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Thomas Joseph Michaels for the degree of Doctor of Philosophy in

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Title: IN VITRO CULTURE AND GROWTH MODELING OF TUBER SPP. AND

INOCULATION OF HARDWOODS WITH T. MELANOSPORUM ASCOSPORES

Abstract approved: _____

Dr. James M. Trappe

In vitro growth of twelve isolates of Tuber spp., the true truffles, was quantified by an agar-melt procedure. All isolates grew poorly on media commonly used for the culture of mycorrhizal fungi, but responded markedly to the addition of nitrate, as well as other inorganic ions, to malt and potato-dextrose basal media. The pH of all media containing nitrate rose during incubation, and maximum rates of growth occurred at these higher pH values. Growth response to eight polycarboxylic acid buffers was investigated; one in particular, β-methylcarballylic acid is both nontoxic and has a buffering range from pH 2.5 - 8.2 and is suggested for use in subsequent in vitro Tuber studies. Phosphate buffer was also found to be non-inhibitory at moderate concentrations. Of the six isolates studied for temperature response, all had optima around 20 C and declined around 26.5 C. The possible response of other hard-to-culture ectomycorrhizal fungi to nitrate is discussed.

A few simple models of fungal growth in shallow Petri dishes were proposed, and the growth data obtained as mycelial dry weight for twelve Tuber isolates were tested for fit. Growth was hypothesied to be uniform and proportional to total biomass, or to occur only at the colony periphery and proportional to the square root of the biomass. Each of these models was altered to include a time-dependent variation: growth was hypothesized to also depend upon the concentration of a metabolite produced by the fungus. Metabolite-dependent models gave better fit than metabolite-independent versions, but differences between peripheral and uniform growth models were less clear.

In inoculation studies of oaks and filberts with T. melanosporum ascospores, viability of spores was maintained either by freezing or 5 C storage of ascocarps. Liming improves mycorrhization, although not necessarily seedling vigor. The effect of soil organic matter is minimal on growth and mycorrhizal development of Corylus avellana. Soil inoculation with spores is an effective method of establishing the symbiosis, but a germling drench is less so.

A method which nondestructively measures seedling root volume was proposed in order to study mycorrhizal colonization on the same seedling over time.

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INOCULATION OF HARDWOODS WITH T. MELANOSPORUM ASCOSPORES

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TABLE OF CONTENTS

PART II - MYCORRHIZAL COLONIZATION STUDIES

LIST OF TABLES

LIST OF FIGURES

IN VITRO CULTURE AND GROWTH MODELING OF TUBER SPP. AND

INOCULATION OF HARDWOODS WITH T. MELANOSPORUM ASCOSPORES

PART I

IN VITRO CULTURE STUDIES

IN VITRO CULTURE OF TUBER,
A GENUS OF MYCORRHIZAL ASCOMYCETES

CHAPTER 1

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ABSTRACT

In vitro growth of twelve isolates of Tuber spp., the true truffles, was quantified by an agar-melt procedure. All isolates grew poorly on media commonly used for the culture of mycorrhizal fungi, but responded markedly to the addition of nitrate, as well as other inorganic ions, to malt and potato-dextrose basal media. The pH of all media containing nitrate rose during incubation, and maximum rates of growth occurred at these higher pH values. Growth response to eight polycarboxylic acid buffers was investigated; one in particular, β-methylcarballylic acid is both nontoxic and has a buffering range from pH 2.5-8.2 and is suggested for use in subsequent in vitro Tuber studies. Phosphate buffer was also found to be non-inhibitory at moderate concentrations. Of the six isolates studied for temperature response, all had optima around 20 C and declined around 26.5 C. The possible response of other hard-to-culture ectomycorrhizal fungi to nitrate is discussed.

INTRODUCTION

Mycorrhizal fungi of the genus Tuber generally grow slowly, if at all, in culture. Media commonly used for the pure culture growth of mycorrhizal fungi do not support good growth of Tuber spp. The present work was undertaken to find suitable media to maintain vigorous isolates of Tuber spp. in our culture collection, increase growth rate to raise sufficient biomass for mycorrhizal colonization studies, increase thallus biomass for sporulation studies, and study nutritional needs and truffle physiology.

Although a number of species of Tuber have been obtained in culture, most reports of their culture media are sketchy (Bonfante et al., 1971; Chaze, 1943, 1947, 1950; Chaze and Mestas, 1939; Chavalier, 1972; Chevalier et al., 1973; Chevalier and Pollacsek, 1973; Fontana, 1968, 1971; Fontana and Palenzona, 1969; Guiochon, 1959; Grente et al., 1972; Palenzona et al., 1970, 1972; Montacchini et al., 1972; Vrot, 1977). Chaze and Mestas (1939), for example, report the formation of perithecia of T. melanosporum Vitt. in pure culture, but decline to describe the medium, saying only that it is one currently utilized in the study of higher fungi. A later report (Chaze, 1950) simply mentions that agar media of "carrot, malt, etc." support perithecial development. Growth parameters for a single strain of T. melanosporum have been reported for which the basal medium is that of Modess (1941), a simple medium containing a few inorganic ions, malt extract and glucose (see Hagem's agar below). Large inoculum plugs, 12 mm diameter, were required for assured growth on transfer to test media (Bonfante and Fontana, 1973). A three- to four-fold increase in dry weight was obtained under optimal conditions: 23 C, pH 6.5-6.7, no agitation. Malt extract as a carbon source gave better growth than any single mono- or polysaccharide tested and ammonium ion was the best nitrogen source, although there were many small weight differences between the various treatments and statistical support for these two conclusions is lacking. Chevalier (personal communication) suggests a similar medium of malt exract agar containing less than 20 ppm added calcium and having pH 6.1-6.5. Good, but unquantified, growth was also obtained on casein hydrolysate-small grain (oats,

millet, or barley) media (Fontana and Palenzona, 1969). Vrot (1977) has proposed a defined liquid medium. Its agar equivalent and the other media just mentioned are compared in this report.

Some of the _Tuber_ cultures used in this study contain gram-negative rod bacteria, observed when plated out on potato-dextrose agar (PDA) and some other media. Fontana (1971) has stated that repeated subculturing on malt extract agar can eliminate the bacteria. However, Chevalier (1972) noted that bacteria-free cultures so obtained would not survive successive subculture. Routine periodic transfers of our cultures to fresh media resulted in mycelial debilitation and eventual loss of five of our 17 _Tuber_ isolates, namely, _T. gibbosum_ Harkn. (S-364), _T. irradians_ Gilkey (S-363), _T. rufum_ Pico:Fries (S-324 and S-325) and _T. aestivum_ Vitt. (U1). Four of these isolates contained no apparent bacterial associate. Also, of the twelve remaining isolates all but two contained a bacterial member (although bacteria-free pure cultures of four of these have since been obtained by hyphal tip transfer onto a new maintenance medium reported below). Vrot (1977) has also reported a beneficial effect from the bacteria, while noting similar responses to a variety of treatments between bacteria-laden and bacteria-free _Tuber_ cultures. We report here work with six strains in pure culture and six maintained less rigorously in dual culture.

Agar media were used throughout these experiments. Palmer (1971) suggests that solid media generally support better growth of mycorrhizal fungi than liquid media. Also, although _T. melanosporum_ is reported to grow better in a liquid medium than on its surface (Bonfante and Fontana, 1973), Chevalier (personal communication) recommends that if submerged culture is desired, the submerged inoculum plug should be positioned close to the surface. Mikola (1948) observed that _Cenococcum geophilum_ Fr., another mycorrhizal ascomycete, grows better in the presence of colloids. In a preliminary study at our laboratory with a buffered (20 mM phosphate) modified Melin-Norkrans medium (Marx, 1969) three _Tuber_ spp. grew radially in agar media better than in liquid media. Although measurement of mycelial dry weight, metabolic substances, etc. is more direct when liquid media are used, it can also be obtained for

colonies extracted from melted and diluted agar (Day and Hervey, 1946). Temperature optima for mycorrhizal fungi typically are within the range found for other soil fungi. Paxillus involutus (Batsch) Fr. strains found in temperate climates had a range of optima from 15 to 25 C (Laiho, 1970). All six mycorrhizal fungi in the study of Hacskaylo et al. (1965) demonstrated optimal or near-optimal growth in a similar temperature range. Harley (1969) observes "...the optimum temperature for the majority of mycorrhizal fungi is usually about 20 C." Some biotypes have optima closer to 30 C, although they do grow over a wide temperature range (Theodorou and Bowen, 1971; Hung and Chien, 1978). Cultures of Tuber spp. for this study were incubated at 20 C unless otherwise noted.

Mycorrhizal fungi are not generally sensitive to strong light (Palmer, 1971). Bonfante and Fontana (1973) noted no particular response to light for Tuber melanosporum.

METHODS AND MATERIALS

Isolates.

Mycelial cultures were selected from the collections of mycorrhizal fungi maintained by Mr. G. Chevalier at the Station de Pathologie Vegetale, Institut National de la Recherche Agronomique (I.N.R.A.), Clermont-Ferrand, France, and Drs. James Trappe and Randolph Molina at the U.S. Forest Service Pacific Northwest Forest and Range Experiment Station, Corvallis, Oregon, for this growth study (Table I). Most isolates were obtained from sporocarp excisions incubated on Chevalier's malt extract agar (ChMA; see p. 10), modified Melin-Norkrans agar (MMNA; Marx, 1969) or potato-dextrose agar (PDA) from one to eight years. Beginning in 1978, all cultures were routinely subcultured every three months on PDMmA (see P. 13 and Table II).

Excisions from Tuber sporocarps, even young specimens, often are contaminated by other fungi and soil microflora (Marletto, F., 1969; Marletto, O. I. O., 1970). The identity of several of our isolates as Tuber spp. has been confirmed by formation in vitro of mycorrhizae

Table I.

Sources of Tuber isolates used in this study

Species	FSL ID#	Collector (ID#)	Date	Sources [1]	Location	Soil pH	Probable host	Bacterial associates
T. aestivum Vitt.	S-321	Chevalier (aesl)	7/71	sporocarp	Aurel, France	7.8	Quercus pubescens Willd.	+
T. aestivum Vitt.	S-322	Chevalier (U2)	12/70	sporocarp	Chemilly-Sur-Serein, France	7.9	Q. sessiliflora Smith	−
T. borchii Vitt.	S-211	Chevalier (A2)	3/74	sporocarp	Urbani cannery, Spoleto, Italy	--	--	−
T. borchii Vitt.	S-393	Michaels	6/78	S-211 sector	--	--	--	−
T. brumale Vitt.	S-323	Chevalier (Gl)	8/70	sporocarp	Chemilly-Sur-Serein, France	7.9	Q. pedunculata Erhr.	+
T. brumale var. moschatum (Ferry) Fischer	S-485	Chevalier (MOSR)	2/74	sporocarp	Southwest France	8.2	Q. pubescens	+
T. melanosporum Vitt.	S-488	Chevalier (a)	1/71	sporocarp	Peyrins, France	8.0	Q. pubescens	+
T. melanosporum Vitt.	S-489	Chevalier (g)	1/71	sporocarp	Peyrins, France	8.0	Q. pubescens	+
T. melanosporum Vitt.	S-490	Chevalier (1017)	-/73	mycorrhizae	--	7.8	Corylus avellana L.	+
T. puberulum Berkeley and Broome	S-394	--	--	--	--	--	--	−
T. rufum Pico	S-487	Chevalier (R3)	12/70	sporocarp	Peyrins, France	8.1	Q. pubescens	−
T. rufum var. nitidum (Vitt.) Fischer	S-362	Molina/Trappe	10/76	sporocarp	Ochoco Divide, Crook Co., Ore.	--	Populus tremuloides Michx.	−

[1] Mycelial cultures obtained from excisions of fresh sporocarps or surface-sterilized mycorrhizae.

6

similar to those produced from spores in non-sterile pot inoculation studies (Chevalier, 1972, 1973, 1974; Chevalier and Desmas, 1975; Fontana and Palenzona, 1969; Fontana and Bonfante, 1971; Grente et al., 1972; Palenzona, 1969; Palenzona, et al., 1972). The isolates not thus verified show these traits in common with verified isolates on PDMmA:

a) a tendency for initial, relatively fast radial growth, followed by hyphal branching behind colony margin to slowly increase colony density.

b) occasional formation of bulging intercalary cells.

c) formation of mycelial clumps of crowded hyphae also with occasional intercalary swellings.

General Procedures.

To increase inoculum, isolates were transferred to 60 x 15 mm Petri dishes containing 8.0 ml of the same medium. After six to eight weeks, agar plugs four mm in diameter (six mm for growth curves in experiment II) were taken from midway between the center and the aerial perimeter of the colony for transfer to test plates. Plates of a given isolate were sealed in Parafilm (American Can Co.) and randomly placed in an incubator for 40 d at 20 C unless otherwise noted. Light was not thought to be critical in these introductory studies with Tuber, a genus with a totally hypogeous life cycle. Inoculum was transferred under flourescent room lights and incubated in darkness.

After incubation pH was measured on two plates of each treatment. Distilled, deionized water (3.0 ml) was added to each plate. After equilibrating for one hour, pH was measured with a surface electrode at ambient temperature. Although a 3.0 ml addition could theoretically shift pH as much as 0.2 units in unbuffered systems, the dilution effect in this instance was considered negligible because the media are all somewhat buffered.

To obtain mycelial weight, agar and mycelum were transferred to a beaker containing 150-200 ml tapwater and steamed for 45-60 min. The entire mixture was aspirator-filtered hot through a stainless steel

support grid, 130 μm mesh, of a Millipore filter apparatus and washed with 200-400 ml of 40-50 C tapwater. The grid retains mycelium while passing bacteria, agar sol and small particulates. Mycelial pads were frozen and lyophilized to constant weight (usually 2 d, 20 μm Hg).

Experiment I.   Growth on different media.

Researchers working with mycorrhizal fungi often employ only a few media formulations for pure culture studies. Elias Melin, credited with the first successful isolation and culture of mycorrhizal fungi in 1922 (Hatch, 1937), found malt extract to be a key undefined substance for growth. "All of the culture media employed contained, or consisted of, malt extract" (Melin, 1925). Although a chemically defined medium is clearly desirable in nutritional studies, most mycorrhizal fungi are rountinely maintained on partially undefined media, often containing malt extract (Marx, 1969; Modess, 1941; Zak, 1971).

For experiment I, six media recommended for mycorrhizal fungi and six other media of general mycological utility were used. In some preliminary studies for pure culture synthesis of these fungi with various trees, it was found that addition of Hewitt's minerals (Hewitt, 1966), a formulation commonly used as a nutrient base in higher plant studies, promoted fungal growth. Accordingly, five additional media formulations with these minerals were also included.

All media except Nos. 5 and 6 (OaA and OaCA) incorporated agar (Difco Bacto-Agar) at 1.5 per cent w/v and were adjusted immediately after autoclaving (20 min, 19 lb psi) to pH 5.7 as determined with an Orion 601A Digital Analyzer equipped with an ATC probe for internal temperature/slope correction for hot solutions.

Each treatment was replicated six times and incubated at 25 C. At the end of the incubation period four of the six replicates were randomly selected for dry weight measurement. Some _Tuber_ isolates, especially those with a long lag phase of growth, do not always grow upon transfer to some media. Of the four replicates selected, those that did not grow were replaced with one of the remaining replicates.

The following media were studied. Their compositions plus those of water and malt agars are compared in Table II. An asterisk (*) indicates it has been used by others in culture of mycorrhizal fungi. All formulations are per liter.

1. Modified Melin-Norkrans agar. MMNA*. (Marx, 1969).

$CaCl_2$	0.05 g
NaCl	0.25 g
KH_2PO_4	0.50 g
$(NH_4)_2HPO_4$	0.25 g
$MgSO_4 \cdot 7H_2O$	0.15 g
Sequestrene	0.02 g
Sequestrene 330Fe	0.02 g

    (Ciba-Geigy, sodium
       ferric diethylene-
       triamine pentaacetate,
       10% Fe)

Thiamine·HCl	0.10 g
Malt extract	3.0 g
d-Glucose	10.0 g
Agar	15.0 g

    (pH 5.5)

2. Hagem's agar as modified by Modess. HaA*. (Modess, 1941).

KH_2PO_4	0.50 g
$MgSO_4 \cdot 7H_2O$	0.50 g
NH_4Cl	0.50 g
$FeCl_3$, 1% soln.,	0.5 ml
d-Glucose	5.0 g
Malt extract	5.0 g
agar	15.0 g

    (pH 4.5-4.7; Hacskaylo et al., 1965)

3. Vrot's medium, ammonium nitrogen source (agar added). $VrNH_4A$*. (Vrot, 1977).

KH_2PO_4, 0.50 mM	11.2 ml

K_2HPO_4, 0.50 mM   8.8 ml

$CaCl_2$ 0.2 g

$MgSO_4 \cdot 7H_2O$ 0.5 ml

$ZnSO_4$, 6.9 mM   0.5 ml

$MnSO_4$, 9.0 mM   0.5 ml

Fe-citrate, 1% soln.   0.5 ml

Thiamine·HCl   0.100 mg

d-Glucose   10.0 g

(pH 6.6)

As nitrogen source Vrot recommends NH_4NO_3. Medium modified to test NH_4^+ and NO_3^- separately. For NH_4^+ include:

NH_4Cl 0.50 g

NaCl   0.55 g

All substances except phosphates autoclaved together; phosphates added after autoclaving.

4. Vrot's medium, nitrate nitrogen source (agar added). $VrNO_3A^*$. (Vrot, 1977). As for previously described medium, except that $NaNO_3$ replaces NH_4Cl:

$NaNO_3$ 0.80 g

NaCl   0.55 g

5. Oatmeal agar (Difco). OaA.

6. Oatmeal/casein hydrolysate agar. $OaCA^*$. (Fontana and Palenzona, 1969).

Oatmeal agar (Difco)   72.5 g

Casein hydrolysate   12.0 g

     (enzymatic, Calbiochem)

Fontana and Palenzona (1969) report culturing T. albidum on a medium consisting of autoclaved cereal grains (millet, barley, or oats) and casein hydrolyzate. However, the amount of the latter is not reported. The quantity used here is in the range of the amount of hydrolyzed proteins in a number of media described in the Difco Manual, 9th ed. (1953).

7. Chevalier's malt agar. ChMA*. (Chevalier, personal communication).

Malt extract	10.0 g
$MgCO_3$	(0.125 g)
$Ca_3(PO_4)_2$	(0.050 g)

Mineral solution is prepared by stirring a finely powdered suspension of the inorganics in distilled water overnight. Insolubles are filtered off before adding malt extract. Chevalier (personal communication) does not specify a pH, although we obtain a post-autoclave pH 6.0 by this preparation method. This medium has been used for maintenance of Tuber isolates.

8. Potato dextrose agar (Difco). PDA.

9. Prune agar (Difco). PrA.

10. Czapek's agar. CzA. (Thom and Church, 1926).

$NaNO_3$	2.0 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Sucrose	30.0 g
Agar	15.0 g
(pH 7.3)	

11. A defined medium for culture of some obligate plant pathogenic fungi. TrA (Trione, 1964).

KH_2PO_4	0.613 g
$K_2HPO_4 \cdot 3H_2O$	0.114 g
$MgSO_4 \cdot 7H_2O$	0.246 g
$CaCl_2$	0.055 g
Sequestrene 330Fe	20.0 mg
$ZnSO_4 \cdot 7H_2O$	3.52 mg

$CuSO_4 \cdot H_2O$	0.38 mg
$MnSO_4 \cdot H_2O$	0.031 mg
$Na_2MoO_4 \cdot 2H_2O$	0.024 mg
Thiamine·HCl	5.0 mg
L-asparagine	3.0 g
Sucrose	20.0 g
(pH adj. to 6.0)	

All substances mixed together prior to autoclaving. Medium stays clear with little visual evidence of the Maillard reaction (Maillard, 1916).

12. Medium TrA, above, with 1.0 g/l yeast extract added. TrYA.

13. A rich mycological medium, "corn meal plus agar." CM^+A.

Corn meal agar (Difco)	17.0 g
Malt extract (Difco)	5.0 g
Casein hydrolysate	5.0 g
(enzymatic, Calbiochem)	
Yeast extract (Difco)	2.0 g
d-Glucose	10.0 g
Hewitt's minerals	
(see Medium 14, below)	
Agar	11.2 g
Post-autoclave pH to 5.7	

14. An agar medium of malt extract and Hewitt's minerals (Hewitt, 1966) at the concentration recommended for hydroponic plant physiology studies. MmA.

Malt extract	10.0 g
Agar	15.0 g
Hewitt's minerals:	
KNO_3	0.505 g
$Ca(NO_3)_2$	0.820 g
$NaH_2PO_4 \cdot H_2O$	0.186 g
$MgSO_4 \cdot 7H_2O$	0.369 g

Sequestrene 330Fe	0.056 g
$MnSO_4$	0.023 g
NaCl	5.85 mg
H_3BO_3	1.86 mg
$CuSO_4$ $5H_2O$	0.24 mg
$ZnSO_4$ $7H_2O$	0.296 mg
Na_2MoO_4 $4H_2O$	0.024 mg
$CoCl_2$ $6H_2O$	0.024 mg

The post-autoclave pH ca. 4.6 is adjusted to 5.7 with a few ml of
0.4 N NaOH.

15. Potato dextrose agar plus Hewitt's minerals, post-autoclaved pH
    to 5.7. PDmA.

16. Potato dextrose malt agar plus Hewitt's minerals,
    post-autoclaved pH to 5.7 PDMmA.

Potato dextrose agar(Difco)	19.5 g
(@ 1/2 recommended conc.)	
Malt extract (Difco)	5.0 g
(@ 1/2 MmA conc.)	
Agar	7.5 g
Hewitt's minerals	(see MmA)

17. Medium PDMmA above plus 0.1 per cent (w/v) yeast extract. PDMYmA.

Experiment II. Growth as a function of time.

Petri plate cultures of isolates were prepared, grown and
harvested as described in General Procedures above. Enough plates of
each isolate were grown to permit random selection of six at weekly
intervals for 8 to 10 weeks (slow-growing isolates were carried longer
than fast-growing).

Experiment III. Growth at Different Temperatures.

Six isolates were grown at different temperatures by placing

Table II.

Concentration of principal moieties in test media of experiment I[1]

MEDIUM	NO3 (mM)	NH4 (mM)	ORG-N (mM N)	PO4 (mM)	K (mM)	Ca (mM)	Mg (mM)	SO4 (mM)	ADDED TRACE ELEMENTS	DEFINED CARBOHYD. (% w/v)	INFUSIONS AND EXTRACTS (% w/v)	ADDED THIAMINE (m)	pH LIT	pH EXP	REFERENCE
1. MMNA	--	3.8	--	3.7	3.7	0.4	0.6	0.6	Fe	d-GLUCOSE 1.0	MALT. 0.3	0.30	5.5	5.7	Marx, 1969
2. HaA	--	9.4	--	3.7	3.7	--	2.0	2.0	Fe	d-GLUCOSE 0.5	MALT. 0.5	--	4.6	5.7	Modess, 1941
3. VrNH4A	--	9.4	--	10.0	14.4	1.8	2.0	2.0	Fe,Mn,Zn	d-GLUCOSE 1.0	--	0.30	6.6	5.7	Vrot, 1977
4. VrNO3A	9.4	--	--	10.0	14.4	1.8	2.0	2.0	Fe.Mn.Zn	d-GLUCOSE 1.0	--	0.30	6.6	5.7	Vrot, 1977
5. OaA	--	--	--	--	--	--	--	--	--	--	OATMEAL. 0.6	--	--	5.7	
6. OaCA	--	--	CASAMINO 100	--	--	--	--	--	--	--	OATMEAL. 0.6	--	--	5.7	Fontana and Palenzona, 1969
7. ChMA	--	--	--	<0.3	--	<0.5	<1.5	--	--	--	MALT. 1.0	--	6.1	5.7	Chevalier, personal comm.
8. PDA	--	--	--	--	--	--	--	--	--	d-GLUCOSE 2.0	POTATO. 0.4	--	5.6	5.7	Difco Manual. 1953
9. PrA	--	--	--	--	--	--	--	--	--	--	PRUNE, 0.9	--	5.6	5.7	Difco Manual. 1953
10. CzA	23.5	--	--	5.7	11.5	--	2.0	2.0	Fe	SUCROSE 3.0	--	--	7.3	5.7	Thom and Church. 1926
11. TrA	--	--	ASN, 68.7	5.0	5.5	0.5	1.0	1.0	Fe,Zn.Cu,Mn,Mo	SUCROSE 2.0	--	14.8	6.0	5.7	Trione, 1964
12. TrYA	--	--	ASN. 68.7	5.0	5.5	0.5	1.0	1.0	Fe,Zn.Cu,Mn,Mo	SUCROSE 2.0	YEAST. 0.1	14.8		5.7	
13. CM+A	15.0	--	CASAMINO 41.5	1.3	5.0	5.0	1.5	1.5	Fe.Zn.Cu,Mn, Mo.Co.B	d-GLUCOSE 1.0	CORNMEAL, 0.2 MALT. 0.5 YEAST. 0.2	--		5.7	
14. MmA	15.0	--	--	1.3	5.0	5.0	1.5	1.5	Fe, Zn,Cu,Mn. Mo.Co,B	--	MALT. 1.0	--		5.7	
15. PDmA	15.0	--	--	1.3	5.0	5.0	1.5	1.5	Fe.Zn.Cu.Mn. Mo.Co,B	d-GLUCOSE 2.0	POTATO, 0.4	--		5.7	
16. PDMmA	15.0	--	--	1.3	5.0	5.0	1.5	1.5	Fe.Zn.Cu.Mn, Mo.Co,B	d-GLUCOSE 1.0	MALT. 0.5 POTATO. 0.2	--		5.7	
17. PDMYmA	15.0	--	--	1.3	5.0	5.0	1.5	1.5	Fe. Zn.Cu.Mn. Mo.Co.B	d-GLUCOSE 1.0	MALT. 0.5 POTATO, 0.2 YEAST. 0.1	--		5.7	
WA[2]	Tr[5]	Tr		1.2	Tr[6]	0.4	1.0	0.6	NA[5]	B = 0.2mM; Fe, Zn.Mn<5μm					
PDA[3]	Tr	Tr		3.6	1.1[6]	6.1	0.7	0.4	NA	B. 0.1mM: Fe. Zn.Mn<30μm					
MA[4]	Tr	Tr		1.4	0.2[6]	0.5	0.7	Tr	NA	B.Fe.Zn.Mn<5μm					

1) See text for ionic form and concentration: concentration of inorganic ions shown represent only the defined portion of the medium; amounts of ions from undefined components can be highly variable. Final concentration of the mineral ions should be determined after quantitative analysis of undefined components.
2) Quantitative analysis for water agar (Bacto-Agar, Difco), 1.5% w/v.
3) Quantitative analysis for potato dextrose agar (Difco), 3.9% w/v.
4) Quantitative analysis for malt extract (Difco), 1.0% w/v.
5) Tr = Trace: NA = Not available.
6) Total phosphorus. expressed as PO4.

inoculated plates at regular intervals on a temperature gradient plate
(Trione and Leach, 1970). The temperature ranged from 8 C to 33 C
over the gradient as measured in the center of a PDMmA-containing
Petri dish. To minimize effects of changes in light and ambient air
temperature on the established temperature gradient, the array of agar
plates was insulated with cotton and covered with aluminum foil
between sampling times. Temperatures of 2 C and 5 C were added for
some fungi by placing replicates in appropriately set incubators.

After inoculation as described in General Procedures, plates were
pre-incubated at 20 C for 7 to 14 d before placement on the tempera-
ture gradient plate or in the low temperature incubators. Only plates
showing uniform growth from the inoculation plug and with similar
mycelial densities and diameters were used. Three plates were
selected at random from each temperature increment at 20, 40, and 60 d
intervals after placement on the gradient plate.

Experiment IVa and b. Effects of minerals on growth.

It was observed in Part I that addition of a complete Hewitt's
minerals formulation stimulates Tuber growth in vitro. To test the
effect of individual ions, components of the formulation were
selectively replaced by a corresponding sodium or chloride ion at the
same concentration in experiment IVa. Trace elements (including S as
sulfate) were replaced as a group. For discussion a medium containing
all the added ions but Ca^{++}, for example, is designated as PDM(m-Ca)A.
Controls consisted of the complete formulation, no.16 (PDMmA), and
also the same medium plus 15 mM NaCl (PDM(m+NaCl)A) to investigate if
Na^{+} and Cl^{-} as the replacement ions affected growth. Finally, a
medium devoid of Hewitt's formulation or additional NaCl (PDMA) was
included. All media were standardized to pH 5.7 after autoclaving.
Treatments were replicated seven times and randomized. Data was
analyzed from viable transfers only, arbitrarily taken to mean those
cultures yielding more than 2.0 mg dry weight after the incubation
period.

Results of experiment IVa indicated need for experiment IVb on

effects of addition of nitrate (as $NaNO_3$). Three media were prepared: PDMA and PDM($+NO_3$)A, the last one being PDMA plus 15 mM $NaNO_3$. Experimental procedures were as for experiment IVa.

Experiment V. Effects of inorganic phosphate buffer on growth.

Buffer solutions were prepared by adjusting aqueous $NaH_2PO_4 \cdot H_2O$ to pH 5.5 with 0.4 N or 5 N NaOH. To lessen the phosphate-mediated conversion of aldoses at elevated temperatures (Englis and Hanahan, 1945), the phosphate solutions of approximately three times the desired final concentration were autoclaved separately from PDM(m-PO_4)A components, which were dissolved in a volume of water necessary to bring them to the standard formulation concentrations upon addition of the buffer solution. Six buffer solutions were prepared in exponentially increasing concentrations of $0.75 \times 10^{n/2}$ mM phosphate, where n = 0, 1, 2,..., 5. A distilled water solution (n = $-\infty$) was also included. After autoclaving, buffers were added hot to their respective volume of PDM(m-PO_4)A solutions and the pH adjusted to 5.7 with 0.4 N NaOH (or 5 N NaOH in the case of the two highest phosphate concentrations). A slight precipitate formed in the 75 mM phosphate solution and a heavy, fine precipitate in the 227 mM solution. These precipitates were fine enough to pass through the stainless steel grid mesh filter used in the work-up procedure. Treatments were replicated seven times and cultures were incubated and weight determinations made as described for experiment IVa. Dry mycelial weights obtained were subjected to a separate analysis of variance (ANOVA) for each isolate. For treatments in which growth was greatly inhibited, variance was very small relative to the other treatments. If a null hypothesis by an F-test for equal variances could not be accepted (α = 0.05) in the ANOVA for a treatment it was separated from the others. The hypothesis that the mean mycelial weight from that treatment was equal to that of another was then tested by a t-statistic which accounts for unequal variance (Dixon and Massey, 1969).

Experiments VIa and b. Effects of polycarboxylate buffers on growth.

Media incorporating the buffers shown in Table X were prepared for experiment VIa by a procedure similar to that of experiment V, with the exception that, because of the larger number of acid equivalents, compounds were solubilized by the addition of solid NaOH before bringing solutions to pH 5.5 with 5 N NaOH. Each buffer except β-MTCarbA was tested at a final concentration of 75 mM; β-MTCarbA, expensive and in short supply, was tested at 65mM. This study was undertaken prior to the establishment of temperature optima -- incubation temperature was 25 C. Weight determination, replication and sampling procedure were as described under General Procedures.

The results of experiment VIa gave reason to determine the concentrations of β-MTCarbA which inhibit growth of Tuber spp. and the range of concentrations over which it can be an effective buffer. Media buffered with β-MTCarbA were thus prepared for experiment VIb in a manner similar to the phosphate series of experiment V. The concentrations employed followed the exponential sequence $0.75 \times 10^{n/2}$ mM, n = 0, 1, ..., 4. The 227 mM concentration (n = 5) was omitted due to the high cost of this buffer. A medium (PDMmA) in which β-MTCarbA was omitted (n = $-\infty$) was also prepared. Incubation, weight determination, etc. were as described in experiment VIa.

RESULTS AND DISCUSSION

Experiment I. Growth on different media.

Growth data (Table III) reveal several general effects of media components on growth. The inclusion of undefined substances in the media usually increased growth. The four defined media[1] (nos. 3, 4, 10, 11) ranked seventh, ninth, thirteenth, and fifteenth respectively

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[1]Although agar is a highly purified product, its composition can vary from one lot to the next (Lilly, 1965). Consequently, these media are not "completely defined."

Table III.
Effect of selected undefined or partially defined media of mycorrhizal and/or general mycological utility on growth of <u>Tuber</u> isolates in experiment I[a]

Medium: Isolate	1 MMNA	2 HaA	3 VrNH$_4$A	4 VrNO$_3$A	5 OaA[c]	6 OaCA[c]	7 ChMA	8 PDA
aes S-321	1.9±0.2	1.5(n=1)	2.2±0.8	7.7±2.7	+	−	1.4±0.2	11.3±0.9
aes S-322	18.2±5.6	0.6±0.1	0.9±0.2	1.7±1.1	+	−	22.3±11.5	3.2±0.7
bor S-211	3.7±1.0	4.0±0.7	3.7±0.6	6.9±2.9	++	++	0.2±0.2	10.4±3.8
bor S-393	2.8±0.2	1.8±0.1	1.1±0.1	4.4±0.5	++	++	1.2±0.1	27.7±0.6
bru S-323	2.6±0.2	2.2±0.3	2.4±0.4	2.1±0.7	+	−	1.4±1.8	5.4±0.5
brum S-485[b]	3.2±2.3	2.0±0.1	1.7±0.3	4.0±2.3	+	−	0.3±0.2	0.5±0.2
mel S-488	1.3±0.1	1.0±0.2	1.1±0.3	1.0±0.3	−	−	0.6±0.1	1.0±0.5
mel S-489	1.0±0.1	1.0±0.2	1.2±0.4	4.4±2.9	−	−	1.5±1.5	4.8±0.9
pub S-394	5.6±0.5	3.8±0.2	2.6±1.4	7.4±4.3	++	++	1.2±0.2	26.6±1.2
ruf S-487	1.3±0.2	1.4±0.2	1.7±0.2	2.8±0.3	+	−	0.6±0.2	6.1±2.4
rufn S-362	1.5±0.2	1.3±0.2	0.1±0.2	7.7±2.7	+	−	4.0±5.4	7.4±3.2
TOTAL	43.1	20.6	19.6	50.1			34.7	104.4
" as % growth on PDMma	17	8	8	19			13	40

Medium: Isolate	9 PrA	10 CzA	11 TrA	12 TrYA	13 CM$^+$A	14 MmA	15 PDmA	16 PDMmA	17 PDMYmA
aes S-321	0	0.7±0.1	3.7±0.6	3.2±0.8	32.6±0.8	9.6±0.8	45.0±3.4	29.6±2.7	27.5±1.3
aes S-322	1.8±0.2	0.3±0.2	1.8±0.1	2.7±1.0	1.6±0.2	10.2±0.6	25.8±8.2	30.2±0.4	28.5±0.7
bor S-211	3.0±1.1	0.4±0.1	1.6±0.1	2.0±0.1	17.1±8.8	9.0±0.3	34.2±22.6	28.6±6.6	17.1±3.8
bor S-393	1.9±0.2	0.4±0.1	2.7±0.2	3.1±0.3	49.0±1.8	9.9±0.3	61.7±1.0	36.2±1.0	31.4±1.9
bru S-323	2.5±0.7	1.6±0.9	5.5±2.0	34.1±10.5	33.9±4.8	8.0±0.6	44.6±2.0	28.7±0.4	29.8±2.5
brum S-485[b]	1.6±0.6	2.4±0.6	11.6±1.5	26.8±1.2	15.6(n=1)	8.2(n=1)	24.3±10.4	16.4±1.2	19.6±4.3
mel S-488	0.6±0.2	0	3.0±1.8	2.6±0.8	1.0±0.6	7.5±1.3	8.6±5.3	16.2±1.1	18.6±4.4
mel S-489	1.2±0.4	1.5±0.5	3.8±1.2	12.4±5.3	1.0±0.1	9.2±0.5	12.3±3.3	21.8±1.5	18.1±1.2
pub S-394	2.2±0.9	0.3±0.2	1.3±0.4	1.2±0.4	37.9±2.5	7.5±0.2	38.2±2.1	22.4±0.9	25.7±0.9
ruf S-487	2.8±0.4	1.8±0.1	3.1±0.1	3.4±0.2	6.4±0.9	6.8±1.2	9.9±2.1	20.1±6.6	11.3±5.3
rufn S-362	0.6±0.2	0.6±0.2	2.9±0.9	1.6±0.5	12.8±11.0	15.5±0.7	6.4±0.9	8.1±1.4	11.7±2.9
TOTAL	18.2	10.0	41.0	93.1	208.9	101.4	311.0	258.3	239.3
" as % growth on PDMmA	7	4	16	36	81	39	120	100	93

a) Mean mycelial dry (lyophilized) weight (mg) of four replicates ± one standard error; cultures incubated 40 d at 25C.
b) For this strain N < 4. Many transfers did not grow in this study.
c) Oatmeal grains interspersed with mycelium on filter. Total dry weight < 3.0 mg = −; 3.0 − 30 mg = +; > 30 mg = ++

among the 15 media for which growth could be quantified. The mean
growth of all species combined on these media was 12 per cent of that
on PDMmA and 23 per cent of that for all the undefined media.
Addition of 0.1 per cent yeast extract to an otherwise defined medium
(nos. 11 vs. 12) significantly (P ≤ 0.05) increased growth of four -
isolates. TrA (no. 11) contains thiamine, for which mycorrhizal fungi
are typically heterotrophic (Melin, 1963). The improved growth with
yeast extract (no. 12, TrYA) thus suggests that these isolates respond
to other substances as well. Malt extract and/or potato infusion are
present in the six best media. Addition of yeast extract to one of
these (no. 17, PDMYmA, vs. no. 16, PDMmA) significantly reduced growth
of five isolates while promoting growth in one other. The differences
are not generally large, but nevertheless indicate that in the more
nutritionally complete media yeast extract contains some inhibitors of
some isolates.

In oatmeal agar, casein hydrolysate did not stimulate growth as
reported by Fontana and Palenzona (1969), and, indeed, inhibited some
strains at the concentration used. At 40 per cent of the
concentration (no. 13, CM$^+$A), however, growth was not appreciably
retarded for most isolates.

The Tuber isolates studied also responded positively to inorganic
media components. Addition of Hewitt's minerals to both malt and
potato-dextrose formulations roughly tripled growth. The moieties
most responsible are investigated in experiment IV. In Vrot's medium
nitrate produced better growth than ammonium in nine of eleven cases.
Although the differences were not significant for some isolates, they
were significant for the mean of all isolates combined (paired t-test,
P ≤ 0.05). This nitrogen response is different from most mycorrhizal
fungi, at least among the higher basidiomycetes, which grow better on
ammonium nitrogen (Lundeberg, 1970; Norkrans, 1950). About two-thirds
of the mycorrhiza-formers in Lundeberg's study, for example, could not
metabolize nitrate well, and even those other isolates that could
still grew better with ammonium N. Nitrate utilization has been
little studied in the mycorrhizal ascomycetes, although Ho and Trappe
(1980) found that Cenococcum geophilum Fr., an ascomycete (Trappe,
1971), had higher nitrate reductase activity than six other

mycorrhizal fungi, all basidiomycetes. However, C. geophilum also prefers ammonium N over nitrate N in pure culture (Mikola, 1948). Our data suggest that Tuber spp. have nitrate reductase activity but it does not necessarily follow that the growth response is due to a preference for nitrate. The pH of all media containing added nitrate rose during incubation. Since the pH fell during incubation in VrNH$_4$A, as it did in all ammonium-containing media (Table IV), the growth response observed with nitrate could also be interpreted as a response to a more alkaline environment. Although most mycorrhizal fungi prefer acidic conditions (Harley, 1969), some Tuber biotypes may be exceptions. All isolates used in our study, possibly excepting T. puberulum and T.rufum var. nitidum, were obtained from sporocarps found in alkaline soils and may therefore be adapted to grow well at high pH. Indeed, a scatter diagram of growth vs. pH (Fig. 1) in PDMmA shows that most growth occurs at the higher pH values.

The primary objective of this experiment was to choose a working medium for culture maintenance and further study of Tuber spp. Of the 17 media studied, the one selected for this purpose was PDMmA. It elicited the most growth, after PDMA, of all the media tested. A t-test of sample means of individual isolates shows that PDmA gave significantly better growth for four of them and PDMmA for two, viz., T. melanosporum S-488 and S-489. This species is the most important economically of all species studied, at least in terms of carpophore production. Perhaps more importantly, the dispersion from the mean was less with PDMmA for 10 of the 11 isolates, a useful characteristic for strengthening statistical inference in further work. Also, in the case of dual cultures, bacterial growth was much less in PDMmA than in other media. A major concern with this medium, however, is that precipitation of Ca and Mn salts occurs above pH 5.8 where many Tuber isolates grow well. Further work on the effects of minerals in this medium is reported later in this paper.

Experiment II. Growth as a funtion of time.

The period of maximum culture growth rate, measured as a change in mean weight per week, occurred as early as the third week for T.

Table IV.
Final pH of media after growth of 40 d by _Tuber_ isolates in experiment I

Medium:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	MMNA	HaA	VrNH$_4$A	VrNO$_3$A	OaA	OaCA	ChMA	PDA	PrA	CzA	TrA	TrYA	CM$^+$A	MmA	PDmA	PDMmA	PDMYmA
Isolate																	
aes S-321	4.5	4.5	4.9	5.0	7.4	8.5	7.9	8.2	6.5	5.6	6.3	6.0	7.9	6.9	7.8	8.4	8.3
aes S-322	4.0	5.8	4.0	6.3	6.5	6.4	7.1	4.8	7.0	6.0	7.7	7.5	7.9	7.0	7.4	7.6	7.9
bor S-211	3.7	3.4	3.3	6.4	5.8	5.2	6.5	5.8	7.4	5.9	·7.0	7.4	5.4	7.7	7.6	8.2	7.5
bor S-393	3.6	3.6	3.9	6.5	6.2	6.6	6.9	6.7	7.4	6.0	8.3	8.5	7.9	8.0	8.3	8.4	8.4
bru S-323	3.9	3.7	3.7	6.3	7.2	8.4	7.6	8.0	7.3	6.6	6.9	7.3	8.4	7.5	8.2	8.0	8.3
brum S-485	4.8	3.9	4.2	6.2	7.2	8.2	7.6	8.2	7.5	6.6	7.1	6.1	7.5	7.2	7.6	7.6	7.5
mel S-488	4.7	4.6	5.0	6.2	7.6	8.5	7.9	8.3	NA[1]	6.6	8.8	8.5	8.1	7.6	7.6	8.0	7.8
mel S-489	4.7	4.6	5.3	6.2	7.6	8.5	7.9	8.5	7.4	6.1	8.6	8.0	8.2	8.1	7.9	8.1	7.8
pub S-394	3.8	3.0	4.1	6.5	6.3	6.4	7.0	6.8	6.8	5.9	7.4	8.0	8.4	8.0	8.4	8.4	8.4
ruf S-487	4.3	4.3	4.3	6.2	6.6	6.3	7.0	5.3	7.0	6.2	4.8	4.5	5.2	7.1	5.3	7.4	7.7
rufn S-362	4.2	4.1	3.9	5.2	6.2	6.3	6.5	5.8	6.4	5.5	5.0	7.1	4.6	5.4	7.2	6.6	6.6

1 NA = not available
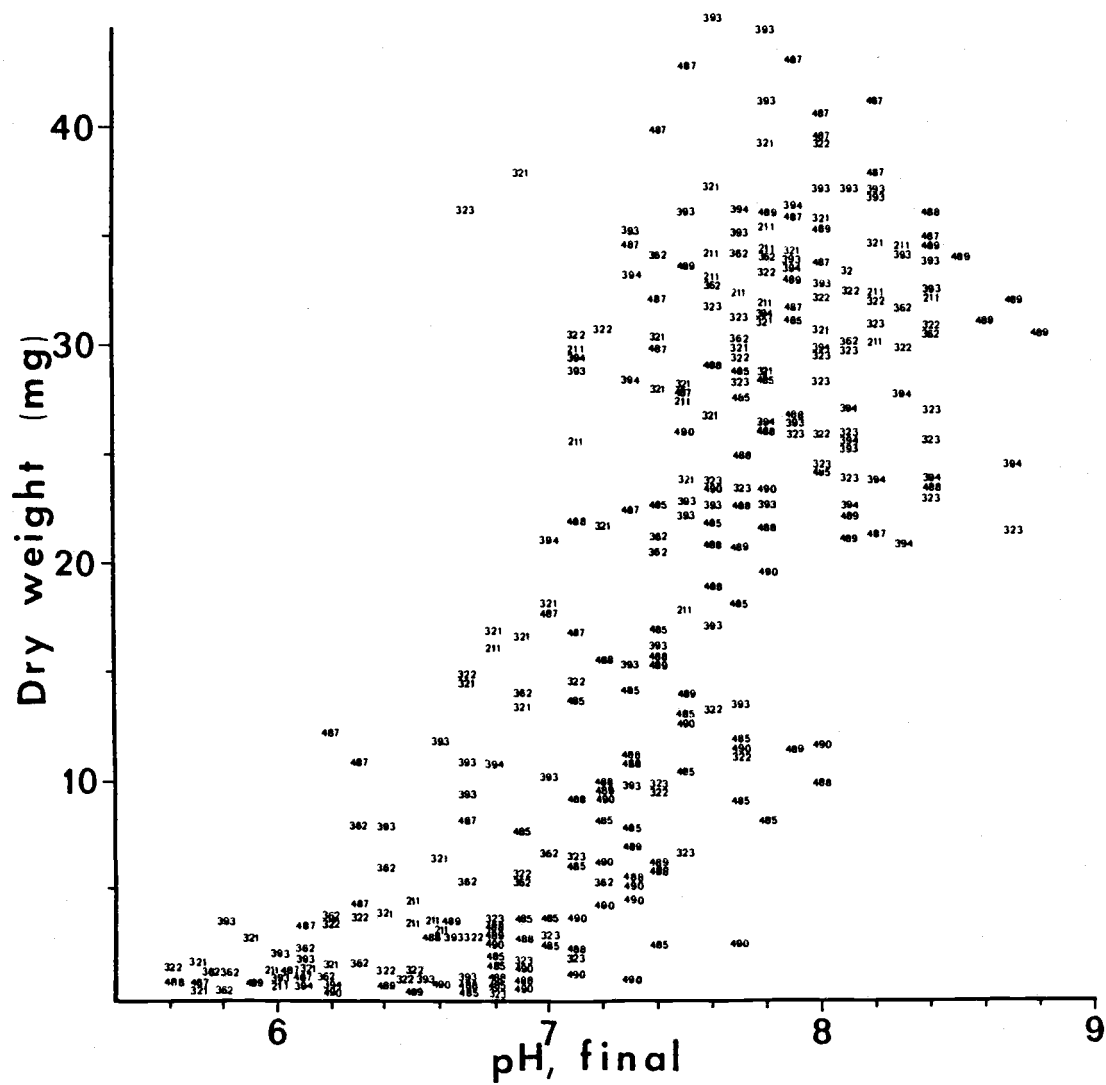
Fig. 1. Scatter diagram of growth vs. final medium pH of 12 isolates of <u>Tuber</u> growing on PDMmA. The three-digit numbers plotted are the culture collection S-number identifiers.
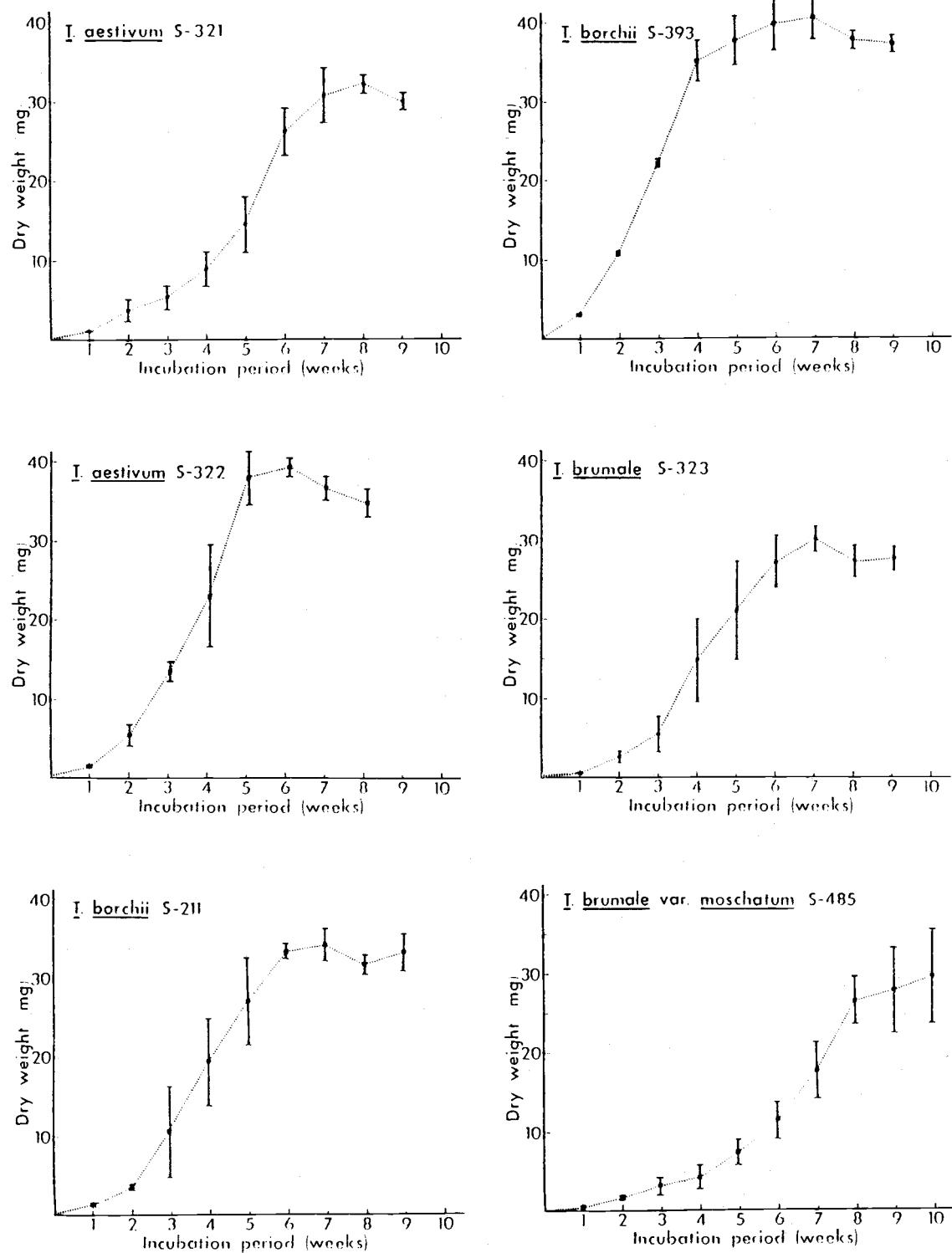
Fig. 2. Growth of 12 *Tuber* isolates on PDMmA at 20 C.
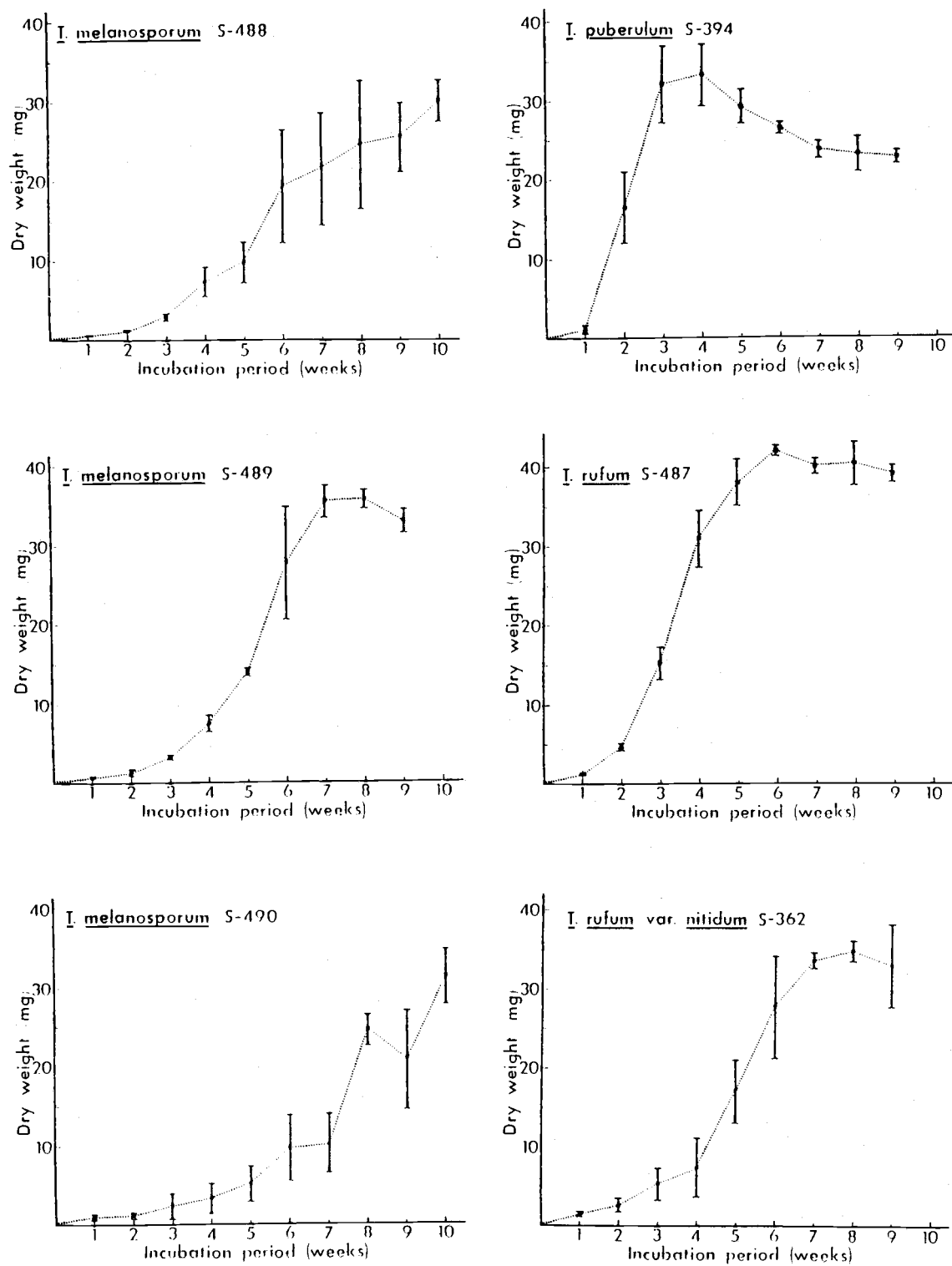
(Fig. 2, cont'd).

puberulum and the fourth week for T. brumale and T. borchii S-211 and S-393 (Fig. 2). T. melanosporum S-490 and T. brumale var. moschatum, the two most sluggish isolates, did not obtain such growth until the eighth week. All isolates achieved maximum culture growth rate at pH greater than 7 (Table V), indicating that, at least in culture, they thrive in the slightly alkaline medium. Maximum rates of culture growth also tended to be greater for those isolates reaching the maximum earlier. For example, T. rufum and T. puberulum increased at a maximum rate of 15.5 and 15.4 mg/week, respectively, while for T. brumale var. moschatum the rate is only 7.6 mg/week.

Average maximum growth varied from 29.3 mg for T. brumale var. moschatum to 42.0 mg for T.rufum, although it must be noted that for three isolates the maximum may not have occurred by the last sampling date. Although these sluggish isolates had not begun their stationary growth phase, the curves do suggest that they had passed from the exponential phase into a deceleration phase by this time (growth phases are defined in the sense of Mandels, 1965).

A number of isolates demonstrated a clear phase of decline, which was noncorrelated with the presence or absence of bacteria. The most dramatic decline occurred in the case of T. puberulum, for which the mean weight by the ninth week was only 68 per cent maximum at week 4. (Streak-plating and microscopic examination of the hyphae, however, failed to show any indication of a hyperparasiste or other organism.) Considering all isolates, both mean and median times to 90 percent maximum growth was 6.4 weeks with extremes of 2.8 weeks (T. puberulum) and 9.4 weeks (T. melanosporum S-488).

Experiment III. Growth at different temperatures.

Growth is shown as a function of temperature for each sampling time in Fig. 3. Optimum temperatures appear to shift to lower values for the longer growth periods, probably because an isolate in its decline phase of growth at a given temperature has a reduced total biomass; the decline phase will have begun sooner at those temperatures where growth was best. One can arbitrarily define an optimum temperature range for growth as that temperature interval in

Table V.

Final pH of working medium (PDMmA) for growth vs. time study in experiment II

Isolate	Age (weeks)									
	1	2	3	4	5	6	7	8	9	10
aes S-321	6.5	6.2	6.7	7.3	7.5	7.9	8.1	7.5	8.2	--
aes S-322	6.1	6.5	6.7	7.5	7.3	7.6	7.8	7.9	--	--
bor S-211	6.0	6.6	6.6	7.5	7.1	7.7	7.8	7.5	8.2	--
bor S-393	6.6	6.9	7.6	7.8	7.4	8.2	7.9	8.1	8.2	--
bru S-323	6.8	6.9	7.0	7.4	7.6	7.8	7.7	8.0	8.2	--
brum S-485	6.7	6.8	7.0	7.2	7.0	7.2	7.5	7.6	7.9	8.0
mel S-488	6.8	6.8	7.0	7.4	7.2	7.5	7.6	7.6	7.6	7.8
mel S-489	6.3	6.3	6.7	7.4	7.5	8.0	7.8	7.9	8.4	--
mel S-490	6.5	6.9	6.9	7.2	7.2	7.3	7.4	7.5	7.8	7.8
pub S-394	6.1	6.9	NA[1]	7.8	7.3	7.9	8.2	7.8	8.3	--
ruf S-487	6.1	6.2	7.1	7.4	7.4	7.9	8.1	8.0	8.1	--
rufn S-362	6.2	6.2	NA	6.9	7.4	7.5	7.8	7.8	8.2	--

[1] NA = not available
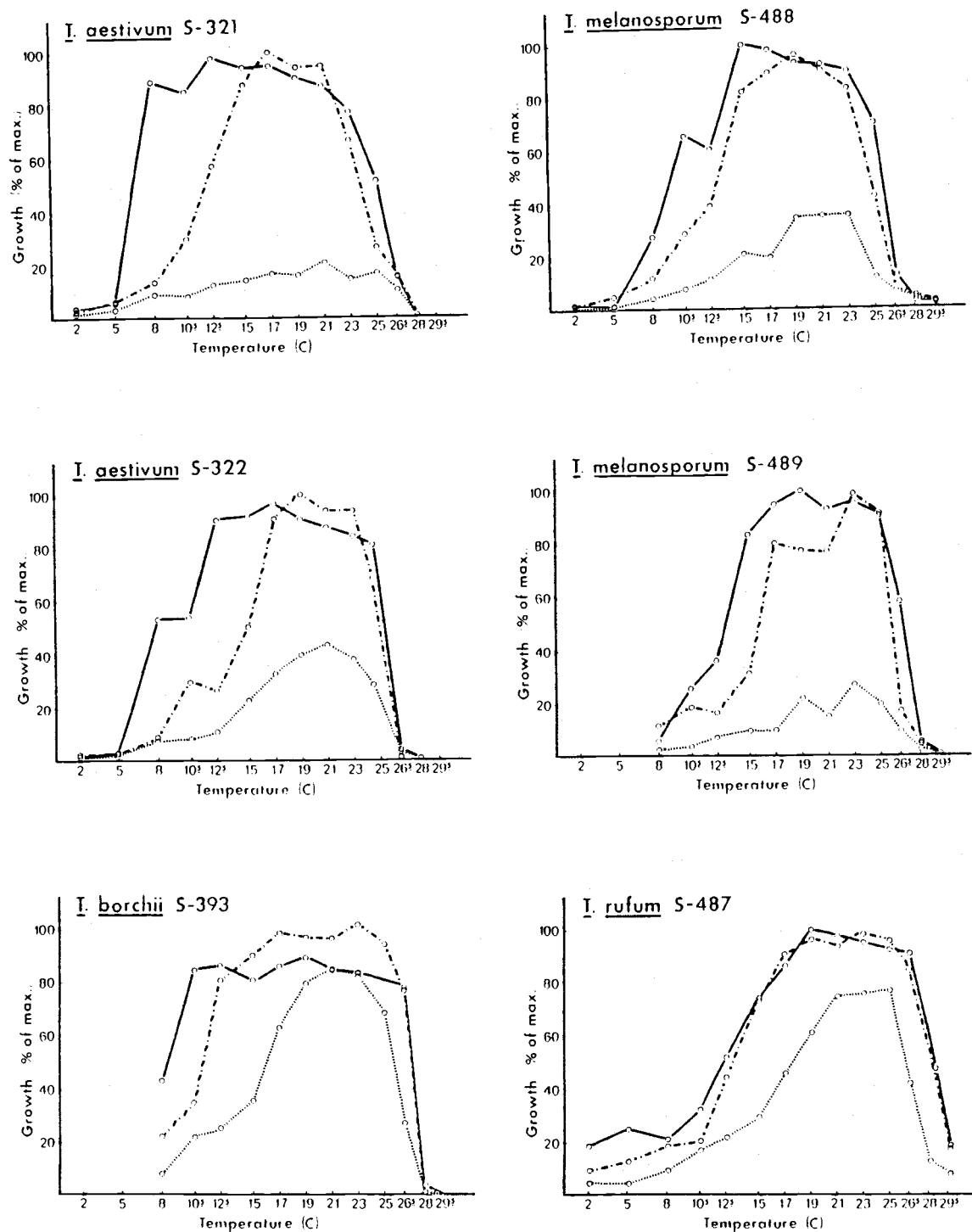
Fig. 3. Effect of temperature on growth of six Tuber isolates on PDMmA: 20 d, ∘⋯⋯∘⋯⋯∘ ; 40 d, ∘—∘—∘ ; 60 d, ∘————∘— .

which growth is greater than 90 per cent of maximum. With that
definition applied to the 40 d data, the following tabulation provides
guidelines for growing some Tuber isolates:

T. aestivum S-321	15-21 C
T. aestivum S-322	17-23 C
T. borchii S-393	15-25 C
T. melanosporum S-488	16-22 C
T. melanosporum S-489	22-25 C
T. rufum S-487	17-26 C

These temperature ranges are similar to those found for other
ectomycorrhizal fungi from temperate regions (Hung and Chien, 1978;
Laiho, 1970; Hacskaylo et al., 1965) although Harley (1969) suggests a
slightly higher range of 18-27 C for most species.

The trend among the Tuber isolates studied to cease growth near
28 C also indicates that their thermal death point in extended culture
is near this temperature. Only T. rufum recovered consistently to
resume growth when returned to a 20 C environment after 20 d of
elevated temperature. The bacteria existing in dual culture with
Tuber isolates did not recover either. It is hazardous to speculate
about survival of vegetative mycelium in root and soil subjected to
periodic intervals of high temperature on the basis of pure culture
studies. However, in Petri dish culture the higher temperature alone
did not stimulate formation of resistant resting structures.

Experiments IVa and b. Effects of minerals on growth.

In experiment IVa, every change in the working medium (PDMmA)
except potassium deletion significantly affected one or more isolates
(Table VI). The use of Na^+ or Cl^- as substitution ions generally had
no effect, except for T. aestivum S-321, for which growth was
depressed 8 per cent. It is interesting that for this isolate growth
was depressed relative to the PDMmA treatment for all ion deletion
trials, suggesting that Na^+ and/or Cl^- might be affecting growth. The
lack of potassium effects is not surprising, because its concentration
at 3.5 mM in the undefined components of the media is in the general
range for good growth of many fungi (Cochrane, 1958). Final pH's of

Table VI.
Effect of deletion of mineral ions from Hewitt's mineral supplement
to PDMA on growth[2] of twelve Tuber isolates in experiment IVa[1]

Isolate	PDMmA (wt,mg)	Medium							
		+NaCl	-K	-Ca	-Mg	-Tr	-PO$_4$	-NO$_3$	PDMA
aes S-321	(37.7) 100 ++	92* ++	84 +	85 +	95 ++	91* ++	60**++	28**	38**
aes S-322	(36.3) 100 ++	97 ++	100 ++	99 +	99 ++	94 ++	98 ++	25**+	52**
bor S-211	(35.5) 100 ++	99 ++	96 ++	97 ++	90 ++	102 ++	106 ++	13**	12**
bor S-393	(44.7) 100 ++	103 ++	98 ++	95 ++	96 ++	94 ++	107* ++	39**	38**
bru S-323	(31.5) 100 ++	97 ++	99 ++	81(n=1)	101 ++	92 ++	66**++	20**++	22**
brum S-485	(10.0) 100	136	83	85	92	84	113	69	68
mel S-488	(31.1) 100 ++	94 ++	85 ++·	24**	98 ++	85 ++	76**++	27**+	23**
mel S-489	(31.7) 100 ++	98 ++	90 ++	47**	106 ++	104 +	80 ++	31**	28**
mel S-490	(18.2) 100 ++	108 ++	115 ++	128 ++	106 ++	131 ++	55 +	34**+	26**
pub S-394	(30.4) 100 ++	95 ++	104 ++	102 ++	89**++	101 ++	71**++	58**	57**
ruf S-387	(40.1) 100 ++	100 ++	NA	NA	98* ++	100 ++	90**++	20**++	16**
rufn S-362	(32.9) 100 ++	94 ++	101 ++	100 ++	96 ++	99 ++	98 ++	21**	18**

1) PDMA, medium PDMmA (see text) less all Hewitt's minerals; +NaCl, PDMmA + 15mM NaCl; -K, -Ca, -Mg, -NO$_3$, -PO$_4$ are media PDMmA with indicated ion replaced by equal equivalents of Na or Cl; -Tr, medium PDMmA with trace elements S, Fe, Mn, SO$_4$, B, Cu, Zn, Mo and Co replaced by Na or Cl.

2) expressed as per cent of growth on PDMmA. *, ** indicates growth significantly different from growth on PDMmA at $P \leq 0.05$, $P \leq 0.01$, resp.; +, ++ indicates growth significantly different from growth on PDMA at $P \leq 0.05$, $P \leq 0.01$, resp., by t-tests of means.

Table VII.
Effect of deletion of mineral ions from Hewitt's mineral supplement to PDMA on
pH change from 5.7 of nutrient medium by twelve Tuber isolates in experiment IVa[1]

Isolate	Medium								
	PDMmA	+NaCl	−K	−Ca	−Mg	−Tr	PO_4	−NO$_3$	PDMA
	Final pH of medium								
aes S-321	8.0	7.8	7.9	7.7	8.0	7.8	7.9	7.6	7.7
aes S-322	7.3	6.8	7.5	7.6	7.3	7.2	7.2	5.4	5.1
bor S-211	7.7	7.2	8.3	8.4	7.9	7.6	7.6	5.8	5.2
bor S-393	7.7	7.6	8.3	8.4	7.6	7.4	7.5	5.9	5.3
bru S-323	7.7	7.8	8.4	7.9	7.8	7.7	7.6	7.4	6.8
brum S-485	7.5	7.6	7.8	8.0	7.5	7.4	7.5	7.5	7.8
mel S-488	7.8	7.9	8.2	8.2	8.0	8.0	8.1	7.6	7.6
mel S-489	7.4	7.3	8.1	8.0	7.7	7.6	7.8	7.4	7.4
mel S-490	7.3	7.4	8.0	8.1	7.4	7.3	7.3	7.4	7.7
pub s-394	7.1	7.0	8.6	8.5	7.9	7.6	7.5	6.1	7.2
ruf S-487	8.0	7.9	7.8	7.7	8.0	8.0	7.9	5.3	5.2
rufn S-362	7.6	7.4	8.4	8.4	7.7	7.6	7.5	4.6	4.8

1) See Table VI for explanation of medium abbreviations.

media of all treatment combinations are presented in Table VII.

Nitrate deletion decreased growth an average of 72 per cent relative to PDMmA. The growth decrease was significant ($P \leq 0.01$) for eleven isolates. Variability was high for all treatments for $\underline{T}$. $\underline{brumale}$ var. $\underline{moschatum}$, so little can be concluded regarding this isolate. In experiment IVb a comparable result obtains for the effect of nitrate addition alone: growth of PDMA is 70 per cent less than on PDM($+NO_3$)A (Table VIII). What, however, is truly the effect of nitrate as a source of nitrogen for the fungus and as a source of OH^- from possible nitrate reductase activity? In weakly buffered media the pH can be expected to rise as a result of nitrate reductase activity. For all media containing nitrate, and for all Tuber isolates studied, the pH rose above 7 by the end of the incubation period (Table VIII). For the two media containing only trace amounts of nitrate, PDMA and PDM($m-NO_3$)A, final pH was not much changed from the initial pH for six isolates. But for the other six isolates, final pH was above 7. Interestingly, these are the six isolates containing the "helper" bacteria for maintaining culture viability. However, fungal growth was not notably greater for the latter six strains in the higher pH environment if deficient in nitrate than for the former six strains.

Two frequent generalizations about growth of mycorrhizal fungi are that slightly acidic substrates and ammonium-nitrogen foster the best growth in pure culture (Palmer, 1971; Lundeberg, 1970; Harley, 1969), although many reports of good growth with ammonium ion are from studies in which pH was not stabilized. It is well known that ammonium uptake tends to lower pH values of a medium. Our Tuber isolates do not fit these generalizations, in that nitrate is a better nitrogen source for them than ammonium, and that good growth and high final pH values are well correlated (Fig. 1).

As already noted most isolates were derived from calcareous soils of pH 7.8-8.2. Nitrifying bacteria are more active in slightly alkaline soils than in acid ones, and rates of nitrification can be high enough to satisfy the nitrogen needs of many crop plants (Brady, 1974; Broadbent and Tyler, 1957). However, fixed nitrogen is limited in the calcareous soils of the truffle grounds, many of which are low

Table VIII.
Effect of nitrate addition as $NaNO_3$ to PDMA on growth of <u>Tuber</u> isolates and final medium pH in experiment IVb.

		Medium		
		PDMmA	PDM(NO$_3$)A	PDMA
Isolate	(wt,mg)			

growth, as % of growth on PDMmA

Isolate	(wt,mg) PDMmA	PDM(NO$_3$)A	PDMA
aes S-321	(16.4) 100a	109a	38b
aes S-322	(33.5) 100a	90b	24c
bor S-211	(32.3) 100a	100a	12b
bor S-393	(37.7) 100a	84b	38c
bru S-323	(30.6) 100a	56b	20c
brum S-485	(22.2) 100a	91a	43b
mel S-488	(23.1) 100a	46b	25b
mel S-489	(29.3) 100a	46b	24c
mel S-490	(19.6) 100a	86b	24c
pub S-394	(25.0) 100a	88b	64c
ruf S-487	NA	NA	NA
rufn S-362	(30.3) 100a	88b	19c

medium pH, final

Isolate	PDMmA	PDM(NO$_3$)A	PDMA
aes S-321	7.8	7.8	7.6
aes S-322	7.8	7.7	5.4
bor S-211	8.4	8.1	5.3
bor S-393	8.2	8.3	6.3
bru S-323	8.0	7.9	7.8
brum S-485	7.5	7.6	7.8
mel S-488	8.2	8.0	8.0
mel S-489	8.2	7.6	7.9
mel S-490	7.8	7.8	7.8
pub S-394	8.7	8.7	7.6
ruf S-487	NA	NA	NA
rufn S-362	8.4	8.4	5.1

1 means for each isolate which share same letters do not differ significantly at $P \leq 0.05$. NA = not available.

in organic matter and herbaceous ground cover (Grente and Delmas, 1974; Delmas and Durand, 1971). Further work to see if some Tuber species could play a role in the limitation of nitrogen loss after it has been converted to a mobile form suggests itself.

Although phosphate levels of 20 mM or more are reported to depress growth of mycorrhizal fungi in pure culture (Giltrap and Lewis, 1981; Marx and Zak, 1965), a certain level of phosphate is, of course, necessary. By calculation from Table II, PDM(m-PO$_4$)A contains about 0.65 mM PO$_4$ and PDMmA about 2 mM PO$_4$ (1.3 mM basis Hewitt's minerals and 0.65 basis the undefined components). Growth decreases significantly at the lower phosphate level for five isolates. The higher phosphate level, however, depresses growth of T.borchii S-393.

Calcium signifantly affected T. melanosporum growth. Fontana and Bonfante (1973) found that additional calcium (1.6 mM - 6.5 mM, as CaCO$_3$) in Hagem's broth approximately doubled growth. Isolate S-489 behaved similarly when growth on PDM(m-Ca)A is compared with PDMmA (Table VII). Growth of S-488 quadruples from the one medium to the other. Unfortunately, isolate S-490 is erratic in culture; its high variability precludes conclusions regarding its greater growth in PDM(m-Ca)A. Although interpreting field behavior on the basis of in vitro studies is hazardous, one might expect that a tolerance of, or need for, high calcium levels could have evolved for a fungus edaphically limited to calcareous soils. However, Cochrane (1958) states that the effect of calcium on growth may not be nutritional per se but rather ameliorative of toxic effects of excess monovalent cations, particularly Na$^+$, K$^+$, and H$^+$.

Effects of magnesium and combined trace elements were only signifcant in three instances; in the case of T. rufum the significant 2 per cent growth depression at the lower magnesium concentration is of doubtful importance.

Many salts of polyvalent metal cations have very low solubility in neutral to slightly alkaline substrates. Chelation compounds such as ethylenediaminetetraacetate (EDTA) used to keep cations (and in particular, trace element cations) available for fungal assimilation have been reported to inhibit some fungi (Li and Aho, 198_; Cochrane, 1958). We found that 10 mM EDTA, added as the sodium salt, would

prevent precipitates from forming in the media above pH 5.8. However, addition of EDTA at this concentration to PDMmA completely inhibited growth of all twelve Tuber isolates. Further work on an amendment to keep PDMmA homogeneous above pH 5.8 is necessary.

Experiment V. Effects of inorganic phosphate buffer on growth.

Small increases in phosphate concentration slightly stimulated growth of some isolates (Table IV). In the control medium, $PDM(m-PO_4)A$, phosphate concentration is about 0.65 mM; five isolates grew significantly better when the concentration was increased to 2.27 mM. Four of the isolates were those reported in experiment III to respond positively to a 1.3 mM phosphate increase; T. melanosporum S-489 replaces S-488 as the fifth. Inhibition of growth due to a small phosphate increase is reproducible in the case of T. borchii S-393.

Growth response was trendless between 2.27 mM and 22.7 mM phosphate levels for all isolates except T. aestivum S-321 which was inhibited at 22.7 mM. Growth responses diverge sharply among the isolates at 75 mM phosphate, however. Growth significantly decreased for 10 isolates, increased for T. borchii S-393, and remained virtually unaffected for T. puberulum. At the highest phosphate concentration tested, 227 mM, growth all but ceased for all isolates except T. puberulum and both strains of T. borchii. The sparse growth of the other isolates was on the inoculation plug only.

Buffering by phosphate at the 22.7 mM concentration was moderate, limiting pH change to no more than 1.3 units (Table IX). At 75mM phosphate, of course, buffering capacity was considerably greater and final pH values were lower still.

It is necessary to note that the precipitates formed after the usual post-autoclave pH adjustment procedure affected the initial pH: upon solidification, pH measured 4.7 and 5.0 in the 75 mM and 227 mM phosphate media, respectively. Given that pH values were different for media with the higher phosphate concentrations, the question of phosphate toxicity at these higher levels cannot be adequately addressed, except in the case of T. puberulum and the T. borchii

Table IX.

Effect of concentration of added phosphate on growth of _Tuber_ isolates and final medium pH in experiment V[1]

		Growth							pH, final						
		Concentration $(0.75 \times 10^{n/2}$ mM$)$							Concentration $(0.75 \times 10^{n/2}$ mM$)$						
	$n = -\infty$ [2]	0	1	2	3	4	5	$-\infty$	0	1	2	3	4	5
Isolate	(wt,mg) 0 mM	0.75 mM	2.27 mM	7.5 mM	22.7 mM	75.0 mM	227 mM	0 mM	0.75 mM	2.27 mM	7.5 mM	22.7 mM	75 mM	227 mM
aes S-321	(22.3) 100a	138ab	162b	155b	115a	15c	2c	7.9	8.0	7.8	7.9	7.9	6.5	5.2
aes S-322	(35.4) 100a	89a	100a	99a	103a	16b	0c	7.2	7.5	7.4	7.6	6.4	5.4	5.2
bor S-211	(36.4) 100a	89a	92a	91a	93a	54b	26c	7.6	7.9	7.6	8.0	6.7	6.0	5.4
bor S-393	(47.6) 100a	84b	87b	88b	90b	101a	59c	7.5	7.7	7.8	7.6	6.8	6.0	5.6
bru S-323	(20.6) 100a	114a	148b	146b	149b	10c	1c	7.6	8.0	7.9	8.0	6.8	4.8	5.0
brum S-485	(11.2) 100a	90a	92a	79a	86a	10b	2b	7.5	7.5	7.0	7.3	6.2	4.8	5.0
mel S-488	(23.4) 100a	111a	130a	107a	115a	1b	0c	8.1	8.0	7.8	8.0	6.6	5.2	5.1
mel S-489	(25.2) 100a	117ab	151b	148b	141b	2c	1c	7.8	8.0	7.8	7.8	7.0	5.2	5.2
mel S-490	(8.6) 100a	193a	179a	140a	148a	5b	0c	7.3	7.3	6.9	7.1	6.2	5.4	5.0
pub S-394	(21.5) 100a	109a	152bc	144b	166c	153bc	202d	7.5	7.7	7.6	7.4	6.2	6.8	5.7
ruf S-487	(35.9) 100a	99a	114bc	113b	119c	34d	1e	7.9	8.1	7.8	8.4	6.4	5.4	5.1
rufn S-362	(32.1) 100ab	91a	100ab	96ab	104b	46c	1d	7.5	7.9	7.3	7.8	6.4	5.9	5.1

1 Growth expressed as percent of growth on PDM(m-PO$_4$)A (control). Means for each isolate which share same letters do not differ significantly by Tukey's test at P ≤ 0.05.

2 PDM(m-PO$_4$)A

isolates. For these fungi high concentrations still elicited good growth in spite of the different final pH values for the media. It should also be noted that a medium containing 227 mM phosphate has a calculated osmotic potential less than -10π and an ionic strength which may affect intracellular electrolyte levels. Physiochemical stresses such as these should not be interpreted as a phosphate toxicity per se.

In vitro culture studies of ectomycorrhizal fungi have often been undertaken with little regard for pH stabilization (Giltrap and Lewis, 1981). Molina and Palmer (1982) suggest that the presence of factors which stabilize pH in experiments on ectomycorrhizae complicates interpretation of results. Palmer (personal communication) proposes use of chemostats designed for bathing a culture at constant hydrogen ion concentration while simultaneously removing pH-destabilizing soluble metabolites from the culture medium. A variety of mycorrhizal fungi have been studied for pH responses in vitro (summarized by Hung and Trappe, 1982), but pH has been allowed to vary during the incubation period in many cases.

Mono- and dibasic phosphate ions are functionally important in in vitro culture of fungi in two ways: they provide the organism with a readily assimilable form of phosphorus and, supplied at the appropriate pH and concentration, are effective buffers. As an essential nutrient phosphate plays a key role in mycorrhiza physiology. Although mycorrhizal fungi facilitate uptake of phosphate from the soil solution, 20 mM to 50 mM concentrations of phosphate inhibit pure culture growth of some mycorrhizal basidiomycetes and Cenococcum geophilum Fr. (Giltrap and Lewis, 1981; Marx and Zak, 1965). Since concentrations of phosphate in the 20 mM range do not inhibit growth of most Tuber isolates, this buffer may be useful in axenic culture studies. Aqueous monobasic phosphate has a pK_a of about 7.2 and might be suitable for study of Tuber growth in weakly alkaline conditions.

Experiment VIa and b. Effect of polycarboxylate buffers on growth.

Numerous buffers have been suggested for pure culture studies

(Child et al., 1973; Mallette, 1967; Good et al., 1966; Malca et al., 1966); many are commercially available. We wanted a buffer with the following properties: a) composed only of carbon, hydrogen and oxygen to avoid confounding interpretation of inorganic nutrition studies and b) able to buffer over a broad pH range into the wealky alkaline range where, as we have already seen, <u>Tuber</u> isolates grow well <u>in vitro</u>. To meet both criteria, polybasic carboxylic acids having pK_as less than 2.0 units apart are logical choices.

Four pairs of compounds were selected from data compiled by Mallette (1967) for the 5.2-7.2 range:

<div align="center">Table X</div>
Polycarboxylic acid buffers tested in experiment VIa.

	Compound	synonym or abbreviation	pK_as	4.0
1a.	2,2-dimethylglutaric acid	2,2-DMGA	4.3;	5.5
b.	1,2,4,5-benzenetetra-carboxylic acid	pyromellitic acid	4.5;	5.6
2a.	<u>trans</u>-aconitic acid	-	4.2;	5.9
b.	1, 2, 3-benzenetricar-boxylic acid	hemimellitic	4.2;	5.9
3a.	propane-1,2,3-tricarboxylic acid	tricarballylic acid (TCarbA)	4.8;	6.2
b.	3, 3-dimethylglutaric acid	3,3-DMGA	-	6.3
4a.	6-endomethylene-1,2,3,6-tetrahydrophthalic acid	EMTA	4.2;	7.0
b.	2-methylpropane-1, 2, 3-tricarboxylic acid	β-methyltricarbal lylic acid (β-MTCarabA)	5.0;	7.2

One such pair of compounds will stabilize pH similarly, so that effects on fungal growth can be related to the buffer per se, rather than change in hydrogen ion concentration. One particular pair, no. 4, should demonstrate an effective buffering capacity to about pH 8. Five compounds (nos. 1a, 2a, 3a, 3b and 4b) are structurally similar to glutaric acid, consisting of a 5-carbon skeleton with a carboxylate group at each end. One (no. 4a) is an aliphatic bicyclic compound which has been reported as a nontoxic buffer useful for growing some fungi (Malca, et al., 1966). The remaining two (Nos. 1b and 2b) are aromatic compounds.

Culture growth varied greatly between isolate x buffer combinations. All buffers strongly inhibited seven of the eleven isolates at all concentrations and pH values tested (experiment VIA, Table XI). Whether this was due to stabilization of pH at values unsuitable for growth or was an effect of some of the compounds per se could not be determined with certainty. The same pattern of growth response, however, obtains over the isolates in this experiment as occurred at high concentrations of phosphate described in experiment V: only T. puberulum, T. borchii S-393 and, to a lesser degree, T. borchii S-211 grew moderately well in media with the lower final pH values reported in this experiment. These three isolates also responded similarly to each of the organic buffers, perhaps thereby indicating which were toxic. β-MTCarbA, 3,3-DMGA, TCarbA, hemimellitic and pyromellitic acid media eleicited the best growth, while 2,2-DMGA was somewhat suppressive and trans-aconitic acid and EMTA completely inhibited growth.

Compounds with similar pK_a values definitely differed in effects on growth. EMTA inhibited growth of all isolates, yet its paired compound, β-MTCarbA, supported growth in seven of eleven cases. Similarly, it can be concluded that pyromellitic acid is not as toxic as 2,2-DMGA, and that hemimellitic acid is a better choice of buffer than trans-aconitic acid.

Of the five best buffers, β-MTCarbA had the least buffering capacity at pH 5.7 (Table XI). Noting that pH rose the most and that the greatest number of isolates grew to some degree in this buffer, it seemed worth examining for further use in studying Tuber growth

Table XI.
Effect of selected buffer on growth of _Tuber_ isolates and on final pH of media in experiment VIa[1]

Isolate	2,2-DMGA	pyromellitic	trans-aconitic	hemimellitic	TCarbA	3,3-DMGA	EMTA	β-MTCarbA
				growth, mg				
aes S-321	0b	0	0	0	0	0.5±0.5	0	0
aes S-322	0	0	0	0	0	1.2±1.0	0	0
bor S-211	0	1.2±1.4	0	2.3±1.7	5.0±5.0	4.2±4.0	0	12.9±9.6
bor S-393	4.6±1.2	23.0±0.9	0	25.1±0.4	23.7±1.4	26.9±0.8	0	27.2±0.6
bru S-323	0	0.2±0.1	0	0	0	0	0	0.4±0.4
brum S-485	0	0.3±0.2	0	0.3±0.2	0	0	0	0.3±0.3
mel S-488	0	0	0	0	0	0	0	0
mel S-489	0	0	0	0	0	0	0	0
pub S-394	0.6±0.1	27.0±1.8	0	28.0±0.71	11.0±2.2	24.0±3.1	0	23.4±1.8
ruf S-487	0	0	0	0	0	1.8±0.4	0	5.3±1.3
ruf S-362	0	0	1.3±1.0	0	2.1±0.3	7.6±1.8	0	6.8±0.5
				pH, final				
aes S-321	5.6	5.7	5.7	6.1	5.9	5.6	5.9	5.8
aes S-322	5.6	5.7	5.7	6.1	5.8	5.9	5.9	5.8
bor S-211	5.6	5.7	5.7	6.1	5.9	5.9	5.8	6.4
bor S-393	5.6	7.4	5.7	6.3	6.1	6.2	5.9	6.7
bru S-323	5.6	5.7	5.7	6.2	5.8	5.7	5.9	6.2
brum S-485	5.6	5.8	5.7	6.3	5.8	5.9	5.9	5.9
mel S-488	5.6	5.7	5.7	6.1	5.8	5.9	5.9	5.8
mel S-489	5.6	5.7	5.7	6.1	5.8	5.9	5.9	5.8
pub S-394	5.6	6.3	5.7	6.7	6.1	6.2	5.9	6.7
ruf S-487	5.6	5.6	5.7	6.1	5.8	5.9	5.9	5.9
ruf S-362	5.6	5.7	5.7	6.1	6.0	6.0	5.9	6.2

1) All buffers at 75mM in PDMmA, except β-MTCarbA at 65 mM. Growth expressed as mean mycelial dry (lyophilized) weight ± one standard error of four replicates.

(experiment VIb). Furthermore, it has three carboxylate groups with pK_a values of 3.5, 5.0, and 7.2, respectively, and therefore could be useful over the entire range of pH supporting growth of many fungi.

At concentrations up to 2.27 mM, β-MTCarbA did not significantly reduce growth with respect to the control. At the next highest concentration, growth was reduced significantly for only one isolate, T. brumale (Table XII). These results suggest that β-MTCarbA is not very toxic to these Tuber isolates (7.5 mM = 1,430 ppm). However, growth of T. brumale was reduced by 36 per cent at 7.5 mM β-MTCarbA and this reduction occurred with a small reduction in final pH. In referring to the phosphate study (experiment V), we find that growth and pH were not much changed between the 2.27 mM and 7.5 mM concentrations for this isolate. Therefore, β-MTCarbA appears to be toxic to T. brumale.

Growth of four isolates was significantly reduced between the 7.5 mM and 22.7 mM concentrations. This may be an effect of pH, because the buffering capacity of this compound is more pronounced at the higher concentrations (Table XII). The pH trend toward lower final values continues from 22.7 mM to 75 mM β-MTCarbA and is similar to what occurred in the phosphate study (experiment V). Growth was severely inhibited for most isolates when tested at a 75 mM concentration of either buffer. As with phosphate buffers, further work is needed to ascertain whether inhibition is due to an inhospitable pH or to toxicity of the buffer. However, a shorter incubation period at a lower buffer concentration, eg., 22.7 mM, is probably a feasible method for establishing growth vs. pH curves for these fungi.

SUMMARY AND CONCLUSIONS

This study was undertaken to improve the growth of Tuber spp. in culture. The twelve isolates studied had many common characteristics. All grew very slowly with poorly defined colony margins on most media tested, including media recommended for mycorrhizal fungi. All responded markedly to the addition of nitrate, as well as other inorganic ions, to malt and potato-dextrose basal media. Indeed, a medium based on this response (no. 16, PDMmA) is suggested for routine

Table XII.

Effect of concentration of 2-methylpropane-1,2,3,-tricarboxylic acid (β-MTCarbA) on growth of Tuber isolates and final medium pH in experiment VIb[1]

Isolate	$n=-\infty$ (wt,mg) 0 mN[2]	Growth Concentration ($0.75 \times 10^{n/2}$ mM) 0 0.75 mM	1 2.27 mM	2 7.5 mM	3 22.7 mM	4 7.5 mM	pH, final $-\infty$ 0 mM	Concentration ($0.75 \times 10^{n/2}$ mM) 0 0.75 mM	1 2.27 mM	2 7.5 mM	3 22.7 mM	4 75 mM
aes S-321	(16.7) 100a	115a	117a	93a	55b	1c	6.9	7.1	7.1	6.5	5.7	5.6
aes S-322	(12.0) 100a	128a	99a	120a	117a	4b	6.8	7.5	6.8	7.2	6.3	5.6
bor S-211	(4.8) 100a	152a	135a	146a	137a	41a	7.1	7.5	7.5	6.8	6.2	5.7
bor S-393	(31.1) 100ab	104a	105a	100ab	96b	108a	7.5	7.4	8.2	8.2	7.7	7.1
bru S-323	(22.7) 100a	106a	84ab	64b	34c	0d	7.9	8.0	8.0	7.8	7.2	5.8
brum S-485	(2.1) 100a	94a	98a	89a	74a	0b	7.2	7.1	7.0	6.7	6.2	5.8
mel S-488	(13.0) 100a	68a	92a	106a	20b	1b	7.2	6.8	7.8	7.7	6.4	6.0
mel S-489	(9.9) 100ab	128a	129a	115ab	81b	0c	7.6	8.2	8.0	7.6	6.8	5.8
mel S-490	(3.6) 100a	229a	208a	227a	132a	4b	8.1	7.9	8.0	7.8	6.6	6.0
pub S-394	(26.8) 100a	97a	92a	92a	90a	89a	8.1	8.4	8.2	8.3	7.4	6.6
ruf S-487	(33.6) 100a	99a	98a	93a	67b	4c	8.0	8.0	7.9	7.6	6.8	5.9
rufn S-362	(4.8) 100ac	267ab	370b	326b	241abc	81c	6.2	7.0	7.5	7.5	7.2	6.5

1 Growth expressed as percent of growth on PDMmA (control). Means for each isolate which share same letters do not differ significantly by Tukey's test at $P \le 0.05$.

2 PDMmA

culture maintenance. Of the six isolates studied for temperature response, all had optima around 20 C, declined around 26.5 C and with one exception died if held at 28 C for 20 d.

The pH of all media rose in response to nitrate addition for all isolates, suggesting significant nitrate reductase activity. Biomass increased at maximum rates at these higher pHs. However, the pH of PDMmA should not be adjusted initially above pH 5.8 due to precipitate formation, which can complicate interpretation of experimental results. But if the pH rises slowly through the action of the fungi, precipitation is reduced dramatically and can only be detected under stereomicroscopic magnification. Addition of 10 mM EDTA prevents the precipitation, but also completely inhibits growth of Tuber spp. Other means of making homogeneous media for well-designed growth studies at neutral to alkaline pH values are needed.

Growth response to a variety of buffers investigated here is complicated in that these compounds may be buffering in the initial 5.7 pH level, a level which could inhibit some isolates. However, by noting growth response to buffers having similar pK_as, some comparisons can be made in the case of those fungi which do grow well at pH 5.7 (T. borchii and T. puberulum). Apparently, buffers which are useful for some fungi are ill-recommended for others. For example, EMTA, suggested for Verticillium albo-atrum (Malca et al., 1966), totaly inhibits Tuber growth; and trans-aconitic acid, suggested for mycorrhizal basidiomycetes, was almost as toxic. One buffer, β-methyltricarballylic acid (β-MTCarbA), though, showed no demonstrable toxicity at moderate concentrations. Because its three carboxylate pK_as are 3.5, 5.0, and 7.2, respectively, it is a desirable buffer for studies over the entire range of physiologically significant pH values. Phosphate, as an inorganic buffer at higher pH values (approximately 6.2-8.2) was also studied and found to be nontoxic at moderate concentrations.

Commonly used mycorrhizal media such as that of Melin and Norkrans (no. 1, MMNA) or Modess (no. 2, HaA) were developed by studying pure culture nutrition of fungi easily isolated from sporocarp excisions on these same media. Most of the nutritional characteristics of such media have been selected as beneficial

specifically for these fungi. That other mycorrhizal fungi do not grow well in culture (i.e., on these media) has been attributed to the supposition that as obligate symbionts their nutritional status is highly heterotrophic for growth factors and moieties of intermediate metabolism of the host. All of our isolates of <u>Tuber</u>, a notoriously hard-to-culture genus, were also failing on these media but perked up by the simple addition of nitrate. Other so-called recalcirant ectomycorrhizal fungi may respond similarly.

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MODELS OF MYCELIAL GROWTH, MEASURED AS
DRY WEIGHT, OF <u>TUBER</u> SPP. ON AGAR

CHAPTER 2

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ABSTRACT

The utility of agar media for quantitative study of fungal
biomass is suggested by a study of growth of <u>Tuber</u> spp., the true
truffles, over time. A few simple models of fungal growth in shallow
Petri dishes are proposed, and the growth data obtained as mycelial
dry weight for twelve <u>Tuber</u> isolates are tested for fit. Growth is
hypothesized to be uniform and proportional to total biomass, or to
occur only at the colony periphery and proportional to the square root
of the biomass. Each of these models is altered to include a
time-dependent variation: growth is hypothesized to also depend upon
the concentration of a metabolite produced by the fungus. For each
model transformed growth data is subjected to least squares or
weighted least squares analysis of variance. Metabolite-dependent
models give better fit than the metabolite-independent versions, but
differences between peripheral and uniform growth models are less
clear.

INTRODUCTION

The kinetics of fungal growth have been studied for numerous
fungi and fermentation systems. The diverse descriptions of growth
depend on many assumptions and hypotheses concerning the fungus under
scrutiny and the constraints of its environment. Factors that may
affect growth include:

a) nutrient composition
b) mechanics of nutrient delivery (chemostats, turbidostats,
    batch culture, continuous culture, etc.)

c)   physical state of the growth medium

d)   geometry of the culture apparatus

e)   the geometry of the fungal biomass itself (single-celled, small agglomerations, pellets, etc.)

f)   relationships between growth and morphologic change during various phases and conditions

g)   number of various physiological states of cells and the corresponding population levels of each one.

The response variables are almost as varied, based on the operational definition of growth dictated by a given system design or upon some effect of growth per se, e.g., the formation of metabolites or various cytological or developmental events. Some systems have been studied in great detail because of their importance in the fermentation industry or because they clarify basic mycological processes, (Bull and Trinci, 1977; Constantinides, et al., 1970; Edwards and Wilke, 1968; Fredrickson, et al., 1970; Koch, 1975; Mandels, 1965; Prosser, 1979; Righelato, 1979, 1975; and Young, et al., 1970).

Fungi are commonly grown on solidified agar in shallow Petri dishes in mycological researach, but growth in such a system is nearly always quantified by measurment of colony diameters. The theme of some of these studies has been to reconcile the assumptions of exponential growth, an accurate description for many organisms at some time in their life cycles, with the constant growth rates observed for hyphal extension in cultures larger than 0.2 mm diameter (Smith, 1924; Plomley, 1959). Growth was quantified by determining distances between hyphal branches and frequencies of hyphal branching. Deppe (1973), as reportd by Koch (1975), observed more directly that an exponential change in colony weight occurs as the colony radius increases linearly with time. However, most determinations of colony growth rates on agar are made indirectly from correlations with growth rate (as time rate of increase of mycelial dry weight) in submerged culture (Morrison and Righelato, 1975; Trinci, 1971). The focus of some studies of this type concerned the localization of growth in the peripheral growth zone of an expanding colony perimeter, a topic we shall also address in this paper.

The most widely applied measure of growth in fungi is increase in mycelial dry weight (Mandels, 1965), and it is surprising that it is not applied more frequently to agar culture where it also has utility (Farries and Bell, 1930; Gillie, 1968; Norrman and Fries, 1967). The extra step of extracting colonies from agar is counterbalanced by advantages over liquid culture: reduced variability by having a single locus of growth and the ease of use and uniformity of disposable Petri dishes, large numbers of which can be stored in a relatively small incubator without need for constant agitation to aerate the colonies. Furthermore, biomass increase must be known in order to understand the relationship between hyphal elongation and colony density, as Gillie (1968) showed in his growth rate studies of Neurospora crassa Shear and Dodge in agar.

The intention of the present work is to show the utility of agar media for quantitative studies of fungal biomass and to propose a few simple models of fungal growth for the shallow Petri dish system. Growth data obtained for cultures of Tuber spp., the true truffles, will be tested for fit.

Fungal growth models.

The modeling of fungal growth has been highly developed in some cases, especially in the fermentation industry where methods and modeling assumptions of chemical engineering have been applied to fungal systems and processes. Our treatment, designed for simpler situations is based on two well-established assumptions: that growth rate is proportional to the number of growing cells and that growth depends on the organism's environment (Fredrickson et al., 1970). The latter assumption entails two salient concepts: a finite volume of nutrient contains a growth-limiting substrate, and an organism alters its environment in ways that affect its growth.

In the models chosen, the separation of biomass into discrete cellular units is ignored and the fungi are assumed to behave as if continuously distributed throughout the colony volume. However, in modifying a logistic model we propose variations which account for different physiological states in the thallus. The first variation has the premise that only cells near the perimeter of the colony are

growing. The second includes a time-dependent function describing a metabolic effect that influences growth rate. Estimation of the model parameters are calculated in each case and data are tested for fit.

"A mathematical model of a system or process is a set of hypotheses concerning the mathematical relations between measured or measurable quantities associated with the system or process." (Fredrickson et al., 1970). By this definition models are usually presented as a set of differential equations, which when solved yield a function containing parameters to be estimated from a set of experimental data. A model should serve both to correlate data and to provide a concise way of thinking about a system (Fredrickson et al., 1970). The model then allows quantitative prediction of performance of a system and also guides one's reasoning in designing further experiments to better understand the system. Such modeling can be either an empirical or theoretical formulation or a combination of both. Certain properties of the derived fitting equation(s) are worth recounting (Table XIII, from Edwards and Wilke, 1968) as guidelines for developing a <u>useful</u> model.

Table XIII

(from Edwards and Wilke, 1968)

Desirable properties of a fitting equation

---

1) Capable of representing all phases of batch culture, growth and metabolism

2) Sufficiently flexible to fit many types of data without introducing distortion

3) At least some of the fittable parameters have direct physical meaning

4) Continuously differentiable, explicit time derivative

5) Derivative zero initially and at very large times

6) Parameters easily evaluated

7) Easy-to-use model once parameters determined

Model 1A: Growth uniform, metabolite independent.

The logistic model (Blumberg, 1968) has been applied to many and diverse population and biomass studies. It has two major elements given that external environmental variables are held constant: 1) The time rate of growth is directly proportional to the total biomass y at any time t, but 2) is limited by the remainder of the substrate to be colonized. The second element is typically represented in terms of y_m, the carrying capacity or maximum growth, as $(y_m-y)/y_m$ and growth rate is usually assumed to be linearly proportional to it. However, staling product formation, nutrient depletion, nutrient diffusion rates through the medium and across the cell interface, enzyme kinetics, and metabolic change are all inferred in the above expression, which suggests that a linear dependence of growth rate on this term may be too simplistic. This oversimplication has been treated by Edwards and Wilke (1968), who generalized the basic logistic formulation and found better fit for data when substituting a fifth-degree polynominal in t for the simpler formulation which is linear in t.

The logistic relationship has been well documented for bacterial systems. However, until it had become widely accepted that mycelial fungi are capable of exponential growth in unrestricted systems (Plomley, 1959; Borrow et al., 1964; Katz et al., 1972; Mandels, 1965), the logistic formulation was seldom applied to fungal systems. Most mycelial growth was typically modelled on cube-root laws (Pirt, 1966; Emerson, 1955). Since then, however, mycelial fungal growth has been reported to fit the logistic model (Constantinides et al., 1970; Hockenhull and Mackenzie, 1968; Edwards and Wilke, 1968). Unfortunately, all cases where mycelial mass (rather than hyphal extension/branching frequency or other measure) was the fitted response variable were for submerged liquid culture systems. Does a logistical model fit mycelial growth, measured as dry weight, in the agar/Petri dish system?

Formulation and statistical treatment of the model is straightforward. The model is specified by two relationships:

$$\frac{dy}{dt} \propto y \tag{1}$$

$$\frac{dy}{dt} \propto 1 - y/y_m \tag{2}$$

where y is mycelial weight at any time t and y_m is the maximum growth. Combining and rearranging,

$$kdt = \frac{dy}{y(1 - y/y_m)} \tag{3}$$

Intergrating growth from y_o to y over time interval 0 to t,

$$\int_0^t kdt = \int_{y_o}^y \frac{dy}{y(1 - y/y_m)} \tag{4}$$

where y_o is the mycelial weight of the initial inoculum plug.

Evaluating the integrals,

$$K_{1A}t = \ln(\frac{y}{y_m - y} \cdot \frac{1}{c_1}) \tag{5}$$

where $K_{1A} = k$ and $c_1 = y_o/(y_m - y_o)$.

To obtain an estimate of K_{1A}, transform y to Y_{1A}:

$$Y_{1A} = \ln (\frac{y}{y_m - y} \cdot \frac{1}{c_1}) \tag{6}$$

and regress Y_{1A} on t:

$$Y_{1A} = K_{1A}'t + B_{1A}'.$$

(7)

To obtain the fitting equation, rearrange (7) to $y = f(t)$:

$$y = \frac{c_1 \exp(K_{1A}'t + B_{1A}')}{1 + c_1 \exp(K_{1A}'t + B_{1A}')} \cdot y_m$$

(8)

Model 1B: Growth uniform, metabolite dependent.

Limited fungal growth in a closed system has sometimes been attributed to other events besides nutrient depletion. It has, for example, been considered as an effect of an accumulation of "staling products," that is, fungal products that inhibit the vegetative phase. Similarly, production of growth-stimulating compounds may be hypothesized to explain, although incompletely, the induction period or lag phase prior to exponential growth of a microbial population. Small amounts of carbon dioxide, for example, have been shown to stimulate growth in a number of fungi (Hartman et al., 1972). A similar example occurs in the system investigated here. The maximum growth rates of the Tuber isolates employed in this study occur at a pH greater than the initial pH of the medium at t_o. However, the pH rises steadily over time, probably as a result of nitrate reductase activity of the fungi (Michaels et al., 1982). Thus the specific growth rate at time t is related to concentrations of self-stimulating and self-inhibiting substances produced, which in turn are related to the metabolism of all active cells present at each time-point over the interval from 0 to t. As a simplification, if direct proportionality is assumed between one limiting factor concentration and growth rate, then

$$\frac{dy}{dt} \propto \int_0^t y(t)dt,$$

(9)

the metabolite dependent condition. Incorporating the two conditions, (1) and (2) above of Model 1A, with (9), a metabolite dependent modification of the pure logistic model is proposed:

$$\frac{dy}{dt} = ky(1 - y/y_m) \cdot \int_0^t y(t)dt \qquad (10)$$

The authors could not find an explicit solution of (10), and a resort to numerical methods seems necessary. However, condition (9) may be approximated by a simpler relationship which can be readily understood from Fig. 4. The righthand side of (9) is represented by the area under the growth curve, which, for the time intervals of interest approximately equals the area of a right triangle formed by the intersections of $y^* = mt$, $x = t_1$ and $y = 0$. Thus condition (9) is approximated as

$$\frac{dy}{dt} \propto (1/2)yt \qquad (11)$$

or,

$$\frac{dy}{dt} \propto (1/2)mt^2 \qquad (12)$$

Replacing (9) by (12) gives a differential equation which has an explicit solution:

$$\frac{dy}{dt} = k \cdot y \cdot (1 - y/y_m) \cdot (1/2)mt^2 \qquad (13)$$

which upon rearranging and integrating from 0 to t and y_o to y gives

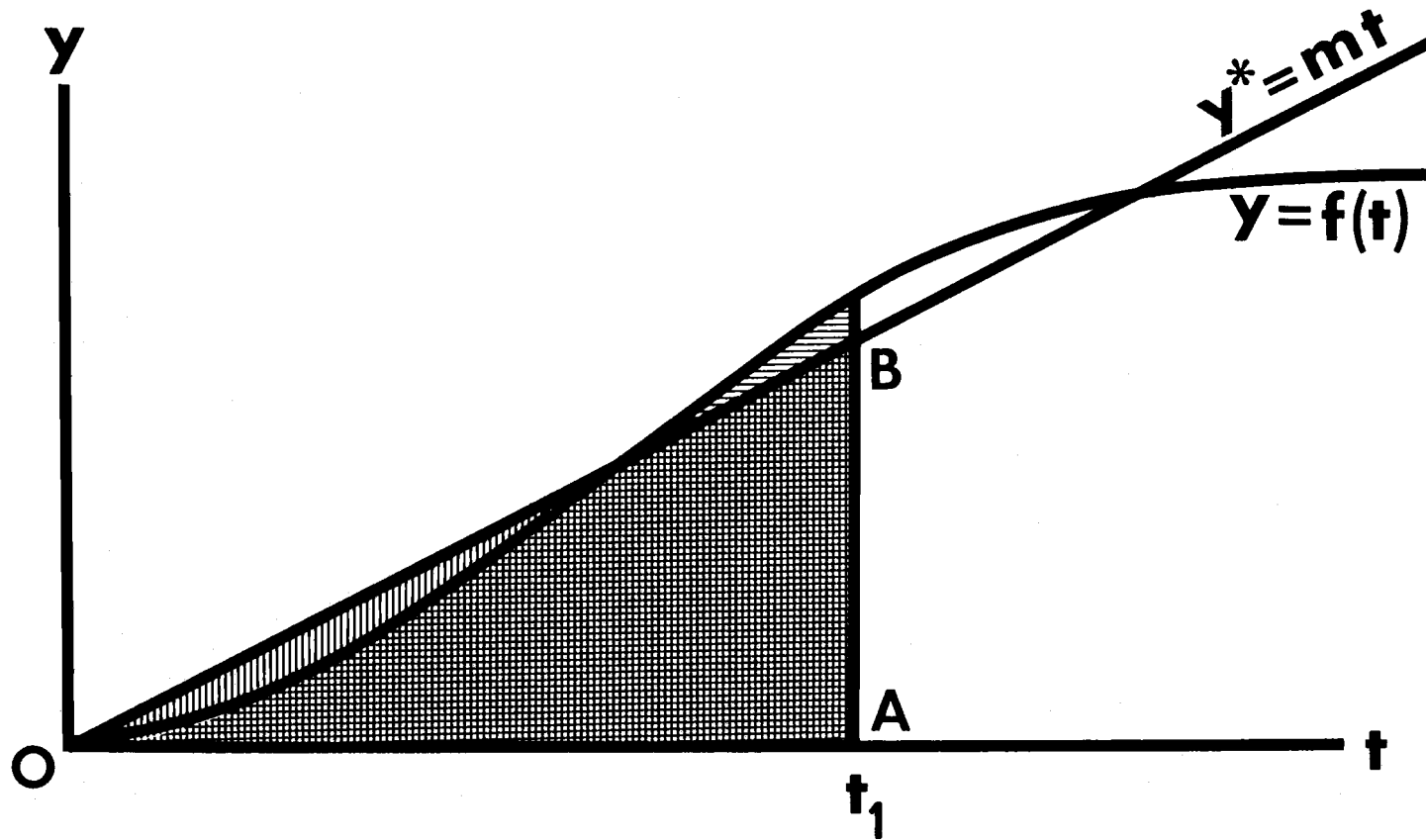$$K_{1B}t = (\ln(\frac{y}{y_m - y} \cdot \frac{1}{c_1}))^{1/3} \qquad (15)$$

Fig. 4. Area under sigmoid curve y = f(t) from 0 to t_1 can be approximated by right triangle OAB.

where $K_{1B}t = (km/6)^{1/3}$ and other constants are as defined for Model 1A. To obtain an estimate of K_{1B}, transform y to Y_{1B}:

$$Y_{1B} = K'_{1B}t + B'_{1B}. \qquad (16)$$

To obtain the fitting equation, rearrange (16) to $y = f(t)$:

$$y = \frac{c_1 \exp(K'_{1B}t + B'_{1B})^3}{1 + c_1 \exp(K'_{1B}t + B'_{1B})^3} Y_m \qquad (17)$$

Model 2A: Growth peripheral, metabolite independent.

Although logistic models describe growth in many systems, much evidence suggests that one condition of the logistic model, uniform growth, does not obtain in a fungal colony larger than 0.2 mm diameter. Rather, effective description of fungal growth requires a segregated model in which the culture is divided into an actively growing area and a more quiescent area. Several studies have shown that growth is greatest in a narrow annulus at the advancing edge of a fungal colony, the so-called peripheral growth zone (Pirt, 1967; Bull and Trinci, 1977: Trinci, 1971; Plomley, 1959; Gillie, 1968). Moreover, on solid media the width of the peripheral growth zone is directly related to the observed mean radial growth rate, with the constant of proportionality being the specific growth rate (Trinci, 1971). For many fungi the width of this annulus is of the order of 1 mm (Pirt, 1967). Righelato (1979) ascribes the phenomenon to a depletion of substrate behind the expanding colony perimeter: "The mycelium in the annulus outside the substrate-limited centre would continue to grow at the maximum specific growth rate but inside the annulus the transition to zero growth rate is likely to be rapid because small changes in the substrate concentration at low growth-limiting levels have a large effect on growth rate. Thus the growth of the colony can be regarded as being solely due to

exponential growth of the mycelium in the outer annulus" (italics added). For systems in which substrate will eventually be limiting, the term exponential growth should perhaps be replaced by logistic growth. The preceeding ideas are formalized for a model of mycelium growing on a thin agar disc.

The time rate of change of growth is proportional to the colony perimeter and is assumed to be linearly proportuional at any time t to the amount of remaining substrate to be colonized, as previously described for Model 1. For simplicity, depth of the thin disc is disregarded, and mycelial density, ρ, is expressed as mass per unit surface area. With a thin disc simplification, growth normal to the plane of colony expansion is also disregarded in the mathematical formalism. For a circular inoculation plug, then,

$$\frac{dy}{dt} \propto \pi d \qquad (17)$$

where d is the colony diameter. Since for a circle of area A, $d = 2(A/\pi)^{1/2}$, and since $\rho = y/A$ by definition, (17) can be rewritten

$$\frac{dy}{dt} \propto 2\pi(y/(\rho\pi))^{1/2} \qquad (18)$$

Since ρ is constant, it can be measured at any time, but most conveniently at the time of maximum growth:

$$\rho = y_m/A_m \qquad (19)$$

or

$$\rho = y_m/(\pi d_m^2/4), \qquad (20)$$

where A_m and d_m are maximum colony area and diameter, respectively. Substituting the right-hand side of (20) for ρ into (18), gives the first proportionality of Model 2A:

$$\frac{dy}{dt} \propto \pi d_m (y/y_m)^{1/2} \qquad (21)$$

And combining with the second condition, viz.,

$$\frac{dy}{dt} \propto (1 - y/y_m),$$ (22)

we obtain after rearranging and indicating the appropriate integration:

$$\int_0^t k\,dt = \int_{y_o}^y (1/\pi d_m) \frac{dy}{(y/y_m)^{1/2} (1 - y/y_m)}$$ (23)

Evaluating (23) gives

$$K_{2A} t = \ln\left(\frac{(y/y_m)^{1/2} + 1}{(y/y_m)^{1/2} - 1} \cdot \frac{1}{c_2}\right),$$ (24)

where $K_{2A} = \pi d_m k / y_m$ and $c_2 = \dfrac{(y_o/y_m)^{1/2} + 1}{(y_o/y_m)^{1/2} - 1}$.

To obtain an estimate of K_{2A}, transform y to Y_{2A}:

$$Y_{2A} = \ln\left(\frac{(y/y_m)^{1/2} + 1}{(y/y_m)^{1/2} - 1} \cdot \frac{1}{c_2}\right)$$ (25)

and regress Y_{2A} on t:

$$Y_{2A} = K'_{2A} t + B'_{2A}.$$ (26)

To obtain the fitting equation, rearrange (26) to $y = f(t)$:

$$y = \left(\frac{c_2 \exp(K'_{2A} t + B'_{2A}) + 1}{c_2 \exp(K'_{2A} t + B'_{2A}) - 1}\right)^2 \cdot y_m.$$ (27)

Model 2B: Growth peripheral, metabolite dependent.

Merging the concepts of peripheral growth developed in Model 2A with the metabolite dependency condition as discussed for Model 1B, we can describe the growth rate by three proportionalities:

$$\frac{dy}{dt} \propto \pi d_m (y/y_m)^{1/2} \tag{28}$$

$$\frac{dy}{dt} \propto (1 - y/y_m) \tag{29}$$

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$$\frac{dy}{dt} \propto (1/2)mt^2 \tag{30}$$

Again combining, rearranging and indicating the appropriate integration:

$$\int_0^t k \cdot (1/2)mt^2 dt = \int_{y_o}^y (1/\pi d_m) \cdot \frac{dy}{(y/y_m)^{1/2}(1 - y/y_m)} \tag{31}$$

Evaluating (31) gives

$$K_{2B}t = \left\{ \ln\left(\frac{(y/y_m)^{1/2} + 1}{(y/y_m)^{1/2} - 1} \cdot \frac{1}{c_2}\right) \right\}^{1/3}, \tag{32}$$

where $K_{2B} = (\pi d_m/y_m) \cdot (km/6)^{1/3}$.

To obtain an estimate of K_{2B}, transform y to Y_{2A}:

$$Y_{2B} = \left\{ \ln\left(\frac{(y/y_m)^{1/2} + 1}{(y/y_m)^{1/2} - 1} \cdot \frac{1}{c_2}\right) \right\}^{1/3} \tag{33}$$

and regress Y_{2B} on t:

$$Y_{2B} = K'_{2B}t + B'_{2B} \tag{34}$$

To obtain the fitting equation, rearrange (34) to $y = f(t)$:

$$y = \left\{\frac{c_2 \exp(K'_{2B}t + B'_{2B})^3 + 1}{c_2 \exp(K'_{2B}t + B'_{2B})^3 - 1}\right\}^2 \cdot y_m \qquad (35)$$

The important equations from the preceeding derivations are summarized in Table XIV.

MATERIALS AND METHODS

The fungi.

The initial impetus for this work came from the attempt to specify nutritional and environmental conditions for maximizing in vitro growth of Tuber spp., the true truffles. Cultures were selected from the collections of mycorrhizal fungi maintained by Mr. G. Chevalier at the Station de Pathologie Végétale, Institut National de la Recherche Agronomique (I.N.R.A.), Clermont-Ferrand, France, and Drs. James Trappe and Randolph Molina at the U.S. Forest Service Pacific Northwest Forest and Range Experiment Station, Corvallis, Oregon, for this growth study: Tuber aestivum Vitt. S-321; T. aestivum S-322: T. borchii Vitt. S-211; T. borchii S-393; T. brumale Vitt. S-323; T. brumale var. moschatum (Ferry) Fischer S-485; T. melanosporum Vitt. S-488; T. melanosporum S-489; T. melanosporum S-490; T. puberulum Berkeley and Broome S-394; T. rufum Pico S-487; and T. rufum var. nitidum (Vitt.) Fischer S-362. Although described in detail elsewhere (Michaels et al., 1982), certain relevant characteristics of these cultures need to be noted:

    a) They grow very slowly in culture. A 0.1 - 0.2 mg (dry wt. basis) mycelial inoculation plug takes from four to ten weeks to grow to maximum weight in 8.0 ml of nutrient medium (described below).

Table XIV.

Summary of assumptions and equations of proposed models[*]

Model	Assumptions	Integrated form; $y \to Y$	Regression model	Fitting equation
1A	$\dfrac{dy}{dt} \propto y$ $\dfrac{dy}{dt} \propto (1 - y/y_m)$	$K_{1A}t = \ln\left(\dfrac{y}{y_m - y} \cdot \dfrac{1}{c_1}\right)$ $Y_{1A} = \quad\quad ''$	$Y_{1A} = K'_{1A}t + B'_{1A}$	$y = \dfrac{c_1 \exp(K'_{1A}t + B'_{1A})}{1 + c_1 \exp(K'_{1A}t + B'_{1A})} \cdot y_m$
1B	$\dfrac{dy}{dt} \propto y$ $\dfrac{dy}{dt} \propto (1 - y/y_m)$ $\dfrac{dy}{dt} \propto \displaystyle\int_0^t y(t)\,dt,$	$K_{1B}t = \left(\ln\left(\dfrac{y}{y_m - y} \cdot \dfrac{1}{c_1}\right)\right)^{1/3}$ $Y_{1B} = \quad\quad ''$	$Y_{1B} = K'_{1B}t + B'_{1B}$	$y = \dfrac{c_1 \exp(K'_{1B}t + B'_{1B})^3}{1 + c_1 \exp(K'_{1B}t + B'_{1B})^3} \cdot y_m$
2A	$\dfrac{dy}{dt} \propto \pi d_m (y/y_m)^{\frac{1}{2}}$ $\dfrac{dy}{dt} \propto (1 - y/y_m)$	$K_{2A}t = \ln\left(\dfrac{(y/y_m)^{\frac{1}{2}} + 1}{(y/y_m)^{\frac{1}{2}} - 1} \cdot \dfrac{1}{c_2}\right)$ $Y_{2A} = \quad\quad ''$	$Y_{2A} = K'_{2A}t + B'_{2A}$	$y = \left(\dfrac{c_2 \exp(K'_{2A}t + B'_{2A}) + 1}{c_2 \exp(K'_{2A}t + B'_{2A}) - 1}\right)^2 \cdot y_m$
2B	$\dfrac{dy}{dt} \propto \pi d_m (y/y_m)^{\frac{1}{2}}$ $\dfrac{dy}{dt} \propto (1 - y/y_m)$ $\dfrac{dy}{dt} \propto \displaystyle\int_0^t y(t)\,dt,$	$K_{2B}t = \left(\ln\left(\dfrac{(y/y_m)^{\frac{1}{2}} + 1}{(y/y_m)^{\frac{1}{2}} - 1} \cdot \dfrac{1}{c_2}\right)\right)^{1/3}$ $Y_{2B} = \quad\quad ''$	$Y_{2B} = K'_{2B}t + B'_{2B}$	$y = \left(\dfrac{c_2 \exp(K'_{2B}t + B'_{2B})^3 + 1}{c_2 \exp(K'_{2B}t + B'_{2B})^3 - 1}\right)^2 \cdot y_m$

*) see text for definitions of variables and constants

b) In many media, including the one employed in this study, most
   Tuber isolates grow with a poorly demarcated colony margin.
   Leading hyphae are sparse and mycelial cell density increases
   irregularly behind the margin. Qualitatively, _T.borchii_
   S-393; _T. aestivum_ S-321 and S-322; _T. melanosporum_ S-489; and
   T. rufum grow with the most sharply defined perimeters. More
   diffuse in growth pattern are _T. brumale_, _T. brumale_ var.
   moschatum and _T. melanosporum_ S-488 and S-490. Of the twelve
   isolates, _T. rufum_ var. _nitidum_ and _T. puberulum_ spread the
   most rapidly. Only in the latter do the hyphae reach the
   sidewalls of a 60 x 15 mm Petri dish when inoculated in the
   center and this it does in less than half the time needed to
   reach maximum mycelial dry weight.

c) Six isolates, _T. aestivum_ S-321, _T. brumale_, _T. brumale_ var.
   moschatum, and the three _T. melanosporum_ isolates, carry a
   small amount of bacteria (gram-negative rods) which some
   workers maintain are necessary for culture viability (Vrot,
   1977; Chevalier, 1972; Fontana, 1971).

d) Calcium is required by at least one species, _T. melanosporum_
   (Fontana, 1971). All isolates except _T. rufum_ var. _nitidum_
   and _T. puberulum_ were derived from ecotypes found in
   calcareous soils of pH 7.8 - 8.2 (Michaels et al., 1982).

e) In the presence of nitrate all isolates raise the pH of the
   growing medium, presumably through nitrate reductase activity
   (Michaels et al., 1982).

f) _Tuber_ spp. are not known to sporulate in culture, except as
   noted in one sketchy report over 40 years ago (Chaze and
   Mestas, 1939). This is useful in growth studies for which
   extraneous physiological states should be minimized.

The culture medium.

A good medium for growing _Tuber_ spp. incorporates Hewitt's
minerals at concentrations recommended for hydroponic plant physiology
studies (Hewitt, 1966) with standard components of many mycological

media (per liter): 19.5 g potato dextrose agar (Difco), 5.0 g malt extract (Difco), 7.5 g Bacto-agar (Difco), KNO_3, 0.505 g; $Ca(NO_3)_2$, 0.820 g; $NaH_2PO_4 \cdot H_2O$, 0.186 g; $MgSO_4 \cdot 7H_2O$, 0.369 g: Sequestrene (Geigy, Fe^{+3} 10 per cent w/w), 0.056 g; $MnSO_4$, 0.023 g; NaCl, 5.85 mg; H_3BO_3, 1.86 mg; $CuSO_4 \cdot 5H_2O$, 0.24 mg; $ZnSO_4 \cdot 7H_2O$, 0.296 mg; $Na_2MoO_4 \cdot 4H_2O$, 0.024 mg; and $CoCl_2 \cdot 6H_2O$, 0.024 mg. The medium was adjusted immediately after autoclaving (20 min, 19 psi) to pH 5.7, as determined with an Orion 601A Digital Analyzer equipped with an ATC probe for internal temperature/slope correction for hot solutions. Aliquots (8.0 ml) were syringed into 60 x 15 mm polystyrene disposable Petri dishes.

Incubation and Weight Determination

Cork-borer inoculation plugs six mm diam, taken about midway between the center and aerial perimeter of six-week-old cultures, were transferred to the center of prepared plates. The freshly inoculated plates of each isolate were sealed in Parafilm (American Can Co.), randomized and incubated at 20 C. Six plates of each isolate were randomly selected at weekly intervals for a period of up to ten weeks (or eight or nine weeks for some of the faster-growing isolates).

To determine mycelial weight, the agar and mycelium were transferred to a beaker containing 150-200 ml tapwater and steamed for 45-60 min. The entire mixture was filtered hot (aspirator assisted) through a stainless steel support grid, 130 μm mesh, of a Millipore filter apparatus and washed with 200-400 ml of 40-50 C tapwater. Mycelial pads were frozen and lyophilized to constant weight (usually 2 d at ambient temperature, 20 μm Hg).

This technique does not, of course, measure the true mycelial dry weight, since most soluble and some colloidal moieties, as well as some cell wall material, are undoubtedly lost in the hot water treatment. The work of Gillie (1968) and Farries and Bell (1930) suggests that this loss, although not neglible, is roughly proportionate to total mycelial dry weight irrespective of culture age.

<u>Statistical analysis.</u>

For transforming y to Y, the maximum average growth obtained for an isolate was chosen as representing $0.99y_m$. Tests on three isolates using other values, viz., $0.98y_m$, $0.97y_m$ and $0.96y_m$, did not appreciably differ from $0.99y_m$ and yielded similar transformation statistics. For those time-points for which growth data points were greater than y_m, where Y is necessarily undefined, only average growth values were transformed (with consequent loss of degrees of freedom at the greater time values).

In testing the models, transformed growth data for each isolate were subjected to linear regression least squares analysis. If a least squares treatment of the data did not allow acceptance of an equal variance hypothesis about each time-point and/or normality of residuals about zero, a weighted least squares method (Neter and Wasserman, 1974) was applied. The weighting method can stabilize a variance which increases at each x-level (time-point), a possibility one might expect for a variable, biomass, itself increasing over time, transformed by a monotone increasing funtion.

RESULTS AND DISCUSSION

Of the four models tested, 2A was eliminated first, because variance at each time-point (within each isolate) and plots of residuals suggested unequal variances and/or non-normality of residuals about zero for at least one time-point for every isolate. Furthermore except for <u>T</u>. <u>rufum</u> var. <u>nitidum</u> and <u>T</u>. <u>puberulum</u>, the attempt to stabilize variance by weighted least squares gave similar results. Therefore, except for these two isolates, Model 2A was judged inappropriate for describing growth in the system under study and was not considered further.

Neither least squares nor weighted least squares analysis gave the necessary variance and residual distribution conditions for <u>T</u>. <u>melanosporum</u> S-489 with any of the models. This isolate is not considered further.

Statistics for the models applied to each of the remaining isolates are listed in Table XV. F-values for goodness of fit tests

Table XV. Estimated parameters and statistics[a] of regressions from transformations y→Y.

Isolate	Model[c]	Regression equation of Y on t	s_K^2	s_B^2	F^b (ν_1, ν_2)	r^2	time to $\overline{}$ by fitting equation .50y_m	.90y_m
aes S-321	1A - W	Y_{1A} = 0.915t + 1.453	0.002218	0.009149	3.35 (5,30)	0.868	4.7 wk	7.1 wk
	1B - W	Y_{1B} = 0.126t + 1.211	0.00005827	0.0002404	4.83 "	0.994	4.6	6.3
	2B - W	Y_{2B} = 0.145t + 0.484	0.00005295	0.0002184	3.00 "	0.968	4.8	7.1
					2.53 = F.95		5.1x	6.8x
aes S-321	1A - W	Y_{1A} = 1.283t + 0.731	0.003675	0.01041	1.29 (3, 19)	0.700	3.5	5.0
	1B - W	Y_{1B} = 0.190t + 1.078	0.00009772	0.0002768	2.83 "	0.995	3.5	4.6
	2B - W	Y_{2B} = 0.213t + 0.416	0.0009389	0.0002660	0.81 "	0.967	3.6	5.2
					3.25 = F.95		3.7x	4.8x
bor S-211	1A - W	Y_{1A} = 1.210t + 0.648	0.003997	0.01447	0.25 (4, 26)	0.492	3.7	5.5
	1B - W	Y_{1B} = 0.174t + 1.065	0.00008522	0.0003086	0.75 "	0.992	3.8	5.0
	2B - W	Y_{2B} = 0.201t + 0.415	0.0001038	0.0003758	0.11 "	0.939	3.8	5.5
					2.75 = F.95		3.7x	5.6x
bor S-393	1A - W	Y_{1A} = 1.405t + 1.361	0.001434	0.004330	2.87 (4, 20)	0.945	2.8	4.4
	1B - W	Y_{1B} = 0.176t + 1.232	0.00003666	0.0001107	18.30 "	0.998	2.9	4.1
	2B - W	Y_{2B} = 0.232t + 0.513	0.00003641	0.0001099	5.07 "	0.990	2.9	4.3
					2.87 = F.95		2.8x	5.7x
bru S-323	1A - U	Y_{1A} = 1.368t + 0.130	0.009506	0.1393	0.38 (4, 26)	0.868	4.1	5.7
	1B - U	Y_{1B} = 0.179t + 1.021	0.0001715	0.002513	5.04 "	0.861	4.3	5.4
	2B - U	Y_{2B} = 0.216t + 0.286	0.0002005	0.002938	0.45 "	0.886	4.1	5.7
					2.75 = F.95 4.1x		4.1x	6.1x

[a] See Table IV for explanation of symbols.
[b] Goodness of fit test statistic.
[c] U indicates analysis by least squares, W by weighted least squares.
[x] Experimental.

70

Table XV. (continued)

Isolate	Model[c]	Regression equation of Y on t	s^2_K	s^2_B	F^b (ν_1, ν_2)	r^2	time to by fitting equation .50y_m	.90y_m
bruvm S-485	1A - W	$Y_{1A} = 0.801t + 0.942$	0.001205	0.006557	2.41 (8, 40)	0.738	5.9 wk	8.7 wk
	1B - W	$Y_{1B} = 0.119t + 1.093$	0.00003776	0.0002055	3.67 "	0.992	5.8	7.5
	2B - W	$Y_{2B} = 0.126t + 0.418$	0.00002583	0.0001406	2.06 "	0.963	6.1	8.8
					2.18 = $F_{.95}$		6.6x	8.2x
mel S-488	1A - U	$Y_{1A} = 0.852t + 0.823$	0.002627	0.07827	0.95 (8, 40)	0.852	5.7	8.3
	1B - U	$Y_{1B} = 0.105t + 1.148$	0.00003511	0.001046	3.52 "	0.867	6.1	8.1
	2B - U	$Y_{2B} = 0.133t + 0.401$	0.00005657	0.001686	1.15 "	0.867	5.9	8.4
					2.18 = $F_{.95}$		5.6x	9.4x
mel S-490	1A - U	$Y_{1A} = 0.706t + 0.780$	0.002303	0.06583	4.23 (8, 39)	0.822	7.0	10.2
	1B - U	$Y_{1B} = 0.926t + 1.126$	0.00003931	0.001124	0.86 "	0.823	7.2	9.4
	2B - U	$Y_{2B} = 0.112t + 0.382$	0.00005402	0.001544	3.66 "	0.831	7.1	10.2
					2.19 = $F_{.95}$		7.7x	9.2x
pub S-394	1A - W	$Y_{1A} = 3.068t - 1.206$	0.03832	0.06992	1.01 (2, 10)	0.634	2.1	2.8
	1B - W	$Y_{1B} = 0.428t + 0.803$	0.001696	0.003095	3.07 "	0.946	2.1	2.7
	2A - W	$Y_{2A} = 1.614t - 1.412$	0.01520	0.02773	4.25 "	0.857	1.9	3.0
	2B - W	$Y_{2B} = 0.503t + 0.116$	0.001329	0.002425	2.19 "	0.317	2.1	2.8
					4.10 = $F_{.95}$		2.0x	2.8x
ruf S-487	1A - W	$Y_{1A} = 1.434t + 0.552$	0.001429	0.004604	1.05 (4, 22)	0.718	3.3	4.9
	1B - W	$Y_{1B} = 0.199t + 1.069$	0.00003144	0.0001013	3.88 "	0.998	3.5	4.5
	2B - W	$Y_{2A} = 0.234t + 0.381$	0.00003324	0.00003324	1.05 "	0.981	3.4	4.9
					2.82 + $F_{.95}$		3.4x	5.0x
rufvn S-362	1A - W	$Y_{1A} = 0.776t + 1.095$	0.006356	0.02197	1.11 (5, 23)	0.661	5.2	8.0
	1B - W	$Y_{1B} = 0.123t + 1.112$	0.0002268	0.0007841	0.15 "	0.983	5.0	6.8
	2A - W	$Y_{2A} = 0.320t - 0.114$	0.001987	0.006868	17.12 "	0.063	5.4	9.0
	2B - W	$Y_{2B} = 0.129t + 0.489$	0.0001726	0.0005968	0.77 "	0.935	5.3	7.9
					3.95 = $F_{.95}$		5.2x	6.6x

have been included, because in a few cases a highly significant regression is generated, although the small error mean square value has an even much smaller pure error component. The goodness of fit F-value quantifies the relationship between the two values. Thus, for example, the highly significant r^2 = 0.998 of Model 1B for T. borchii S-393 must be interpreted with caution. Furthermore, in deciding which model best describes growth, one must test the predictive capac-ity of the model by "doing a different, independent experiment to see if the data so obtained are fitted by the model with the same set of parameters obtained in the previous stage of testing." (italics mine; Fredrickson et al., 1970). The goodness of fit F-value is useful for fitting means at each time-point in deciding if a model is to be accepted or rejected. In this discussion we shall consider both.

The time-dependent B models which take into account metabolites produced by the fungus generally described growth better than the A models, although for T. brumale, T. melanosporum S-488 and S-490 models 1A, 1B, and 2B did not differ significantly. However, within the metabolite-dependent models, differences between the uniform growth (1B) and peripheral growth (2B) modifications were less clear. If one accepts a threshold r^2 = 0.900 and F-tests for goodness of fit at = 0.05, both 1B and 2B describe growth for T. aestivum S-322, T. borchii S-211 and T. rufum var. nitidum, but T. puberulum is best described by the uniform model and T. brumale var. moschatum and T. rufum by the peripheral growth model. The real world of fungal behavior in the Petri dish probably lies somewhere between these two descriptions: growth rate decreases with distance behind the advancing perimeter. Trinci (1971) reports that the width of the annulus of growing hyphae at the colony perimeter multiplied by the specific growth rate obtained from submerged culture yields the radial growth rate. For T. puberulum the predominantly uniform mode of growth coincides well with observation; its leading hyphal tips reach the plate sidewalls in less than two weeks, yet colony biomass is less than half maximum at that time. The specific growth rate of T. puberulum is undetermined, but such growth behavior indicates a growing annulus of great width. T. rufum var. nitidum grows similarly but less rapidly. Determination of the width of the peripheral growth

annulus certainly suggests itself as an independent corroborative test of the models proposed in this paper.

It was originally hoped that the proposed models would indicate at a glance what growth pattern was occurring in a particular system. For _Tuber_ spp. on a malt extract-potato-dextrose-mineral salts agar, the isolates grew to their respective maxima according to the metabolite-dependent equations (the 2 models). The distinction of growth primarily at the edge vs. throughout the colony was less discernable. It was thought that those isolates having sharply demarcated perimeters (for example, _T. aestivum_ S-321 and 322, _T. borchii_ S-393 and _T. rufum_) would tend toward a fit by model 2, and those with a wide outer annulus of sparse mycellium (such as _T. puberulum_, _T. rufum_ var. _nitidum_, _T. brumale_ and _T. brumale_ var. _moschatum_) would be better fitted by model 1. Apparently, however, the growth modelling as presented here is not sensitive enough with the system studied to select for perimeter (peripheral) vs. areal (uniform) growth habit, because fit by more than one model can be quite good for the same isolate.

This analysis does, however, suggest that growth in the system studied is metabolite-dependent. The observable effect of such growth is lengthening of the lag and deceleration phases and a higher growth rate at the inflection point near half maximum growth, i.e., the fitting equations from the B models plot as steeper, more "S-shaped" curves than those from A models. If, for example, a pH change in the medium resulting from metabolism is suspected as significantly affecting growth rate a more buffered medium might be used to better stabilize pH over the growth period. This could be useful as an independent test of the model.

Considering further the shape of the fitted curves to the untransformed growth data (Fig. 5), a surprisingly consistent tendency towards congruency of curves is generated from Model 2B and the logistic Model 1A (Fig. 5h). Without regression analysis of the transformed data, the segregated growth behavior implied by Model 2B could be readily mistaken for the nonsegregated behavior described by the pure logistic model.
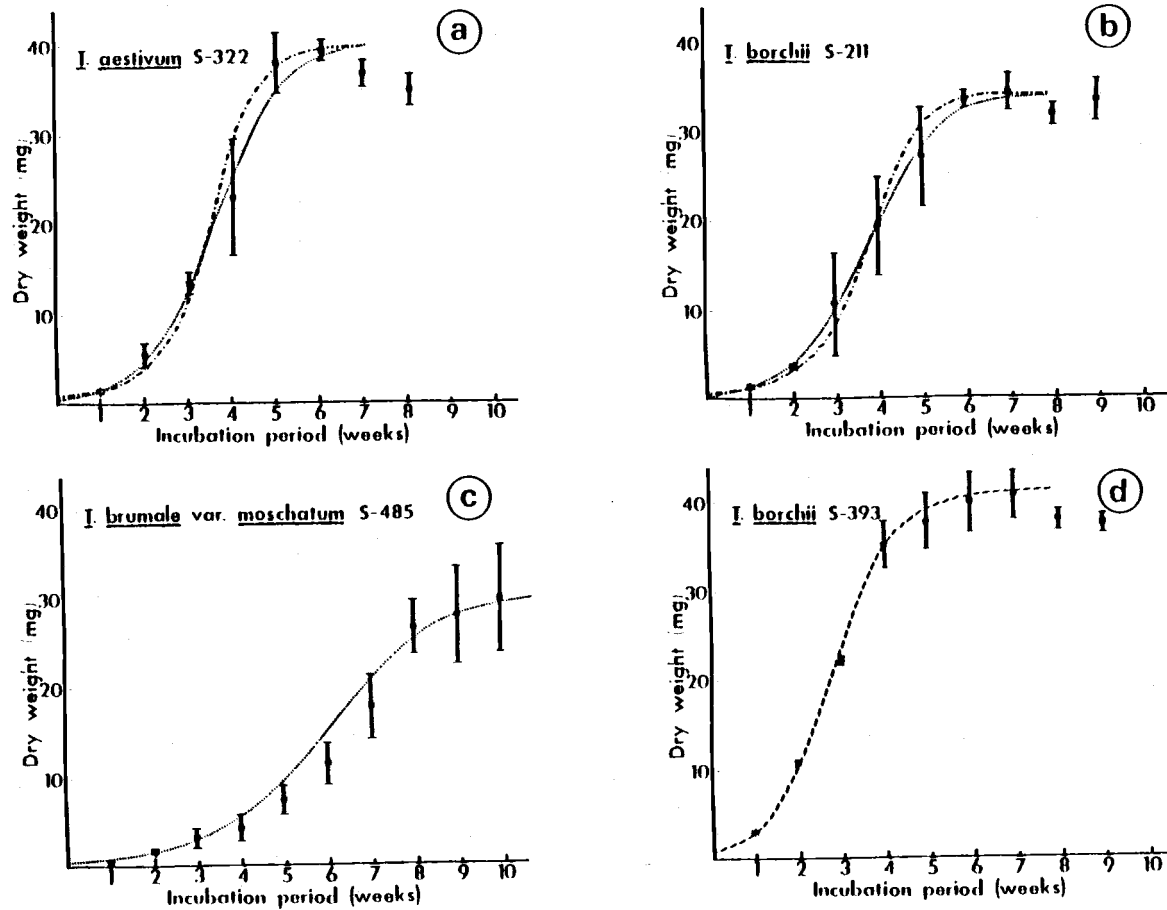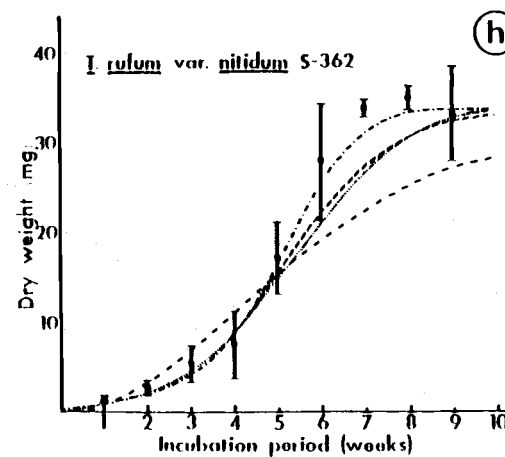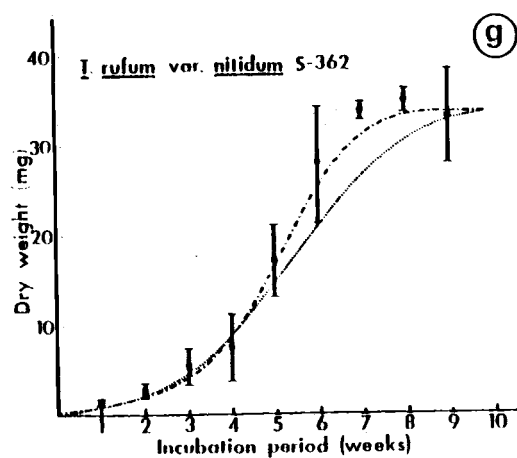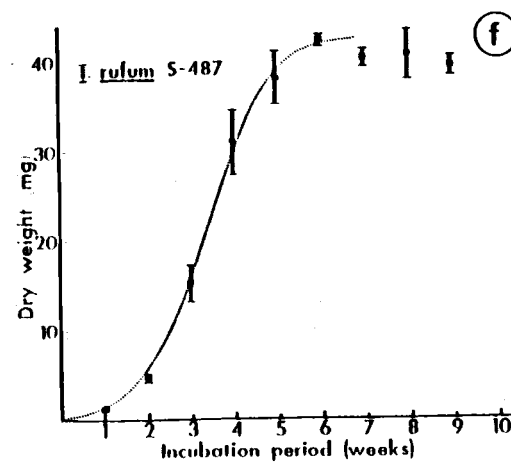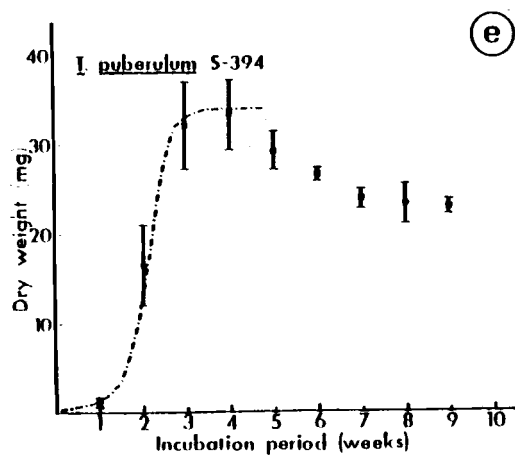
Fig. 5. a-g. Fitted growth curves for Tuber spp. for which models have $r^2 \geq 0.900$ and are significant for goodness of fit ($\alpha = 0.05$); h. T. rufum var. nitidum fitted by all four models. Curve 2B at less than 4 weeks is nearly congruent with 1A. Legend for fitting curves: 1A -------- ; 1B -------- ; 2A ------ ; 2B ....

(Fig. 5, cont'd).

SUMMARY

Fungal growth as a function of time is most frequently reported for liquid culture systems in which growth is measured as filtered mycelial dry weight. Although dry weights have been determined for fungi growing in agar, we found no reports applying the technique to studies of growth as a function of time. Our work confirmed that the patterns of dry weight-based growth curves found for liquid culture systems also obtained in an agar system.

A few simple models are presented for fungal growth measured as dry weight in an agar/Petri dish system to rationalize the sigmoid growth pattern observed.

ACKNOWLEDGEMENTS

Table XVI

Definition of symbols

A colony area

A_m maximum colony area

B'_{--} statistical estimator of Y_{--}-intercept

c_1 constant obtained from evaluation integrals of Model 1A, 1B

c_2 constant obtained from evaluating integrals of Model 2A, 2B

d colony diameter

d_m maximum colony diameter

F F statistic

k constant of proportionality

K_{--} constant of proportionality for growth Model --

K'_{--} statistical estimator of K_{--}

m slope of line approximating sigmoid curve

r^2 coefficient of determination

s_B^2 sample variance of intercept estimator B'_{--}

s_k^2 sample variance of slope estimator K'_{--}

t time

Y_{--} transformed growth datum by Model --

y growth as mycelial dry weight

y_m maximum growth or carrying capacity

y_o dry weight of initial mycelial inoculum at $t = 0$

α "is proportional to"

ν_1 degrees of freedom of numerator mean square of F ratio

ν_2 degrees of freedom of denominator mean square of F ratio

ρ colony density expressed as mass per surface area

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PART II

MYCORRHIZAL COLONIZATION STUDIES

A SIMPLE, NONDESTRUCTIVE METHOD
FOR MEASURING SEEDLING ROOT VOLUME AND ESTIMATING DRY WEIGHT

CHAPTER 3

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INTRODUCTION

In many studies of roots, growth has been defined as a change in dry weight. Dry weight determination is necessarily destructive so successive measurements of root growth are not possible on a single plant. Consequently, to study a particular treatment over time, different individuals in a population had to be sampled at each time-point. If, however, the same individuals can be followed over the course of an experiment, statistical inferences will be stronger (Dixon and Massey, Jr., 1969). The objective of this work was to develop a fast, accurate, nondestructive technique to measure root volume of individual plants at successive intervals of time and to correlate volume data with corresponding dry weight data for the same roots.

Most determinations of root volume involve submersion of roots in water and measurement of the water displaced (Bohm, 1979; Andrew, 1966; Musick et al., 1965; Pinkas et al., 1964; Novoselov, 1960; Priestley and Pearsall, 1922). Typically the water is displaced into an overflow apparatus. The technique requires careful monitoring of fluid levels before and after root submersion, and is limited in accuracy to the reading of a water meniscus in a calibrated glass tube. Accuracy of $\pm$ 0.1 cm^3 is typical.

Measurement can be simplified by applying the Principle of Archimedes: A body wholly or partly immersed in a fluid is buoyed up by

a force equal to the weight of the fluid displayed (Hodgman et al., 1962). Or, more succinctly,

$$F = \rho g V, \tag{1}$$

where F is the buoyant force, V is the volume of the fluid displaced, ρ its mass density, and g is the acceleration due to gravity.

Ignoring the slight buoyant effect of air, the buoyant force on a submerged (or partially submerged) object can be measured by noting the difference in its weight in air, W_1, and when submerged, W_2. That is,

$$F = W_1 - W_2. \tag{2}$$

Combining the above relationships and solving for V, we have:

$$V = \frac{(W_1 - W_2)}{\rho g}. \tag{3}$$

In the study we used a top-loading laboratory balance which compares masses rather than deterimes weights _per se_. Consequently, equation (3) simplifies to

$$V = \frac{1}{\rho} (m_1 - m_2). \tag{4}$$

At 25 C. $\rho = 0.997$ g cm^{-3}, and

$$V = 1.003 \text{ cm}^3 \text{g}^{-1} (m_1 - m_2). \tag{5}$$

If the weighings are accurate to about one part in one hundred (as in our study) the submerged volume in cm^3 simply equals the difference of balance readings in grams before and during submersion.

Only the volume of the submerged portion of the seedling is determined. This is convenient for root volume measurement, because the rigid stem can be clamped to a balance pan and serve as the support for submerging the roots in the water (Figure 6).

Fig. 6. A simple apparatus for measuring root volume. a) Seedling
first weighed in air. b) Seedling weighed with roots submerged.

Fig. 6

MATERIALS AND METHODS

To test this gravimetric approach, 1-0 hardwood seedlings grown in 500 ml capacity bullet tubes ("Deepots") containing a vermiculite: sand::1:1 potting mix were lifted, washed and patted dry in paper toweling. Fifteen trees from each of four groups of seedlings were randomly selected: Quercus garryana Dougl. ex Hook., (Corvallis)[a], Q. garryana, (Ashland)[b], Q. douglasii Hook. and Arn., and Corylus avellana L.

A Sartorius Model 2357 top-loading balance (1600 g cap., $\pm$ 0.005 g. prec.) was used for the weighings. A small three-fingered clamp to hold seedlings for root immersion was attached to a balance pan with raised sides (Fig. 1). Unaerated tapwater was used as the submerging fluid. Since small bubbles dislodged readily from the root mass with a slight shaking of the seedling, addition of surfactant seemed unnecessary.

Whole fresh weight, m_1, was determined first. Seedlings were then submerged to the soil line for m_2 determination. After removal from water, roots were cut at the waterline, frozen and lyophilized to constant weight for determinations of dry weight.

RESULTS AND DISCUSSION

The response variable, root dry weight, was plotted against predictor variable, root volume, for each group of seedlings, and the appropriate linear regression equation found by the method of least squares (Figure 7).

Root volume and dry weight correlated very well, particularly for oaks. These first-year seedlings typically have a long thick taproot with relatively few laterals and feeder roots. Errors due to inadvertant weighing of mucilage, soil particles, microflora and other

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[a]Seed collected under Q. garryana canopy, Oregon State University campus, Corvallis, OR, September 1979.
[b]Seed collected south of Ashland, OR on Ashland Grade, old Hwy 99, ca. 800 m elev., September, 1979.

adhering features of a root system are diminished due to the smaller surface-to-volume ratio of such a root configuration. Seedlings of C. avellana, by contrast, have fibrous root systems, which may explain the reduced correlation of volume to dry weight. The deviation from the regression is also greater for larger roots, as indicated in the scatter diagrams (Fig.7). Weighing excess surface water will tend to put points below the regression line, whereas inadvertant weighing of attached soil particles moves data points above the line.

The slope of the regression line is a measure of root density expressed as grams of dry matter per cubic centimeter of fresh root volume. Interestingly, root densities differed significantly, (= 0.05) for the two Q. garryana biotypes. The reason for the difference was not investigated, but it is curious that the biotype from the more moderate climate (Corvallis) yielded more root biomass per unit volume than that from the harsher environment (Ashland) when both were raised in mild greenhouse conditions.

Theoretically the regression lines obtained should pass through the origin. A t-test shows that the sample y-intercept is not significantly different from zero (= 0.05) for C. avellana and Q. garryana (Corvallis). However, for the other two population samples, the y-intercepts are significantly different from zero. The roots of smaller Q. garryana (Ashland) and Q. douglasii seedlings, were lighter and had smaller volumes causing their means, (X,Y) to be closer to the origin. As to be expected, then, the fitted curves (which must pass through (X,Y)) have correspondingly smaller standard errors for the y-intercepts (Neter and Wasserman, 1974). As a practical matter, though, changes in weight (volume) or relative weight (volume) are usually the concern of the researcher, and the absolute weight at the y-intercept is of little consequence.

The tendency toward a negative y-intercept may indicate either a systematically low measurement of root dry weight and/or fresh weight, or a systematically high measurement of submerged fresh weight (or both). Nonetheless, the correlation between root dry weight and root
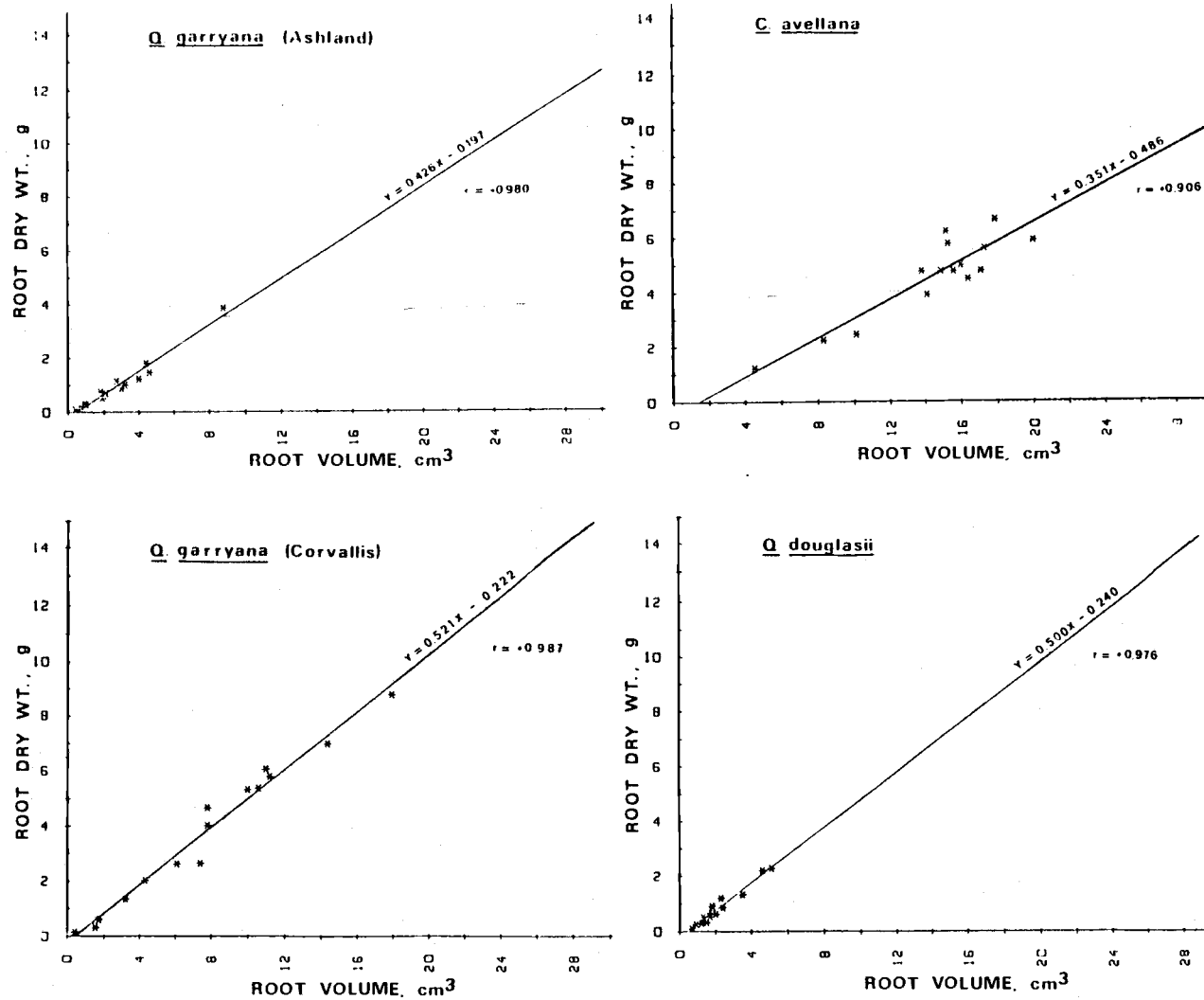
Fig. 7. Linear regression equations of dry weight as a function of root volume obtained for four groups of 15 one-year-old greenhouse-grown seedlings.

volume of oaks investigated is high enough that for most purposes a standard curve need not be constructed.

SUMMARY

A simple method is proposed for measuring root volume on the basis of Archimedes' Principle. Correlations with root dry weight suggest that this nondestructive method can replace dry weight measurements for quantifying root growth of an individual plant over time.

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INOCULATION OF HARDWOODS
WITH ASCOSPORES OF <u>TUBER MELANOSPORUM</u>:
SOME SIMPLE EXPERIMENTS

Chapter 4

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European workers have reported mycorrhizal synthesis from ascospores of <u>Tuber melanosporum</u> Vitt. in nonsterile pot culture with various Fagaceae and Betulaceae: <u>Quercus pubescens</u> Willd., <u>Q. pedunculata</u> Erhr., <u>Q. sessiliflora</u>, Smith, <u>Q. ilex</u> L., <u>Q. coccifera</u> L., <u>Fagus silvatica</u> <u>L.</u>, <u>Castanea sativa</u> Mill., <u>Tilia cordata</u> Mill. and <u>Corylus avellana</u> L. (Chevalier and Grente, 1979; Chavalier et al., 1973; Palenzona, 1969; Delmas and Poitou, 1979). We initiated similar syntheses with two species common in western Oregon and Washington, <u>Q. garryana</u> Dougl. ex Hook., the Oregon white oak, and <u>C. avellana</u>, the European filbert of commerce. Acorn yields of Oregon white oak vary highly from year to year (Silen, 1965); consequently, not all the experiments described could incorportate this species. The California blue oak, <u>Q. douglasii</u> Hook. and Arn., is also included in one experiment. The experiments were designed to determine if <u>T. melanosporum</u> could be obtained on these hosts regularly and reliably.

MATERIALS AND METHODS

Experiment I. Two methods of spore storage treatment by two fertility
levels.

For inoculation, spores of T. melanosporum are usually obtained
from fresh ascocarps surface-disinfected with alcohol and stratified
in moist sand at 5 C (Grente et al., 1972; Palenzona, 1969). However,
imported fresh truffles in this country, less fresh and more readily
decomposed by spoilage organisms, did not stratify well or keep their
integrity by this technique. We therefore tried our first
inoculations with spores from fresh truffles purchased in December and
stored for 3 months by (1) freezing one whole at -10 C and (2) keeping
a second one of similar appearance and ripeness in a plastic container
at 5 C. The latter one, although very soft and having an odor and
visual signs of spoilage, was still somewhat firm to the touch at the
end of the storage period. Microscopic examination showed most asci
intact.

Inoculum was prepared by homogenizing a truffle in a Waring
blender at high speed with an ice-water slurry for 5 min. Autoclaved
vermiculite was added and homogenized with the spores for another 5
min to produce a slurry containing 1.5×10^5 spores ml^{-1}. Calcium
carbonate, 2.5 per cent (w/v), was added to autoclaved potting mix of
50:50::sand:coarse vermiculite and a portion of the spore slurry
diluted in water was added with thorough mixing to give a final
density of 2.5×10^3 spores cm^{-3}.

Untreated Q. garryana acorns from Finley Wildlife Refuge near
Corvallis, OR were planted in the fall in a sandy loam and placed in
an unheated greenhouse, where they slowly germinated over the winter
months (germination was 81 per cent). In early March germlings with
unbroken taproots and first leaves not fully expanded were washed 20
min under running tapwater, soaked 10 min in Tween 20 solution (0.1
per cent, v/v) then washed again for 20 min in running tapwater.

Filbert nuts from the previous fall crop were stored at 2 C
unshelled in partially open plastic bags. Nuts were shelled by
squeezing the pericarp with a small pair of Vise-grips until it
cracked. The use of this tool greatly aids in keeping the seed coat

intact and preventing contaminants from lodging in the endosperm.
Seeds were washed for one hour with cold tapwater, soaked for 24 h at
2 C in 25 ppm gibberellic acid (GA-3, 90 per cent, Sigma Chemical Co.)
to hasten germination, surface-sterilized with 10 per cent H_2O_2 for
one hour, rinsed, covered with moist vermiculite and placed in a warm
greenhouse for germination which occurred in about 8 d.

Seedling roots and cotyledons were dipped in the spore slurry and
transplanted in the potting mix in 3" x 3" x 3" plastic pots and set
in the greenhouse without auxiliary lighting. A soluble complete
mineral formulation (Hewitt, 1966) was added at full strength, 50 ml
per pot, once every four weeks as the high fertilizer treatment level.
This corresponds to 10.5 mg N (as NO_3), 2.0 mg P (as PO_4), 9.8 mg K,
10 mg Ca, 1.8 mg Mg, 2.4 mg S (as SO_4) plus minor nutrients. A low
fertility regime consisted of the above-mentioned major nutrients
added at 25 per cent the high level plus minor nutrients at full
strength. Each treatment was replicated eight times and pots were
randomized on a greenhouse bench.

Hardwood mycorrhizae of T. melanosporum are highly branched and
have two unique identifying characteristics; the presence of
"spinules" (Palenzona, et al., 1972), trichome-like hyphal projections
extending radially from the mycorrhiza mantle, and a mantle with an
interdigitated cellular structure appearing not unlike a jigsaw puzzle
on squash-mounted slide preparations. In this experiment four methods
are compared for the extent of formation of such structures;
uninoculated seedlings were not germane to this objective, and
therefore not included.

Surviving plants (24 oaks, 30 filberts) were harvested in late
November and percentages of mycorrhizae determined by direct counting
of root tips. Plants showing any foreign mycorrhizae were discarded.
Because percentage data is binomially rather than normally
distributed, an arcsin(percent mycorrhizae)$^{1/2}$ transformation of the
data is recommended, especially if the data includes both very high
and low percentages; data was so transformed before proceeding with
the analysis of variance (Steel and Torrie, 1980).

Experiment II. Effect of liming.

The effect of lime on mycorrhiza formation was studied with three
hosts: Q. garryana, Q. douglasii, and C. avellana. Lime treatment
consisted of dolomitic lime added to pasteurized
sand:vermiculite:peat::5:4:1 at either 5 per cent (v/v) or not at all
(control).

Freshly fallen acorns with green pericarps of Q. garryana from
the 800 m elevation just south of Ashland, OR and Q. douglasii from
Red Bluff, CA were washed with cold tapwater for one hour. Acorns
that sank in the water were soaked in 10 per cent H_2O_2 containing a
small amount of Tween 20 surfactant for one hour and washed again with
cold tapwater for another hour. Seed was stored at 2 C in moist
coarse vermiculite in covered plastic containers over the winter.
Most Q. garryana and many Q. douglasii acorns germinated after a few
months in such conditions, and many grew radicals too long for
transplanting. In March ungerminated acorns were transferred to
greenhouse flats containing moist vermiculite, where most germinated
in two to three weeks. Filbert seed was germinated by the method
described in experiment I.

A truffle spore suspension stored on a vermiculite matrix at 2 C
for three months was resuspended in water and thoroughly mixed into
the potting mix at the time of addtion of the lime and fertilizer at
the rate of 2.5 x 10^3 spores cm^{-3}. Young germlings with radicals 1 to
10 cm long were dipped in a kaolin suspension of 1.5 x 10^5 spores cm^{-3}
and planted in 2.5" x 10" plastic "bullet tubes" (Deepots) containing
about 500 cm^3 of potting mix each. Twenty tubes with one germling
each were prepared per treatment. Soluble 20-19-18 NPK (Peter's
"Peat-lite Special") was applied as a drench at manufacturer's
recommended concentration for greenhouse plants (473 ppm N) once every
two weeks. Seedlings were harvested in late November after growing in
the greenhouse for one season with regular watering and pest control
with organophosphate insecticides. All seedlings weighing more than
2.0 g fresh and showing no evidence of contaminating mycorrhiza were
measured for colonization by T. melanosporum and growth. Mycorrhiza
colonization was determined in the top, middle, and bottom thirds of

the root system of each seedling with the following rating scale based on visual estimation by observation of rootlets under the binocularscope: no mycorrhiza, 0; 0-5 per cent, 1; 5-30 per cent, 2; 30-50 per cent, 4; 70-95 per cent, 5; and 95-100 per cent, 6. Fresh weight, stem diameter at the soil line, root volume by immersion to soil line (Michaels, 198_) and root volume to fresh weight ratio were determined for each seedling.

Experiment III. Effect of organic matter.

Organic matter may profoundly affect mycorrhiza formation, but the effect is little understood (Slankis, 1974) and should be studied to some degree for each fungus-host combination investigated. Generally, the presence of organic matter tends to promote mycorrhiza formation. An experiment to test three levels of organic material, added in the form of sphagnum peat, on growth and mycorrhiza formation of C. avellana was designed. Three pasteurized potting mixes were prepared having different rations of vermiculite (V), sand (S), and peat moss (P): mix A, 60V:20S:20P; mix B, 65V:25S:10P; and mix C, 70V:30S:0P. Each component was pasteurized separately prior to mixing to avoid interactions during heating. To each mix was added 5 per cent (v/v) lime and 1 per cent (v/v) Osmocote 18-6-12 NPK with 8 to 9 month release period. Each mix was inoculated with spores at the rate of 2.5 spores cm^{-3} by the method of experiment II, placed in the bullet tubes (experiment II) and randomized. Young C. avellana germlings were dipped in a kaolin-spore suspension, 1.5×10^5 spores ml^{-1}, planted one per tube, and watered in with a solution of Hewitt's minor nutrients. Due to severe infection of filbert by contaminating mycorrhizal fungi in the greenhouse in previous experiments, these treatments were placed outdoors under 50 per cent shade screen immediately after sowing (mid-May). Every two weeks seedlings were treated with a soil drench of minor nutrients and sprayed for pests with either diazanon or malathion as needed. Seedlings were harvested in mid-November and assayed for mycorrhizal colonization and growth by the methods described in experiment II, except that mycorrhizal colonization was evaluated for the top one-third vs. the bottom

two-thirds of the root system.

Experiment IV. Effect of soil inoculation vs. germling drench.

Mycorrhizal colonization is affected not only by the type of inoculum but also by where it is placed relative to seedling roots and especially in the case of spores, by the density of propagules (Alvarez, et al., 198_; Marx, 1976; Marx, et al.,1979; Theodorou and Bowen, 1971). Spores of T. melanosporum have been inoculated around the root ball at the time of transplanting from the germination bed (Chevalier and Grente 1979) or into the first 3-4 cm of soil around the seed at the time of planting (Palenzona, 1969). Results from experiment III showed that, at the spore concentrations used, germling dip together with soil inoculation produced a highly mycorrhizal seedling. To determine relative effectiveness of germling dip vs. soil inoculation vs. the two combined, a 2 x 2 experiment was conducted with all combinations of treatments.

Filbert seed germination, potting mix preparation, germling drenches and growing conditions were as described in experiment I and III, except that seedlings were inoculated in early June and harvested in early December.

RESULTS AND DISCUSSION

Experiment I. Two methods of spore storage treatment by two fertility levels.

Seedling feeder root colonization averaged 44.7 per cent over all treatments with no significant differences between treatments (Table XVII). At the inoculum levels employed, fresh, but putrefying truffles gave similar levels of mycorrhizal colonization, and normal levels of plant nutrients were adequate to obtain this level of infection. The nutrient level is probably not critical for oaks in the first season, because they have a large nutrient reservoir in

Table XVII

Effect of spore storage method and fertility on mycorrhizal colonization of $\underline{Q}$. $\underline{garryana}$

| Fertility level | Storage method | |
	Fresh (5 C)	Frozen (-10 C)
	per cent mycorrhiza	
High[1]	52.8[2]	46.7
Low	36.8	43.2

[1]High rate is Hewitt's minerals applied once every four weeks, 50 ml per seedling; low rate is similar except macronutrients plus Mg and SO_4 supplied at 25 per cent the high rate.

[2]No significant differences ($P \leq 0.05$) in per cent mycorrhiza between or within treatments by analysis of variance after transformation of per cent data to arcsin(per cent)$^{1/2}$.

the endosperm.

Indeed, Chevalier has obtained mycorrhizae of oaks with $\underline{T}$. $\underline{melanosporum}$ with no added fertilizer in the first season (personal communication), although the degree of colonization was not specified. Although treatments did not differ significantly, the overall results are encouraging. We have shown that spore inoculation can be a useful procedure for establishing $\underline{T.melanosporum}$ on Oregon white oak. A serious concern with the technique, though, is that colonization percentage was highly variable in all treatments: extreme values are 4 and 98 per cent.

A similar experiment with $\underline{C}$. $\underline{avellana}$ failed, in that contaminating mycorrhizal fungi (mostly $\underline{Thelephora}$ $\underline{americana}$ Lloyd as well as an ectendomycorrhiza with dematiaceous hyphae surrounding the mantle) colonized the roots to some degree in each pot and thoroughly colonized them in a few pots. The difference between $\underline{C}$. $\underline{avellana}$ and $\underline{Q}$. $\underline{garryana}$ in susceptibility to contaminants may be partially explained by the geometry of the root systems. The latter species sends down a strong taproot and forms most of its laterals and feeder

roots at the bottom of the pot, thereby keeping the roots somewhat isolated from air-borne propagules percolating through the soil mix; C. avellana roots, however, forms a network throughout the entire soil volume.

A similar experiment the following year with seed sources of Q. garryana from Ashland, Grants Pass, and Corvallis, OR gave similar results: no significant differences among the biotypes and a highly variable colonization rate.

Experiment II.   Effect of liming.

For Q. douglasii and Q. garryana, significantly more mycorrhizae developed overall in the lime treatment than in the control, although at each pot depth the differences were not significant (Table XVIII). No significant differences from liming were observed in growth parameters of Q. douglasii, although for Q. garryana overall growth, as well as root growth relative to the whole seedling is depressed in the limed treatments. It should be emphasized that on the basis of what subsequent experiments have shown, that the mycorrhizal ratings for both species are quite low. The poorer growth response of Q. garryana in lime treatments lessens its desirability as a truffle host, while the response of Q. douglasii is more encouraging.

As in experiment I, the filbert seedlings were overrun with contaminating mycorrhizal fungi, principally Thelephora americana, which was positively identified from the presence of sporocarps in the pots.

Experiment III.   Effect of organic matter.

Mycorrhizal colonization was excellent in the upper third of the root ball and slightly less so in the lower two-thirds (Table XIX). Per cent organic matter correlated with mycorrhizal rating at both soil depths, suggesting that the effect of peat on mycorrhiza formation may be more than just one of increased soil aeration.

Table XVIII

Ectomycorrhiza development and growth of two oak species inoculated
with T. melanosporum spores with or without lime[1].

Treatment[1]	Mycorrhiza rating[2]				Growth parameters			
	Top	Middle	Bottom	All	Fresh wt. (g)	Root vol (cm^3)	R.V. / F.W.	Collar dia. (mm)
				Q. douglasii				
+ lime	1.8a	1.1a	2.2a	1.9a	4.16a	2.63a	0.658a	2.03a
- lime	1.3a	0.8a	1.2a	1.1b	3.97a	2.75a	0.686a	1.71a
				Q. garryana				
+ lime	2.2a	1.5a	1.9a	1.9a	2.95a	2.23a	0.748a	1.99a
- lime	1.1b	0.9a	1.4a	1.1b	4.04b	3.00a	0.815a	2.63b

[1] Means not showing a common letter within a column for the
respective species differ significantly (P ≤ 0.05) by t-test.

[2] Rating scale of 0 through 6 by visual estimation of per cent feeder
roots mycorrhizal: 0, none; 1, 0-5 per cent; 2, 5-30 per cent; 3,
30-50 per cent; 4, 50-70 per cent; 70-95 per cent; 6, 95-100 per
cent.

Neither peat nor mycorrhiza levels significantly affected growth
of the host. However, total weights and root volumes were highly
variable in this experiment. But interestingly, the data indicated a
constancy of the ratio of the two from seedling to seedling, since the
of R.V.:F.W. is quite small (around 4-6 per cent of the mean).

Experiment IV. Effect of soil inoculation vs. germling drench.

As in the previous experiment, significant differences between
mean mycorrhizal ratings at various pot depths and treatments are
common, but again no significant differences appear between the means

of growth parameters (Table XX). Either the contaminant mycorrhizal colonization in the noninoculated control (-I, -D) was sufficient to induce a growth response or filbert seedlings may not respond to any level of mycorrhiza colonization.

Table XIX

Ectomycorrhiza development and growth of C. avellana inoculated with T.melanosporum spores at three levels of organic matter.

Treatment[1]	Mycorrhiza rating[2,3]		Growth parameters[3]			
	Top 1/3	Bottom 2/3	Fresh wt. (g)	Root vol. (cm^3)	R.V. F.W.	Collar dia. (mm)
A (20% P)	5.6a	5.4a	20.3a	16.2a	0.789a	6.0a
B (10% P)	5.5a	4.1b	18.4a	13.9a	0.766a	5.9a
C (0% P)	4.9a	3.4b	28.1a	22.6a	0.787a	6.1a

[1] A = 60% vermiculite, 20% sand, 20% peat. B = 65% vermiculite; 25% sand; 10% peat. C = 70% vermiculite; 30% sand.

[2] Rating scale of 0 through 6 by visual estimation of per cent feeder roots mycorrhizal: 0, none; 1, 0-5 per cent; 2, 5-30 per cent; 3, 30-50 per cent; 4, 50-70 per cent; 70-95 per cent; 6, 95-100 per cent.

[3] Means not sharing the same letter in each column differ significantly (P ≤ 0.05).

An analysis of variance shows that in the top third of the root system, the differences in mean mycorrhizal rating between germling drench treatments within uninoculated soil treatments are significant, but are insignificant in inoculated soil (Table XX; Fig. 8).

Analysis of the middle third of the root system shows that mean mycorrhizal ratings between germling drenches with and without spores are not significant, both within and among infestation treatments. For soil inoculation, however, significant differences appear between and within the germling drenching treatments. Also, if germlings are spore drenched there is no significant increase in colonization if the soil is inoculated with spores. However, in the middle and lower
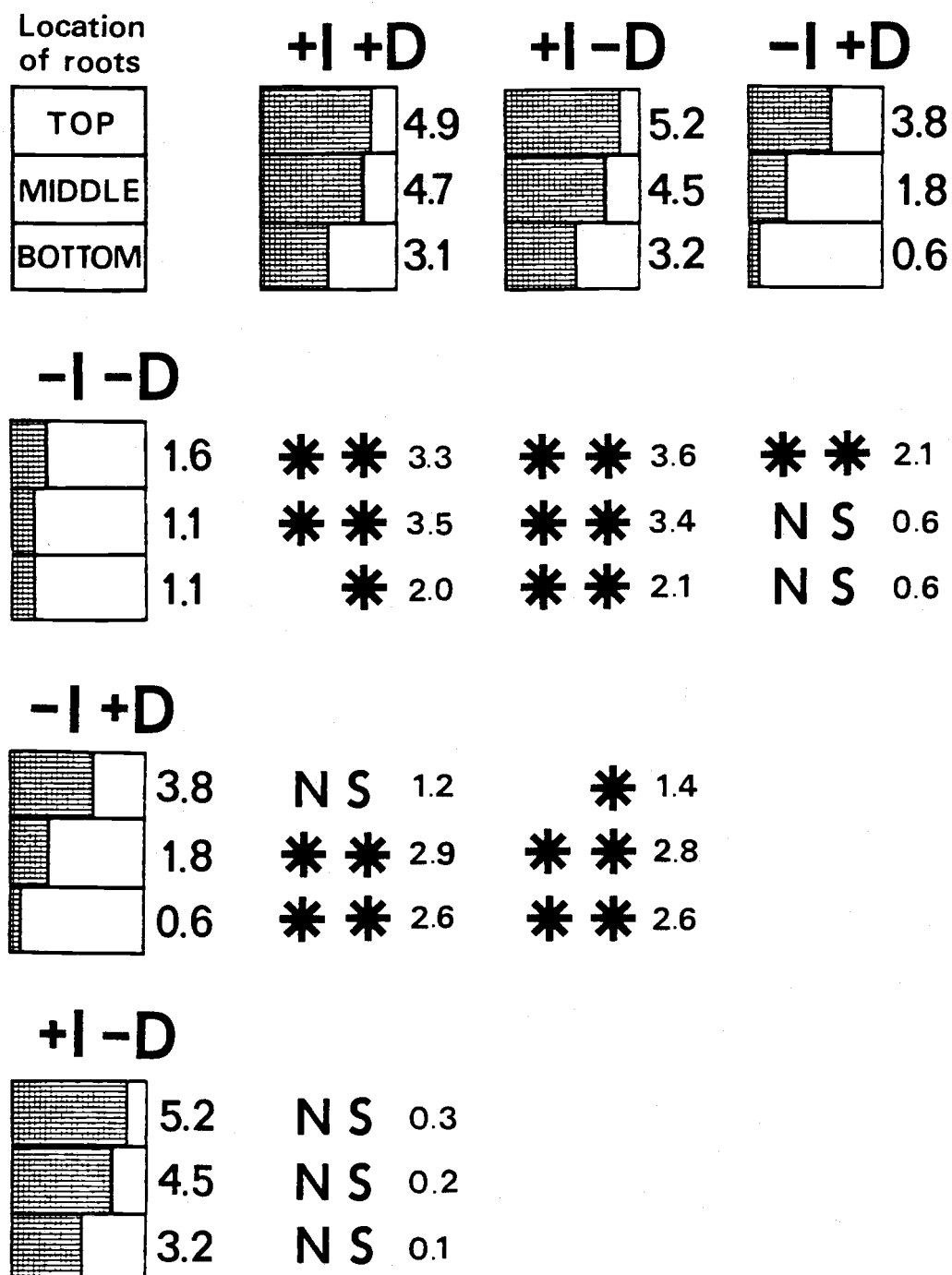
Fig. 8. Differences of mean mycorrhizal ratings at three pot depths between treatments in experiment 4. **, * denote significance, P≤0.01, P≤0.05, respectively, by t-test; NS = not significant.

thirds the soil inoculation does increase colonization, even in the germling drenching treatments, suggesting that the downward spread of the fungus from its initial colonization point near the germinated seed is limited. The two important conclusions from this experiment are that (1) spore inoculation of the soil alone produces good mycorrhization that is not improved by additionally drenching germlings with spores, and (2) spread of the mycorrhiza colonization from an inoculation area around a spore-drenched germling is limited to the upper few cm of the pot during the first year.

Table XX

Ectomycorrhiza development and growth of C. avellana inoculated with T. melanosporum spores in soil vs. germling drench.

Treatment[1]	Mycorrhiza rating[2,3]			Growth parameters[3]			
	Top	Middle	Bottom	Fresh wt. (g)	Root vol. (cm^3)	R.V. F.W.	Collar dia. (mm)
+I, +D	4.9ab	4.7a	3.1a	17.8a	12.3a	0.688a	6.8a
+I, -D	5.2a	4.5a	3.2a	17.4a	11.0a	0.615a	6.9a
-I, +D	3.9b	1.8b	0.6b	17.9a	11.9a	0.661a	6.9a
-I, -D	1.6c	1.1b	1.1b	18.7a	12.5a	0.681a	6.9a

[1] I = soil inoculated at 2.5×10^3 spores cm^{-3}; D = germlings coated with kaolin-spore slurry, 1.5×10^5 spores ml^{-1}.

[2] Rating scale of 0 through 6 by visual estimation of per cent feeder roots mycorrhizal: 0, none; 1, 0-5 per cent; 2, 5-30 per cent; 3, 30-50 per cent; 4, 50-70 per cent; 70-95 per cent; 6, 95-100 per cent.

[3] Means not showing a common letter within a column differ significantly (P ≤ 0.05) by t-test.

SUMMARY

The experiments reported here have demonstrated a few simple points about T. melanosporum spore inoculations. The spores remain viable between fall harvest and spring planting either by freezing ascocarps whole or by storing them moist at 5 C (allowing a certain amount of putrefaction to set in), or by dispersing ascocarp homogenate on vermiculite and storing at 2 C. Liming may be important for establishment of the mycorrhizae; although liming improves mycorrhization of Q. douglasii and Q. garryana, seedling growth is somewhat retarded by liming in the latter species. The effect of organic matter is minimal on C. avellana seedling growth and mycorrhiza development, although there is a trend toward more mycorrhization when some is present. Finally, soil inoculation at 2.5×10^3 spores cm^{-3} is effective for establishing the symbiosis but spore drenches of seedlings are less so.

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