Chapter 22

PHYSIOLOGICAL STUDIES ON MYCORRHIZAL FUNGI PRODUCTION

A.A. De Araujo[1], M. de los A. Aquiahuatl Ramos[2], S. Roussos[1]

[1]*Laboratoire de Microbiologie de l'IRD, IFR-BAIM; ESIL Case 925, Universités de Provence et de Méditerranée, 163, Avenue de Luminy, F-13288 Marseille Cedex 9 France;* [2]*Departamento de Biotecnologia; UAM-I, Avenida Michoacan y la Purisima; Apartado Postal 55-535, Mexico DF C.P. 09340, Mexico*

Running Title: Technic for mycorrhizal fungi

1. Introduction

Micro-organisms are present in large numbers on and near the feeder roots of trees, and they play vital roles in numerous physiological processes. Pelmont (1993) stated that these dynamic processes are mediated by associations of micro-organisms participating in symbiotic root activities as nitrogen fixation and phosphorus mobilization. Mousain (1993) and Strullu (1991) confirmed that the major symbiotic associations on tree roots have been bacterial with *Rhizobium* or fungal in mycorrhizas. Mycorrhizas are defined as durable unions based in reciprocal exchanges between plant roots and fungi (Marx and Cordell, 1994). Each one optimizes its development due to this association. Mousain *et al.* (1998) opined that ectomycorrhizal fungus such as *Lactarius* Pers., *Pisolithus* Alb. & Schwein. and *Suillus* P. Karst. should be considered as fundamental micro-organisms for qualitative improvement of the trees and reforestation programs. De Araújo *et al.* (1008) studied the effect of culture media, initial pH, and salt concentration on the apical growth of four ectomycorrhizal fungi. However, not much information is available about ectomycorrhizal fungi biomass production and their metabolites. Because of this, a non-destructible technique was development to evaluate biomass production and

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analyse metabolites as pigments, enzymes, sugar, and organic acids present in agar. Baar *et al.* (1997) determined mycelium biomass by dissolving agar media in hot water and filtering the solution before drying (100°C, 24 h) and determining dry weight as described by Oort (1981). As discussed by Jongbloed and Borst-Pauwels (1990), this method could cause loss of water-soluble compounds amounting to approximately 35% of biomass with little variation between isolates and no effect of age of cultures and composition of media on the amount of loss. Dry weight loss of biomass of *L. bicolor* as estimated by Jongbloed and Borst-Pauwels (1990) was approximately 19%. Gibson and Deacon (1990) described mycelium development of ectomycorrhizal fungi *in vitro* by radial growth and biomass production. Jongbloed and Borst-Pauwels (1990) hypothesized that radial growth reflected exploitation of resources, whereas biomass production was a measure of accumulation of carbon and nutrients.
Attempts were made to present some modifications introduced in the technique employed by Baar et al. (1997) and analysed by Jongbloed and Borst-Pauwels (1990) in order to evaluate glucose, pH, and produced biomass profiles after 5, 10 and 15 days of incubation at 25°C for *Suillus collinitus* and *Pisolithus tinctorius* strains in different culture media (PDA, BAF, MNM, MP, GM8, and MG). This non-destructible technique is being employed now to evaluate biomass production and analysis of metabolites such as pigments, enzymes, sugar, organic acids present in the agar.

2. Experimental

2.1. CULTURES AND MEDIA

Mycelia of mycorrhizal species were obtained from sporophores in *Pinus*. *Pisolithus tinctorius* (Pers.) Coker and Couch (PF 26) was isolated from Murcia region of Spain in 1991 and *Suillus collinitus* (Fr.) Kuntze (Sc 24) from La Grande-Motte (south-west region of France) in 1994. The mycelia of the strains were grown and maintained on Potato-Dextrose-Agar (PDA), pH 5.6. Six synthetic media as shown in Table 1 were used.

2.3. ANALYSIS

Colony diameters were measured at regular intervals up to 15 days. Results were expressed as means of diameter on three replicate plates. Average diameter was taken from tree replicates. Colony description was made in terms of its mycelium type, colour, margin aspect and characteristic features of the mycelium. Diffusive pigments presented in agar media were also described. Sugar consumption was determined according to Miller (1959).

Table 1. Media composition for ectomycorhizas nutritional studies.

Compounds	Culture media					
(g/l)	PDA	BAF	MNM	MP	GM8	MG
D-Glucose	20.0	30.0	10.0	10.0	31.25	10.0
Peptone	-	2.0	-	2.0	2.5	-
Yeast extract	-	0.2	-	-	-	-
Potatoes	200	-	-	-	-	-
Malt extract	-	-	3.0	-	-	-
$CaCl_2$. 2 H_20	-	-	0.2	0.13	-	-
$Ca(N0_3)_2$. 4 H_20	-	-	-	-	2.0	-
NaCl	-	-	0.025	-	-	-
KH_2PO_4	-	0.5	0.5	0.5	-	-
$(NH_4)_2HPO_4$	-	-	0.5	-	-	-
$MgSO_4$. 7 H_20	-	0.5	0.15	-	-	-
Ammonium tartrate	-	-	-	-	1.0	-
$FeCl_3$	-	0.005	0.005	0.01	-	-
L-aspargin	-	-	-	-	1.0	-
Thiamine HCl	-	-	100 µg	-	-	-
Myo inositol	-	0.05	-	-	-	-
Oligoelement solution*	-	1.0 ml	-	-	-	-
Vitamin solution**	-	1.0 ml	-	1.0 ml	-	-
Agar	-	15.0	15.0	15.0	15.0	15.0
pH	5.6	6.0	6.0	7.5	5.0	6.5

*Oligoelement solution ($g.l^{-1}$): $CaCl_2$, 100; $MnSO_4$, 5l; $ZnSO_4$ $7H_20$, 1

**Vitamin solution ($mg.l^{-1}$): Thiamine HCl, 500; Biotin, 10; Folic Acid, 100.

2.2. INOCULATION AND INCUBATION

A sterilized cellophane disc was placed on medium surface contained in Petri dishes (50-mm diameter). These were inoculated centrally with a mycelial block (3x3x2-mm) cut from the advancing margin of the 15 days old colony on PDA medium. The plates were wrapped in Parafilm and incubated at 25°C in dark.

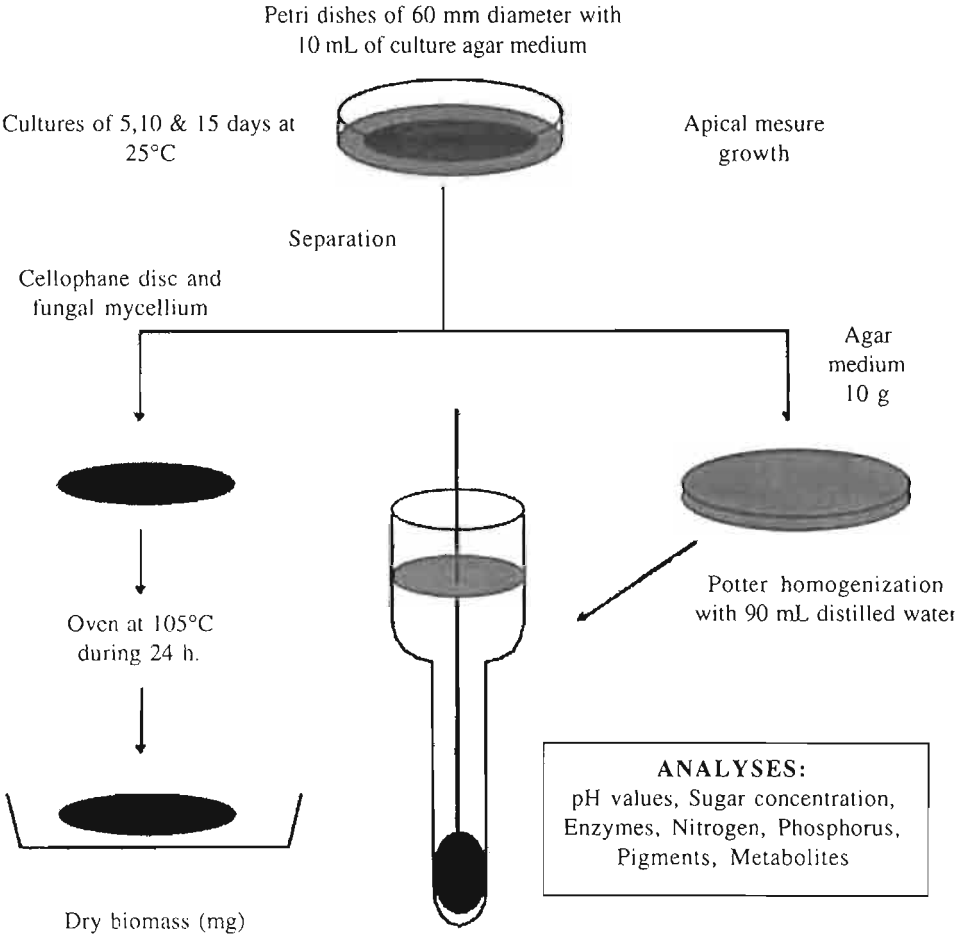

Figure 1. Scheme of fungi mycelia culture on agar surface containing a cellophane film and sampling treatment for biomass determination and metabolites produced analysis during colony development on solid surface.

2.3.1. Treatment of samples:

Figure 1 shows the treatment scheme of the samples. Agar medium (10 ml) was suspended with 90 ml of distilled water and homogenized with the Potter during 30 seconds at 4°C. Colonies growing on cellophane disc were used to measure biomass produced by drying the cellophane sheet containing the mycelium at 105°C during 24 hours. Controls were made with the cellophane sheet's weight determined similarly from un-inoculated Petri dishes. It represents an average of 15 samples.

3. Results and Discussion

Tables 2 and 3 show the consumption of glucose, biomass produced, colony diameter, and pH evolution for *S. collinitus* and *P. tinctorius* strains.

Table 2. Evolution of pH, biomass production and glucose used after 15 days incubation at 25°C for *Suillus collinitus* strain.

Culture Media	Glucose used (%)	Biomass production (g/l)	Colony diameter (mm)	pH
PDA	96	7.1	45	5.0
BAF	69	8.6	50	4.2
MNM	93	3.9	45	3.5
MP	27	1.3	24	5.8
GM8	0	1.6	18	4.8

Table 3. Evolution of pH, biomass production and glucose used after 15 days incubation at 25°C for *Pisolithus tinctorius* strain.

Culture Media	Glucose Used (%)	Biomass production(g/l)	Colony diameter(mm)	pH
PDA	52	5.4	23	5.8
BAF	41	7.6	37	4.3
MNM	77	4.5	43	3.4
MP	31	0.9	16	6.6
GM8	0	2.7	20	6.0
MG	24	1.4	33	5.7
MG	42	1.6	32	4.9

3.1. COMPARISON BETWEEN DIFFERENT CULTURE MEDIA FOR THE *S. COLLINITUS* GROWTH

Figure 2 shows different culture media, which were screened for maximum biomass production and sugar consumption. Evidently PDA medium was best for *Suillus collinitus* (Sc 24). After 15 days of growth, sugar consumption was 96% and biomass production was 7.1 $g.l^{-1}$ (Table 2). BAF medium was favourable for biomass production, which suggested that strain Sc 24 needed yeast extract, oligo-elements and vitamins to grow. Growth patterns in MNM medium showed that this strain did not need vitamins to grow and that the decrease in the pH value after 15 days of incubation was due to the presence of PO_4^{3-} ions in its composition. Sugar consumption in this medium was 93% and nitrogen source was the limiting component.

Growth in GM8 medium showed that both the strains did not utilize glucose and little biomass was produced. It was, hence concluded that nitrate at a concentration of 2 $g.l^{-1}$ had a negative effect for both strains. Similar results have been reported previously De Araújo *et al.* (1998).

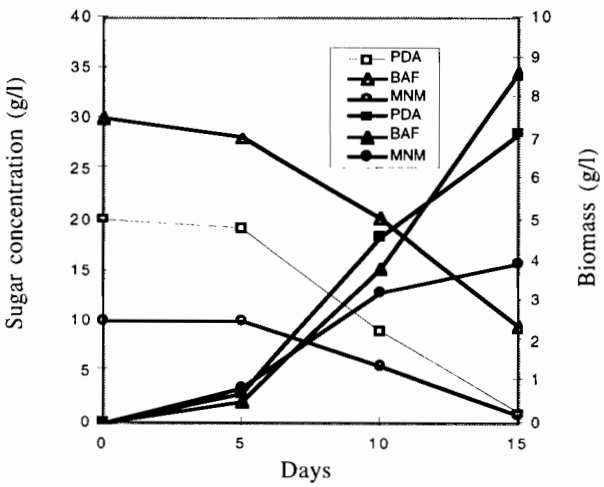

Figure 2. Glucose consumption (empty symbols) and biomass production (full symbols) for *Scuillus collinitus* grown on PDA, BAF, and MNM media at 25°C for 15 days.

3.1.1. Sugar consumption and biomass production profiles for Suillus collinitus

Sugar consumption in MG medium was 42% and very little biomass was produced. Mycelial growth was very poor due to absence of any nitrogen source. In MP medium only 27% sugar was consumed and almost no biomass was produced. Although GM8

medium contained nitrate in its composition, apparently Sc 24 for growth did not utilize this (Figure 2).

In view of these results, PDA media was selected for further studies. Torres and Honrubia (1991) studied the influence of culture media and pH on the growth of several strains of ectomycorrhizal fungi such as *S. collinitus*, *S. granulatus*, *Rhizopogon roselus*, *R. luteolus* and *Amanita muscaria* isolated from Murcia and Albacete (Spain). They cultivated the strains in different media such as MNM, PDA, MEA (2%), and the pH range was 5.5-7.5. For *S. collinitus*, after 60 days of incubation at 24°C on different culture media, they found that PDA was the best media and the colony presented 8.5-cm diameter.

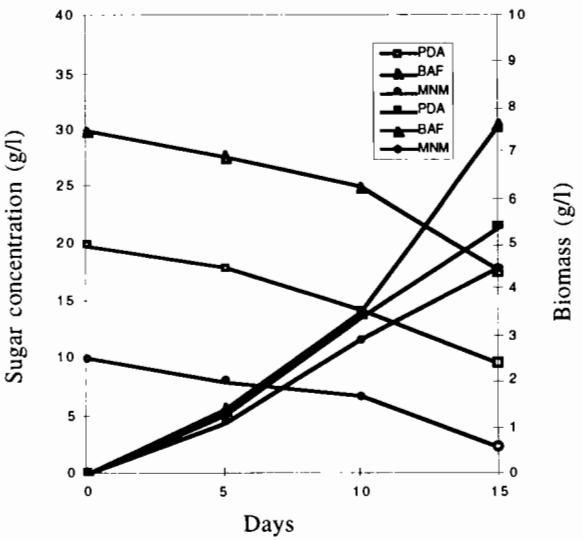

Figure 3: Glucose consumption (empty symbols) and biomass production (full symbols) for *Pisolithus tinctorius* grown on PDA, BAF, and MNM media at 25°C for 15 days.

3.2. COMPARISON BETWEEN DIFFERENT CULTURE MEDIA FOR THE *P. TINCTORIUS* GROWTH

As is evident from the Figure 3 and Table 3, better development was observed on PDA, BAF, and MNM media. The strain PF 26 did not utilize glucose at same level as the strain Sc 24. After 15 days of culture on PDA medium, 48% glucose was still present. Lower consumption of glucose by PF 26 was due to mainly its apical growth, which was slower in the medium. Perhaps nitrogen was limiting factor for this strain in these

media. Apparently strain PF 26 strain tolerated the presence of nitrate, as it produced 2.7 $g.l^{-1}$ biomass in GM8 medium.

4. Summary and conclusions

A technique for studying the mycorrhizal fungi culture parameters on agar media was established. Two strains *viz. Suillus collinitus* and *Pisolithus tinctorius* were screened for biomass production and sugar consumption patterns in different media such as PDA, BAF, MNM, MP, GM8 and MG. Fungal biomass analysis was carried out by drying the biomass grown on cellophane sheet. Evolution of glucose, pH and produced biomass after 5, 10 and 15 days of incubation at 25°C. PDA medium appeared best. Data obtained for sugar consumption, pH values and fungal biomass production permitted to optimize culture conditions and study the physiology and metabolism of ectomycorrhizal fungi grown on solid agar media. One main advantage of this new technique was based in the fact that it was not destructible method.

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