a proper root system and improve the initial colonization by AM.
- In the following phases, a slight nutrient deficiency is recommended, especially low levels of Phosphorus (associated with increased AM colonization and spore production). When the plant detects it, it is predisposed to carry out a symbiosis with another microorganism to overcome the lack of nutrients. This way, the penetration of the fungus hyphae into the root cells of the plant is facilitated.
- The timing of nutrient addition will influence colonization levels and propagule production. While high availability of P often suppresses colonization, addition of P at a later stage could enhance growth and sporulation of the AM fungus.

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SUBSTRATE-BASED. Nutrient

- Many other factors may indirectly influence AM fungal colonization and spore production. Among them we can find the factors that influence photosynthesis or the distribution of carbon in the roots.
- Soil characteristics such as pH, temperature, and water content can also influence the system. For example, some types of mycorrhizae are sensitive to pH, and changing from acidic to basic soil can reduce AM growth. Therefore, once a suitable host plant and substrate combination has been found for our mycorrhizal species, changing it is not recommended.
- Another aspect to take into account is the level of humidity. Plants, like fungi, must have access to sufficient water, but avoid flooding.

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SUBSTRATE-BASED. Nutrient solutions

Hoagland's nutrient solution	
Salt	mg/L
$KN0_3$	606.6
$Ca(N0_3)_2 \cdot 4H_20$	944.8
$NH_4H_2PO_4$	34.53
$MgSO_4 \cdot 7H_2O$	493
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$Zn\ SO_4 \cdot 7\ H_2O$	0.22
$Cu\ SO_4 \cdot 5\ H_2O$	0.08
$Na2MoO_4 \cdot 2\ H_2O$	0.02
FeEDTA	9.4
NH_4NO_3	28

Long Ashton nutrient solution	
Salt	mg/L
KNO_3	505
$Ca(NO_3)_2$	656
$Na\ H_2PO_4 \cdot 2\ H_2O$	52
$Mg\ SO_4 \cdot 7\ H_2O$	369
Fe citrate$\cdot 5\ H_2O$	24.5
$Mg\ SO_4$	2.23
$Cu\ SO_4 \cdot 5\ H_2O$	0.24
$Zn\ SO_4 \cdot 7\ H_2O$	0.296
$(NH4)6Mo_7O_{24} \cdot 2\ H_2O$	0.035
NaCl	5.85

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SUBSTRATE-BASED. Characteristics

- Isolated spores or a mixture of spores and root pieces of previously inoculated plants can normally be used to initiate production.
  - Spores can be obtained by dissolving the soil in water and straining the mixture through filter paper. The roots could be used as inoculum after being dried and cut into small pieces. The inoculated soil can also be used directly as a starter.
- The most widely used technique is growing in substrate using pots, substrate bags, or on the ground in raised beds. It does not require large infrastructures or expensive materials.
  - It is the most natural system and can work correctly for the multiplication of many mycorrhizal species, individually or in combinations of species.
  - If inert substrates are used, nutrient levels can be controlled to favor AM inoculation and propagation.

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SUBSTRATE-BASED. Drawbacks

- The difficulty of guaranteeing cross-contamination by other microorganisms.
- Also, the necessary setup can take up a lot of space, and the host plants require additional care, such as pest control.
  - To get spores, the substrate must be diluted in water and filtered, which increases the complication as the volume of substrate increases.
  - To use the roots as inoculum, these can be difficult to prepare due to the remains of the substrates (clays or organic remains). Inert substrates such as perlite or vermiculite can be used, with which cleaner roots are obtained that can be cut directly into pieces.

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SUBSTRATE-FREE. Introduction and methods

- Also called hydroponic techniques, the roots are not anchored in a substrate, but grow freely in a nutrient solution.
- The simplest method is the mixed, in which the host plant grows on an inert substrate that only serves as an anchor and does not provide any nutrients. The plants are watered with a solution that contains all the nutrients
- Can be further divided into two types.
  - The static method: The nutrient solution does not flow, it remains in the same container. The main problem is it can cause a lack of oxygen to the roots (anoxia) and it must be kept aerated by means of an air pump (it moves the nutrient solution and can harm the extraradicular hyphae). To avoid it, aeration can happen for short periods to minimize vibrations and develop hyphae.
  - Growth by nutrient flow: The plants are arranged on channels where roots and AM develop and the solution flows in a thin layer through channels. The nutrient solution flows in a foil, it does not need additional aeration.

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SUBSTRATE-FREE. Introduction and methods

- Aeroponics method: the roots are completely exposed and are not covered with any type of substrate or nutrient solution.
  - Nutrients are provided by spraying a solution directly on the roots with a pump with an atomizing nozzle or with a nebulizer.
  - Not suitable for all types of mycorrhizae. It has been successfully tested on several species of the glomus genus, other species have not shown good results. Lack of growth and propagation of mycorrhizae may be associated with excess humidity (despite good aeration, the roots are in constant humidity).
  - It may be useful for mycorrhizae associated with aquatic plants.
- Successfully used on many plant species, such as sorghum, wheat and maize.
- The choice of the host plant is important since the nutritional requirements will vary depending on the species. The type of nutrient solution will be adjusted.
- It can influence the levels of colonization obtained with some species of AM fungi and possibly also affect sporulation.

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SUBSTRATE-FREE. Introduction and methods

Detail of hydroponics system with aeration pump

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SUBSTRATE-FREE. Features

- Proper handling of the nutrient solution is vital. The plants capture the nutrients from the nutrient solution. As a starter any of the conventional nutrient solutions can be used, such as Hoaghland or Knop, full or diluted.
- Phosphorus levels, crucial for the establishment of mycorrhization, are lower than usual to favor the establishment of the fungus.
- Recommended to add iron in the form of a chelate to avoid the chlorosis of the plants, as well as molybdenum to improve the mycorrhization of the fungus.
- The nutrient solution must be constantly refreshed before nutrient levels drop to harmful levels. Frequent change of the nutrient solution to avoid contamination by bacteria, fungi and algae, or the accumulation of toxins by other microorganisms.
- The nutrient solution must be adapted to the needs of the crop, so besides the nutrients available in an assimilable form for the plants, the pH and temperature must be taken care of. Ideally, the pH should be in a range between 6.5 and 7.2 in which both plants and microorganisms can develop without problems.

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SUBSTRATE-FREE. Advantages and drawbacks

- Advantages:
  - The production of inoculum free of soil particles. The roots obtained with this technique can be cut into small pieces and used directly as inoculum, or stored. Similarly, spores can be obtained without interrupting the fungus life cycle.
  - It possesses the ability to monitor the pH and the nutrients that are applied and adapt them to the crop. These depend only on the nutrient solution and it is much easier to modify than the substrate.

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SUBSTRATE-FREE. Advantages and drawbacks

- Drawbacks:
  - Only serve to multiply the roots of the inoculated plants, not for an initial inoculation. It is necessary to inoculate and grow the plants for a couple of weeks in a substrate production system. Then, they can be transferred to the hydroponic or aeroponic system where the inoculated roots will multiply.
  - It is very prone to contamination by other unwanted microorganisms or algae. Especially dangerous in hydroponic nutrient film crops as the continuous flow of nutrient solution can carry contamination from one plant to another.
    - It could be partially solved by using inert substrates during pre-inoculation.
  - On the other hand, the speed at which roots develop in medium without substrate can cause low inoculation rates during the first cultivation periods.
  - It requires suitable facilities and precise control of the nutrient solution to be applied, so its cost in infrastructure and qualified personnel is higher.

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SUBSTRATE-FREE. Staining of mycorrhiza in roots

It can be used to check if the roots of the host plant are correctly inoculated.

- The roots are cleared (removing cytoplasmic contents from cells) using hot 10% KOH. Different approaches are used, from autoclaving cassettes for 5-10 minutes to boiling them in some container on the lab bench.
- After clearing, the roots are boiled (95C) for at least 3 min a 5% ink diluted in vinegar (5% acetic acid).
- Following staining, roots are rinsed several times during more than 20 min with acidified tap water (add several drops of acetic acid to the water). If the water for rinsing is not acidified and has a high pH (neutral is high), roots will destain.
- Roots are ready to visualize.
- If desired, the stained fungus can be completely destained by reincubating the root in KOH.

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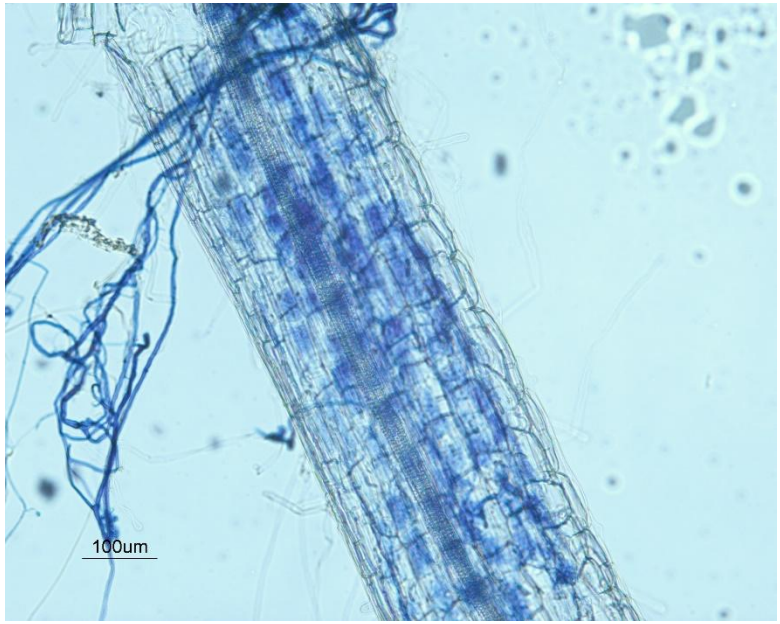
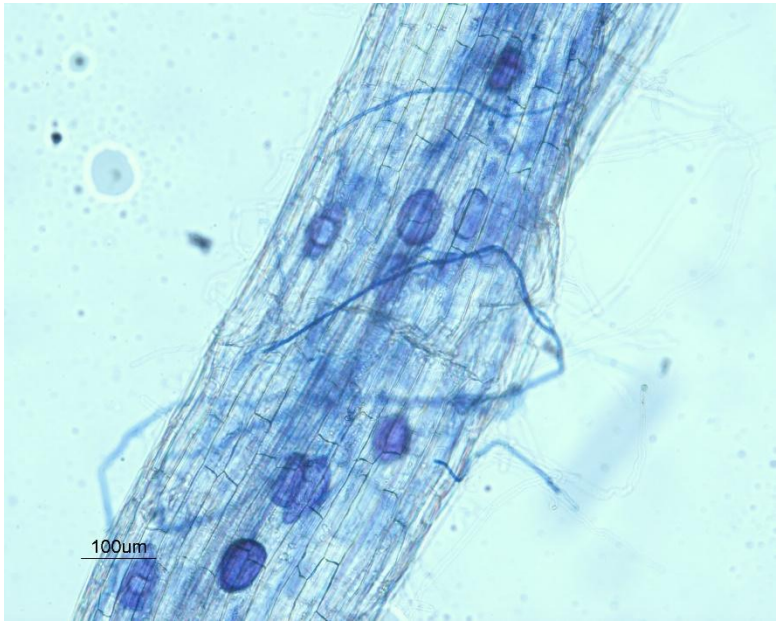
SUBSTRATE-FREE. Staining of mycorrhiza in roots



Root with hyphae and arbuscles



Root with hyphae and vesicles

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IN VITRO CULTURE. Introduction

- It is technically the most complicated. A purely in vitro culture has not yet been achieved because these fungi are obligate biotrophs.
- The inability of mycorrhizae to grow in axenic cultures, without the presence of a host, gave rise to the development of monoxenic crops using root fragments as hosts. Among them, the most used is the culture system with carrot roots (Dacus carota L.) transformed using Agrobacterium rhizogenes (known as the ROC system).
    - Useful for the study of mycorrhizal development, since the roots grow quickly without the need for growth regulators, and nutritional requirements are low.
    - Limitations: the absence of photosynthetic tissues, hormonal balance and normal source to sink physiological relations, the improvement of sucrose in the culture medium can modify the biochemistry of the plant-fungus interaction.
- This system has been successfully tested on dozens of AM species and is used as a colony maintenance method. However, very few species grow fast enough to be able to use this method to produce inoculum. Among them, the Glomus intradices, one of the most productive and most used species to date.

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IN VITRO CULTURE. Introduction

- When speaking of an in vitro mycorrhizal multiplication system, it refers to the inoculation of plants in a sterile agar medium with a mycorrhiza. However, the establishment of mycorrhizal symbiosis under these conditions has some drawbacks:
  - Inoculum contamination, host behavior and the obligate biotrophic nature of the symbiont.
- Although the first successful attempts to cultivate AM in vitro were achieved with the ROC system, this system was extended to other species: alfalfa, potato or chicory.
- Ideally, for this type of cultivation, the use of herbaceous plants is recommended since their handling in vitro is much simpler than that of woody plants.
- In general, laboratory sugar culture media such as minimal medium or modified Stullu Roman medium, which contain macro and micronutrients, vitamins and vitamins, are used for in vitro production.
- When the culture system has the complete plant, it is not necessary to add vitamins and sucrose to the culture medium, since the plant supplies them.

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IN VITRO CULTURE. Advantages, drawbacks and applications

- Advantages
  - The absence of counterinfection by other microorganisms, which makes them suitable for the production of high quality inoculum.
    - It may happen that during the handling of the system we have cross-contamination, either by some microorganism or by another AM. These can occur both in the establishment of the system and in later stages.
    - It is very important to keep a surveillance of the culture.
  - The minimum space necessary to establish the system
  - The possibility of controlling the dynamics of spore production and calculating the optimal harvest time, since these parameters can be detected and controlled more easily in vitro.

IN VITRO CULTURE. Advantages, drawbacks and applications

- Drawbacks
  - The diversity of fungi that have been cultivated by this system is less than that which can be cultivated in systems with substrate.
  - A much higher cost of infrastructure and personnel than systems, since it requires laboratories prepared for in vitro culture that can maintain sterile conditions, qualified personnel, incubators...
- Applications:
  - The maintenance of pure strains.
  - To improve the production of metabolites for the pharmaceutical industry.

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