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An Analysis of Growth and Respiratory Changes with Age of Mycelial Mats of Alternaria solani Ell and G Martin) L R Jones and Grout

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AN ANALYSIS OF GROWTH AND RESPIRATORY CHANGES WITH AGE OF MYCELIAL MATS OF *ALTERNARIA SOLANI* (ELL. AND G. MARTIN) L.R. JONES AND GROUT

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ABSTRACT

A fungal mat was developed to facilitate the study of cellular aging in the fungus Alternaria solani. The mat produced was analyzed for similarity of results within itself and in comparison to other mats of the same age using a nested analysis of variance design and various physiological responses, and to determine its qualifications as a biological tool. The growth pattern of the fungus on the mat, respiratory changes with age, and residual glucose and pH of the medium were determined.

The mycelial mat exhibited the characteristic four phase growth curve (lag, log, stationary, and death) during a 156 hour period. The residual glucose at the time of death showed 60% of the initial concentration still present. The pH decreased during the lag and log phases, stabilized during the stationary phase, and increased at the death phase.

Each mat was physiologically nonhomogenous and dissimilar to other mats prior to the onset of the stationary phase of growth. After the onset of the stationary phase, homogeneity, in most cases, was noted within mats but not between mats, indicating a new culture technique for use in the study of cellular age related changes of fungi.

The rate of respiration (QO_2), both endogenous and exogenous, of the A. solani mats decreased with age. A young mat showed significant differences between endogenous QO_2 and exogenous QO_2 at the same age, but as the age of the mat increased, the differences became insignificant. This agrees with previous work of fungal respiratory changes with age.

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INTRODUCTION

Age or aging has been defined many ways, notably, as the changes which occur in the post-reproductive period of an organism resulting in its decreased capacity for survival (23), or as the length of time a cell, tissue, or organism has existed as a distinct entity (8). Aging has also been found to be associated with functional changes in cellular processes, specifically, protein synthetic systems (9). Consequently, any multicellular organism whose growth can be defined; that has a decreased capacity for differentiation; that does not produce cell turnover within its tissue; and whose cell's age can be accurately determined is useful for studies on aging. Fungi adapt themselves well to these criteria. A fungal colony can be initiated from a homogeneous source, its growth is produced only at a hyphal tip (33), so that the age of the cells behind the tip can be precisely determined. In many cases, intercellular differentiation is limited to the production of reproductive structures. Porous septa provide cytoplasmic continuity between cells and thus allow transfer of metabolites, and hyphae also have the ability to undergo anastomosis. Essentially, fungi have the ability to produce undifferentiated cell populations of uniform ages.

Many biochemical and physiological aspects of aging have been studied on various fungi: most notably, morphological change (31,33), respiration (QO_2) (18,20, 24,27), phosphorous metabolism (30), nitrogen metabolism (3,18), biochemical changes (8), protein and protein synthetic changes (6,9,19), and changes in enzyme systems (14,27,32). These studies have varied in their procedures to allow harvesting of a large quantity of the organism at specific ages. They have thus varied in their precise ability to delimit specific ages on a cellular basis. The single cell preparation of Zalokar (33) is the most precise method, but it is limited by its small quantity for each harvest. Preparations on agar (9) and liquid media (3,6,8,18,19,20,24,27,32) increase the harvest but also introduce the problem of three dimensional growth and thus provide a less accurate estimate of the average age of any one subset of material, as it has been observed that there are biochemical differences between submerged and aerial hypha (34). This three dimensional growth has been significantly reduced by Gottlieb (6,8,27) due to Rhizoctonia solani's (Kuehn) growth characteristics on the liquid's surface, and by Yanagita and Kogane (30,31) by the introduction of a cellophane membrane between the medium and the organism, Aspergillus niger van Tiegh. However, even though the membrane eliminated submerged hyphal growth, Yanagita and Kogane's system was complicated by A. niger's differentiation after a period of time from pure vegetative hyphae into reproductive structures. A further qualification

is the range of age associated with the sampling of large quantities of hyphae. The age of a harvest would be the combined ages of the cells making up the harvest, thus the average age would be between the age of the youngest and oldest cells in a harvest, depending on the amount of branching. This interval of time would be carried within the system and would constitute the individual system's variability in age. Gottlieb's method of removing concentric rings from a point-inoculated colony has produced a minimum reproducible age and it has thus allowed studies to be made on the age changes related to a single fungal colony.

It would be most appropriate to utilize the best aspects of the above systems while eliminating those aspects which offer disadvantages to studies on aging in fungi. Thus a system should eliminate differentiation of any sort so that only vegetative growth occurs. It should eliminate submerged hyphal growth thus producing a mat as close to a monolayer of hyphae as possible. It should decrease as much as possible the range of ages associated with each sample, while keeping the quantity of sample available for harvest, at any one time, high. It should be a system in which no limitations are made on the amount of nutrients available and which does not allow metabolites and possible staling substances to build up.

To accommodate the above criteria in a biological tool for the study of cellular age-related changes rather than

colonial age-related changes, a modified system is introduced. The theory of unlimited growth in fungi has been questioned (5) so it is proposed that on a confined surface (petri plate) flooded with either spores or mycelial fragments of a fungus, the organism will grow to a certain point and stop, either as a consequence of the organism's self limitation or through physical or chemical barriers, or both. The time involved between the initiation of growth and the cessation of growth will be the greatest possible time a hypha could grow in a given medium, under a given set of conditions. Thus the average age of the total mycelium will be slightly less than 1/2 that time due to branching. If the hyphae are equated to the whole system, then the total system will have an average of slightly less than 1/2 the time involved between inoculation and growth cessation, as it is assumed that the cessation of growth in dry weight assumes the cessation of new cell production in this case. The average age would be "noise" carried in the system for as long as the system remained viable. In addition, if anastomoses were known to occur between adjacent filaments, each point on the mat could conceivably be continuous with all other points on the mat.

Alternaria solani, (Ell. and G. Martin) L.R. Jones and Grout, is a septate fungus belonging to the order Deuteromycetes. It was used in this new system for the study of changes related to age in fungi. It has been shown that conidia productions by A. solani can be inhibited (13)

and its growth requirements have been well worked out (11,12,15,16). It has also been shown that it has the ability to undergo anastomosis (22). The system, then, involves the vegetative growth of A. solani on a membrane covered agar, which eliminates submerged (three dimensional) growth, and thus allows the organism to undergo vegetative aging in the absence of differentiation. The purpose of this investigation is, in part, to examine A. solani grown on membrane covered agar for use as a possible biological tool for the study of aging by determining its growth in terms of dry weight changes, its respiration, and its ability to incorporate amino acids.

There has been a considerable amount of work involving QO_2 changes associated with fungal aging (18,20,24,27). In all cases noted, the predominant change was a decreased QO_2 with increased fungal age. This physiological response is not only useful as an age-related change in itself, but is also useful for subsequent tests on the functionality of a new system as a tool. Thus a further point in this research is the age related changes of endogenous and exogenous QO_2 of A. solani. The effect of unlimited nutrients and the accumulation of waste products was not considered to allow for a deeper study of the fungal mats produced by the organism within a static system.

MATERIALS AND METHODS

Alternaria solani, (Ell. and G. Martin) L.R. Jones and Grout, was obtained from stock cultures maintained on potato dextrose agar (PDA) in the laboratory of R.J. Lukens at the Connecticut Agricultural Experiment Station, New Haven, Connecticut. Throughout this study, continuous stock cultures were maintained on PDA slants stored at 3° C. Basic media employed in both liquid shake cultures and agar petri plates were composed of the following: ammonium sulfate 3.0 g; magnesium sulfate 0.25 g; glycine 1.0 g; monobasic potassium phosphate 3.0 g; thiamine-HCl 20 ug; niacin 20 ug; biotin 1.0 ug; d-inositol 200 ug; pyroxidine 10 ug; folic acid 10 ug; and distilled water to one liter. In addition, shake liquid cultures contained 20 g of glucose (2%) while the solid medium contained 50 g of glucose (5%) as well as 15 g of Bacteriological Agar (1.5%). The pH of all media was adjusted to 6.0 by the addition of 1.0 N KOH. Sterilization of all components was accomplished by autoclaving at 15 psi and 121° C for 15 minutes.

Stock shake cultures were produced by transference of the organism from stock PDA slants to 500 ml Erylenmyer flasks containing 200 ml of 2% glucose medium. After a

period of seven days, these shake cultures were removed and used for the inoculation of petri plates. Nine-cm petri plates containing 50.0 ml of basic 5% glucose medium and 1.5% agar were covered with sterilized cellulose xanthate membrane (Dupont PUD-o-193) in such a way that the membrane created a cup extending 2-5 mm up the sides of the interior of the plate in order to eliminate direct contact between the fungus and the medium. Air trapped between the membrane and the medium was eliminated.

Inoculum for this plate/membrane complex was prepared by removing 5.0 g wet weight of A. solani from a 2% shake culture and homogenizing it in 100 ml of chilled potassium phosphate buffer (pH 6.0) for 30 seconds in a Sorval Omnimixer (Ivan Sorval Inc., Newton, Connecticut) at 6000 RPM. The resultant slurry was then added to 50 ml of chilled buffer in a 300 ml tilting assembly and shaken vigorously. Eight ml of this slurry were dispensed on each plate/membrane complex. These were covered with standard Coors covers, and placed under a small fan set on a rheostat (The Superior Electric Co., Bristol, Connecticut) reading of 40.0 volts to evaporate excess water. This latter procedure will subsequently be termed "drying procedure". After a period of 48 hours, the plates containing developing fungal mats were removed, Coors covers were replaced by standard glass covers, and the cultures were incubated in a Percival Growth Chamber (Percival, Boone, Iowa) at 30° C with a continuous light

sequence. At various time periods between the initial inoculation (0 hr) and the termination of the experiment (156 hr) random plates (mats) were removed for study.

The growth of the organism was established by taking a mat every 12 hours for a period of 156 hours, removing nine #7 1 cm diameter cork bore discs, washing them three times in phosphate buffer, drying them at 60° C for 36 hours, and weighing them in sets of three on a Cahn electrobalance (Cahn Instrument Co., Paramount, California). Two additional discs were removed from the same mat and reinoculated into 125 ml Dulong culture flasks containing 25 ml of liquid basic medium containing 5% glucose. These were allowed to grow on a rotary shaker for 24 hours, harvested, washed with phosphate buffer, dried at 60° C for 36 hours, and weighed. This procedure gave an indication of the ability of the fungus to promote regrowth and was calculated as the percent increase in dry weight. Regrowth, as used here, is defined as an increase in dry weight of a disc over the original weight of a disc after a 24 hour incubation in fresh growth medium containing 5% glucose. It is recognized that this is an inaccurate measure of viability as one is not sure exactly where new growth is being originated, but it can give one some idea of the relative viability of the cultures at different ages. The remaining original mat was washed, dried, and weighed. The medium of each of the plates was measured to determine its pH and residual glucose concentration. The pH was determined on a Corning pH meter by inserting the electrodes

directly in the medium. The residual glucose was removed from the agar by dicing the agar, inserting it into 200 ml of distilled water, shaking it for one hour, and then filtering the water into a collecting vessel. This procedure was repeated until the agar had been rinsed with a total of 1,000 ml of water. Residual glucose removed by this procedure was determined by the Anthrone procedure of Scott and Melvin (4) using D-glucose as a standard. Known standard glucose agar preparations were run through this procedure and it was established that 90% of the glucose was removed. Agar medium without glucose was examined and found to have no significant affect on the test. The average growth curve was determined by combining the data from six separate experiments. The initial weight of inoculum of each replicate was determined by collecting hyphae from three individual 8 ml samples of the original slurry in centrifuge tubes, drying them at 60° C for 36 hours, and weighing them. From this weight, and the ratio of the weight of a disc to the weight of the whole mat, the weight a corresponding disc would have weighed at zero time was calculated and used for the initial weight in the growth curve. At no time in this procedure, nor in any of the following procedures, was a disc removed within 5 mm of the edge of the mat. For the determination of similarity within and between mats at any one time, the nested (hierarchal) analysis of variance design (21, 28) was employed. Dry weight measurements were incorporated

into this design by taking four discs from each of four sections within each of eight mats at the 72 hour period, weighing them and determining the components of variance.

Respiratory changes with age were made by taking a whole mat, randomly removing nine discs, rinsing them in phosphate buffer, and introducing three discs into each of three 15 ml Warburg flasks. A mat from each inoculation was measured at each 24 hour interval for as many intervals as possible. Measurements were made every 24 hours from 36 hours to 156 hours using standard Warburg techniques at 30° C (25). The assay systems always included 2.4 ml of potassium phosphate buffer, 0.1 ml of 10% KOH (center well) and 0.5 ml of basic 2% glucose medium (side arm). Each run was conducted on two mats of different ages and different inoculations with three replicates for each mat. Because of the high endogenous oxygen consumption associated with fungi (2), the preparations were starved for 30 minutes, followed by a 1.5 hour reading of endogenous QO_2. This latter was then followed by a 1.5 hour reading of exogenous QO_2 after the addition of 2% glucose substrate from the side arm. At the termination of each run, the discs were removed, washed, dried at 60° C for 36 hours, and weighed. The total number of replicate mats for each time interval varies because of the increased incidence of contamination associated with the increase in the time the mats were incubated. Concurrent with the above measurements of age related QO_2 changes, the statistical

nested design was run. This involved three QO_2 readings from each of three sections within each of six mats for each of the 48 and 120 hour samples. Both endogenous and exogenous QO_2 were measured with this design.

Measurements on the incorporation of amino acids using the nested design were made with L-phenylalanine-U1-C^{14} (International Chemical and Nuclear Corp., Irvine, California). Number 7 discs were removed from the mats and incubated in small syracuse dishes containing 9.5 ml of potassium phosphate buffer to which was added an additional 0.5 ml of 1.0 uc/ml L-phenylalanine-U1-C^{14} (total volume 10.0 ml). The cultures were incubated at 30° C for 60 minutes on a reciprocal shaker. The reaction was terminated by the addition of 10 ml of cold 5% trichloroacetic acid (TCA). The discs were then washed twice for 20 minutes in 10 ml of cold 5% TCA and twice for 20 minutes in hot 5% TCA. After drying and weighing, they were flattened by pressing "scotch tape" on one side and allowing them to adhere sufficiently to produce a more rigid uniform surface. The discs were placed on planchets and counted for five minutes with a gas flow counter using Geiger gas (Tracerlab, Waltham, Massachusetts) and a flow rate of 70%. Counts per minute (CPM) were determined for each disc and equated to cpm/mg dry weight. The nested design for this procedure involved four discs from each of four sections within each of four mats for each of the 48 and 120 hour samples. It was noted that the presence

of glycine seemed to hinder the rate of incorporation of phenylalanine as did the use of a 5% basic medium, so these incubation media were discarded in favor of phosphate buffer alone. In addition to the nested results, the average uptake for the two sample times was determined.

RESULTS

A. solani grew from the initial randomly placed mycelial slurry to form a uniformly thick (2-3 mm) fungal mat on the surface of the membrane. Most of the thickness was produced during the 48 hour period of drying and was accompanied by a large production of aerial hyphae. After transfer of the mats to the incubation chamber, each mat showed individual morphological changes. These changes took the form of pigmentation and were manifest in such a way that some mats would be darkly pigmented at the same time other mats from the same inoculation would be translucent. During this period, most aerial hyphae became incorporated horizontally into the body of the mat. Throughout the period of incubation, observable changes in the coloration of the agar below the membrane were noted, changing from an opaque to a slightly yellow tinge. Throughout the entire experiment, neither conidiation nor membrane digestion was noted, nor was there any observable difference between points within a single mat, except at the periphery where a thinning of the mat could be detected.

Because the growth characteristics of the fungus in the system are of prime importance, a growth curve

was determined to establish the time at which each of the four phases of growth (lag, log, stationary, and death) occurred. It should be noted here that the initial weight of the inoculum introduced on the membrane from different subcultures showed no significant differences between inoculations (Table 1). It has been shown by others that changes in DNA concentration could be used as a criterion for increase or decrease in cell number (7). It has also been shown in this laboratory that the total concentration of DNA in A. solani follows the growth curve (17); so it is reasonable to assume that the initiation of the stationary phase of growth corresponds to the cessation of cell reproduction. This may in fact occur prior to stationary phase (7) but, in this report, stationary growth will be equated to a stationary cell population. It can be seen from the results (Figure 1) that the growth of A. solani in this system shows the four characteristic phases. Lag phase occurs from 0-12 hours, log phase from 12-72 hours, stationary phase from 72-144 hours, and death phase after 144 hours. It should also be noted that there is a marked increase in the variation about the means as age increases, indicated by 95% confidence intervals about those means. Concurrent with the growth curve, residual glucose and pH changes in the media were measured (Figure 2). There was a gradual decrease in residual glucose throughout the experimental period, but at no time did the glucose level fall below 60% of the initial

TABLE 1

ANALYSIS OF VARIANCE FOR THE ORIGINAL INOCULATIONS (29)

Source of Variance	df	Mean Square	F
Inoculations	5	5.8026	1.01^{ns}
Within Groups	12	5.7305	

ns = not significant at the 0.05 probability level.

FIGURE 1

The growth of Alternaria solani mats for 156 hours indicated by the dry weight of replicate one-cm discs removed from the mats. Variation is indicated by 95% confidence intervals.

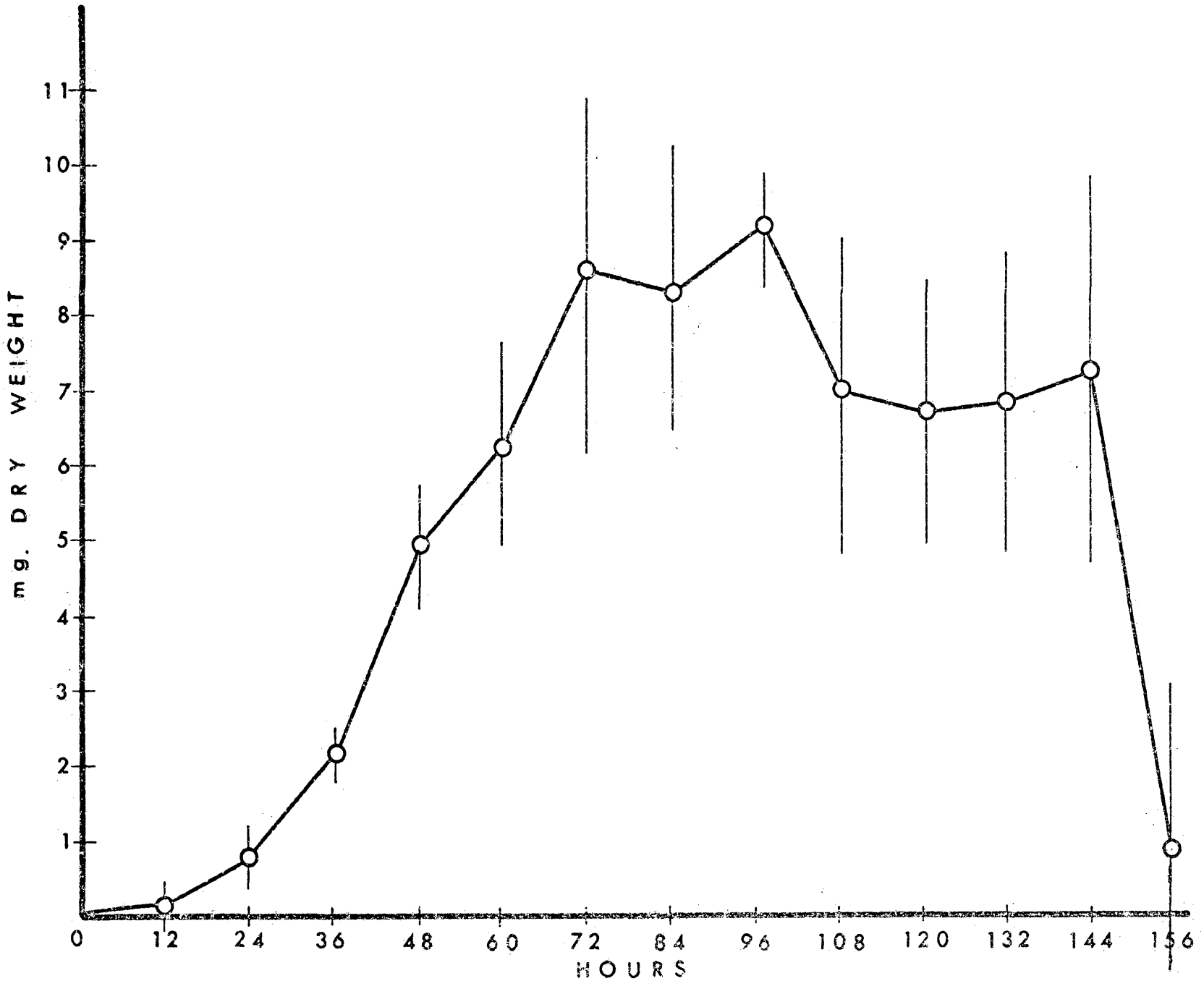
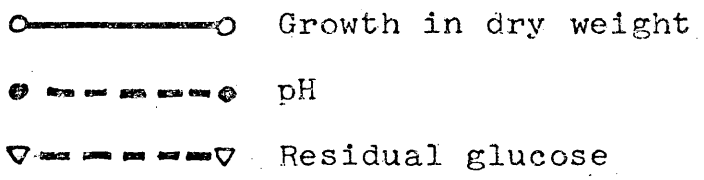
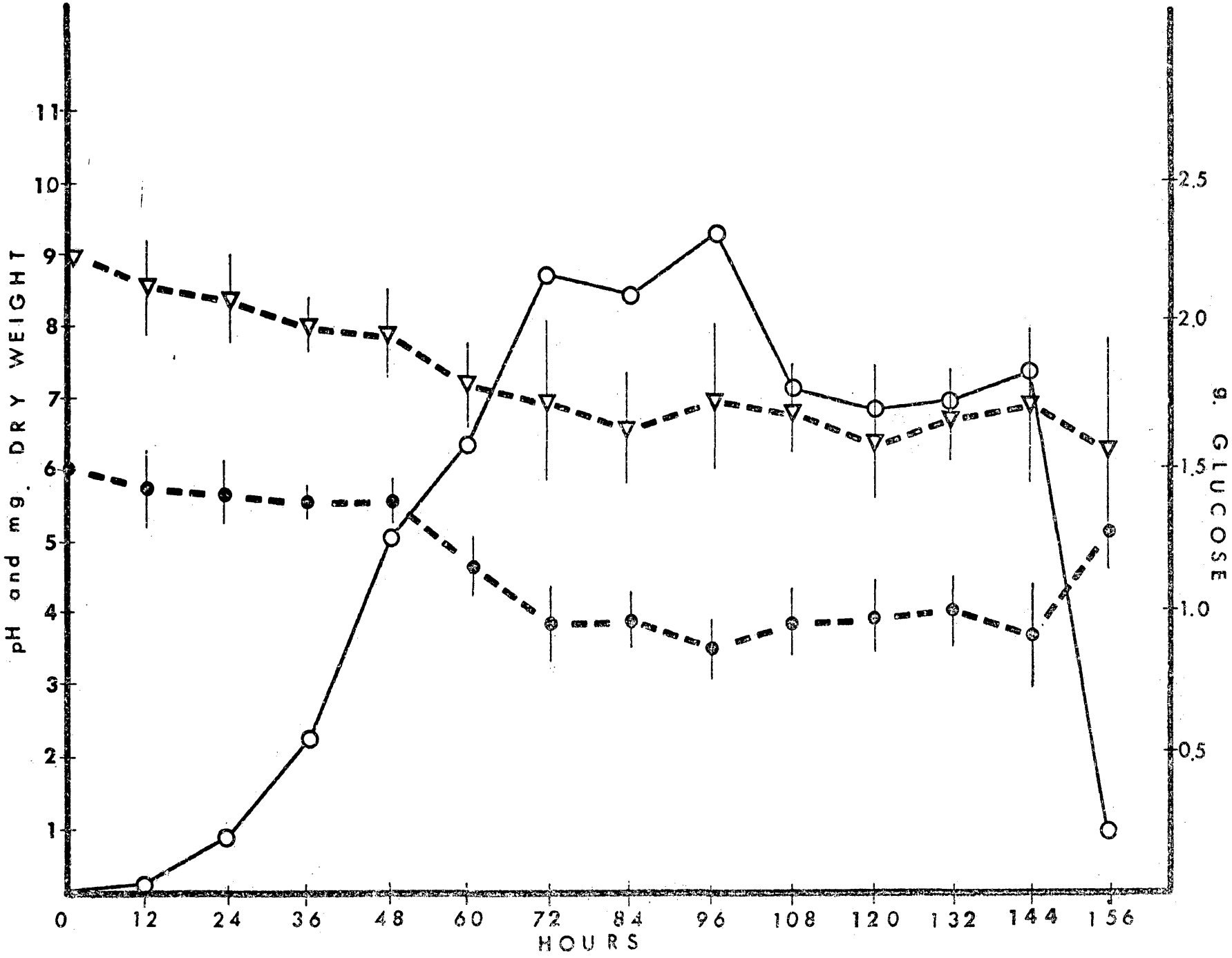

mg. DRY WEIGHT
11
10
9
8
7
6
5
4
3
2
1
0
12
24
36
48
60
72
84
96
108
120
132
144
156
HOURS

FIGURE 2

A comparison of the growth of Alternaria solani mats with residual glucose concentration and pH of the medium at the corresponding times. Residual glucose was determined by the Anthrone procedure and pH by direct insertion of electrodes into the medium. Variation is indicated by 95% confidence intervals.




pH and mg. DRY WEIGHT
g. GLUCOSE
HOURS
0
12
24
36
48
60
72
84
96
108
120
132
144
156
1
2
3
4
5
6
7
8
9
10
11
0.5
1.0
1.5
2.0
2.5

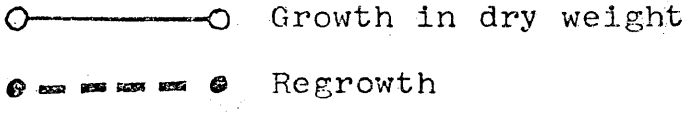
concentration. This suggests death was not caused by a deficiency in carbon or energy storing compounds. It is difficult to tell if this residual glucose was vertically stratified in the petri dish. From an initial of 6.0, pH decreased gradually for 48 hours, at which time a sharp drop occurred to a low pH of about 3.1 at 96 hours. From 72 hours to 144 hours it stayed relatively constant. It is seen during the death phase (144 hours and beyond) that the pH increased to 5.0. Apparently, there is an inverse relationship which exists between pH and dry weight. That is, as the dry weight (growth) increases, pH decreases; but as dry weight decreases (as in death), the pH increases.

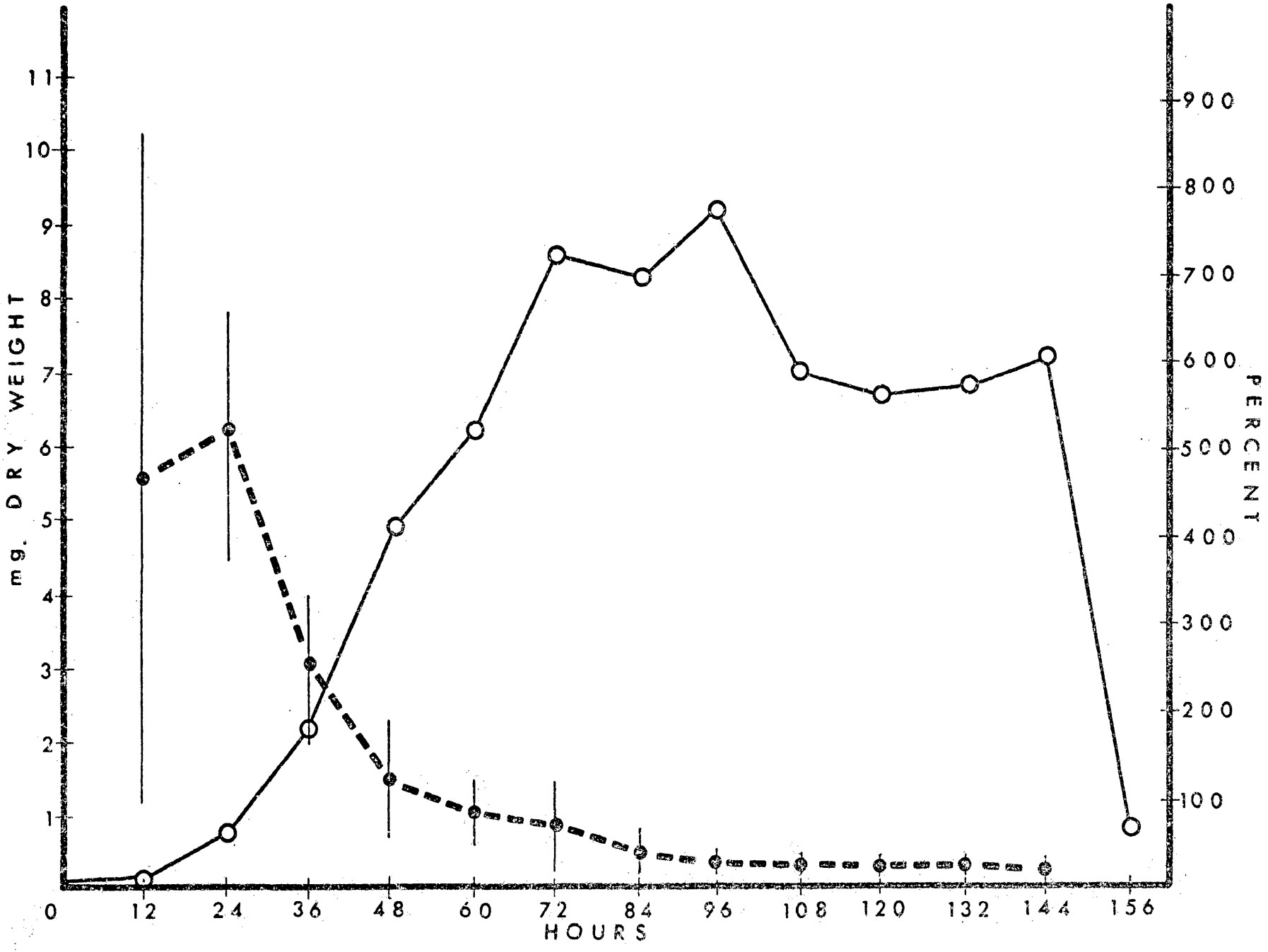
Viability studies (Figure 3) are reported as the percent increase in dry weight of a disc over the original dry weight of a disc during a period of 24 hours in a fresh growth medium. In essence, these studies show the mat's ability at the time of harvest to promote regrowth (ie. generate new growth in fresh media). Results indicate a definite decrease in the rate at which new growth is promoted by samples taken from 12 hour to 72 hour cultures. Beyond this, the rate at which new growth is promoted stays relatively constant at around 25%. Readings at 156 hours were not made so one is unable to tell if this decrease is due to an increase in the number of dead cells, or rather a real decrease in a viable cell's ability to initiate branching and, thus, regrow.

Homogeneity, ie. physiological similarity between and within mats, was measured by various physiological

FIGURE 3

A comparison of the growth of Alternaria solani mats with the percent of regrowth of similar discs taken at the indicated times and allowed to grow in a 5% basic medium for 24 hours. Variation is indicated by 95% confidence intervals.




mg. DRY WEIGHT
PERCENT
HOURS
0
12
24
36
48
60
72
84
96
108
120
132
144
156
1
2
3
4
5
6
7
8
9
10
11
100
200
300
400
500
600
700
800
900

criteria using a nested analysis of variance design. Homogeneity in this context is not synonymous with physiological unity. This design not only enables one to determine whether an added variance component shows a significant change in the system, but also allows one to determine at what point in the system the greatest percentage of variability within the total system is introduced. This allows one to determine, using a physiological response, whether a single fungal mat responds at all points in a similar manner and whether replicate mats respond with insignificant variability. The expected mean square expressions add a variance component to each of the levels as the levels of the design increase. Thus the total variance includes a random variance component or within section variance(σ_e^2), a variance component from between sections within a mat (σ_s^2), and a variance component from between mats (σ_m^2). This allows the precise determination of each component of variance as a percent of the total variance associated with the system.

In determining points of time during the growth of the mat which would produce the most interesting results in conjunction with the determination of the components of variance of the procedure, the times of 48 hours (middle log phase) and 120 hours (middle stationary phase) were chosen. Hypothetically, any difference between or within mats would be magnified during the log phase of growth simply because of the great amount of change associated

with short time intervals in this stage. Samples measured at 120 hours would enable one to determine whether these differences were continued after growth and reproduction had ceased, or if they had diminished significantly, both between or within mats.

The physiological responses used were endogenous and exogenous oxygen consumption (QO_2), dry weight, and incorporation of C^{14} labeled phenylalanine after cold and hot TCA washings. Due to the various techniques involved, the most precise response was that of QO_2, so more emphasis will be placed on this criterion. It can be seen in Tables 2 and 3 that both endogenous and exogenous QO_2 measurements at 48 hours showed significant differences between and within mats, but in both cases, 94.7% and 51.3% of the respective variances in the total systems were introduced by the variance component incorporated by the difference between mats. It should also be noted that there was a greater similarity between the random variance (σ_e^2) and the variance between sections (σ_s^2), than there was between either of these and the variance between mats (σ_m^2). Tables 4 and 5 show the QO_2 results at 120 hours. Here there seems to be a loss of significance for endogenous and exogenous QO_2 between sections within a mat ($F=2.07$ and $F=1.39$, respectively) but a comparison between mats still shows significant differences ($F=199.$ and $F=450$). This is further documented by the fact that in both cases over 95% of the total

TABLE 2

ANALYSIS OF VARIANCE NESTED DESIGN OF 48 HOUR ENDOGENOUS RESPIRATION (21,28)

Source of Variance	df	Mean Square	F	Expected Mean Square Expressions
Mat	5	155.78173	71.1**	$\sigma_e^2 + \sigma_s^2 + \sigma_m^2$
Section	12	2.19041	6.54**	$\sigma_e^2 + \sigma_s^2$
Within Section	36	0.33491		σ_e^2

COMPONENTS OF VARIANCE

Source	Component	Percent
Mat	σ_m^2	94.7
Section	σ_s^2	3.4
Within Section	σ_e^2	1.8

** = significant at the 0.01 probability level.

TABLE 3

ANALYSIS OF VARIANCE NESTED DESIGN OF 48 HOUR EXOGENOUS RESPIRATION

Source of Variance	df	Mean Square	F	Expected Mean Square Expressions
Mat	5	18.80289	6.18**	$\sigma_e^2 + \sigma_s^2 + \sigma_m^2$
Section	12	2.94324	2.83**	$\sigma_e^2 + \sigma_s^2$
Within Section	36	1.03715		σ_e^2

COMPONENTS OF VARIANCE

Source	Component	Percent
Mat	σ_m^2	51.3
Section	σ_s^2	18.4
Within Section	σ_e^2	30.1

** = significant at the 0.01 probability level.

TABLE 4

ANALYSIS OF VARIANCE NESTED DESIGN OF 120 HOUR ENDOGENOUS RESPIRATION

Source of Variance	df	Mean Square	F	Expected Mean Square Expressions
Mat	5	201.32734	199.6**	$\sigma_e^2 + \sigma_s^2 + \sigma_m^2$
Section	12	1.08824	2.07*	$\sigma_e^2 + \sigma_s^2$
Within Section	36	0.48601		σ_e^2

COMPONENTS OF VARIANCE

Source	Component	Percent
Mat	σ_m^2	91.7
Section	σ_s^2	0.7
Within Section	σ_e^2	2.1

** = significant at the 0.01 probability level.
* = significant at the 0.05 probability level.

TABLE 5

ANALYSIS OF VARIANCE NESTED DESIGN OF 120 HOUR EXOGENOUS RESPIRATION

Source of Variance	df	Mean Square	F	Expected Mean Square Expressions
Mat	5	448.69438	450.0**	$\sigma_e^2 + \sigma_s^2 + \sigma_m^2$
Section	12	0.99539	1.37ns	$\sigma_e^2 + \sigma_s^2$
Within Section	36	0.72203		σ_e^2

COMPONENTS OF VARIANCE

Source	Component	Percent
Mat	σ_m^2	98.4
Section	σ_s^2	0.1
Within Section	σ_e^2	1.4

** = significant at the 0.01 probability level.
ns = not significant at the 0.05 probability level.

variance is produced by the between mat variance component. This suggests that during the stationary phase, the differences within mats are removed but that differences between mats are carried through. This point is further shown by the fact that at 72 hours (Table 6) a dry weight analysis shows no significant variation between sections within a mat ($F=0.95$), but the between mat variance component is quite significant ($F=18.7$). Again, the percentage of each component supports this conclusion. Tables 7 and 8 show results produced through the incorporation of C^{14} labeled phenylalanine at 48 hours and 120 hours after hot and cold TCA washings. The results at 48 hours show no significant differences both within and between mats ($F=2.08$ and $F=2.42$, respectively), while at 120 hours both within and between mat variance was significant ($F=5.12$ and $F=17.5$). This trend looks like a complete reversal to that found in QO_2 at both 48 hours and 120 hours, but it should be noted that in both 48 hour and 120 hour incorporation samples the variance component introduced between mats still represents 79% or greater of the total variance. Again, a comparison of the variance within a section to that between sections shows a greater similarity than when either is compared to the between mat variance. In this report, less importance has been given to the results of phenylalanine incorporation. In essence, the homogeneity results seem to show that points within mats tend to become more similar as the

TABLE 6

ANALYSIS OF VARIANCE NESTED DESIGN OF 72 HOUR DRY WEIGHT ANALYSIS

Source of Variance	df	Mean Square	F	Expected Mean Square Expressions
Mat	7	8.38457	18.7**	$\sigma_e^2 + \sigma_s^2 + \sigma_m^2$
Section	24	0.44796	0.95ns	$\sigma_e^2 + \sigma_s^2$
Within Section	96	0.46930		σ_e^2

COMPONENTS OF VARIANCE

Source	Component	Percent
Mat	σ_m^2	65.2
Section	σ_s^2	0.0
Within Section	σ_e^2	34.7

** = significant at the 0.01 probability level.
ns = not significant at the 0.05 probability level.

TABLE 7

ANALYSIS OF VARIANCE NESTED DESIGN OF 48 HOUR PHENYLALANINE INCORPORATION

Source of Variance	df	Mean Square	F	Expected Mean Square Expressions
Mat	3	880,841	2.42 ns	$\sigma_e^2 + \sigma_s^2 + \sigma_m^2$
Section	12	362,912	2.08 *	$\sigma_e^2 + \sigma_s^2$
Within Section	48	173,993		σ_e^2

COMPONENTS OF VARIANCE

Source	Component	Percent
Mat	σ_m^2	82.6
Section	σ_e^2	4.6
Within Section	σ_e^2	12.7

* = significant at the 0.05 probability level.
ns = not significant at the 0.05 probability level.

TABLE 8

ANALYSIS OF VARIANCE NESTED DESIGN OF 120 HOUR PHENYLALANINE INCORPORATION

Source of Variance	df	Mean Square	F	Expected Mean Square Expressions
Mat	3	10,519,968	17.5**	$\sigma_e^2 + \sigma_s^2 + \sigma_m^2$
Section	12	616,681	5.12**	$\sigma_e^2 + \sigma_s^2$
Within Section	48	120,360		σ_e^2

COMPONENTS OF VARIANCE

Source	Component	Percent
Mat	σ_m^2	79.3
Section	σ_s^2	11.9
Within Section	σ_e^2	8.6

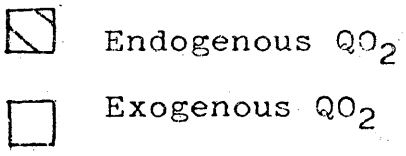
** = significant at the 0.01 probability level.

growth curve approaches stationary phase, but different mats inoculated at the same time do not show the same trend and are thus the greatest source of error. This latter point suggests that each mat is an entity unto itself and its total growth with respect to time is not comparable to that of all other mats. This is further confirmed by the large variability associated with each mean in the growth curve.

Figure 4 shows the QO_2 changes associated with fungal mats ranging in age from 36 hours to 156 hours. It is apparent that endogenous and exogenous QO_2 decreases as the age of the mat increases. It is also apparent that the endogenous and exogenous QO_2 of a mat at any one age becomes increasingly similar as the age of the mats increase. In fact, from 60 hours to 156 hours there is no significant difference between endogenous and exogenous QO_2 at any one hour based on the 95% confidence intervals. It is interesting that the QO_2 changes with age are correlated with the changes occurring in the viability studies evident in Figure 3. It should also be noted (Table 9) that the average rate of incorporation of phenylalanine seems to decrease from 3594 ± 300 cpm/hr at 48 hours to 1198 ± 392 cpm/hr at 120 hours. However, this is based only on two sample times and therefore, may not be an accurate picture of the actual changes associated with fungal age.

FIGURE 4

The relationship of fungal mat age of Alternaria solani and QO_2 in the presence and absence of glucose. Discs were starved for 30 minutes, endogenous respiration measured for 1.5 hours followed by the addition of 2% basic glucose medium and a 1.5 hour exogenous QO_2 determination. Variation indicated by 95% confidence intervals.



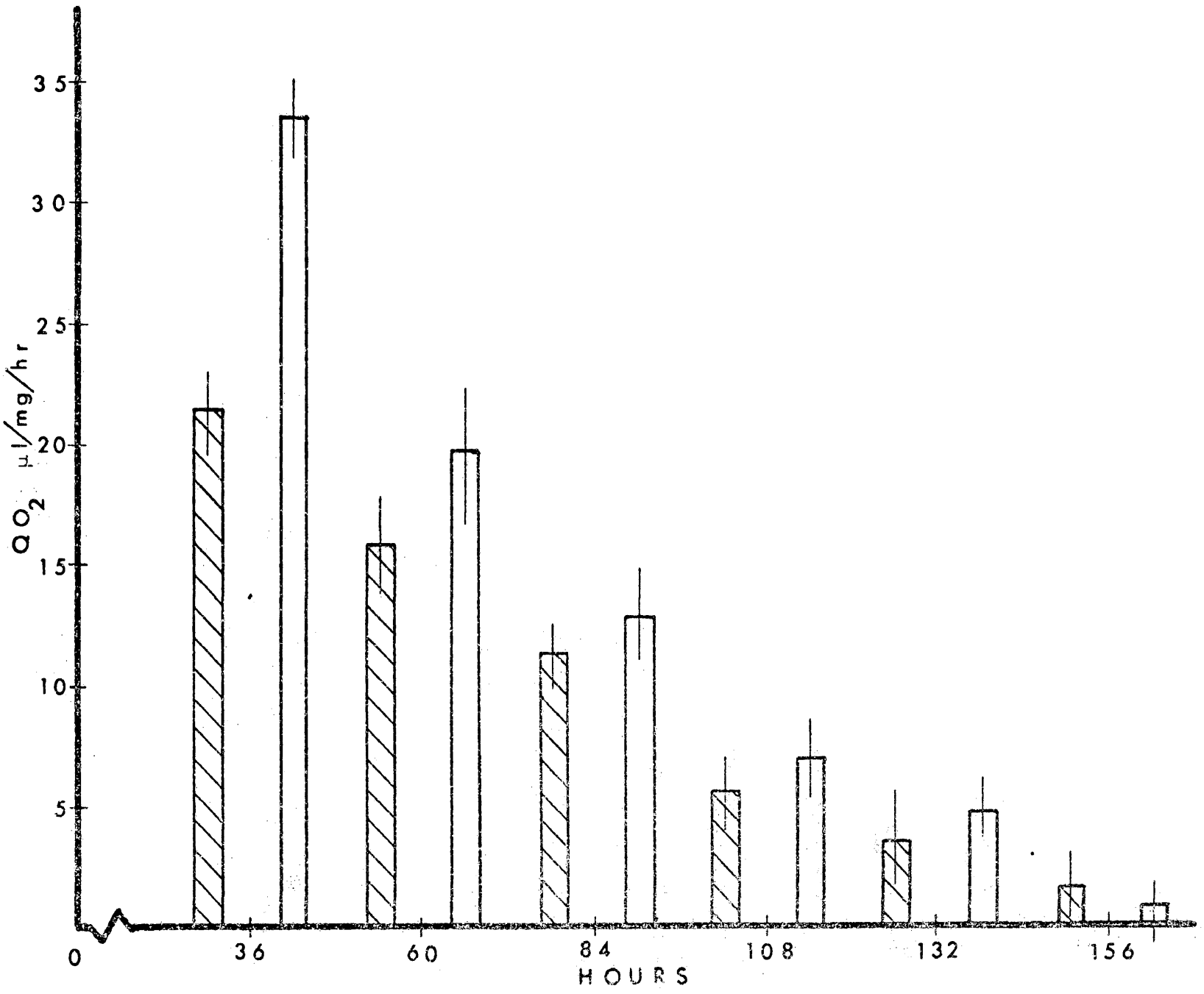

35
30
25
20
15
10
5
0
Q_{O_2} µl/mg/hr
36
60
84
108
132
156
HOURS

TABLE 9

48 HOUR AND 120 HOUR PHENYLALANINE INCORPORATION

WITH 95% CONFIDENCE INTERVALS

Hour	Mats	CPM/Hour/Disc
48	4	3594 ± 300
120	4	1198 ± 392

DISCUSSION

An analysis of the mats produced using membrane covered agar and A. solani can be broken down into two categories; what happens on a mat and what happens between mats. The former involves the actual growth characteristics of an A. solani mat in the single confined area of a petri dish; the latter involves the determination of growth and subsequent age characteristics of two or more distinct mats with respect to their similarity at a specific time after inoculation. The growth characteristics of an individual unit in the system directly determines whether cellular aging (aging of uniform cell populations) or colonial aging (aging of a complete fungal colony) is being observed.

It seems logical that three types of units could be formed through the characteristics of A. solani in particular and fungi in general. Noting that anastomosis does occur in A. solani (22), it is quite possible that during the aging procedure, the growth of the fungus includes a great percentage of hyphal fusions between adjacent hyphae. This system would then be a morphologically continuous unit from one point to another. Assuming also that a bidirectional translocation of materials through porous septa could occur (2,10), the biochemical and thus, physiological characteristics of each cell in a hypha

would be more similar than one would find in cells of a single hyphal strand or a point inoculated colony and one could then call this system a continuum of fungal cells. Yanagita and Kogane, using A. niger, compared a mat produced by a random spore dispersion with a unit produced from a point inoculation and found this to be the case (31). Buller, using Coprinus sterquilinus Fr., noted that during its development its hyphae would undergo anastomosis and produce only one reproductive structure (1). Thus, one possibility is a mat of interconnected cells which could act as a single physiological unit (System I). In such a system there would be minimal hyphal tips present at the time of growth cessation.

At the opposite end of a range of possibilities, the unit mat could undergo little or no anastomosis (System II). Each fragment from the mycelial slurry inoculum would produce a distinct microcolony and the mat would consist of a fixed number of discrete staled microcolonies randomly dispersed on its surface. Observations on this mat would show changes related to microcolonies, not to a continuum of cells, so that during growth and at cessation of growth, all microcolonies in System II would be at the same relative stage of development and the results would mimic a single colony. At the cessation of growth there would be a large number of staled hyphal tips, unlike that found in System I. This resultant system would be somewhat similar to that observed by Gottlieb

and Van Etten (8), in that colonial fungal aging would be observed.

Having taken both extremes of a possible range, the third possibility is a midpoint between the two (System III). This would show the properties of anastomosis and staling and would be the most complicated on which to determine the meaning of subsequent observations, as neither colonial aging nor cellular aging phenomenon would predominate.

The term age, and subsequent observations of aging on each of these possibilities should be clarified. In System I the age related changes of cells would be of primary interest after growth had ceased and the intercellular mixing through bidirectional translocation had occurred. Hypothetically, this would not occur completely until the unit as a whole had attained a stationary period of growth. Hence, the initiation of the stationary period of growth would be the point at which the mat would be considered to be at physiological age time zero. On the other hand, a staled culture of microcolonies (System II) would consist of a mixture of old and young cells up to the onset of stationary phase similar to that found in Gottlieb's point inoculated culture; but after stationary onset, this similarity would cease. The age related changes in this system would be those of the colony as a whole, and would be initiated at the time of inoculation. The observations would show the influence of the growing tips on the older cells and vice versa. The range of fungal

ages in System II would get progressively greater during the lag and log phases of growth so that the accuracy for any age would get progressively less precise. During the stationary period of growth, this "noise" would continue for as long as the unit existed. The observations would be those of colonial age, not cellular age. A culture which exhibits both the properties of staling and anastomosis (System III), would have hypothetically both types of aging interrelated.

Homogeneity studies showed, for the most part, that prior to stationary phase sections within mats differed, but during stationary phase these sections did not differ. This does not allow one to distinguish between Systems I and II. In System I, homogeneity would result from cytoplasmic continuity, whereas, in System II, the lack of apparent homogeneity would be a result of the dispersal of discrete microcolonies and the random assortment of intermixed hypha of all ages over any one point on the mat. In order to detect differences between Systems I and II, increasingly smaller samples would have to be taken.

The 72 hour period up to the stationary period of growth introduces a large error in either system due to its duration when compared to the total time of 156 hours of mat existence. If System I existed, the study of aging should commence after the cells have stopped reproducing and completed undergoing anastomosis. This mixing of

cellular constituents would involve a range of cells from zero to something less than 72 hours. If a staled system of microcolonies existed (System II), this 72 hour range of cellular ages would constitute the average range of age of a sample of cells in the system after growth had ceased. This range, even though the absolute value would increase, would never be lost from the system. There would always be a problem associated with this range, unless the mat remained viable for an extended period of time. A staled culture (System II) grown in this manner to show colonial aging is not as precise as that grown by the method of Gottlieb and Van Etten (8). Death would seem to occur in this system as a gradual phenomenon of individual microcolonies, but in the cellular system (System I) as a simultaneous cellular phenomenon. Death did seem to occur simultaneously as seen in the growth curve results and because at 156 hours there is such a drastic drop in the growth curve, this tends to suggest that the system was a continuum of cells. However, the fact that the pH and color of the media changed, indicating the possible presence of a staling substance, combined with the fact that after the drying procedure the mat was quite thick, indicating a number of stacked hyphal strands, suggest System II.

In a study of viability, samples taken prior to stationary phase of growth would include growing hyphal tips regardless of the system involved, so that a

determination of what system is present at this time is rather difficult. The fact that between the hours of 72 and 144 the data (Figure 3) were relatively constant suggests that regrowth is initiated by branching after placement into new growth media. Again, it is difficult to determine which system is existing as there is no real basis to allow a comparison between the ability of either System I or System II to promote branching. It is possible that the actual decrease in the percent viability is essentially an increase in the total number of dead cells, suggesting a System II culture. It should be stressed that this is an inaccurate measure of the true cellular viability, but it does show that at 144 hours the mats have the ability to promote branching and thus must be viable to some extent.

The results on respiration may be explained by either system. However, in comparing the data of Gottlieb and Van Etten on the colonial age related changes of R. solani and Sclerotium bataticola Taub. (8) with those of A. solani for the 36 and 60 hour readings, the changes with age are quite similar. This suggests that the fungus during this period of time appears to be acting as a series of microcolonies. Further, there are no abrupt QO_2 changes before or after the stationary phase such as would be expected in System I. A gradual decrease in metabolic activity throughout the mats of A. solani with increase in age suggests that death is occurring gradually through

the whole mat and the decrease in the regrowth may therefore be a measure of an increased number of dead cells. This supports the idea of staled microcolonies. On the other hand, this does not eliminate the possibility that a continuum of interconnected hyphae could exist since the same results could be explained by this system if there is a gradual decrease in intracellular metabolic activity as cellular age increases.

In recapitulation, the actual mat existing on the surface of the membrane seems to fit both System I and System II possibilities equally well. The fact that anastomosis is known to occur in A. solani combined with the possibility that staling substances are eliminated, as indicated by a pH decrease and color change in the media, suggest that System III, a combination of the two extreme possibilities, may actually be the true description of the mat. Stall noted that anastomoses could occur to a great extent, but not exclusively (22). Consequently, the results reported here would most closely resemble those expected from a number of physiologically and biochemically interrelated colonies that have undergone partial anastomoses. This resultant system would be a less precise way of measuring either cellular age related phenomenon or colonial age related phenomenon, than would either System I or System II, respectively. However, as most fungal aging studies have been done on cultures comparable to System II, they would be measuring colonial

aging rather than cellular aging. System III, therefore, appears to be a better means of investigating phenomenon related to cellular aging than previous culture techniques.

The second characteristic of the tool concerns the reproducibility of replicate mats at a single point in time. Two things should be specifically noted: first, the homogeneity results in mostly all cases show significance between mats and second, the confidence intervals about the growth curve means are quite large. This suggests that each mat has its own rate of growth regardless of the similarities of the inoculation. Thus the growth curve (Figure 1), is a combination of mats which appear to be growing at different rates. This introduces the phenomenon of time slippage between replicate mats. If one assumes that the time from the initiation of stationary period to the point where the death phase takes predominance is finite for each mat, but that the rates of growth up to the stationary period differ, then one could have in any set of replicate mats variation in the times at which individual mats enter into stationary and death phase. At any one point in time, the physiological and biochemical constituents of replicate mats would be dissimilar. This fact renders the reproducibility of the system with respect to the changes with age suspect since one would never be sure that any two mats were the same age at the same time after inoculation. This error in the system seems to occur during the lag and log phase of growth, so one

must assume that the reason for this fault lies in the drying procedure which occurs during the 48 hours immediately following inoculation. This is supported by the observation that directly after the drying period, there was a notable difference in the color and the moisture content of different mats. As the drying procedure was necessary using this technique, there seems to be no way to remove the problem unless an entirely different inoculation procedure is used.

The results obtained on the endogenous and exogenous QO_2 for a large number of mats under the conditions known to exist with this procedure, give one a relative picture of the age related QO_2 changes in A. solani. From 60 hours to the end of the experimental period, there was no significant difference between endogenous and exogenous QO_2 at any one hourly interval. This could be due either to the inability to absorb substrate or to an increased use of stored material by an enzymatic shift associated with age. Van Etten and Gottlieb alluded to the fact that as R. solani increased in age there was a possible decrease in the permeability of the membrane (26). They also noted that a number of respiratory enzyme concentrations shifted with an increase in fungal age (27). Either of these facts would cause a shift from exogenous substrate utilization to stored fat or carbohydrate utilization and result in insignificant differences between endogenous and exogenous QO_2 results, as well as an overall decrease

in respiratory rate.

In general, the system used in this report has a number of inherent problems. Aside from the fact that once a mat is used for a specific time period it is unusable for any other period, individual mats inoculated with mycelial fragments at the same time do not show similarity at any specific time after the inoculation. Further, prior to stationary phase, there is no similarity of response within mats. Another complication is the fact that since hyphal anastomoses and colonial staling may be occurring simultaneously, one is unsure whether cellular or colonial fungal aging is being measured within the system. However, it would seem that the existing system is a better measure of cellular aging than previous culture techniques. In any case, the amount of time needed to reach stationary phase, when compared to the amount of time the total mat is viable, is much too great to allow accurate delineations of fungal age and thus, increases the inaccuracy of this specific system as a tool. The system, as it has been reported, could be improved by the elimination of a solid finite nutrient source in favor of a liquid infinite nutrient medium. Nevertheless, endogenous and exogenous QO_2 changes of A. solani decrease with increasing age, and the significant differences between endogenous and exogenous QO_2 during linear growth disappear during the stationary growth. This indicates a marked similarity with previous work on fungal respiratory changes with age.

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