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A Technique for Mycelial Development of Ectomycorrhizal Fungi on Agar Media

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Abstract

A technique was established to study ectomycorrhizal fungi on agar media. Petri dishes, 60 mm in diameter, containing 10 mL of culture medium covered with a cellophane disk were used for easy collection of the mycelium after growth. For analysis of fungal biomass production, a sterilized cellophane sheet was placed on the medium's surface. Inoculation was achieved by placing a mycelial block onto the center of the cellophane sheet and then incubating at 25°C in the dark. Colony radial growth was measured and biomass dry wt was determined. Fresh mycelia were homogenized with 10 mL of acetate buffer (pH 5.5) for enzyme analysis. A crude extract was obtained by adding all culture medium to 90 mL of distilled water and homogenizing in a Potter. Reducing sugars, enzyme concentration, and pH were determined. Three fungal strains, *Suillus collinitus*, *Pisosithus arrhizus*, and *Hebeloma cylindrosporum*, were grown in different culture media (potato dextrose agar or Pintro's medium). Parameters measured over time included glucose concentration, phosphatase activity, biomass, and pH.

Index Entries: Ectomycorrhizal fungi; physiology; apical growth; *Pisolithus*; *Suillus*; *Hebeloma*; biomass; phosphatase; agar media.

Introduction

Microorganisms present in great numbers on and near the feeder roots of trees play vital roles in numerous physiological processes. Pelmont *(1)* stated that these dynamic processes are mediated by associations of microorganisms participating in symbiotic root activities, such as nitrogen

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fixation and phosphorus mobilization. Mousain *(2)* and Strullu *(3)* affirmed that the major symbiotic associations on tree roots are bacterial with *Rhizobium* or fungal with mycorrhizas. Marx and Cordell *(4)* defined mycorrhizas as durable unions based on reciprocal exchanges between plant roots and fungi. Each one optimizes its development thanks to this association. Mousain et al. *(5)* reported that ectomycorrhizal fungi, such as *Lactarius* Pers., *Pisolithus* Alb. & Schwein., and *Suillus* P. Karst., are considered fundamental microorganisms for qualitative improvement of trees in reforestation programs.

De Araújo et al. *(6)* have studied the effect of culture media, initial pH, and salt concentration on apical growth of four ectomycorrhizal fungi. However, little information is available in the literature regarding biomass production as well as metabolites of such microorganisms. Therefore, a nondestructive technique for enzyme determination was developed. This technique also enables the valuation of biomass production and metabolite production (pigments, sugar, organic acids).

Baar et al. *(7)* determined mycelial biomass by dissolving agar media in hot water and filtering the solutions before drying them (100°C, 24 h). Dry weight was determined according to Oort *(8)*. As discussed by Jongbloed and Borst-Pauwels *(9)*, this method may cause loss of water-soluble compounds amounting to approx 35 % of biomass. In addition, Jongbloed and Borst-Pauwels *(9)* estimated the dry wt loss of *Laccaria bicolor* biomass, which was approx 19%.

Gibson and Deacon *(10)* have described mycelial development of ectomycorrhizal fungi in vitro by radial growth and by biomass production. Jongbloed and Borst-Pauwels *(9)* hypothesized that radial growth reflects exploitation of resources, whereas biomass production is a measure of accumulation of carbon and nutrients; thus, both parameters are important in describing mycelial development.

The aim of the present work was to set up a technique that will enable easy monitoring of a wide range of parameters during ectomycorrhizal fungal growth.

Materials and Methods

Microbial Strains

Mycelia of mycorrhizal species were obtained from sporophores in *Pinus. Pisolithus arrhizus* (Scop.) S. Rauschert (PF 26) was isolated in the Murcia region (Spain) in 1991, *Suillus collinitus* Fr. Kuntze (Sc 24) at La Grande-Motte (southwest region of France—Hérault Department) in 1994, and *Hebeloma cylindrosporum* Romagnesi (Hb 12) at Lacanau-Océan (southwest of France—Gironde Department) in 1990.

Culture Media

Mycelia of the strains were cultured and maintained on potato dextrose agar (PDA), pH 5.6. Another synthetic medium, Pintro's medium *(11)*

was used for fungal culture. Pintro's medium consists of the following: 10.0 g/L of Glucose, 0.45 g/L of KNO_3,0.107 g/L of NH_4Cl, 0.25 g/L of $MgSO_4$, 0.15 g/L of $CaCl_2$, 5.85×10^{-3} g/L of NaCl, 74.55×10^{-3} g/L of KCl, 13.6×10^{-3} g/L of KH_2PO_4, 0.101×10^{-3} g/L of Thiamine-HCl; 0.5 mL of Ferric citrate 1%, 0.2 mL of trace element solution (3.0 g/L of $MnSO_4 \cdot H_2O$, 4.4 g/L of $ZnSO_4$ $7H_2O$, 2.8 g/L of H_3BO_4, 0.97 g/L of $CuSO_4 \cdot 5H_2O$, 0.29 g/L of $Na_2Mo_4 \cdot 2H_2O$) *(12)*, and 15.0 g/L of agar (pH 6.0).

Inoculation and Incubation

A sterilized cellophane disk was placed on the surface of the culture medium contained in Petri dishes (60 mm diameter). The dishes were then inoculated centrally with a mycelial block (3×3×2 mm) cut from the advancing margin of a 15-d-old colony grown on PDA medium. The plates were wrapped in Parafilm. Cultures were carried out at 25°C in the darkness.

Analysis

Colony diameters were measured with a ruler at regular intervals for 21 d. Results are expressed as the means of the diameter of three replicate plates. Colony description was made in terms of its mycelial type, color, margin aspect, and characteristic features of the mycelium. Diffusible pigments present in agar media are also described. Sugar consumption was determined according to Miller *(13)*. Phosphatase activities were determined according to Tabatabai and Bremner *(14)*. Biomass produced is expressed in grams of dry matter/L of medium, and phosphatase activities are expressed in enzymatic units (EU)/millimeter of culture medium. One enzymatic unit corresponds to the amount of enzyme required to produced 1 μmol para-nitrophenol (product of the reaction).

Results and Discussion

Technique of Fungal Growth and Sample Treatment

A technique was developed for ectomycorrhizal fungi mycelia culture during colony development on a solid surface containing a cellophane film (Fig. 1). Moreover, sampling treatment for biomass determination as well as analysis of metabolites produced was developed.

After inoculation and incubation, sampling consisted of separating the cellophane disk, containing the grown colony, from the agar medium. Duplicate Petri dishes were used: one to measure colony dry biomass produced, and another to analyze mycelial enzymes from fresh grown colony.

The initial mycelial block (3 × 3 × 2 mm) was removed and polyvinylpolypyrrolidone (PVPP) (Sigma, St. Louis, MO) was added to the fresh mycelium in a proportion of 10% (w/w). PVPP bound polyphenols that are known to inhibit enzymatic activities *(11)*. The mycelium was then homogenized at 4°C with 10 mL of 50 m*M* acetate buffer (pH 5.5) using an Ultraturax homogenizer (IKA Labortechnik) at 16,000 rpm. The resulting extracts were used to determine mycelial enzyme activities.

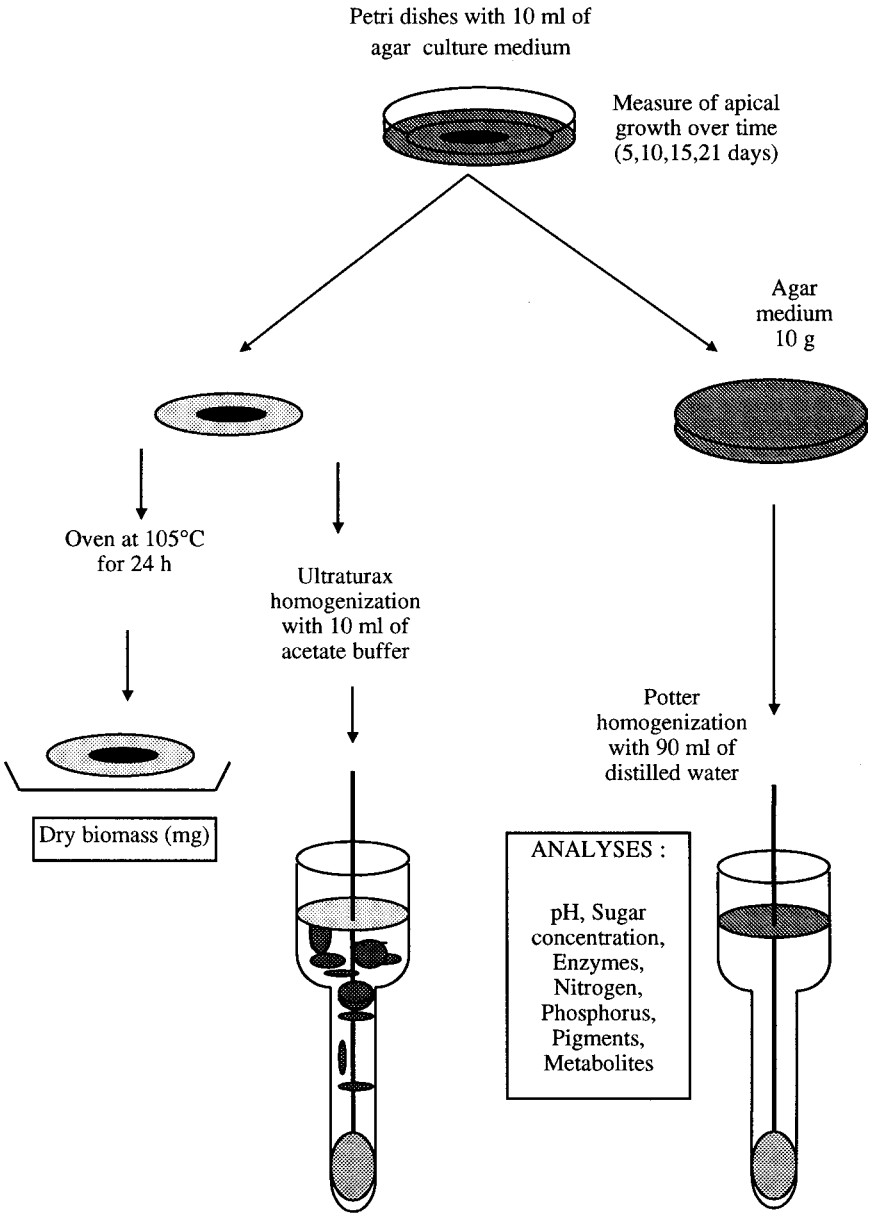

Fig. 1. Schematic representation of steps involved in biomass determination and metabolite production analyses from fungal culture on agar surface.

Biomass (dry matter) was determined by placing the cellophane sheet containing the colony into an oven at 105°C for 24 hours followed by cooling down in a dry atmosphere. Dry biomass was determined by calculating the weight difference between the cellophane disk containing mycelia and the average weight of the cellophane's disk (15 sheets were dried at 105°C for 24 h and weighed).

Table 1
Evolution of Colony Diameter, Ph, Biomass, Phosphatase Production, and Glucose Consumption During *S. collinitus* Growth at 25°C on PDA Surface Culture Media Containing a Cellophane Film

	Incubation time (d)				
Parameter	0	5	10	15	21
Colony diameter (mm)	0	9.0	14.0	24.0	29.0
pH	6.0	4.8	5.5	6.4	5.2
Glucose (g/L)	19.5	13.7	12.1	9.8	4.1
Biomass (g/L)	0	0.45	3.89	4.43	5.72
Excreted phosphatase (EU/mL)	0	0	0.02	0	0.06
Mycelium phosphatase (EU/mL)	0	0.29	0.53	1.97	2.03

After cellophane removal, agar medium (10 g) was suspended in 90 mL of distilled water and homogenized with a Potter (Bioblock Scientific) for 30 s at 4°C. The samples thus obtained were used for determination of sugar concentration, pH, and soluble phosphatase activities.

Mycelial Development of S. collinitus on PDA

S. collinitus was grown over a period of 21 d and various parameters were monitored, as described in Table 1. Phosphatase activity is expressed in enzymatic units. One enzymatic unit corresponds to the quantity of enzymes necessary to produce 1 µ*M* paranitrophenol (product of the enzymatic reaction)/min.

The results showed that the utilization of a cellophane sheet on the surface of PDA medium does not prevent fungal mycelial growth. Colony size as well as colony aspects were very similar to those obtained on agar surface without a cellophane disk (data not shown). The grown mycelium on the surface of the cellophane disk was easily separated from the culture media. Fresh biomass was also easily harvested and could be used directly either for enzyme and metabolite extraction and analysis or for biomass determination. Over a period of 21 d, the microorganism grew, consuming glucose and producing biomass. Phosphatase accumulates over time in and around the mycelium, but a substantial increase in the excreted enzyme was only observed after 15 d of culture. The important quantification of phosphatase is based on the fact that phosphatase enzymes function to hydrolyze phosphorus (the least available mineral nutrients for plant growth) in orthophosphate anion (P_i). In addition, P_i not only plays a vital functional role in energy transfer and metabolic regulation, but also is an important structural constituent of many biomolecules. Consequently, the assimilation, storage and metabolism of P_i are of critical importance to plant growth and development.

Table 2
Comparative Evolution of Colony Diameter, pH, Biomass, Phosphatase Production and Glucose Consumption by *H. cylindrosporum*, *P. arrhizus* and *S. collinitus* Grown on PDA Surface Culture Medium for 21 d at 25°C

Parameters	*H. cylindrosporum*	*P. arrhizus*	*S. collinitus*
Colony diameter (mm)	35.0	25.5	29.0
pH	7.2	5.0	5.2
Glucose (g/L)	0.3	6.3	4.1
Biomass (g/L)	7.87	6.37	5.72
Excreted phosphatase (EU/mL)	0.09	0.06	0.06
Mycelium phosphatase (EU/mL)	1.2	0.43	2.03

Ectomycorrhizal Mycelial Development on PDA medium

To verify the validity of the proposed technique, other ectomycorrhizal strains (*H. cylindrosporum* and *P. arrhizus*) were studied. The same parameters as in Table 1 were considered. However, data from each strain are reported after a 21-d culture. Table 2 shows that *H. cylindrosporum* grew faster than the other two strains. For *H. cylindrosporum*, glucose consumption and biomass production were higher than for *P. arrhizus* and *S. collinitus*. *S. collinitus* produced more mycelial-associated phosphatase than the other two strains.

Ectomycorrhizal Mycelial Development on Synthetic Culture Medium

Because PDA is a natural culture medium, containing potato extracts, it is difficult to obtain a mass balance. To establish such a mass balance for ectomycorrhizal fungi, three strains were cultivated on a synthetic Pintro's medium containing KH_2PO_4, (13.6 mg/L) as the source of phosphorus.

Table 3 shows that the strains grow slowly on this synthetic culture medium and utilize the sugar source in different proportions. On this synthetic medium, *S. collinitus*, *H. cylindrosporum*, and *P. arrhizus* presented apical growth similar to that obtained on PDA medium. Colony size was similar, but, its aspect was different. On PDA medium, the strains developed better and were denser than on the synthetic medium. Sugar consumption was about 40% for *H. cylindrosporum* and *P. arrhizus* and about 75% for *S. collinitus*. This was linked to the fact that a smaller quantity of biomass was obtained for *H. cylindrosporum* and *P. arrhizus* than that obtained for *S. collinitus*. By comparing the biomass produced, when the strains were grown on PDA or on the synthetic medium, it was observed that on PDA medium, greater amounts of biomass were obtained with higher consumption of glucose (almost 20 g/L for *H. cylindrosporum*). Pintro's synthetic medium contained 10 g/L of glucose. We can conclude

Table 3
Comparative Evolution of Colony Diameter, pH, Biomass, Phosphatase Production and Glucose Consumption by *H. cylindrosporum*, *P. arrhizus* and *S. collinitus* Grown on Pintro's Synthetic Medium with 100 µ*M* Phosphorus from KH_2PO_4 after 21 d at 25°C

Parameters	*H. cylindrosporum*	*P. arrhizus*	*S. collinitus*
Colony diameter (mm)	38.0	24.0	27.5
pH	5.79	4.02	4.56
Glucose (g/L)	5.93	6.31	2.40
Biomass (g/L)	1.91	1.46	2.33
Excreted phosphatase (EU/mL)	0.28	0.07	0.001
Mycelium phosphatase (EU/mL)	1.28	0	0.27

that at this concentration of synthetic medium, some compounds important to the metabolism of these strains are not present in comparision to PDA medium, which is a complete medium.

The greater amount of exocellular phosphatase produced was detected for *H. cylindrosporum*. The same observation was detecetd by Pintro *(11)*, comparing some strains of *Suillus*, *Hebeloma*, *Paxillus*, and *Rhizopogon*. Otherwise, pH values determined on synthetic medium cultures were lower than those determined on PDA medium.

Conclusion

We have demonstrated that the cellophane membrane deposited on the surface of PDA culture medium enabled easy separation of fresh mycelia from culture medium. A great advantage of this new technique is based on the fact that it is a nondestructive method for enzyme detection. Both exo- and endoenzymes can be measured precisely. Results showed that the technique for mycelial growth of ectomycorrhizal fungi (*Suillus*, *Pisolithus*, and *Hebeloma*) on agar surface is well established. Data obtained for sugar consumption, enzyme concentration, pH values, and fungal biomass production enabled optimization of culture conditions. The present study enabled the development of a simple but precise tool to study the physiology and metabolism of ectomycorrhizal fungi grown on solid agar media.

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