A Shake Culture Method for Pythiaceae Applicable to Rapid, Small-Scale Assay of Vegetative Physiology

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ABSTRACT

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A method is described for growing uniform mycelial suspensions of *Pythium ultimum* and *Phytophthora megasperma* var. *sojae* in a defined medium in shake culture. Dry weight doubling times of these fungi were 5 to 6 hr and about 12 hr, respectively. Incorporation of radioactive leucine, adenine, glycerol, and acetate into proteins, nucleic acids, and lipids by 3-ml cultures could be measured easily with 10-min pulse periods. These synthetic activities were blocked in a predictable fashion if the cell samples were pretreated for 10 min with a variety of inhibitors known to impair specific cytoplasmic and mitochondrial functions. The cultures were maintained easily, and after about 12 hr from the seeding of fresh cultures they were ready for use. The rapid growth and the ease and reproducibility of sampling make possible short-term manipulations of small amounts of cell material as well as the rapid production of large amounts of uniform, actively growing mycelium.

The utility of liquid culture methods for physiological studies of filamentous fungi often is restricted by one or more factors (5, 8). Preparation of test cultures from a mycelial mat or tight mycelial clumps leads to sampling difficulties owing to nonuniformity of culture material. In addition, many fungi grow slowly in liquid culture. Although slow growth and cell heterogeneity may be overcome to some extent by seeding media with hyphal fragments and mechanically shaking the cultures, many fungi still exhibit extensive mycelial aggregation when grown in shake culture.

In particular, these problems are common with *Pythium* spp. and *Phytophthora* spp. Many of the latter species typically grow very slowly, and in shake culture hyphal seed pieces of both groups of fungi give rise to tight pellets of mycelium. Information regarding some of the unusual features of these fungi, such as their sensitivity to antibacterial drugs (3), is limited, in part because their growth habit precludes certain types of experimentation. The results of several studies indicate the advantage of shake culture for increasing the growth rates of these fungi (2, 4). Tolmsoff (9) reported that two *Pythium* spp. could be induced to grow in shake culture on natural media as individual "budding" hyphae by blending liquid cultures and seeding hyphal fragments into fresh medium on several successive days. Once established, these cultures could be maintained, without further blending, by periodic transfer to fresh medium. This procedure would seem to solve all of the above problems.

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In our work with an isolate of *Pythium ultimum* Trow, one of the species used by Tolmsoff (9), we found the establishment of such cultures to be possible but time-consuming and their maintenance was especially difficult at high cell densities. Further, we were unable to adapt a *Phytophthora megasperma* Drechs. var. *sojae* Hildb. isolate to such hyphal suspension growth owing to difficulty in satisfactorily fragmenting the mycelium without extensive destruction. Even though Tolmsoff (9) did not study *Phytophthora* spp., he noted that the blending conditions and transfer interval were critical and that they varied with the organism. In this report we describe a shake culture method which is applicable to a fast-growing *P. ultimum* isolate and a slow-growing *P. megasperma* var. *sojae* isolate, that provides for rapid growth in a defined medium, ease of culture production and maintenance, near-uniformity of culture material, sampling ease with good reproducibility, and rapid assay of metabolic activities. A brief report of part of this work has been made (7).

MATERIALS AND METHODS

Culture conditions.—The *Pythium ultimum* and *Phytophthora megasperma* var. *sojae* isolates were supplied by Paul Allen and James Partridge, respectively. The fungi were grown in a defined medium formulated as follows: 10 g glucose, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.2 ml Vogel trace element solution (10), 0.24 g arginine, 0.175 g aspartic acid, 0.33 g glutamic acid, 0.69 g glycine, 0.13 g lysine, 30 mg histidine, 60 mg isoleucine, 27 mg methionine, 48 mg threonine, 15 mg tryptophan, 69 mg tyrosine, 95 mg valine, 10 mg cystine, 1 mg cholesterol,

1.36 g KH_2PO_4, 1.74 g K_2HPO_4, and a vitamin mixture that provided 2.5 units α-tocopherol, 450 units vitamin A acetate, 50 units calciferol, 22.5 mg ascorbate, 37.5 mg choline, 1.5 mg pantothenate, 2.5 mg inositol, 1.1 mg menadione, 2.25 mg niacin, 2.5 mg *p*-aminobenzoate, 0.5 mg pyridoxine, 0.5 mg riboflavin, 0.5 mg thiamine, 10 μg biotin, 45 μg folate, and 0.75 μg vitamin B_{12} per liter of medium. The phosphate salts were autoclaved separately and added after cooling to complete the medium. No precautions were taken to protect heat-labile vitamin factors. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO 63178, except for the vitamin mixture (United States Biochemical Corp., Cleveland, OH 44122).

Shake cultures were initiated by transfering several pieces of mycelium from an agar culture into 100 ml of medium in a 250-ml Erlenmeyer flask and shaking the flask at 150 rpm (rotary shaker, 2.5 cm diameter path) at 25 ± 2 C for 3 to 5 days, by which time large mycelial clumps had formed. The entire culture was blended twice in a 400-ml Sorvall Omnimixer canister (Dupont Instruments, Newton, CT 06470) at top speed for 15 sec. A suspension of broken cell material (10 to 20 ml) was seeded into 100 ml of fresh medium, and the culture was shaken 24 to 48 hr. The entire culture then was blended in the same fashion, and 10 to 20 ml were seeded into 100 ml of fresh medium. This blend-and-seed procedure was repeated over a period of 5 to 7 days, until a dense mycelial suspension was obtained. The fungi were maintained in this state by repetition of the blend-and-seed step every 24 to 48 hr. Cell material used in growth rate and pulse-label tests was prepared by seeding 20 to 30 ml of blended cell material into 150 ml of fresh medium in a 500-ml Erlenmeyer flask.

For dry weight measurements two or three 10-ml samples were taken from triplicate flasks with a 10-ml wide-tipped pipette and were filtered on 2.3 cm diameter Whatman 3MM paper disks. The small mycelial mats were peeled from the filters and dried to constant weight at 100 C.

Pulse-label experiments.—Radio-labeled [1,2-^{14}C]-acetic acid (45-60 mCi/mmole), [^{3}H(G)]-adenine (20.2 Ci/mmole), and [2-^{3}H(N)]-glycerol (200 mCi/mmole) were purchased from New England Nuclear, Boston, MA 02118. [4,5-^{3}H]-L-leucine (46 Ci/mmole) was purchased from ICN Pharmaceuticals, Irvine, CA 92715. These precursors were diluted to appropriate concentrations with the growth medium. Triplicate 3-ml culture samples were withdrawn from flasks with a 10 ml wide-tipped pipette and placed in scintillation vials. One-tenth ml of the precursors (equivalent to 0.5 μCi ^{14}C; 0.4 μCi ^{3}H in *Pythium* tests, 0.8 μCi ^{3}H in *Phytophthora* tests) was added, and the vials were shaken for 10 min as described above. After the pulse period, trichloroacetic acid (TCA) was added to a final concentration of 5%. The samples were transferred to 2.3 cm Whatman 3MM paper disks, washed three times with 5 ml of 5% TCA and twice with 5 ml of ethanol:ether (1:1), and then dried. The disks were placed in 10 ml of an Omnifluor (New England Nuclear)-toluene solution (4 *g*/liter), and their radioactivity was measured by scintillation spectrometry. Counting efficiencies were 7% with ^{3}H and 50% with ^{14}C.

In some experiments 3-ml triplicate culture samples were incubated for 10 min with various metabolic inhibitors and then pulse-labeled for 10 min with the radioactive precursors, after which they were killed with TCA and processed as above. All data reported are typical of results obtained in three or more experiments.

RESULTS

Growth characteristics.—The repetition of blend-and-transfer steps over a period of about 7 days yielded cultures which were easily maintained in a state of very rapid growth. Dry weights of these adapted cultures increased several fold within 24 hr of seeding fresh medium with mycelial fragments (Fig. 1). Typically, growth was logarithmic. The early slight departure of the *P. ultimum* curve from logarithmic growth rates probably reflects the dry weight contribution of fragments killed in the blend step. Indeed, this factor undoubtedly accounted for an apparent lag period of variable length found with both fungi in occasional tests. Such a lag was found more often with *Pythium* than with *Phytophthora*, which is consistent with our observation that the *Phytophthora* mycelium was much less readily fragmented than was that of *P. ultimum*.

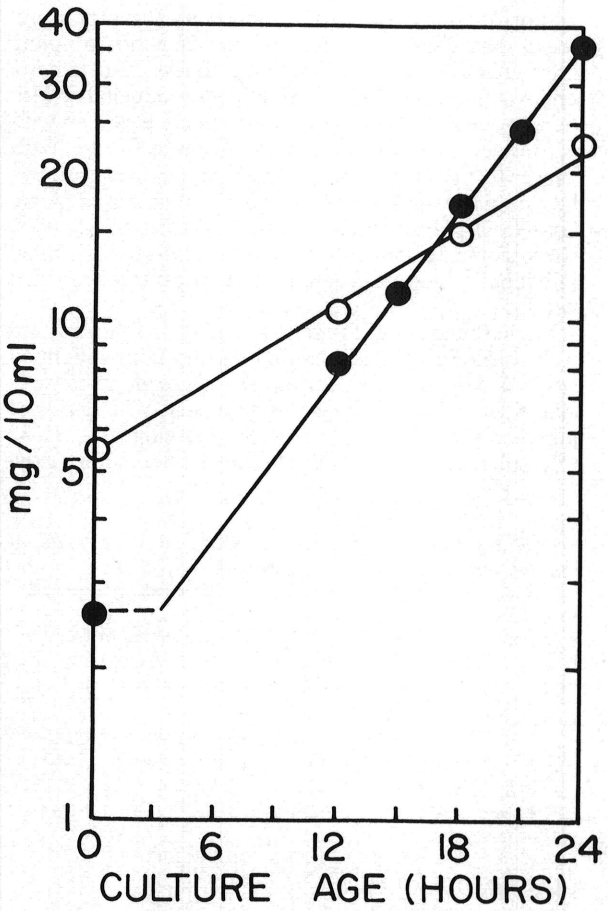

Fig. 1. Dry weight increase of *Pythium ultimum* (●) and *Phytophthora megasperma* var. *sojae* (○) in shake culture. Fresh medium was seeded with hyphal fragments at time zero. Points are means of two or three 10-ml culture samples from triplicate flasks in each of three tests.

Regardless of any early variation in growth rate, growth during the period 12 to 24 hr was consistently logarithmic (Fig. 1). *Pythium ultimum* doubled in about 5 hr, and *Phytophthora* in about 12 hr. In separate tests it was found that the growth rate did not begin to decrease until culture densities exceeded about 4 mg/ml dry weight. This stationary phase was not thoroughly characterized and, therefore, is not shown in Fig. 1.

Culture densities of 1 to 3 mg/ml dry weight were quite satisfactory for physiology experiments, as described below. Such actively growing cultures were easily produced for routine work. For example, a 24-hr-old *P. ultimum* culture with a density of 2 to 3 mg/ml was blended in late afternoon; 15 ml of fragmented mycelium was placed in 135 ml of fresh medium. By the following morning the culture was in the 12- to 24-hr period of reproducible growth rate and could be used for several hours. From the foregoing it is obvious that culture density can be manipulated easily through selection of appropriate inoculum densities and volumes.

The lack of mycelial aggregation of the culture material is reflected by the precision with which it could be sampled with a wide-tipped pipette (Table 1). The experiments shown were selected to cover the range of culture densities routinely obtained by the procedure. These data illustrate three points: (i) good sampling reproducibility within individual flasks and among replicate flasks, (ii) similar sampling reproducibility with the two fungi, (iii) no change in sampling precision with culture densities which varied as much as 8-fold. With respect to the third point, the variance of dense cultures did not differ significantly ($P = 0.05$) from that of sparse cultures, as judged by a test for homogeneity of variances. Microscopic examination of the cultures revealed individual hyphae and small bits of profusely branched medium.

Neither fungus was found to sporulate in shake culture in this medium. The formation of a ring of mycelium on the flask wall occurred infrequently, except in cultures shaken two or more days without transfer. When a ring of adherent mycelium was observed in young (0 to 12 hr old) cultures, it was dislodged easily without altering the sampling uniformity. Although several days were required for the transition from the initial mycelial seed pieces to rapidly growing mycelial suspensions, no rigid schedule was necessary to produce or maintain the latter. The features reported in Fig. 1 and Table 1 held if the cultures were blended and transferred to fresh medium every 24 to 48 hr. Visual inspection of culture density generally was the best guide for determining when to blend a culture. Once adapted to shake culture, mycelium could be transferred to fresh medium several times without blending, or cultures could be shaken without transfer for several days, without loss of the capacity to generate the actively growing suspensions with a single blend and transfer step.

Pulse-label experiments.—Both fungi readily incorporated several radioactive precursors into TCA-insoluble materials (Table 2). In these experiments, triplicate 3-ml samples were pulse-labeled for only 10 min; yet reproducibility within tests was similar to that of dry weight measurements with 10-ml samples. The magnitudes of incorporation indicate that metabolic activities can be assayed with smaller amounts of the precursors by selection of a suitable culture density.

The nature of some of the TCA-insoluble radioactive products was examined. Presumably 2-^{3}H-glycerol is incorporated into triglycerides, phosphatides, or complex lipids, since any other fates of this precursor would result in the loss of tritium as water (1). Samples pulse-labeled for 10 min with ^{3}H-leucine were filtered, without TCA addition, and disrupted in buffer [0.04 M tris-(hydroxymethyl)-aminomethane, 0.033 M $NaCH_3COO$, 0.5 M NaCl, pH 7.0] in an MSK mechanical homogenizer (Bronwill Scientific, Inc., Rochester, NY 14601). The resultant brei was clarified by centrifugation and dialysis. Treatment of the dialysate with 50 μg nuclease-free Pronase (Sigma Chemical Co.) per ml rendered TCA-soluble 94% of the originally TCA-insoluble radioactivity. Thus, in a 10-min pulse leucine was incorporated selectively into protein. Samples pulse-labeled for 10 min with ^{3}H-adenine were filtered, without TCA addition. Treatment of nucleic acid extracts (6) of the cells with 0.3 M NaOH at 25 C for 16 hr rendered TCA-soluble 98% of

TABLE 1. Variation in sampling of shake cultures of *Pythium ultimum* and *Phytophthora megasperma* var. *sojae*[a]

Source of variation in test	*Pythium* dry weight (mg/10 ml)	C.V.	*Phytophthora* dry weight (mg/10 ml)	C.V.
Variation within flasks				
1	22.0 ± 1.45	6.6%	19.9 ± 0.69	3.5%
2	17.3 ± 0.53	3.1%	13.0 ± 0.34	2.6%
3	9.3 ± 0.67	7.2%	9.5 ± 0.47	4.9%
4	3.4 ± 0.21	6.2%	4.2 ± 0.25	6.0%
Variation among flasks				
1	25.1 ± 1.11	4.4%	26.8 ± 1.48	5.5%
2	15.9 ± 0.59	3.7%	17.5 ± 1.35	7.7%
3	7.2 ± 0.35	4.9%	12.0 ± 0.42	3.5%
4	3.1 ± 0.21	6.8%	5.5 ± 0.15	2.7%

[a]Culture dry weight data are means and standard deviations, with coefficients of variation (C.V.), obtained with triplicate 10-ml samples from triplicate flasks. Cultures were sampled at 21, 18, 12, and 0 hr after transfer in tests 1, 2, 3, and 4, respectively.

TABLE 2. Incorporation of radioactive precursors into trichloroacetic acid-insoluble material by *Pythium ultimum* and *Phytophthora megasperma* var. *sojae* in shake culture[a]

Organism	Precursor	Counts/min: Mean ± standard deviation	Coefficient of variation
P. ultimum	^{3}H-leucine	3,076 ± 118	3.8%
	^{14}C-acetate	41,576 ± 3,515	8.5%
	^{3}H-glycerol	9,557 ± 443	4.6%
	^{3}H-adenine	1,656 ± 53	3.2%
P. megasperma	^{3}H-leucine	540 ± 41	7.6%
	^{14}C-acetate	17,753 ± 882	5.0%
	^{3}H-glycerol	649 ± 35	5.4%
	^{3}H-adenine	4,167 ± 291	7.0%

[a]Triplicate 3-ml samples from 15-hr-old cultures were pulsed 10 min with 0.1 ml of precursor (555,000 CPM ^{14}C; 75,000 CPM ^{3}H for *P. ultimum*, and 150,000 CPM ^{3}H for *P. megasperma*). Data represent incorporation by 1.5 mg/ml culture samples (dry weight).

TABLE 3. Effects of metabolic inhibitors on incorporation of radioactive precursors by *Pythium ultimum* and *Phytophthora megasperma* var. *sojae* in shake culture[a]

	Percent inhibition				
	Pythium		*Phytophthora*		
Inhibitor	^{3}H-leucine	^{14}C-acetate	^{3}H-leucine	^{14}C-acetate	^{3}H-glycerol
3.5×10^{-6} M Cycloheximide	94	3	72	31	2
10^{-4} M KCN	95	98	42	92	56
10^{-2} M Malonic acid	95	99	72	40	35
10^{-3} M NaN_3	89	92		Not Assayed	
10^{-4} M 2,4-dinitrophenol	94	97		Not Assayed	
5.5×10^{-4} M p-fluorophenylalanine	72	0		Not Assayed	

[a]Data are means obtained in three or more tests in which triplicate 3-ml samples from 15- to 18-hr-old cultures were incubated 10 min with inhibitor, then pulsed 10 min with precursor. Trichloroacetic acid-insoluble radioactivity was then measured.

the originally TCA-insoluble radioactivity. Thus, in a 10-min pulse adenine was incorporated selectively into ribonucleic acids. The products labeled with ^{14}C-acetate were not characterized; since pythiaceous fungi presumably have an active glyoxylate cycle (3), acetate probably was incorporated into a variety of macromolecules.

That shake cultures of these fungi can be quickly and easily manipulated for assay of metabolic activities is shown also by the responses to several metabolic inhibitors (Table 3). The inhibitions, which were obtained within 20 min, are consistent with the established modes of action of these agents. Cyanide, malonate, sodium azide, and 2,4-dinitrophenol, which interefere with mitochondrion energy generation in various fashions, substantially reduced incorporation of all three precursors used. The inhibitors of protein synthesis, cycloheximide and *p*-fluorophenylalanine, selectively blocked leucine incorporation in *P. ultimum*. Cycloheximide also reduced acetate incorporation in *Phytophthora*, an indication that some acetate is incorporated into protein in this fungus. In general, the degree of inhibition was less with *P. megasperma* var. *sojae* than with *P. ultimum*. This is consistent with the difference in their growth rates (Fig. 1) and isotope incorporation rates (Table 2). However, the data indicate that, in very short experiments, cytoplasmic and mitochondrial activities in both fungi can be manipulated easily and in predictable ways.

DISCUSSION

The seeding of mycelial fragments into liquid media is a commonly used device for the production of mycelium in a readily harvestable form. Shaking of such cultures can increase the growth rates substantially (4). The essential feature of the method described here is a gradual increase in the number of seed pieces coupled with a gradual decrease in their size. This is accomplished by repeated blending and transfer, with sufficient time between blend steps to allow an increase in mycelial mass. The practical result is an extremely large population of actively growing propagules whose metabolic activities can be assayed easily. The ultimate result, as Tolmsoff (9) reported, is uniform growth as a suspension of individual hyphae which pinch off new hyphal segments. Maintenance of this state requires rigid control of culture conditions. Our *P. ultimum* isolate could be adapted to such growth, but it frequently reverted to clumped mycelial growth, especially at the culture densities best suited for physiology studies (dry weight = 1 to 3 mg/ml). Further, based on our observations with *P. megasperma* var. *sojae*, many *Phytophthora* species may not be adaptable to growth as suspensions of individual budding hyphae. Although the advantages of such growth are several, the present method seems more suitable for many types of study in that it offers greater procedural flexibility in both production and maintenance of cultures.

The growth medium was devised specifically to permit the use of various radioactive precursors. For other purposes, the amino acid complement can be replaced by commercial peptone or yeast extract (in concentrations of about 3 *g*/liter), which provide excellent growth. In fact, the amino acid composition of the medium was designed to approximate that of a peptone medium on which we initially adapted this *P. ultimum* isolate to shake culture.

The two fungi used in this investigation were selected because of their contrasting growth rates on agar media. However, the doubling time of the normally slow-growing *P. megasperma* var. *sojae* was only about twice that of the fast-growing *Pythium* in shake culture. It is likely that other pythiaceous fungi can be adapted to this type of shake culture.

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