STUDIES OF THE GROWTH RESPONSES OF FUNGI TO BORON, MANGANESE, AND ZINC.

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INTRODUCTION

The importance of growing fungi in artificial cultures has been recognized for a long time. These cultural studies have been undertaken to determine taxonomic relationships, the effects of the substratum on the metabolic products, and to obtain theoretical concepts of growth and development. The composition of the substratum used for artificial cultures has varied greatly. Plant decoctions have been employed but they contain numerous unknown chemicals and cannot be easily or even accurately duplicated. Synthetic culture solutions devised by the early workers were often very complex. Raulins' fluid which is still much used by mycologists is a good example of this type of medium.

The later work of Nageli (34), Molisch (32), and Benecke (3) has shown that more simple synthetic media can often be successfully used in culturing a variety of fungi. The results of these investigators showed that after a fungus had been supplied with available sources of nitrogen and carbon, four elements, only, were needed for growth; those elements being sulphur, phosphorus, magnesium and potassium. When highly purified chemicals were used to supply these essential nutrients, some investigators found that growth could be increased by the addition of minute traces of other chemicals. Raulin (39) included a trace of zinc in his classical culture fluid because he was convinced that it gave increased yields. Later workers have found approximately eighteen ions which act in a similar manner when added to cultures of Aspergillus niger. The careful work of Steinberg (47),

Bortels (6), and Roberg (44) indicate that some of these ions are essential for the growth of Aspergillus niger. Richards (41) was convinced that the response to such elements would vary with the organisms cultured.

Elements which are essential or stimulating in minute quantities become important factors whenever cultural studies are undertaken. It is desirable to know how more of the economic fungi respond to such elements. Most of the studies on growth stimulation, in the past, have dealt with non-pathogenic fungi, while very few of the important pathogens were included. Few fungus genera, if any, have in recent years been cultured more than the Fusaria. In these studies dealing with the expression of various morphological characters, and physiological activities, no definite studies have been made of their growth responses to those chemical elements and compounds commonly considered as growth stimulants. The main problem of the present investigation has been to compare the growth responses of several Fusaria with other pathogenic and non-pathogenic fungi to the three elements, boron, manganese and zinc, and if possible to make some contribution to our knowledge of growth stimulation. A minor, yet essential, phase of this problem has involved a study of the growth responses to certain salts containing the so-called essential elements when supplied in varying amounts under different conditions. This study was necessary first to secure a suitable culture medium and second to contrast the growth responses due to the essential elements with the responses initiated by the so-called stimulating elements.

METHODS AND MATERIALS

CLEANING OF GLASSWARE: -

All glassware used in the preparation of the nutrient solutions were first washed in hot soap water, then rinsed in tap water, allowed to drain and dry, and finally rinsed three times in hot distilled water. All the culture flasks were first rinsed with a cleansing acid followed by three rinsings in distilled water. The final operation was to boil about 25 cc. of distilled water in each flask and drain while hot. This procedure was sufficient to remove all traces of soluble residues.

INNOCULATION OF CULTURES: -

Spore suspensions in distilled water were first prepared. A uniform number of droplets were transferred with a sterile platinum wire loop to each culture flask. With the Fusaria vary uniform innoculations were obtained in this manner. However, the Aspergillus spores presented a slightly different problem since they always float on the surface of the liquid. Care must be taken to obtain a fairly uniform distribution of the spores before final incubation. The effects of using extreme differences in the amount of innoculum with Fusarium lini are presented in Table 1 and Figure 2.

FORM OF CULTURE USED: -

In all these experiments a liquid culture medium was employed. The composition of the nutrient solution has been stated under each table of experimental data. When different concentrations of chemicals were used in a series of experiments

dilutions were made from stock solutions. Pyrex Erlenmeyer flasks of 125 cc. or 250 cc. were used, especially when dry weight determinations of the mycelium and spores were desired. A large series of cultures just after innoculation, in the 125 cc. flasks are shown in Figure 1. In some of the experiments where a rather large number of cultures were essential and an economy of incubator space required, test tubes contain-

Fig. 1. A series of the 125 cc. Pyrex Erlenmeyer culture flasks containing 40 cc. of the nutrient solution. (Just after innoculation). In the background are the glass funnels, filter paper and weighing cups used in the dry weight determinations of the mycelium.

ing 6-10 cc. of the culture solution, and arranged in a vertical position were found to be very convenient. All cultures were stoppered with clean cotton plugs, (Non-absorbent cotton, bleached, packed by Seabury and Johnson, East Orange, N. J.). All cultures were steam sterilized at 15 pounds pressure for 8 minutes. More intense heating was unnecessary and sometimes caused the formation of precipitates.

QUANTITATIVE DETERMINATIONS OF THE MYCELIAL MATS: -

The weight of the dried mycelium was considered as one of the most reliable criterions for determining the response of the fungus to the medium on which it has been grown. For such determinations single cultures were filtered on a previously weighed and dried filter paper placed in a glass funnel (See Figure 1.). Here the mycelium and spore masses were washed with distilled water to free them from as much of the medium as possible. In some cases the amount of washing depended upon the quality of the mycelium. Usually about 100 cc. of water were used in the washing of each culture. As soon as the washing was complete the filter paper containing the fungus mat was partially folded up, dropped in an aluminum weighing cup and placed in a drying oven ranging from 85-90°C. After one day in the drying oven the containers were weighed, then dried again for several hours and weighed again. A check within 4 milligrams of the previous weight was considered close enough because fluctuations within this range were difficult to overcome.

In some experiments the amount of mycelium was estimated and scored, taking into consideration the thickness and uniformity

of the mats. Such estimations have been restricted largely to test tube cultures.

pH DETERMINATIONS: -

The pH of the filtrates was determined colorimetrically using color standards and indicator solutions as described by Medalia (31). There is some error in such determinations but they checked reasonably close with known standards. The ease and speed of manipulation is of prime importance when 25-50 determinations must be made in a short time.

CHEMICALS USED IN PREPARATION OF CULTURE MEDIA: -

The chemicals used in the preparation of the culture media varied in kind and amount according to the purpose of the investigation. The distilled water was prepared with a Certified Barnstead Water Still. The same source of water was used by Johnston (21) in some of his studies on the boron relations of the tomato. No special methods of purifying the chemicals as used by Steinberg (47) or Roberg (44) were employed. Chemicals of high purity were used and in general the results obtained can be compared with the work of other investigators using similar chemicals. The following list of salts were specially prepared by J. T. Baker Chemical Company for the Committee on Salt Requirements of the National Research:

Magnesium sulphate	$MgSO_4 \cdot 7H_2O$
Potassium nitrate	KNO_3
Potassium acid phosphate ..	KH_2PO_4
Magnesium nitrate	$Mg(NO_3)_2$
Calcium nitrate	$Ca(NO_3)_2$
Ammonium nitrate	NH_4NO_3

The following chemicals were C. P. products of J. T. Baker Chemical Company:

Urea, crystals ..... $CO(NH_2)_2$
Manganous sulphate ..... $MnSO_4.2H_2O$
Boric acid, U. S. P. X. crystals
Sodium borate ..... $Na_2B_4O_7.10H_2O$
Zinc sulphate ..... $ZnSO_4.7H_2O$

The other chemicals were from different sources.

Iron tartrate, (ferric), Mallinckrodt Chem. Works.
Dextrose, Merks, C. P.
Sucrose, crystals, Merks, C. P.
Sucrose, Commercial Brand, "Crystal Domino".
Sucrose, Commercial Brand, "Quaker Brand".

NAME AND SOURCE OF FUNGI; -

The following Fusarium sp. were obtained from Dr. Freeman Weiss, Associate Pathologist, U. S. D. A. : -

Fusarium	tricothecioides,	strain	141
"	eumartii	"	528
"	sulphurum	"	244
"	oxysporum	"	529
"	solani	"	658
"	radicicola	"	701

The following strains and species of Aspergillus were obtained from Dr. Charles Thom, Mycologist, U. S. D. A. : -

Aspergillus albus candidus - 117 (In the albus candidus group but not definitely named to strain).
Aspergillus niger -- 111 (From Westerdyk).
Aspergillus niger -- 4247w (R. A. Steingerg's strain w)
Aspergillus fuscus Schiemann -- 3534c (Same as A. Schiemanni, Sch.,)Thom.

The strain of Rhizopus nigricans was isolated from a culture contamination in the laboratory.

The Altenaria sp. was isolated from diseased strawberry plants and has not been definitely classified as to species.

The strain of Fusarium lycopersici -- 100 was isolated from infected tomato plants.

The conidial stage of Gibberella saubinettii-- 200 was isolated from diseased wheat grown at the Maryland Experiment Station.

PRESENTATION OF RESULTS

A. Growth responses to "essential elements".

1. GLUCOSE AND SUCROSE REQUIREMENTS.

A great variety of carbon sources have been used in culturing fungi, some of which seem to be almost omnivorous while others show decided preferences. Sucrose and glucose are generally used in the numerous nutrient solutions listed in the literature. The amount of each carbon source in such solutions is usually sufficient to support a fairly good vegetative growth but in the final analysis the amounts seem to have been determined in an empirical manner. The development of reproductive structures, according to Klebs (23), Coons (11), and Leonian (25) are often hindered by very vigorous vegetative growth which can usually be controlled by the carbon supply. Reynolds (40) working with a strain of Fusarium lini has tested a variety of sugars and found that with a suitable carbon source, within rather large limits, the vegetative growth was almost proportional to the amount of sugar present.

As a preliminary experiment a study was made of the effects of using a small and a large number of spores in the innoculation with two concentrations of glucose. In the light innoculations from 5-8 spores were introduced into each culture while several hundred spores were used in the heavy innoculations. The dry weight of the mats and the pH of the culture liquids at different time intervals are given in Table 1. Each weight represents the average of triplicate cultures. These extreme differences in the amount of innoculum did not

materially influence the maximum vegetative growth. The medium in each case became alkaline differing only in the rate of change. The growth curves are presented in Figure 2. From these

Table 1. The effects of a light and a heavy innoculation with spores of Fusarium lini in media containing a high and a low concentration of glucose. (Dry weights and pH values given are the averages from triplicate cultures).

Age of cultures when harvested	Molar concentrations of glucose used							
	M/10 glucose				M/30 glucose			
	Amount of innoculum used				Amount of innoculum used			
	Light		Heavy		Light		Heavy	
	dry wts. mgs.	pH of fil.*	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.
4-days			28	4.1			17	4.0
6-days			131	4.9			66	5.3
8-days	33	4.1	181	7.1	56	4.8	83	7.5
10-days	149	5.3	183	7.6	86	6.9	79	7.6
13-days	193	7.6	204	8.4	82	8.4	78	8.2
16-days	206	8.4	184	8.6	74	8.6	70	8.4
20-days	189	8.6	188	8.6	70	8.6	72	8.6

($MgSO_4$-2, CaH_2PO_4-2, KNO_3-10) **

curves it is evident that these extreme differences in innoculation resulted only in differences of the growth rate. This difference in time required to attain the maximum growth was about four days. The difference in the number of spores taken on a wire loop from a well shaken spore suspension, should not be sufficient to cause much variation within a series of cultures.

* pH of filtrates
** Salts used in the media expressed in grams per liter.

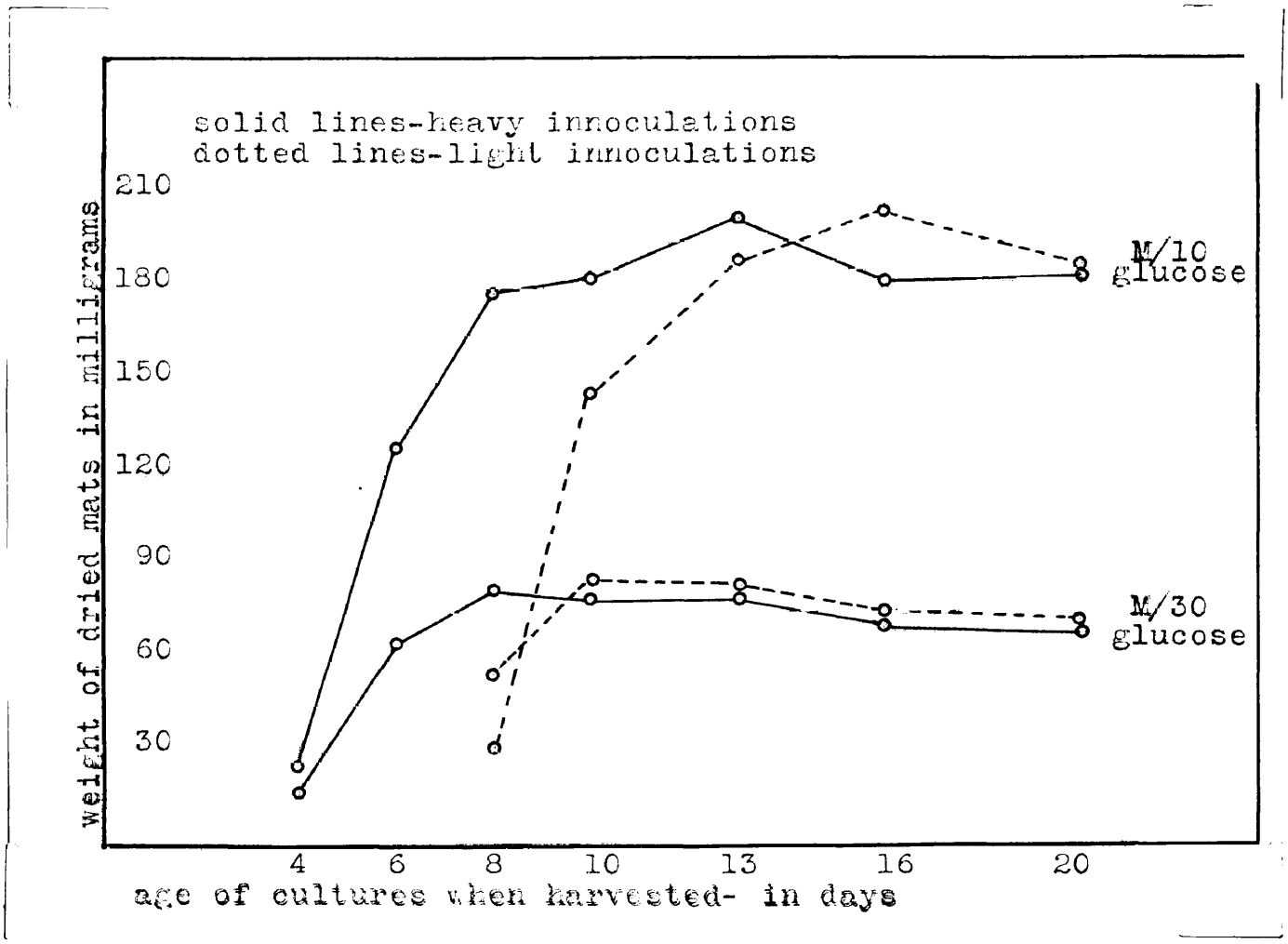

Fig. 2. Growth curves of Fusarium lini on two concentrations of glucose when light and heavy innoculations were used.

The growth of Fusarium lini on different molar concentrations of glucose is almost directly proportional to the amount of available carbon present. However, there seems to be a slightly more efficient utilization in the lower concentrations. In the culture media used for this experiment no zinc or other stimulants were used. The maximum dry weight was reached when the cultures were about three weeks old. The results are presented in Table 2. The weight of the cultures gradually recedes

from the maximum probably due to autolytic reactions. Similar losses in weight have been reported by other investigators. The growth curves obtained by Mayoue (30) whenever a good carbon source was used, reached a maximum point from which there was a downward trend. Camp, (9) using Duggar's solution slightly modified, with a variety of fungi in most cases obtained similar

Table 2. The growth of Fusarium lini on different molar concentrations of glucose over a period of four to five weeks. (Dry weights of the mycelium given are average of duplicate cultures).

Age of Culture in weeks	Molar concentrations of glucose used				
	M/10 dry wts. mgs.	M/15 dry wts. mgs.	M/20 dry wts. mgs.	M/30 dry wts. mgs.	M/60 dry wts. mgs.
1	152	126	108	86	31
2	228	175	161	111	61
3	316	200	162	98	58
4	295	163	129	81	46
5	225	162			

($MgSO_4$-2, CaH_2PO_4-1, KNO_3-10. 40 cc./culture in 125 cc. flasks).

results. Klotz, (24) also using Duggar's solution in the culture of Aspergillus niger, Spaeropsis malorum, and Diplodia natalin obtained similar types of growth curves. Sideris (46) working with Fusarium cromyphthoron found that after the fungus had absorbed all of the available sugar of the culture medium, its mycelium undergoes autolysis.

There is little, if any, information regarding the maximum concentration that any of the Fusaria can tolerate with reference to any of the common carbon sources. In Table 3 some

growth responses of Fusarium radicicola to different molar concentrations of cane sugar are presented. Zinc sulphate was added to the medium. Some observations were taken regarding the general abundance and type of spores present in the cultures containing different amounts of sugar. These observations showed that very few spores of any kind were present in the lowest concentration, and in the 1.5M cultures no spores were found. The abundance and preponderance of microspores over macrospores increased from the M/85 to 1M cultures. The mycelium appeared

Table 3. Some growth responses of Fusarium radicicola to different molar concentrations of cane sugar.

Cane sugar as molar concentrations and as grams per 100 cc. culture	Age of harvested cultures				Maximum culture yield and pH of their filtrates	Percentage ratio of dry weight to amount of sugar in max. yields
	13-days		20-days			
	Weight of dried mats in grams	pH of filtrates	Weight of dried mats in grams	pH of filtrates		
M / 342			0.040	6.0	0.052	
0.100 gms./ C	0.052	6.0	0.041	6.0	6.0	52.0%
M / 85			0.133	6.8	0.176	
0.400 gms./ C	0.176	6.6	0.140	6.8	6.6	44.0%
M / 16			0.783	8.0	0.833	
2.200 gms./ C	0.833	7.6	0.789	8.0	7.6	38.0%
M / 8			1.484	8.0	1.640	
4.300 gms./ C	1.640	7.4	1.444	8.0	7.4	38.2%
M / 4			2.102	6.4	2.102	
8.600 gms./ C	1.813	6.4	2.065	6.4	6.4	24.4%
M / 2			2.643	5.8	2.643	
17.100 gms./ C	1.932	6.2	2.566	5.8	5.8	15.4%
M / 1			2.110	5.6	2.198	
34.200 gms./ C	2.179	5.8	2.198	5.6	5.6	6.4%
1.5 M			0.464	5.0	0.464	
51.300 gms./ C	0.280	5.4	0.458	5.0	5.0	0.9%

($MgSO_4$-4, KH_2PO_4-3, KNO_3-6, and $ZnSO_4$-0.050)

normal in all but the 1.5M concentrations where the cells were short and large usually irregular in outline. The filtrates were clear in the M/342, M/85, and 1.5M cultures while the others contained a yellowish-brown coloration which increased slightly with the culture age.

The sequence and magnitude of the responses of Fusarium radicicola to the different molar concentrations of sucrose are illustrated in Figure 3. The maximum culture yield in dry weight increased with but not in direct proportion to the concentration up to the M/2 series. The M/1 concentration was somewhat less favorable than the M/2 as apparently too high an osmotic pressure existed. Decided toxicity arises in the 1.5M series (see curve A). The efficiency curve B varies indirectly with the amount of sugar present in the culture. These percentages were obtained by dividing the grams of sugar in the culture by the maximum dry weight obtained in that series. There are several apparent reasons why this efficiency decreases with an increase in sugar concentration. The surface area remains constant thus forming a limiting factor. Although rather high concentrations of salts were used there might have been more growth if still larger amounts had been employed with the high concentrations of sugar. This hypothesis draws some support from the experiments dealing with variations in the amounts of magnesium sulphate supplied.

The pH changes in the presence of different amounts of sugar are interesting. It must be borne in mind that more harvest dates and a little longer duration of the experiment would

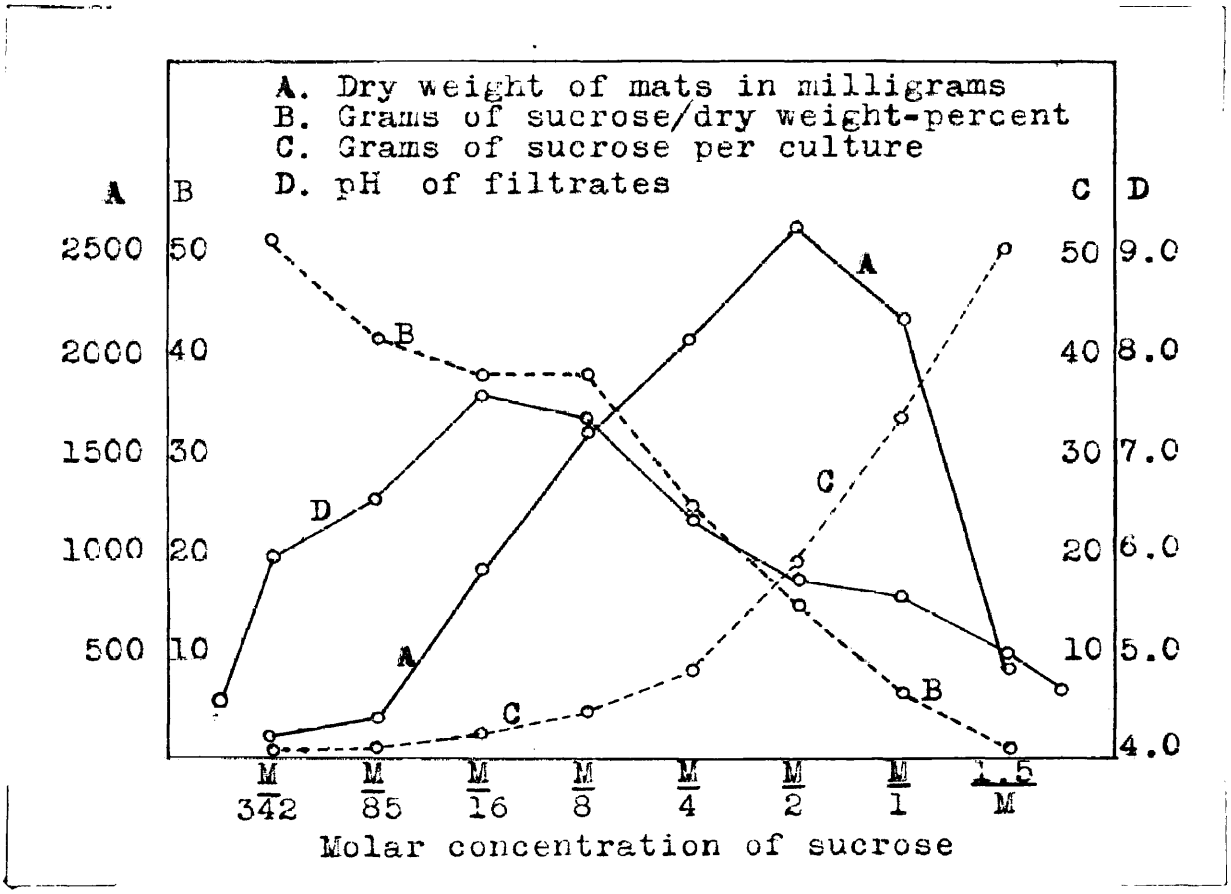

Fig. 3. Responses of Fusarium radicicola to different molar concentrations of sucrose. (The original pH of each series was 4.6).

have given a more complete record of the rate and extent of these changes. The active acidity of the media was not increased beyond the original condition. In general there was a tendency to decrease the pH of the medium. When the exhaustion of the sugar supply was a limiting factor to increased growth, the reduction of the active acidity was closely correlated with the amount of dry weight produced. It is assumed that unequal ionic absorption and autolytic processes tend to bring about an alkaline condition. When 8.6 grams or more of cane sugar was supplied in each culture the alkaline trend of the media was depressed, in direct proportion to the increase in the sugar present, and indirectly proportional to the amount of vegetative growth. The odor of the filtrates from those cultures containing 8.6 grams or more of sucrose suggested the presence of alcohol and acetic acid. The produc-

tion of organic acids and the lessened autolytic action seems responsible for the acid condition of these filtrates. The presence of acids was not detected in the cultures containing less than 8.6 grams of sucrose. Quite similar results were obtained by Herrick and May (17) which show that the condition which most favors the production of gluconic acid is an excess of glucose over the salts available so that vegetative growth is not at the maximum. It is not to be assumed that the same concentration and proportions of the nutrient components will cause identical responses for a number of different organisms.

2. NITROGEN RELATIONS.

The kind and amount of the nitrogen source used in a nutrient solution will depend upon the purpose of the investigator and the type of organism cultured. There have been disagreements as to the relative nutritive value of organic and inorganic nitrogen sources. This problem has not been solved. Comparison of nitrogen compounds based on the number of grams supplied per liter is misleading since the percent of total nitrogen is not constate. Substances which may be supplied in amounts equivalent on the basis of total nitrogen may lead to erronous conclusions of zinc which may cause marked growth stimulation. The kind and concentration of the other nutrient materials certainly will be important factors in determining the growth response to any variation made in the nitrogen source. There have been no such thorough experiments with fungi as with the higher plants. No one has carefully used the "triangle systems" of Shive or Tottingham, nor has there been careful com-

parisons made of the relative nutritive values of the cations or anions as made by Johnston (19 & 20) in his studies with the potato.

Several experiments have been reported in which variations were made in the concentration of the nitrogen source while the other components were kept constant. Pratt (38) used Richard's solution modified to omit the ammonium nitrate in culturing a Fusarium sp. The amount of potassium nitrate was varied with the following results:

Gms. KNO_3/liter	10 gms.	5 gms.	2.5 gms.	1.25 gms.
Max. growth	0.303 "	0.241 "	0.279 "	0.223 "

Steinberg (44) using a strain of Aspergillus niger varied the concentration of the ammonium nitrate in Pfeffer's solution with these results:

Grams NH_4NO_3/liter	25 gms.	15 gms.	0.1 gms.	0.01 gms.
average yield	0.376 "	0.359 "	0.061 "	0.029 "

In my culture studies with F. lini a series of experiments were carried out which involved different concentrations of potassium and sodium nitrates. The maximum yields are given in Table 4.

Table 4. The influence of variations in the nitrogen sources on the dry weight of the mycelium produced by Fusarium lini.

Nitrogen Sources			
potassium nitrate		sodium nitrate	
grams/liter	yield	grams/liter	yield
21	0.614 gms.	21	0.516 gms.
18	0.632 "	18	0.472 "
12	0.610 "	-	--
6	0.595 "	6	0.465 "
3	0.570 "	3	0.464 "
0.5	0.334 "	0.5	0.257 "

($MgSO_4$-2, CaH_2PO_4-1. glucose, M/10) note: grams of sodium nitrate means the amount used was equivalent to grams of KNO_3 on the basis of total nitrogen.

From these results it is evident under the conditions of the experiment that only minute increases in growth were obtained by adding more than 3.0 grams per liter.

The concentration as well as the kind of nitrogen source may influence the changes brought about by the fungus in the liquid medium. The most conspicious of these changes are the formation of pigments and the increase or decrease of both the titratable and active acidity. Palladin (36) at an early date called attention to the production by higher plants of an alkaline condition in the nutrient medium when the nitrogen salts of such alkaline metals as sodium, potassium, or calcium were employed. Arrhenius (2) studied the adsorption of nutrients by higher plants in relation to the hydrogen ion concentration. He found that the presence of Palladins "alkaline nitrogen" salts favored the production of an alkaline condition while the presence of ammonium salts resulted in an acid condition. As an explanation he favors the theory of unequal ionic adsorption as being responsible for these acidity changes. Klotz (24) working with several fungi came to similar conclusions regarding the role played by the nitrogen sources in the development of either alkaline or acid conditions in the culture medium. He suggests that in case a salt such as potassium nitrate is used, the nitrate ion is absorbed faster and in greater amounts than the potassium ion. This ion combines with the carbon-dioxide resulting in the formation of alkaline bicarbonates. When either ammonium nitrate or ammonium sulphate are present in the medium an acid condition prevails since the ammonium ion (NH_3) is more rapidly absorbed thus forming nitric or sulphuric acid in the liquid medium.

The formation of organic acids by certain fungi will cause the medium to become, often, very acid regardless of the nitrogen source. Many investigators have studied the formation of pigments in the culture medium by fungi and in general the assumption is that acid and alkaline conditions are important factors.

Two experiments were designed to study the pH changes brought about by Fusarium radicicola with culture media containing different nitrogen sources when used singly and in combination. It seemed logical to assume that if one nitrogen source led to an alkaline condition and another to an acid condition, that on mixing the two sources some neutralization would occur, and prevent extreme changes in the pH of the medium. In the first experiment eleven combinations of potassium nitrate and ammonium nitrate used singly and in combinations of different proportions were used so that at one end of the series there was a predominance of potassium nitrate and at the other end ammonium nitrate predominated. This series is described in Table 5. The use of five grams per liter as a standard was an arbitrary choice. The pH values given in the following table (Table 5.) are the numerical averages of determinations made on triplicate cultures. Twelve test tube cultures were set up for each of the eleven combinations making a total of 132 individual cultures. pH measurements were made at four different times extending over a period of 12 days. At the 8 and 12 day periods dry weight determinations were made so as to get a quanitative measurement of the growth response. The weights given are the values obtained by weighing together the fungous mats from three cultures and dividing by 3.0 (in milligrams).

Table 5. The effects of the nitrogen sources, used singly or in combinations of different proportions, on the pH changes brought about in the culture media and the weight of the mycelium produced by Fasarium radicicola.

Part I pH changes.

Amount and proportion of each salt for each series											
series number	1	2	3	4	5	6	7	8	9	10	11
Grams of KNO_3/L	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0.0
grams of NH_4NO_3/L	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Age	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH
Start	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
2-days	5.2	4.6	4.6	4.1	4.0	4.0	4.0	4.0	3.9	3.8	3.8
5-days	6.6	6.4	6.1	3.9	3.4	3.2	3.0	2.6	2.6	2.4	2.4
8-days	8.4	8.2	7.4	6.6	3.8	3.6	3.4	3.0	2.8	2.6	2.4
12 days	8.6	8.2	7.6	7.2	4.6	4.0	3.4	3.0	2.6	2.4	2.4

Part II Weight of dried mycelial mats

series number	1	2	3	4	5	6	7	8	9	10	11
Age of culture	dry wts. mgs.	dry wts. mgs.	dry wts. mgs.	dry wts. mgs.	dry wts. mgs.	dry wts. mgs.	dry wts. mgs.	dry wts. mgs.	dry wts. mgs.	dry wts. mgs.	dry wts. mgs.
8-days	56	63	62	62	50	46	46	--	37	33	28
12-days	77	91	92	100	74	71	73	46	39	37	38

($MgSO_4$-2, KH_2PO_4-1, sucrose, Cry. Dom.-40, zinc sulphate -0.050)
(All cultures in test tubes containing about 8 cc. of solution)
(All dry wts. and pH values are the average of 3 cultures)

The appearance of the mats and the liquid media in this culture series was interesting due to the range in pigment formation. In series 1 (potassium nitrate only) there was neither red nor blue pigments but there was a yellowish-green color in the liquid. Blue pigment was formed in small amounts in series 2, 3, and 4. In series 5 first there was a red pigment but as the cultures aged patches of blue appeared in the mycelium. From series 6 to 11 there was a deep pink to red pigment formed in the mycelium which soon diffused out into the underlying liquid.

The presence of this pigment relates directly to the acidity of the medium. Blue being favored in the alkaline cultures and red in the acid cultures. This pigment could be changed from red to blue by the addition of a few drops of a NaOH solution.

Differences in the vegetative growth were conspicious. In series 1 to 4 the mats were mostly white having a luxuriant and fluffy aerial growth over the entire liquid surface. Beginning with series 5 there was a gradual decrease in the total growth along with a corresponding increase in a red appearance and patchy, crinkled growth. The dry weights show that the acid media were unfavorable for a good vegetative growth. In those cultures where an alkaline condition was reached vegetative growth was best. Judging from the weights obtained when the cultures were 12 days old it seems evident that the best growth was possible in a partially buffered medium as in series 4 where neither an extreme alkaline or acid condition prevailed.

The rate and extent of the pH changes in the different series are shown in Figure 4. From these curves it is evident that the media in which there was a preponderance of potassium nitrate became alkaline and this condition increased with time. The acidity shows a close correlation with the ammonium nitrate content. On the basis of gram weights, ammonium nitrate produces an acid condition which similar amounts of potassium nitrate cannot neutralize. This relationship is lessened as growth increases and as the cultures age. The pH of five of the series of combinations is plotted against time in Figure 5. In series 4 there is a slight increase in the active acidity for the first

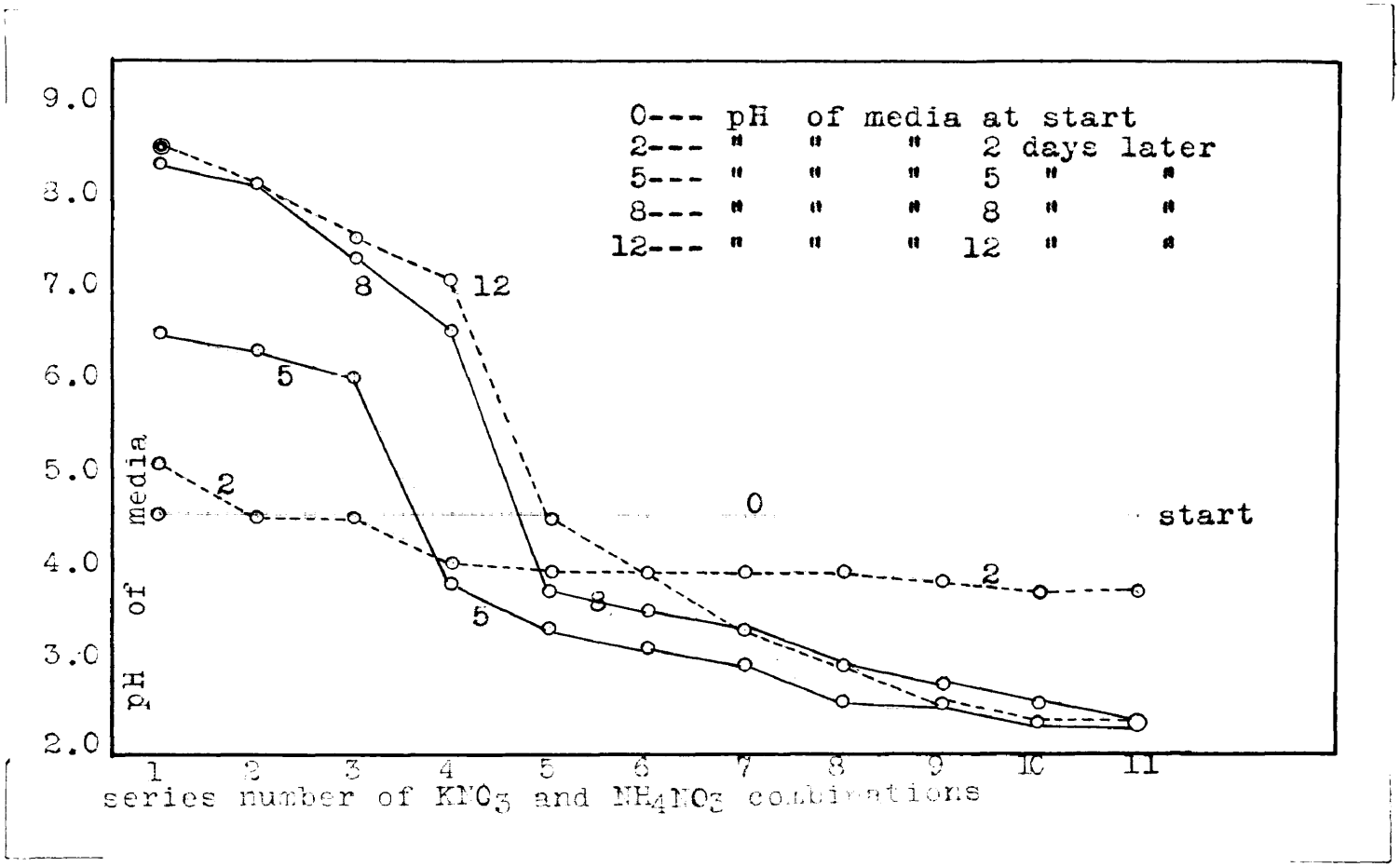

Fig. 4. The rate and extent of the pH change caused by Fusarium radicicola in media containing potassium nitrate and ammonium nitrate when used alone and when used together in different porportions. (See Table 5).

five days after which the pH shifts towards alkalinity. A similar course is followed in series 5 but does not reach the same final pH. If the fungus showed a preference for the nitrate ion rather than the ammonium ion the above results would be difficult to explain. When ammonium nitrate is used alone the (NH_3) and (NO_3) ions should be present in equal numbers. In series 5 where there is 2.5 grams of each nitrogen salt present, there are almost two ions of nitrate ot one ammonium ion yet the fungus does not produce an alkaline condition which certainly suggests a prefenential absorption of the ammonium ion. The

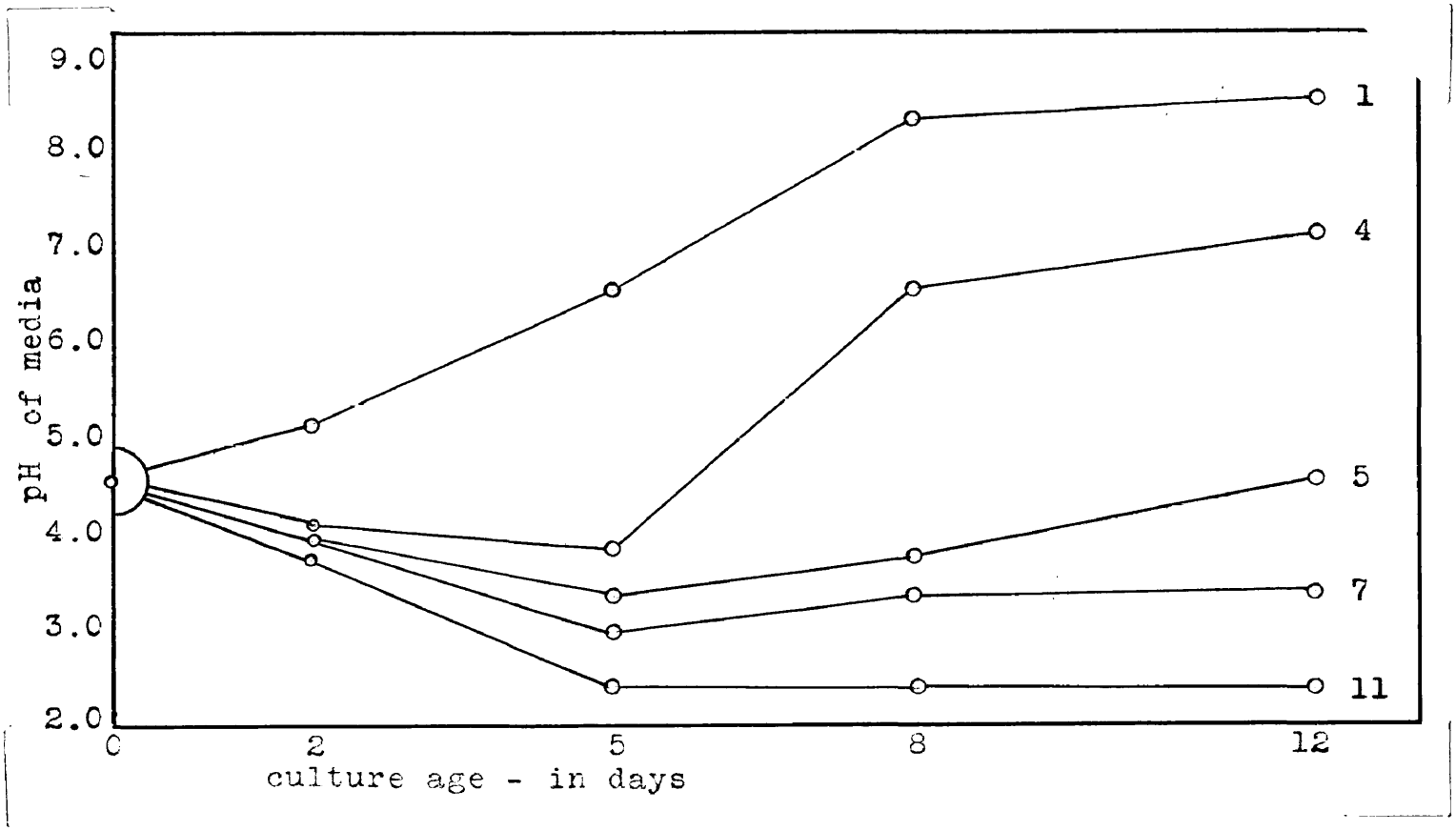

Fig. 5. The influence of culture age on the pH changes brought about by F. radicicola in media containing potassium nitrate and ammonium nitrate when used alone or together in different proportions.

trend towards alkalinity as in series 4 seems logical if it is assumed that as soon as the supply of ammonia has been exhausted the utilization of nitrates is increased.

In a similar manner ammonium nitrate was used in a series of combinations with sodium nitrate, calcium nitrate, and magnesium nitrate. The three nitrate salts seemed equally available to the fungus. These salts possibly were not as good a source of nitrogen as is potassium nitrate. The justification for this assumption lies in the fact that greater H ion concentration was developed in series 3 and 5 than occurred in the same series when potassium nitrate was present. The development of the red pigment and poor vegetative growth were identical with the results obtained when potassium nitrate was used.

Table 6. The effects of the nitrogen sources, used singly or in combinations of different proportions on the pH changes brought about in the culture medium by Fusarium radicicola.

Nitrogen sources	series number	Age of cultures when harvested - days					
		0	4	6	8	10	11
		pH	pH	pH	pH	pH	pH
Sodium nitrate and Ammonium nitrate	1	4.6	6.6	6.6	7.0	7.2	7.4
	3	4.6	2.4	2.4	4.4	5.4	6.4
	5	4.6	2.2	2.2	2.2	2.4	2.4
	7	4.6	6.0	6.2	6.3	6.4	6.4
Calcium nitrate and Ammonium nitrate	1	4.6	6.0	6.2	6.3	6.4	6.4
	3	4.6	2.8	4.8	5.6	6.0	6.2
	5	4.6	2.6	2.2	2.0	2.0	2.2
	7	4.6	3.2	2.2	2.0	2.0	2.2
Magnesium nitrate and Ammonium nitrate	1	4.6	6.4	6.8	7.0	7.1	7.2
	3	4.6	2.8	4.4	5.8	6.2	6.4
	5	4.6	2.6	2.2	2.4	2.4	2.6
	7	4.6	2.4	2.2	2.4	2.4	2.6

($MgSO_4$-2, KH_2PO_4-1, sucrose, 6ry. Dom.-40 zinc sulphate 0.050)
(All cultures in test tubes containing about 8cc. of solution).
(Series numbers indicate the same proportions and total amounts as in Table 5).
(Amounts of each salt used as equivalent to 5 gms. of the anhydrous form: magnesium nitrate - 8.47 gms., calcium nitrate - 7.14 gms., ammonium nitrate and sodium nitrate - 5 gms. each).

Under the conditions of these experiments and with the organism used it is evident that ammonium nitrate is undesirable as a nitrogen source since the growth of the organism brings about an unfavorable acid condition. Unequal ionic absorption offers a partial explanation of the pH changes. Part of the acidity developed may be due to the secretion of organic acids, however, this has not been carefully studied.

3. MAGNESIUM SULPHATE RELATIONS.

Two experiments were conducted to study the influence of variations in the amount of magnesium sulphate supplied on the growth responses of F. radicicola. Steinberg (47) found that his strain of Aspergillus niger showed an increase in growth over a great range in the amounts of magnesium sulphate supplied. This led him to suggest that traces of zinc or other impurities might in part be responsible to some extent for such growth responses. However, some of his results are not conclusive and do not agree with some of his deductions. His results in Table 8 will illustrate this point in a series of variations in the components of the Pfeffer solution.

Table 8. Steinberg (47) (rearranged) The effect of a decrease in concentration of one or more of the salts of the Pfeffer solution.

(Culture period - 7 days)

Grams of each used per liter of solution			Average of yields
NH_4NO_3	KH_2PO_4	$MgSO_4$	
10 gms.	5 gms.	2.5 gms.	0.300 gms.
10 gms.	5 gms.	1.0 gms.	0.365 gms.
10 "	2 "	2.5 "	0.284 "
10 "	2 "	1.0 "	0.299 "
4 "	5 "	2.5 "	0.218 "
4 "	5 "	1.0 "	0.265 "
4 "	2 "	2.5 "	0.190 "
4 "	2 "	1.0 "	0.183 "

He states, "In these experiments we note that only a decrease in yield follows a decrease in concentration of one or more of the inorganic constituents of the Pfeffer solution, ---". Attention should be called to the fact that in three of the above experiments a decrease in the magnesium sulphate actually resulted in an increased yield, and in the last experiment he included an

Table 7. Growth responses of Fusarium radicicola to different amounts of magnesium sulphate in the culture medium. (Cultures 13 days old at harvest).

Grams of $MgSO_4$ per liter	Data on 13 day old cultures			
	Weight of dried mats mgs.	pH of filtrates	Comparative estimate of yellow color in filtrates	Presence of crinkling in surface mats
0.000	no growth	4.6	0.0	none
0.005	100 96 77 91	6.4 6.4 6.0 6.3	0.0	none
0.025	230 237 253 240	6.4 6.4 6.6 6.5	1.0	none
0.100	313 294 311 306	5.6 5.6 5.6 5.6	0.0	none
0.500	408 439 371 406	6.2 6.4 6.2 6.2	1.0	yes
2.000	606 621 605 611	6.8 7.2 7.0 7.0	2.0	yes
10.00	664 664 650 659	7.6 7.6 7.6 7.6	3.0	yes
20.00	692 681 686 686	7.6 7.6 7.6 7.6	4.0	yes
Two grams only of K_2SO_4/L	no growth	4.6	0.0	none

(KNO_3-5, KH_2PO_4-2, Sucrose, Cry. Dom. -40, $ZnSO_4$-0.050)
(40.0 cc. of solution per culture in 125 cc. flasks)

abnormally large yield to get an average of 0.190 grams. Also a decrease in the KH_2PO_4 causes but a very small decrease in the average yield, (in one instance the average yield difference is 0.001 gms.) One of the chief faults of such experimentation is

lack of data over a longer culture period.

In my experiments dealing with the growth responses of Fusarium radicicola zinc sulphate has been added in excess of the required amount so as to avoid any variations due to its presence as an impurity in the magnesium sulphate. Triplicate cultures were permitted to grow for 13 days before harvesting but

Table 8. Growth responses of Fusarium radicicola to different amounts of magnesium sulphate in the culture medium.

Age of cultures at harvest	Grams of magnesium sulphate used per liter							
	A 0.100		B 0.500		C 1.000		D 20.000	
	dry wts. mgs.	pH of filt.	dry wts. mgs.	pH of filt.	dry wts. mgs.	pH of filt.	dry wts. mgs.	pH of filt.
4 days	74	6.2	130	6.8	153	7.0	273	7.6
	84	6.2	116	6.6	127	6.8	239	7.4
	84	6.2	122	6.6	127	6.8	223	7.4
	81	6.2	123	6.7	136	6.9	245	7.5
6 days	205	6.4	379	7.2	482	7.4	585	8.0
	222	6.4	350	7.0	459	7.4	602	8.0
	228	6.6	340	6.8	424	7.3	620	8.0
	218	6.5	356	7.0	455	7.3	602	8.0
8 days	357	6.8	506	7.2	600	7.4	714	8.4
	334	6.8	508	7.2	606	7.4	727	8.6
	322	6.8	493	7.2	618	7.4	718	8.6
	338	6.8	503	7.2	608	7.4	719	8.5
11 days	444	7.2	557	8.0	636	8.2	706	8.8
	478	7.6	570	8.0	641	8.2	716	8.8
	428	7.2	563	7.6	631	8.2	720	8.8
	450	7.3	563	7.9	636	8.2	714	8.8
13 days	488	8.0	553	8.4	617	8.6	720	8.8
	459	8.0	553	8.4	590	8.6	722	8.8
	462	8.0			598	8.6		
	470	8.0	553	8.4	602	8.6	721	8.8

(KH_2PO_4-2, KNO_3-5, Sucrose-40, $ZnSO_4$-0.050. 40 cc./ 125 cc. flask)

there is no assurance that maximum yields were obtained. The average yields increased with the concentration of the salt, but it was only when less than 2 grams per liter were present that the growth increase was in any close correlation with the concentration. It was found that K_2SO_4 could not replace the $MgSO_4$. (See Table 7).

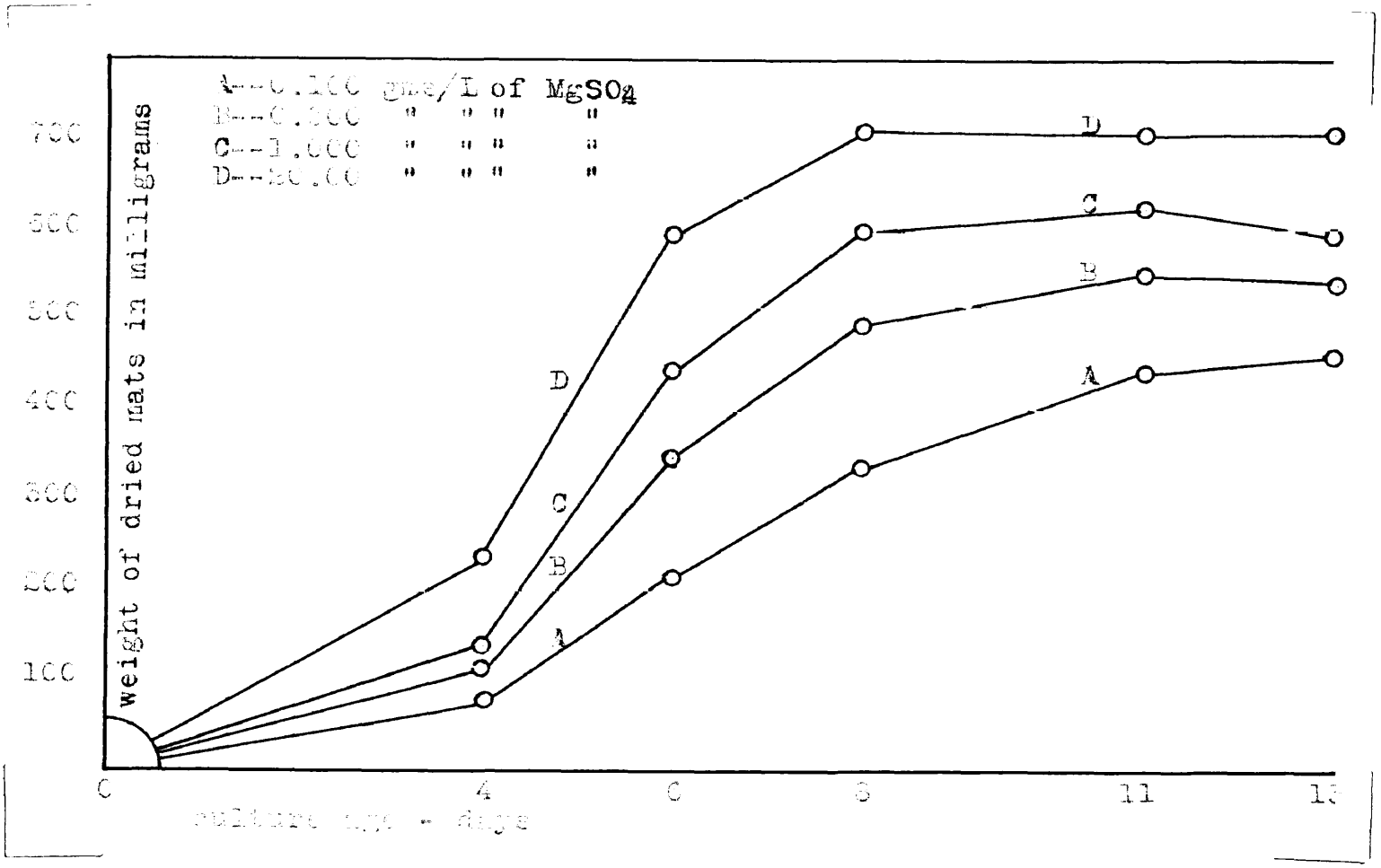

Fig. 6. Growth curves of Fusarium radicicola in media containing different amounts of magnesium sulphate per liter of solution.

In the next experiment the variations in concentration were fewer but measurements were taken at five intervals during a culture period of 13 days. The results are given in Table 8. The growth curves in Figure 6 show that one gram of the salt per liter is not sufficient to give a maximum growth in such a culture solution. The amount of sugar used as well as the concentration of the other nutrient components are important factors in determining the response of an organism to a variation in any one component. If the carbohydrate supply is sufficiently limited then the addition of more magnesium sulphate, it seems would result only in a faster growth rate and not materially influence

the final maximum yield. From these growth curves it is evident that a limitation in the supply of such a salt as magnesium sulphate influences both the dry weight of mycelium produced and the growth rate.

The culture medium tended to become alkaline in all cultures and this tendency was closely correlated with the total growth and the concentration of the magnesium sulphate. The rate and extent of the pH changes are shown in Figure 7.

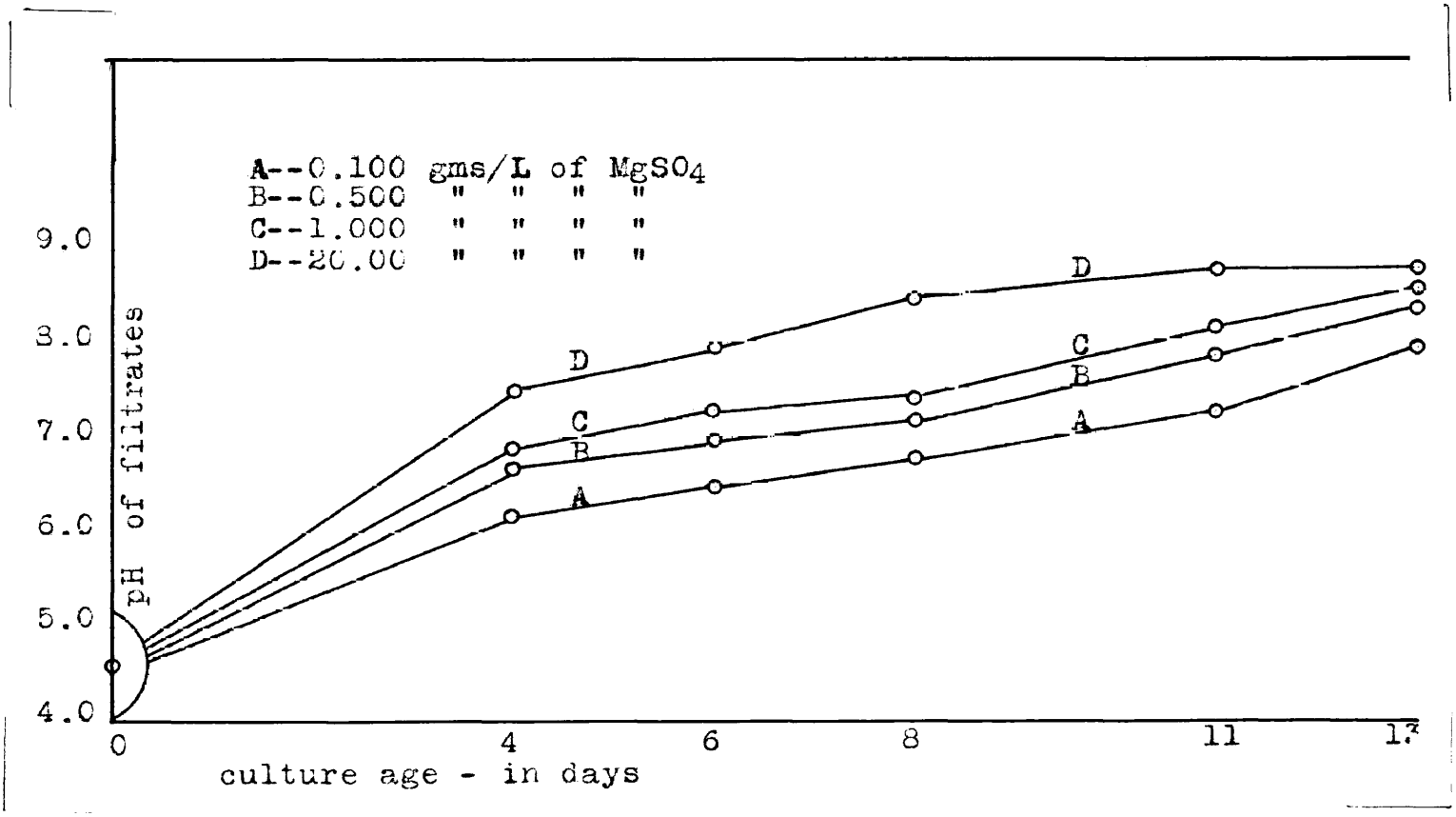

Fig. 7. The rate and extent of the pH changes in media containing different amounts of magnesium sulphate by F. radicicola. (See Table 8).

From the results in these two experiments it seems evident that under these cultural conditions the addition of more than two grams of magnesium sulphate is not necessary to obtain the maximum dry weight yield. However, if larger cultures

were used containing more carbon and more of the inorganic salts then undoubtedly it would be necessary to make corresponding increases in the supply of magnesium sulphate to obtain optimum growth conditions.

4. POTASSIUM ACID PHOSPHATE RELATIONS.

Under a given set of experimental conditions there is little if any data available that would enable one to predict the growth responses of one or more organisms to variations in the amount of monopotassium acid phosphate. An experiment was conducted to compare the responses of two fungi to such variations. The dry weights and the pH changes are presented in Table 9.

In a previous cultural experiment in test tubes it was found that several fungi made practically no growth when the KH_2PO_4 was omitted. The amounts of this salt used in the standard nutrient solutions varies from one to five grams per liter. In most of the solutions the writer has used from one to two grams per liter of either KH_2PO_4 or CaH_2PO_4 and obtained fairly good growth. Secondary potassium acid phosphate (K_2HPO_4) has always caused a heavy precipitate upon steam sterilization. By using Aspergillus niger 4247w and Fusarium radicicola which differ widely in appearance, taxonomic position, and the production of certain metbolic products, in media containing different concentrations of KH_2PO_4 it was hoped that some general conclusions might be reached. As in the previous experiments zinc sulphate was added to all cultures to insure good growth regardless of its possible presence as an impurity.

Table 9. Growth responses of Fusarium radicicola and Aspergillus niger according to the dry weight (expressed in milligrams) of mycelial mats produced and the H-ion concentration of the filtrates to variations in the concentration of KH_2PO_4.

Amount of KH_2PO_4 in gms./liter	Original pH of media	Age of cultures when harvested: 4 days — F. radicicola dry wts. mgs.	4 days — F. radicicola pH of fil.	4 days — A. niger dry wts. mgs.	4 days — A. niger pH of fil.	8 days — F. radicicola dry wts. mgs.	8 days — F. radicicola pH of fil.	8 days — A. niger dry wts. mgs.	8 days — A. niger pH of fil.	12 days — F. radicicola dry wts. mgs.	12 days — F. radicicola pH of fil.	12 days — A. niger dry wts. mgs.	12 days — A. niger pH of fil.
0.000	4.8	16 17 16	5.2	15 16 15	4.4	17 18 17	7.0	17 20 18	4.2	25 24 24	7.2	24 21 22	4.2
0.005	4.8 4.8	30 32 31	6.4	35 33 34	4.0	49 36 42	6.6	43 43 43	3.6	79 74 76	6.0	53 65 59	3.8
0.025	4.8	81 75 78	6.8	80 88 84	3.8	106 112 109	6.8	105 104 104	3.4	164 168 166	6.4	126 136 131	3.2
0.100	4.8	176 171 173	7.2	186 189 187	3.4	392 397 394	7.4	323 350 336	2.4	509 517 513	7.2	411 390 400	2.2
0.500	4.6	166 176 171	7.6	323 315 319	3.8	642 642 642	7.4	741 747 744	2.0	645 633 639	7.6	703 692 697	2.2
1.000	4.4	167 160 163	7.2	329 314 321	4.0	658 645 651	7.4	772 752 761	2.2	665 665 665	8.0	721 725 723	2.2
3.000	4.2	192 186 189	6.8	342 333 337	4.2	615 598 606	7.4	776 789 782	2.2	656 660 658	7.4	718 715 716	2.2
6.000	4.0	193 202 197	6.6	356 342 349	4.0	596 627 611	7.0	792 778 782	2.2	630 624 627	7.2	717 731 724	2.2
10.00	3.8	208 204 206	6.4	344 334 339	4.0	666 663 664	6.8	790 788 789	2.2	665 644 654	6.6	750 730 740	2.2
20.00	3.8	221 211 216	6.0	344 337 340	3.8	634 660 647	6.6	820 821 820	2.2	642 642 642	6.2	761 768 764	2.4

(KNO_3-5, $MgSO_4$-2, sucrose (Cry. Dom.) -40, $ZnSO_4$-0.050. and a trace of ferric tartrate) (40 cc./culture in 125 cc. flasks).

In Figure 8 the maximum dry weight of mycelium and the corresponding pH of the filtrates have been plotted so as to bring out more clearly the nature of the results obtained. The addition of twenty grams per liter did not bring about a condition which was toxic to either fungus. The addition of more than 0.500 grams caused but little increase in growth. The growth rates in the different concentrations varied a little but was scarcely significant in the higher five concentration series. The A. niger was able to make a greater total vegetative growth than the F. radicicola but both exhinit a similar salt requirement. The pH changes brought about by F. radicicola are of interest. The absence of the phosphate as a buffer seems to be responsible for the marked shift towards neutrality in the check series where only a very limited vegetative growth was possible. The pH change towards an alkaline condition in the media containing less than 3.00 grams per liter can be explained on the basis of unequal ionic absorption which in extent, is proportional to the total vegetative growth and the absence of a sufficient amount of the phosphate to make an effective buffer action. When more than three grams were present a buffer action occured. Whatever buffer action was present did not, according to the dry weights, bring about an acid condition which was unfavorable. In general, experimental evidence has shown that the Fusaria can tolerate rather a wide range in acidity and alkalinity.

The pH changes resulting in the A. niger cultures were of a different nature. They became decidedly acid due to the formation of organic acids. No determinations were carried out to

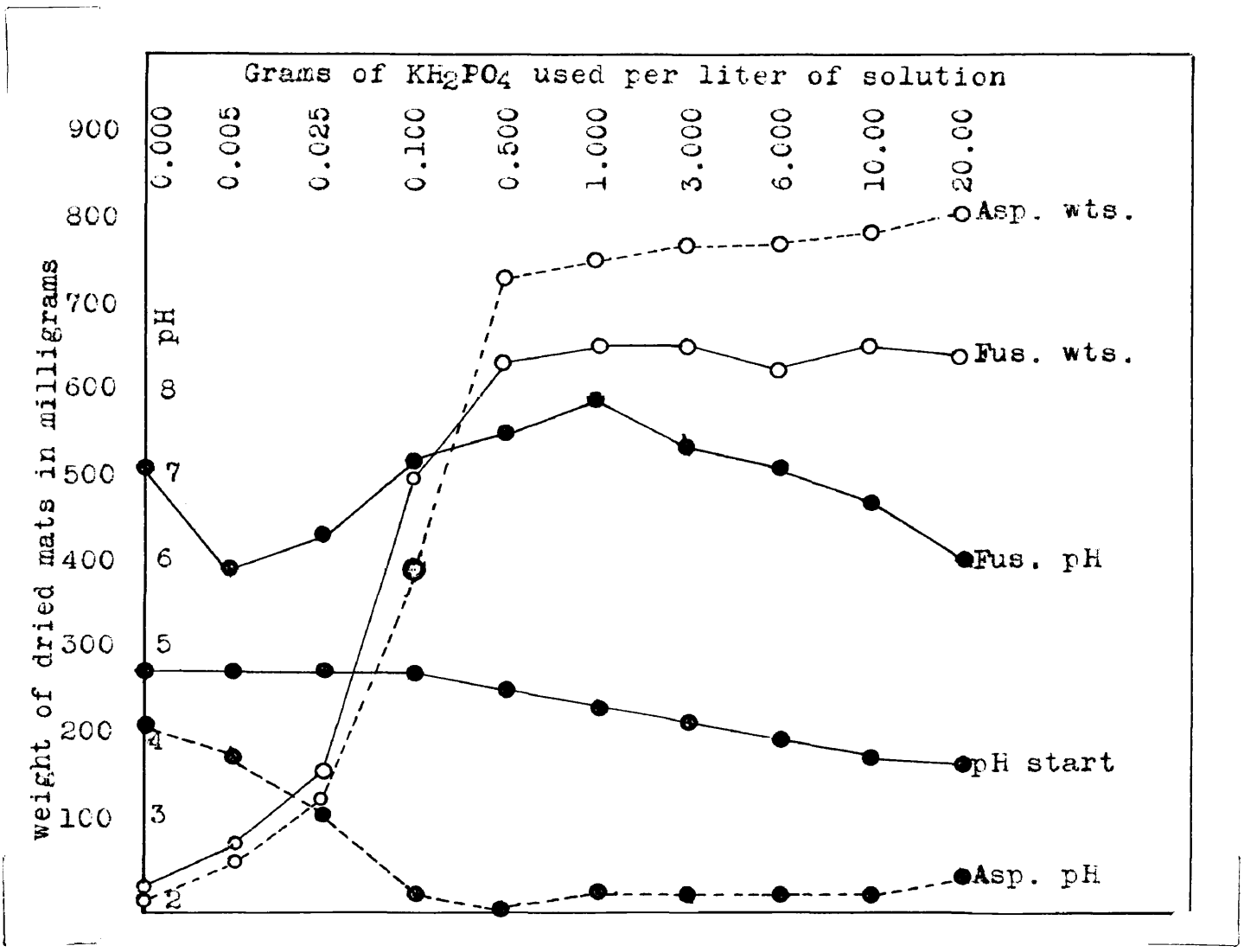

Fig. 8. The average maximum yields and the pH change by Aspergillus niger 4247w and Fusarium radicicola in media containing different amounts of mono-potassium acid phosphate. (See Table 9.)

determine the kind and amount of acid present. A restricted vegetative growth suggests a decrease in the acid production as indicated by the pH in the series low in the phosphate.

The use of 5.0 grams of potassium acid phosphate as in the Pfeffer solution does not lead to a toxic condition but a much smaller amount could be employed without materially affecting the maximum yield. The growth rate and the maximum dry weight yield are influenced by the amount of the different nutrient components used. Differences in the growth rate do

not necessarily mean corresponding differences in the final maximum yield. The maximum yields in vegetative growth or the production of different acidity conditions as influenced by variations in one or all of the nutrients can only be determined accurately by using a sufficient number of cultures that will permit measurements at different times during the culture period.

B. Growth responses to some elements listed as stimulants.

The action of various salts in a synthetic medium has been the subject of an intensive study. As a result some elements were listed as absolutely essential, others unessential, and some as stimulants. The results obtained by various investigators have depended upon the conditions of their experiments, namely, the organisms used, and the purity of the salts and water. Only by the use of the most carefully purified chemicals can one definitely classify an element as essential or unnecessary.

The distinction between a necessary element and one which causes a growth stimulation is in most cases a matter of definition in terms of the experimental conditions. An essential element might be called a stimulating element. In general the so-called stimulants are effective in very small amounts which in some respects suggests that they may function as catalysts. If the addition of an element to the medium causes only an increase in the growth rate but does not change the final total growth it would function as a stimulant. In Figure 9 are three hypothetical growth curves, A, B, and C which will aid in picturing the writer's concept of a possible difference between essential and

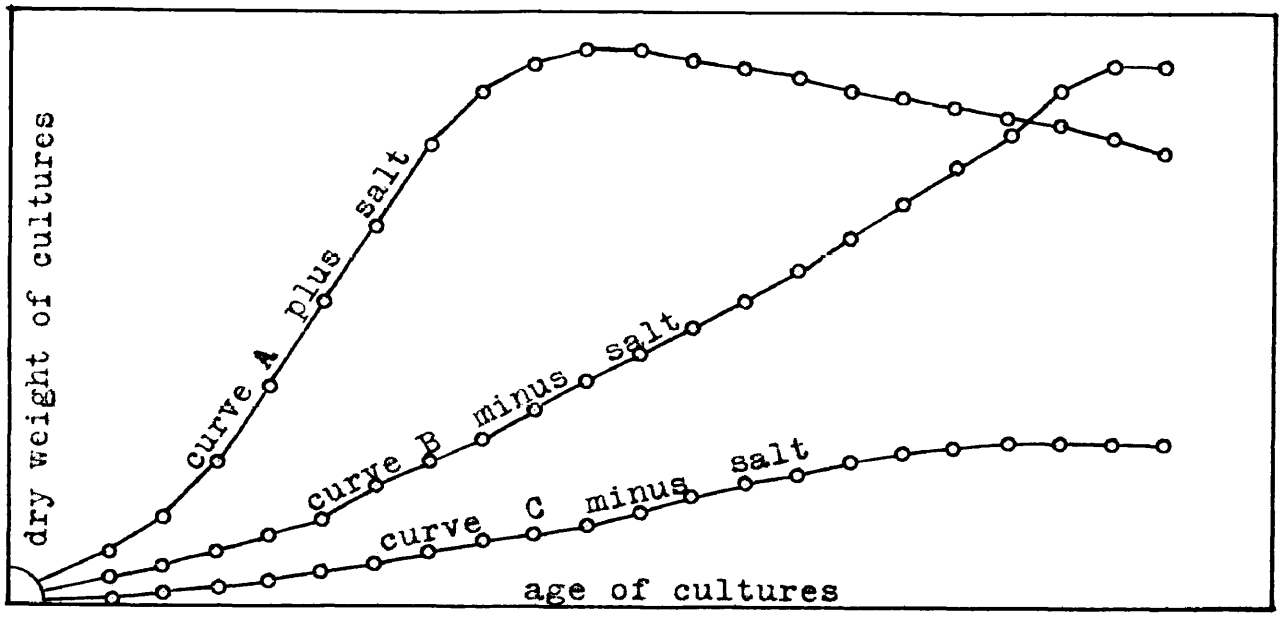

Fig. 9. Theoretical growth curves to illustrate a concept of a chemical stimulation of growth.

stimulating elements. If the curve A is obtained in the presence of the salt containing the element in question and curve B obtained when the salt is absent then the element would function only as a growth stimulant. While on the other hand if in the absence of the salt a curve similar to C was obtained then the element might be considered as essential for good growth.

In the following experiments dealing with boron, manganese, and zinc no special methods of salt purification were employed. The object has been to study the responses of several fungi especially some Fusaria to the presence of these elements in media containing chemicals of standard purity. Most of the culture work with fungi has been with standard C. P chemicals hence the present series of experiments can be compared with the results of other workers using similar experimental conditions. No doubt somewhat different results would have been obtained by

the utilization of special glassware and more highly purified chemicals. However, it seems logical to assume that only minute quantities of boron was present in either the salts or the distilled water since Johnston (21) used the same salts and distilled water and obtained positive responses of tomato plants on the addition of one to two parts of boron per million.

5. RESPONSES TO BORON.

Our knowledge of the action of boron on the higher plants is more definite and complete than in regard to the fungi. Although necessary only in minute traces, the work of Johnston (21), and Brenchley, (7 & 8), makes it clear that boron is an essential element in the nutrition of several crop plants. Collings (10), however, did not find boron essential to grow soy bean plants to maturity in nutrient solutions. Boric acid has been considered by some workers as a possible fungicide. Morel, (33), found that boric acid in weak solutions completely arrested the development of several fungi and suggested that it might be used in disease control measures. Edmondson, et al (16), and Powell, (37), have studied the fungicidal action of boron salts and in most cases have found them more or less ineffective. Agulhon, (1), working with yeasts, bacteria, and molds found no evidence of stimulation with small quantities. With the molds little toxic action was observed until approximately a concentration of 5.0 grams per liter of boric acid was used. Cusumano, (14), claims that the development of Aspergillus niger was favored at strengths below 0.01 per cent boron (boric acid). His results are scarcely conclusive due to the slight increases in

growth, the variability of the culture weights and the lack of growth measurements over a longer period of time.

Davis, et al. (15) used a medium containing C. P. chemicals and another medium for which the salts had been carefully recrystallized and purified. They report, "When Dothiorella sp. was employed yields about one half, and with the addition of one and one half parts to a million restored yield to 90 per cent of that obtained with the unpurified salt". They believe that boron is to be regarded as an essential element.

In a series of preliminary experiments using test tube cultures with different amounts of boric acid in media of varying composition, several fungi were cultured to detect possible increases in growth. Qualitative estimations from such cultures did not give consistent results. It was impossible to detect any benefit from adding small amounts of boric acid or sodium borate.

Steinberg (47) showed quite conclusively that Aspergillus niger was able to secure appreciable quantities of zinc from certain types of glass used for culture flasks. The possibility of the fungi to obtain small quantities of boron from the Pyrex and other flasks containing traces of boron has not been overlooked. However, glass ware known to be free from boron was not used. A series of cultures were prepared in bottles which had contained "Bakers Analyzed Chamicals" of 250 cc. capacity. The following four series of media were prepared:

A. - $MgSO_4$-2.0, KH_2PO_4-2.0, KNO_3-5.0, Glucose C.P. -32.0

B. - Same as A plus 25 mm. of ferric tartrate.

C. - Same as A plus 25 mm. of boric acid.

D. - Same as A plus 25 mm. each of boric acid and ferric tartrate.

Five cultures in each medium were innoculated with Fusarium radicicola and observed at intervals for a period of 15 days. No evidence of stimulation or any other growth responses due to the presence or absence of either boron or iron used singly or together could be observed at any stage of development. Either the fungus can make but little response to these chemicals or sufficient amounts were included as impurities in the medium or the necessary quantities were available from the glassware.

Table 10. Comparative growth responses, based on the dry weight of the mycelial mats and pH changes in the culture medium, of three fungi to the presence of boron and zinc in the culture medium.

Organisms used and age at harvest	Culture series A Check, plus iron		B Plus iron and zinc		C Plus iron and boron	
	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.
Fusarium radicicola 701. 9 days	291	6.8	767	8.2	525	7.8
	286	6.8	788	8.2	480	7.8
	297	6.8	758	8.2	522	7.8
	291	6.8	771	8.2	509	7.8
Aspergillus niger 4247w 9 days	402	*3.4-	738	4.8	475	3.4-
	397	3.4-	782	4.8	492	3.4-
	363	3.4-	775	4.8	488	3.4-
	387	3.4-	765	4.8	485	3.4-
Fusarium tricothecioides 141 21 days	297**	4.0	482	8.0	333	8.0
	316	7.0	500	8.2	195+	6.8
	310	7.2	556	8.4	362	8.2
	313	7.1	513	8.2	347	8.1

($MgSO_4$-2, KH_2PO_4-2, KNO_3-5, Sucrose, Cry. Dom. -30. Ferric tartrate - 0.025). Original pH of medium was 4.6. Cultures in 250 cc. Pyrex Erlenmeyer flasks with 60.0 cc. of culture solution.

** Contaminated with A. niger and not included in average.

+ Flask overturned and part of medium lost.

* pH 3.4 - means below pH 3.4.

A positive response to boron was obtained with three fungi grown in the large culture flasks. The dry weights are given in Table 10. From the dry weights of the mycelium it is evident that zinc caused an increase in the growth of each fungus. Two organisms made some response to the boron but not like they did to the zinc. Fusarium tricothecioides 141 grew slowly and the harvest was delayed until the cultures were three weeks old. It was benefitted somewhat by the zinc while it is doubtful if the slight increase in weight due to boron is significant.

The following descriptive notes were taken at the time of harvest:

Fusarium radicicola 701.

check-cultures A
Liquid medium clear, traces of blue in the mycelium, rather poor surface growth.

plus zinc-cultures B
Liquid medium deep yellow, large blue spots in mycelium, mycelium heavy and much wrinkled.

plus boron-cultures C
Liquid medium faintly yellow, only a few traces of blue in mycelium, surface growth better than in checks.

Aspergillus niger 4247w.

check-cultures A
Medium clear, irregular surface growth, abundant crop of very black spores, no bag-like projections of surface mats down in the liquid.

plus zinc-culturesB
Liquid medium deep yellow, surface growth quite regular, spore masses not so black as in check cultures, much sinking and folding of surface mat forming bag-like projections down into the medium, unquestionably increased growth due to zinc.

plus boron-cultures C
In all respects similar to check cultures other than a slightly heavier surface growth.

Fusarium tricothecioides 141.

check-cultures A
Liquid medium clear, mats pink and water soaked.

plus zinc-cultures B
Liquid medium slightly yellow, mats faintly pink and water soaked, definite growth increase due to zinc.

plus boron-cultures C

Like check cultures, only with less pink color in mats.

The increase in dry weight of the mycelium of the fungi in the above experiment due to the presence of boron did not seem

Table 11. Growth responses of Fusarium radicicola to small amounts of Boric acid in the nutrient medium.

Age of cultures	Concentration of Boric acid in mgs./liter					
	A none		B 10		C 30	
	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.
6 days	68	5.4	89	5.8	65	5.4
	66	5.4	77	5.6	74	5.6
	51	5.4	85	5.8	53	5.4
	74	5.6	67	5.6	61	5.4
	71	5.6	91	5.8	55	5.4
	66	5.5	82	5.7	62	5.4
12 days	297	6.6	329	6.8	314	6.6
	292	6.6	337	6.8	302	6.6
	332	6.8	335	6.8	298	6.6
	290	6.6	336	6.8	298	6.6
	303	6.6	311	6.6	298	6.6
	303	6.6	329	6.8	302	6.6
18 days	366	7.0	381	7.4	373	7.2
	381	7.6	374	7.4	372	7.2
	356	7.0	369	7.2	378	7.6
	365	7.0	377	7.2	352	6.8
	377	7.2	383	7.6	366	7.2
	369	7.2	377	7.3	368	7.2
26 days	326	7.4	339	7.6	342	7.8
	328	7.4	332	7.8	349	7.6
	337	7.8	339	7.6	339	7.8
	323	7.6	334	7.8	331	7.6
	334	7.8	343	7.8	343	7.6
	329	7.6	337	7.7	341	7.7
33 days	323	7.8	309	7.9	314	7.8
	316	7.8	323	7.9	326	7.8
	310	7.8	311	7.8	320	7.8
	312	7.8	328	7.9	322	7.8
	315	7.8	318	7.9	320	7.8

($MgSO_4$-2, KH_2PO_4-1, KNO_3-4, Baker's C. P. Glucose -40)
(40.0 cc. of solution per culture in 125 cc. Pyrex flasks).

to agree with the observed appearance of the cultures. The small number of cultures with only one harvest did not seem to justify a definite conclusion that boron was beneficial. Another experiment was made with Fusarium radicicola using a larger number of cultures and more harvest periods. From the results obtained in Table 11 the beneficial effects of adding boric acid are slight. The addition of 10 mm. of the acid per liter gave some increase in yield over the checks while the addition of 30 mm. per liter did not increase the yield.

As stated before Fusarium radicicola under some conditions forms red and blue pigments which diffuse out into the liquid medium. The acidity developed in a medium containing ammonium nitrate seemed to favor the developments of such pigments. In one of the preliminary tests in test tube cultures, different

Table 12. Effect of boric acid on the vegetative growth and pigment formation of Fusarium radicicola.

Culture description	Grams of Boric acid used per liter					
	0.000	0.010	0.050	0.100	0.500	2.000
quality of growth	poor	poor	poor	fair	good	poor
color of mats	red	red	red	red	white	gray
pH of media	3.4	3.4	3.6	3.8	4.4	4.4

($MgSO_4$-2, KH_2PO_4-1, NH_4NO_3-4, glucose C. P. -32, no zinc).

amounts of boric acid were added to a medium containing ammonium nitrate. In those cultures containing 0.500 gms. of boric acid per liter there was a decided increase in growth, no pigment was formed and the mycelium was white with a luxuriant, fluffy surface growth. The cultures containing less amounts of the boric acid

made a poor growth, with red, hard and crinkled surface mats. The amount of acid used in series 6 was toxic. The original pH of the media was 4.4-4.6. The pH of the culture media after two weeks growth are given in Table 12. These results suggest that the beneficial action is due to something other than the presence of the required amount of boron. In some way the boric acid has prevented the development of an intense acid condition when 0.500 grams per liter had been supplied. The boric acid in such a concentration might have acted as a buffer or may have had a more complex action such as influencing the ratio of ionic absorption or the possible production of organic acids. In test tube cultures these results have been duplicated several times.

In a similar type of experiment the concentration of glucose was varied with different amounts of this acid present while the inorganic salts were the same as stated in Table 12. The beneficial effect of the acid varying from 0.300 to 750 grams per liter was greater in a medium containing 24.0 grams of glucose than one containing 6.0 grams per liter. In the low sugar medium there was little pigment formed in the mycelium and the acid condition persisted only for a short time after which the medium moved towards alkalinity. Urea, peptone, and potassium nitrate were used as nitrogen sources under the same conditions but without a corresponding beneficial action of the boric acid. In the presence of such nitrogen sources this fungus decreased the acidity of the medium. Hence this type of beneficial action of boric acid seems to be restricted to a medium containing ammonium nitrate and an abundant supply of glucose.

Table 13. Growth responses of Fusarium radicicola on an ammonium nitrate medium containing combinations of boron, iron and zinc.

Variations in culture medium	Age of cultures when harvested							
	14 days		21 days		28 days		34 days	
	dry wts.	pH	dry wts.	pH	dry wts.	pH	dry wts.	pH
check	94	4.4	176	4.8	256	5.4	205	5.0
minus boron, iron and zinc	112	4.4	169	4.8	253	5.4	204	5.0
	105	4.4						
	104	4.4	172	4.8	255	5.4	204	5.0
plus 0.300 gms. boric acid/L	101	4.0	191	4.4	172	4.4	183	4.4
	120	4.0	184	4.4	175	4.4	183	4.4
	108	4.0						
	110	4.0	187	4.4	174	4.4	183	4.4
plus 0.400 gms. boric acid/L	116	4.2	186	4.2	165	4.2	158	4.2
	137	4.2	163	4.2	166	4.2	160	4.4
	124	4.2						
	126	4.2	174	4.2	165	4.2	159	4.3
plus 1.000 gms. boric acid/L	148	4.2	145	4.2	155	4.0	144	4.0
	127	4.2	149	4.2	149	4.0	149	4.0
	115	4.2						
	130	4.2	147	4.2	152	4.0	147	4.0
no boric acid plus iron and zinc	471	3.6	389	8.4	341	8.8	305	8.8
	477	3.6	379	8.4	348	8.8	295	8.8
	481	3.6						
	476	3.6	384	8.4	345	8.8	333	8.8
plus 0.300 gms. boric acid/L plus iron and zinc	388	3.6	338	8.0	316	8.2	285	8.2
	371	3.6	343	8.0	308	8.2	292	8.2
	362	3.6						
	373	3.6	381	8.0	312	8.2	288	8.2
plus 0.400 gms. boric acid/L plus iron and zinc	267	3.6	336	8.0	307	8.2	288	8.2
	275	3.6	328	8.0	301	8.2	282	8.2
	258	3.6						
	267	3.6	332	8.0	304	8.2	285	8.2
plus 1.000 gms. boric acid/L plus iron and zinc	88	4.2	268	3.8	326	5.0	230	5.4
	97	4.2	271	3.8	317	5.0	229	5.4
	97	4.2						
	94	4.2	270	3.8	322	5.0	229	5.4

($MgSO_4$-2, KH_2PO_4-1, NH_4NO_3-4, Glucose C. P. 32)
(40 cc. of solution per culture in 125 cc. Pyrex Erlenmeyer flasks).

The conditions governing the production of pigment by Fusarium radicicola are complex. The size and shape of the culture vessel may influence the results. In Table 13 are the results of an experiment with this fungus cultured in Pyrex Erlenmeyer flasks containing 40.0 cc. of solution. The medium

used was the same as stated in Table 12, with slightly different amounts of boric acid. The series of boric acid variations were divided in two lots. To one series 25 mm. of zinc sulphate and 25 mm. of ferric tartrate were added. The presence of the zinc greatly increased the growth in the check cultures. The toxicity of the boric acid was increased in the zinc cultures. Contrary to expected results only faint traces of pigment were formed in the cultures lacking zinc and iron, nor was there any definite beneficial action of boric acid. The cultures containing boric acid, iron and zinc at the end of two weeks had developed an intense amount of red pigment which turned to a bluish-purple color as the cultures aged. The change from red to blue corresponded closely with the changes in the acidity of the culture medium, an alkaline condition causing the pigment to become blue. The results do not seem to line up with the results obtained in the previous experiments. However, there is a great difference in the ratio of volume of liquid to area of liquid surface between a culture in a test tube and one in an Erlenmeyer flask. A large growing surface and a shallow depth of the culture medium without much question favor vegetative growth more than would the reverse conditions. Zinc and iron may play some role in the formation of such pigments either directly or indirectly. Zinc was not added to the test tube cultures but these tubes were not Pyrex hence there is a possibility that enough zinc could have been obtained from such glass. Time has not permitted a further study of the factors which function in the development of pigments by this fungus nor have other organisms been cultured under similar conditions.

The toxic action of boric acid has been given some consideration. No outstanding increases in vegetative growth was consistently obtained by the addition of relatively small amounts of boron. It seemed important to determine what concentrations of boric acid were actually necessary to hinder the growth of several fungi and at what concentration growth was inhibited. The results of such a study are given in Table 14. The cultures were all innoculated on the same day and stored under the same conditions of light and temperature. The results agree, in general with those of previous investigators, in that boric acid is not uniform in its toxic action on a variety of fungi, and that relatively strong concentrations are necessary to inhibit growth. The Fusaria used differ widely in their tolerance of boric acid. It was interesting to note that two species F.242 and F.141 which are slow in growth and never make as large a dry weight yield (See Table 20 and Figure 11) as some of the other species, were able to withstand greater concentrations of boric acid than some more rapidly growing species. The white-spored Aspergillus 117 was unable to make more than a trace of mycelium in any cultures. Aspergillus 4247w and 5534c were able to grow over a wide range of the acid concentration. Both Aspergilli were retarded in spore production and eventually inhibited in concentrations where a fairly good vegetative growth was possible. (See Figure 10). The pH of the filtrates varied somewhat with the different organisms but in general the decrease in acidity was in direct proportion to the total growth. However, Aspergillus niger 4247w was an exception apparently due to the formation of organic acids. The increse in the H^{+}ion concentration due to

Table 14. Toxicity of Boric Acid to different fungi when grown under similar conditions.

Gms. B. A. per L.	0	1	2	4	6	8	10	12	14	16	18	20
series number	1	2	3	4	5	6	7	8	9	10	11	12
original pH	4.6	4.6	4.6	4.6	4.4	4.4	4.4	4.4	4.4	4.2	4.2	4.2
Fungi												
	5.4	5.2	5.2	4.6	4.4	- final pH of media						
Asp.	s	s	s	s	s	-type of growth-submerged only						
117	¼	½	¼	¼	¼	-estimated total growth						
	7.4	6.2	5.2	4.6								
Fus.	m	m	s			m - surface mats formed						
528	4	4	1	0								
	6.6	6.0	5.4	4.6								
Fus.	m	m	m									
658	5	4	1	0								
	7.2	6.4	5.8	4.6								
Fus.	m	m	m									
701	5	4	1	0								
	7.4	7.2	6.8	6.4	5.0	4.4	4.4					
Fus.	m	m	m	m	s	s						
200	5	5	5	2	1	½	0					
	7.0	7.2	6.2	5.8	5.2	4.4						
Fus.	m	m	m	m	s							
100	4	4	3	2	1	0						
	6.8	6.4	6.2	6.2	5.8	5.4	5.2	4.4	4.4			
Fus.	m	m	m	m	m	m	m	m	s			
244	4	4	4	4	3	1	1	½	0			
	6.4	6.4	6.6	6.6	6.0	5.0	4.8	4.6	4.6	4.6	4.4	4.2
Fus.	m	m	m	m	m	s	s	s	s	s	s	
141	3	3	3	3	2	1	½	½	½	½	¼	
	4.8	4.2	4.2	2.6	2.4	3.8	4.2	4.4	4.4	4.2		
Asp.	m	m	m	m	m	m	m	m-	m-			
4247w	5	5	5	4	2	2	1	½	½	0		
	7.0	7.0	6.8	6.8	6.4	6.2	6.0	5.6	5.2	4.6	4.2	4.2
Asp.	m	m	m	m	m	m	m	m	m	m	m	s
3534c	5	5	5	5	4	4	4	3	2	2	1	½

($MgSO_4$-3, KH_2PO_4-2, KNO_3-5, sucrose, Quak.-30, zinc sulphate-0.050) (All cultures in test tubes, containing the nutrient solution to a depth of 3.5 cm.) Duplicate cultures used.

the presence of boric acid does not seem to explain the nature of the exhibited toxicity since A. niger 4247w in cultures 4 and 5 increased the active acidity more than did any concentration of the added acid. The spores of this fungus always float on the

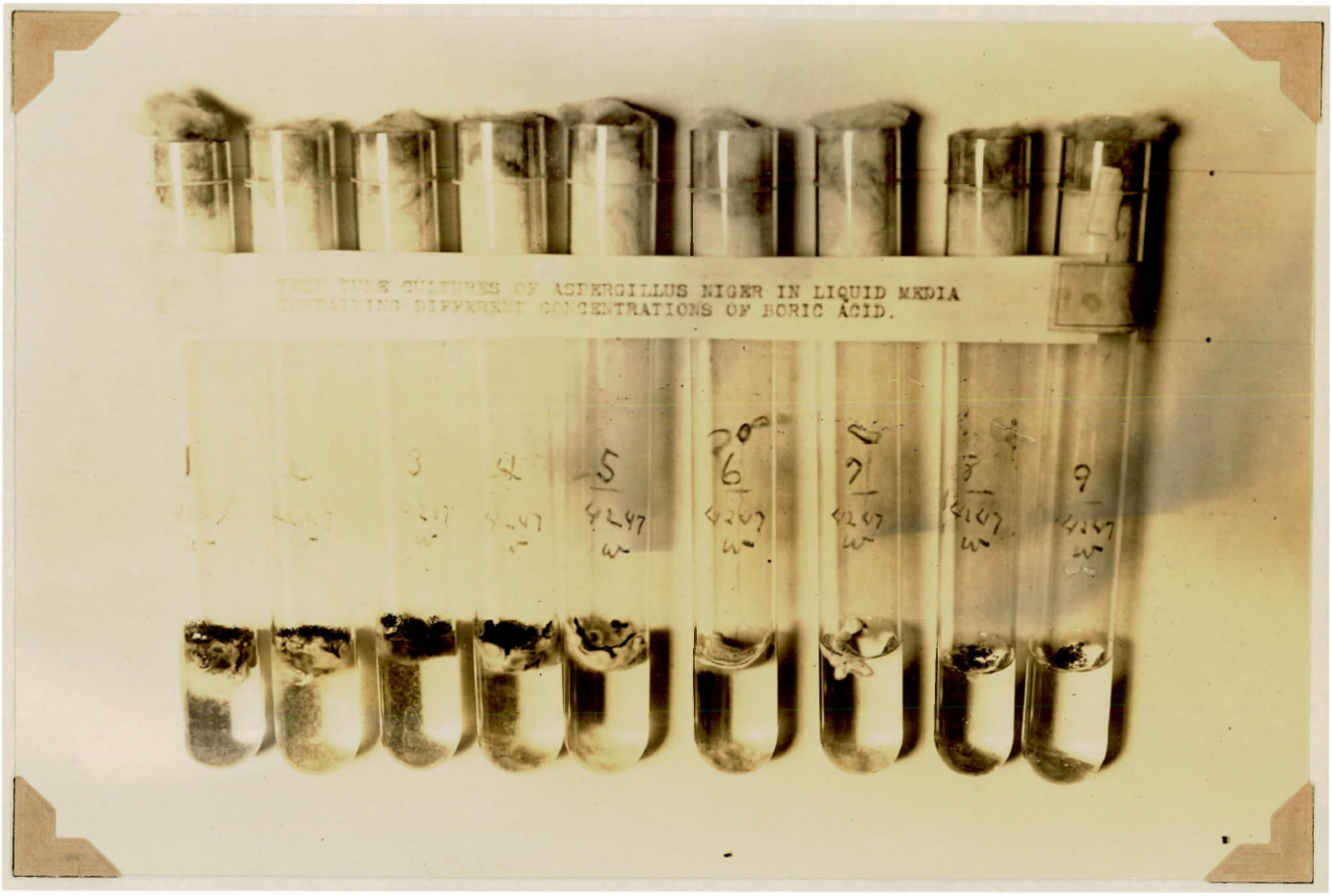

Fig. 10. Test tube cultures of Aspergillus niger 4247w in media containing different concentrations of boric acid. (See Table 14) Note that in cultures 6 and 7 spore production has been inhibited.

surface of the liquid and as a result cultures 8 and 9 in Figure 10 show black masses of spores used in the innoculation. These spores germinated but made only a weak growth and did not produce a new crop of spores.

Since vegetative growth is so closely related to the supply of available carbohydrates and a suitable nitrogen source, it seemed likely that the tolerance of boric acid might vary with the amounts of these food materials in the culture medium.

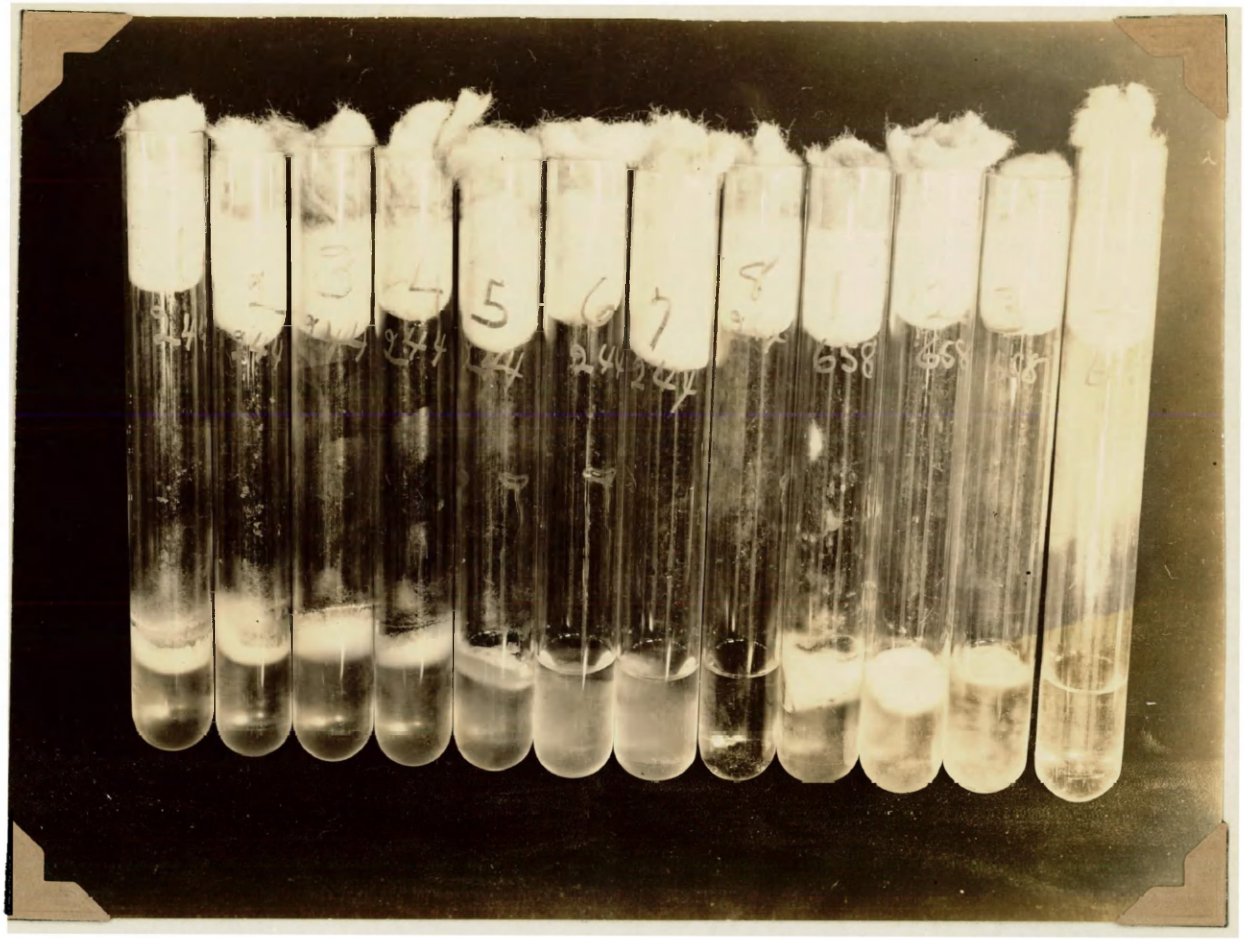

Fig. 11. Test tube cultures of Fusarium sulphurum 224 and Fusarium solani 658 in media containing different concentrations of boric acid. (See Table 14). Note that F. 244 was able to tolerate a higher concentration of boric acid, than F. 658.

Aspergillus niger 4247w was cultured for 21 days in media containing high and low concentrations of glucose and potassium nitrate. The results as indicated by the dry weights of the mycelial mats produced in test tube cultures are presented in Table 15. These figures definitely prove that boric acid tolerance is in a large measure influenced by the composition of the substratum, especially the supply of available carbon. These results suggest

Table 15. The effect of the nutrient solution on the tolerance of boric acid by Aspergillus niger 4247w, as indicated by the weight in milligrams of the dried mycelium.

Media used	Boric acid in grams per liter						Totals
	0.00	1.00	4.00	6.00	8.00	10.0	
glucose 6.0 grams/L KNO_3-1.0 grams/L	65	69	59	51	9	10	212
glucose-24.0 KNO_3-1.0	163	157	161	117	109	13	720
glucose-6.0 KNO_3-10.0	97	86	88	65	10	11	357
glucose-24.0 KNO_3-10.0	243	229	233	200	155	54	1014
vertical totals	568	541	542	434	283	88	

($MgSO_4$-2, KH_2PO_4-1, no zinc added)
(cultures in test tubes containing 10 cc. of soln.)
(all cultures in triplicate)

that the amount of any toxic substance required to inhibit the growth of fungi is probably influenced by the nutritive value of the substratum.

6. RESPONSES TO MANGANESE.

There has been some disagreement in regard to the manganese requirements of fungi. Richards (38) found that Aspergillus niger Penicillium glaucum, and Botrytis cinerea responded to traces of manganese chloride. These organisms did not show an equal response and he concluded that many fungi may be stimulated by manganese but it is not to be expected that all fungi will respond to the same degree. Loew and Sawa (29) were not able to detect any beneficial action of manganese. Bertrand and Javillier (4,5) using manganese sulphate found that one part of manganese in 25,000,000 substantially increased the yields of Aspergillus niger. They also

found that manganese and zinc used together gave better yield than when either was used alone. In a four days old culture series, the following results were obtained: Check-- 1.31 gms.; zinc, 1-500,000 --3.18 gms.; manganese, 1-10,000 -- 2.10 gms.; zinc and manganese -- 3.72 gms. (dry weight of mycelium expressed in grams). Sauton (45) claims that manganese is essential for normal spore production by Aspergillus fumigatus. No satisfactory explanation has been found regarding the specific function of manganese in fungus metabolism.

In the time available it has not been possible to make a very thorough study of the responses of the Fusaria to the presence of minute traces of manganese. A positive response was obtained with Fusarium radicicola and Fusarium sulphureum, to the addition of 50 mm. of manganous sulphate. According to the results presented in Table 16, zinc was more effective than manganese in increasing the dry weight of the mycelium. The cultures to which zinc had been added formed mats with a greater toughness and a more wrinkled suface than in the other two culture series. Although the two fungi are different in their growth rate and utilization of the sugar in dry weight production, their responses to the manganese and zinc are of the same order of magnitude.

An experiment was designed to study the effects of using different amounts of manganous sulphate in a medium plus and minus zinc since Bertrand and Javillier found that a better growth could be obtained if these elements were both present. The different culture series are explained in Table 17. During the steam sterilization precipitates appeared in some of the series of cultures.

Table 16. Growth responses of Fusarium radicicola and Fusarium sulphureum to the presence of zinc and manganese in the medium.

Organisms used cultures 14 days old	Culture media employed					
	plus manganese		check		plus zinc	
	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil
FUSARIUM RADICICOLA	827	8.0	717	7.2		
	863	8.2	638	7.0		
	848	8.0	691	7.2	926	8.0
	868	8.0	688	7.2	936	8.0
	839	8.0	651	7.2	901	8.0
	849	8.0	677	7.2	921	8.0
FUSARIUM SULPHUREUM	344	6.2	251	5.8		
	331	6.2	241	5.8		
	326	6.2	241	5.8	391	6.8
	338	6.2	196	5.4	400	6.8
	340	6.2	249	6.0	409	6.8
	336	6.2	236	5.8	400	6.8

($MgSO_4$-2, KNO_3-5, KH_2PO_4-2, sucrose Cry. Dom. 40)
(Manganous sulphate - 50 mm./L. zinc sulphate-50mm./L.)
(cultures with 60 cc. of soln. in 250 cc. Pyrex flasks)
(original pH of media-4.4-4.6)

This precipitate was readily disolved with a little hydrochloric acid, however, it was not possible to treat the culture flasks with acid. The original plan was to use Fusarium radicicola as the test organism but this suggested a further complication due to the tendency of this fungus to decrease the acidity of the medium. It seemed probable that Aspergillus niger, due to the production of organic acids, would be able to dissolve the precipitates and thus prevent a serious error in making dry weight determinations of the mycelium. As predicted this fungus was able to completely dissolve all the precipitates within eight days.

The dry weights of the mats in Table 18 show that manganese caused an acceleration of growth when used alone. There is no evidence that growth is increased by using both manganese and zinc

Table 17. Composition of the cultures series used in studying the response of Aspergillus niger (4247w) to zinc and manganese.

Series number		S-1	S-2	S-3	S-4	S-5
Media minus zinc	Gms. $MnSO_4$/Liter	0.000	0.005	0.050	0.200	1.000
	Number of cultures	12	12	12	12	12
	pH of media before steam sterilization	5.0	5.0	5.0	4.8	4.2
	pH of media after steam sterilization	7.0	6.8	6.6	6.4	6.2
	Precipitate after sterilization	none	trace	faint trace	fairly heavy	heavy
Series number		S-6	S-7	S-8	S-9	S-10
Media plus zinc	Gms. $MnSO_4$/Liter	0.000	0.005	0.050	0.200	1.000
	Number of cultures	12	12	12	12	12
	pH of media before steam sterilization	4.6	4.6	4.6	4.4	4.4
	pH of media after steam sterilization	6.4	6.4	6.4	6.4	6.2
	Precipitate after sterilization	trace	trace	fairly heavy	fairly heavy	heavy

($MgSO_4$-2, Urea -4, KH_2PO_4-2, zinc sulphate -0.050, and Sucrose, C. P. Merk, -30).
(40 cc. of soln. per culture in 125 cc. Pyrex flasks.)

in the same medium. However, the presence of the precipitates may have had some effect on the growth of the fungus. Zinc causes a greater acceleration and a larger production of dry weight than manganese. At the third harvest period autolysis is evident in all series except the checks (S-1). In all series the pH of the medium was sharply increased during the early period of growth and later decreased. This decrease in acidity is probably due to a partial utilization of the organic acids as well as a process of neutralization caused by ammonia released during autolysis.

Table 18. Growth responses of Aspergillus niger 4247w to zinc and manganese when used alone and together in the medium.

CULTURE SERIES	Age of cultures when harvested					
	8 days		15 days		22 days	
	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.
S-1	215	2.8	403	2.8	448	4.6
S-2	336	2.6	439	2.6	441	6.0
S-3	316	2.6	485	2.4	451	6.0
S-4	309	2.6	511	2.4	452	6.0
S-5	312	2.6	508	2.6	444	6.4
S-6	610	3.2	542	6.4	450	7.2
S-7	565	2.8	565	6.0	457	7.2
S-8	550	2.8	591	6.0	447	7.2
S-9	527	2.8	637	6.0	438	7.2
S-10	550	4.0	511	6.6	454	7.2

(Both weights and pH values are the averages of triplicate cultures).

The results given in Tables 16 and 18 lead to the conclusion that manganese supplied as manganous sulphate will increase the growth rate and the total dry weight of the mycelial mats to approximately the same degree with either Fusarium radicicola, Fusarium sulphureum, or Aspergillus niger. The role that manganese plays in the metabolism of these fungi is still obscure.

7. RESPONSES TO ZINC.

It has been definitely proven that some fungi make a better vegetative growth when zinc is present in the culture medium. In most of these culture studies strains of Aspergillus niger were used as the test organisms. Some of the results of the early investigators do not agree on the zinc requirements of this fungus. However, this difference of opinion may be due to the fact that several physiological strains of Aspergillus niger are known to exist as well as possible differences in the purity of the culture media employed.

Raulin (39) considered zinc as an essential element for fungi and was convinced that 0.070 grams of zinc sulphate in 1500 cc. of culture solution was sufficient to meet the normal requirements. Richter (43) reports a growth stimulation of Aspergillus niger with 1/700,000 gram molecule of zinc sulphate, while concentrations above 1/600 are toxic. Coupin (12) was not able to duplicate the results of Raulin and Richter and concluded that zinc and iron were not necessary for Sterigmatocystis nigra. (Sterigmatocystis nigra in the early literature is probably the same as Aspergillus niger). Lepierre (26, 27, 28) claimed that aeriation was the deciding factor in determining the amount of growth in cultures of Sterigmatocystis. He was able to obtain normal growth without zinc, provided the ratio of volume of liquid to surface exposed was greater than two to one. The results of Richards (41) show definite increases in the growth of Aspergillus niger, Penicillium glaucum, and Botrytis cinerea with a 0.002% concentration of zinc sulphate in a variety of culture media. More recent work by

Javillier (18), Steinberg (47), Bortels (6), and Roberg (44) prove that zinc, when added in small amounts, gives marked increases in vegetative growth and that this action is even greater in the presence of such elements as iron, manganese, and copper.

The manner in which minute traces of zinc function in the growth and development processes is not thoroughly understood. Zinc has been considered as both an essential element and a stimulant. Unfortunately, most of the investigators report growth measurement based on one harvest of compratively young cultures. Due to differences in the growth rate and the rather sharp decrease from the maximum weight caused by autolysis, the one harvest method does not give a complete story. Measurements should be taken on a culture series at frequent intervals during the growing period of the organism. The action of zinc either as an essential or a stimulating element depends upon the purity of the chemicals used, and the peculiarities of the test organisms. The data presented by Roberg (44) on the responses of Aspergillus niger indicate that zinc is essential for a large and rapid growth while the absence of zinc practically inhibits growth. Javillier (18) thought that zinc favored the production of the enzyme, sucrase, but his data fail to prove whether the increased production of the enzyme is the cause or the effect of accelerated growth. Steinberg (47) suggests that zinc salts are effective owing to their ability to hydrolize in aqueous solutions thus causing a beneficial increase in acidity. He supports this view with a series of experiments in which he obtained growth responses similar to those with zinc, by the addition of acids to the culture medium. Other theories have been advanced which suggest

Table 19. Growth responses of five fungi to the presence of fifty milligrams of zinc sulphate per liter of culture solution.

Name of fungi used		Age of cultures at first and second harvest							
		10 days				15 days			
		minus zinc		plus zinc		minus zinc		plus zinc	
		dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.
Rhizopus nigricans		22	3.6	36	3.6	128	3.6	397	6.4
		23	3.6	40	3.6	113	3.6	392	6.4
	av.	22	3.6	38	3.6	120	3.6	394	6.4
Fusarium solani		400	7.8	426	8.2	454	7.8	345	8.8
		414	7.8	456	8.4	475	7.8	335	8.8
	av.	407	7.8	442	8.3	464	7.8	340	8.8
Fusarium sulphur-um		91	5.8	427	7.8	82	6.8	417	8.6
		76	5.4	436	7.8	88	6.8	397	8.6
	av.	83	5.6	431	7.8	85	6.8	407	8.6
Altenaria sp.		159	6.8	348	8.0	326	7.2	369	8.4
		171	6.8	310	8.0	299	6.8	384	8.4
	av.	168	6.8	329	8.0	312	7.0	376	8.4
Asp. 117 white spores		111	7.2	20	5.8	225	7.4	40	6.2
		117	7.2	24	5.8	184	7.4	46	6.2
	av.	114	7.2	22	5.8	205	7.4	43	6.2

($MgSO_4$-2, KH_2PO_4-2, KNO_3-5, Sucrose, Cry. Dom.-32, -$ZnSO_4$-0.050.) (40 cc. of soln. per culture in 125 cc. Pyrex flasks).

that zinc acts as a catylist either inside or outside the cells and that zinc gives a proper physiological balance by the phenomenum of antagonism.

In the present series of experiments one purpose was to obtain more information regarding the nature of the action of zinc and secondly to get a comparison of the growth responses of several fungi grown under the same conditions to zinc when added to the culture medium. The first experiment included five fungi, of which duplicate cultures were harvested at ten and fifteen days after the innoculation. It is evident from the weights of the dried mats given in Table 19 that the organisms are not equally sensitive to

zinc. The strain of Aspergillus albus candidus was not benefitted, in fact, the growth in the check cultures were greater than in the zinc cultures which suggest that zinc at the strength applied was toxic. Fusarium solani produced but slightly heavier mats in the zinc cultures, however, the presence of zinc seemed to cause the cultures to mature earlier as indicated by the differences in the pH of the culture media and a loss in weight by the last harvest. R. nigricans, F. sulphureum, and the Altenaria sp. showed definite increases in dry weight in the zinc cultures.

The results of a more detailed and extensive comparative study are presented in Table 20. Again it is evident that the

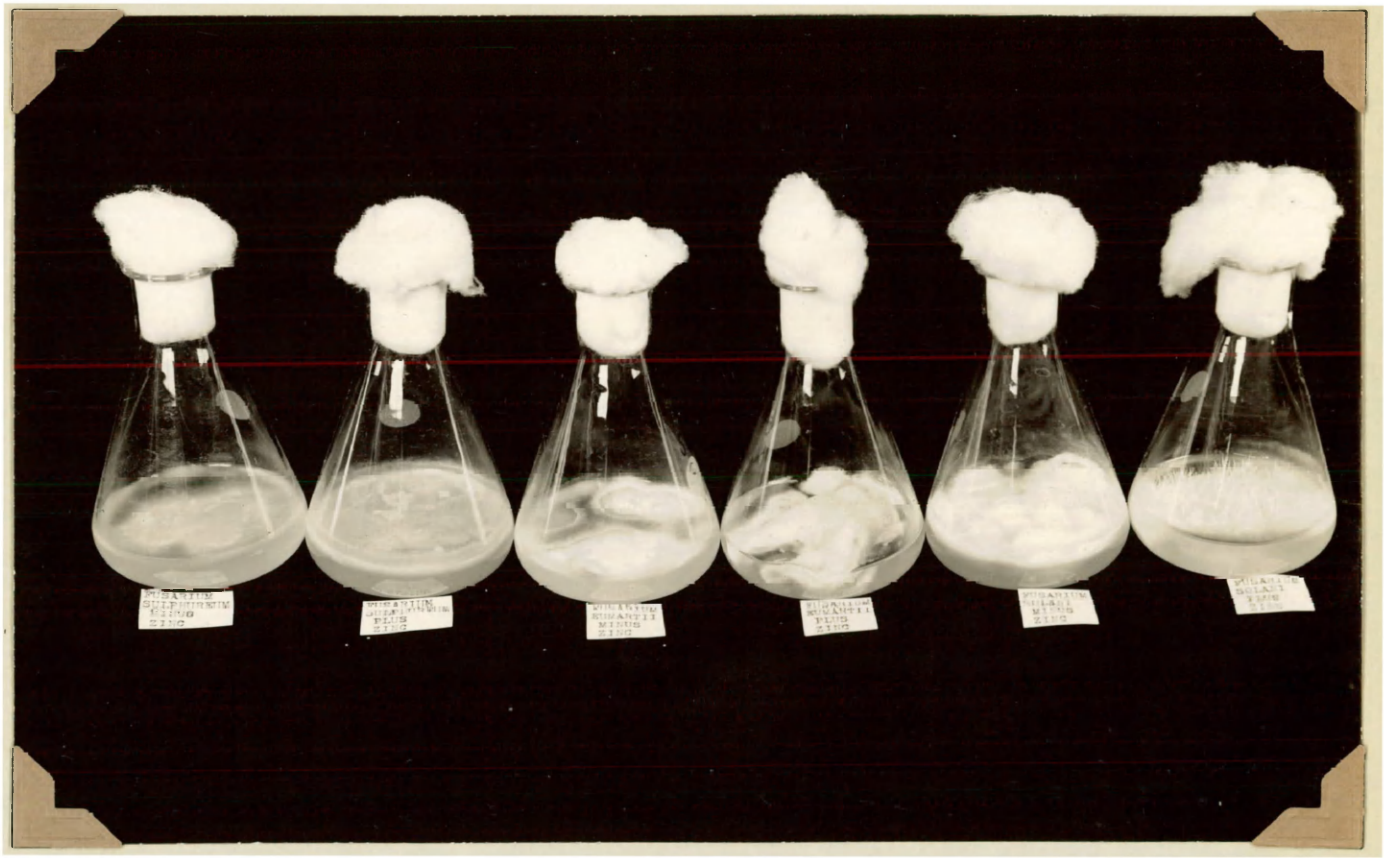

Fig. 12. Fourteen day old cultures of three Fusaria in media with and without the addition of 50 mm. of zinc sulphate per liter of culture solution.

Table 20. Growth responses of six Fusaria (F) and two Aspergilli (A) in a medium plus and minus zinc.

Fungi used plus and minus zinc	Age of cultures at each harvest							
	7 days		12 days		18 days		24 days	
	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.
F. lycop.	354	7.4	506	7.4	537	8.0	548	8.2
plus Z	273	7.2	476	7.4	619	8.0	573	8.6
F. lycop.	91	6.2	92	6.2	113	6.6	133	7.0
minus Z	94	6.0	96	6.4	116	6.6	128	7.0
F. 528	127	6.6	452	6.6	657	7.8	626	8.8
plus Z	140	6.6	535	7.2	633	7.8	625	8.8
F. 528	49	5.8	151	5.0	273	4.8	181	4.6
minus Z	75	5.8	133	5.0	259	4.8	251	4.6
F. 658	372	7.0	464	6.3	489	6.3	621	8.0
plus Z	403	7.0	519	6.4	523	6.4	644	8.0
F. 658	167	6.8	340	6.4	458	7.2	550	7.8
minus Z	223	6.8	348	6.2	454	7.4	537	7.8
F. 701	409	6.4	592	6.6	600	8.4	563	8.2
plus Z	401	6.4	638	7.0	595	8.4	558	8.2
F. 701	115	6.0	342	6.4	467	7.6	443	7.6
minus Z	122	6.0	261	6.4	480	7.4	437	7.6
F. 244	87	5.8	228	6.4	341	7.4	368	7.2
plus Z	81	5.8	232	6.8	358	7.4	358	7.4
F. 244	94	5.6	130	5.4	143	5.8	222	7.0
minus Z	82	5.6	107	5.2	150	6.0	225	7.0
F. 141	227	5.6	205	4.2	337	5.6	426	8.4
plus Z	186	5.6	241	4.4	350	6.4	418	8.0
F. 141	121	5.2	133	5.6	205	6.6	254	7.8
minus Z	109	5.2	125	5.6	194	6.6	244	7.4
A. 3534c	293	5.6	634	6.6	589	8.0	567	8.4
plus Z	301	5.6	635	6.6	584	8.0	572	8.4
A. 3534c	147	4.2	314	3.8	507	3.8	544	5.0
minus Z	164	4.2	360	3.8	499	3.8	537	4.4
A. 111	597	2.6	560	2.6	478	2.8	473	2.8
plus Z	668	2.6	571	2.6	492	2.8	481	3.0
A. 111	202	2.4	345	2.4	409	2.4	371	2.4
minus Z	225	2.4	329	2.4	421	2.4	380	2.4

($MgSO_4$-4, KH_2PO_4-2, KNO_3-4, Sucrose, Comm. Quaker brand -40, zinc sulphate -0.050.) (40 cc. per culture in 125 cc. Pyrex flasks).

quanitative increase in dry weight in the zinc cultures varies greatly with the organisms. Under the conditions of this experiment, zinc, in several cases, seems to function largely as an accessory growth promoting agent. Traces of zinc as an impurity may be present in sufficient amounts for a slow yet apparently normal growth. In all cases there was a visible difference in the appearance of the check and test cultures. The differences in weight of mats produced by F. solani in the check and test cultures were not great but the aerial hyphae in the zinc cultures grew in clumps, giving a distinctive appearance to the cultures. (See Figure 12). Heavy, tough, and much distorted mats were developed in the zinc cultures by F. eumartii in sharp contrast to the thin, smooth surface colonies in the check cultures. The appearance of the cultures of F. eumartii 528 were quite similar to the cultures of F. radicicola 701. The main difference between the check and test cultures of F. sulphureum 244 and F. tricothecioides 141 was in the thickness of the mats and a more uniform surface growth in the presence of zinc. The two species of Aspergillus produced heavier mats in the zinc cultures but the best sporelation occured in the minus zinc cultures.

It is interesting to note that none of the eight fungi listed in Table 20 developed a greater acidity in the zinc cultures than in the test cultures. The general tendency of the Fusaria was to decrease the acidity in close correlation with the amount of growth and the age of the culture. In both check and test cultures F. lycopersici decreased the acidity, while a slightly different response occured in the case of Aspergillus fuscus 3534c. There was a marked increase in the acidity in both culture series of

Table 21. Growth responses of Fusarium radicicola to different amounts of zinc sulphate supplied in the culture medium.

Grams of $ZnSO_4$ used per liter	pH of media at start	Age of cultures at first, second and third harvest: 5 days dry wts. mgs.	5 days pH of fil.	7 days dry wts. mgs.	7 days pH of fil.	11 days dry wts. mgs.	11 days pH of fil.
0.000	4.6	70	6.4	194	7.0	265	7.4
		82	6.6	163	6.6	261	7.4
		61	6.2	161	6.6	258	7.4
		71	6.4	173	6.7	261	7.4
0.005	4.6	375	8.2	515	8.4	402	8.8
		379	8.2	528	8.4	411	8.8
		387	8.2	511	8.4	407	8.8
		380	8.2	518	8.4	407	8.8
0.025	4.6	347	7.8	516	8.0	441	8.6
		354	7.8	517	8.0	453	8.6
		367	7.8	513	8.0	440	8.6
		356	7.8	515	8.0	445	8.6
0.100	4.6	359	7.8	505	8.0	420	8.6
		351	7.8	510	8.0	420	8.6
		360	7.8	500	8.0	394	8.6
		357	7.8	505	8.0	411	8.6
0.500	4.4	336	7.6	510	7.8	430	8.6
		321	7.6	504	7.8	432	8.6
		334	7.6	496	7.8	434	8.6
		330	7.6	503	7.8	432	8.6
1.000	4.2	180	6.8	452	7.4	438	8.6
		198	6.8	463	7.4	417	8.6
		179	6.8	435	7.4	424	8.6
		186	6.8	450	7.4	426	8.6
4.000	3.6	26	4.2	29	4.8	153	5.2
		25	4.2	31	4.8	146	5.2
		26	4.2	33	4.8	145	5.2
		26	4.2	31	4.8	148	5.2
8.000	3.6	27	3.8	28	3.8	30	3.8
		28	3.8	27	3.8	33	3.8
		27	3.8	25	3.8	36	3.8
		27	3.8	27	3.8	33	3.8

($MgSO_4$-2, KH_2PO_4-2, KNO_3-5, sucrose, Merk-32, ferrous sulphate-trace)
(40.0 cc. of solution per culture in 125 cc. flasks)

Aspergillus niger 111. The nature of these changes will be discussed later but it should be noted that an increase in acidity of the medium does not of necessity precede growth acceleration.

Time and equipment did not permit a large culture series test of the responses of several organisms to different amounts of

zinc sulphate in the culture medium. The results of one experiment are reported in Table 21 in which Fusarium radicicola 701 was cultured for a period of eleven days in a medium with varying amounts of zinc sulphate. The presence of 0.005 grams of zinc sulphate per liter (1.13 part zinc per million) was found to be sufficient to give as good a growth as much larger amounts. The cultures containing from 0.005 to 0.500 grams of zinc sulphate per liter showed no marked differences in the growth rates or general appearance of the mats produced. The use of 0.050 grams of zinc sulphate per liter in previous experiments supplied the amount necessary but not in a concentration that was toxic.

Average values of the dry weight of mats and pH changes throughout the growing period for each concentration are presented in graphic form in Figure 13. (Calculation of average dry weight and pH for check cultures -- 71,173, and 261 mm. give an average value of 168 mm. -- also 6.4, 6.7, and 7.4 give an average pH value of 6.8). There is some evidence of toxicity when one gram or more of this salt was present in a liter of culture solution. Eight grams per liter caused a considerable increase in the acidity of the medium. The effect of an increase in the pH of the medium is not clearly understood as the writer has never grown this organism in a carefully buffered medium so as to give a considerable range in pH. Here, as in the studies on the boric acid, tolerance and ammonium nitrate requirements it is difficult to distinguish between metallic ion toxicity and the concentration of hydrogen ions. It is interesting to note that this organism was able after seven days to make a decided growth in the presence of 4.0 gms./L. of zinc sulphate, and to decrease the acidity of the medium. The pH

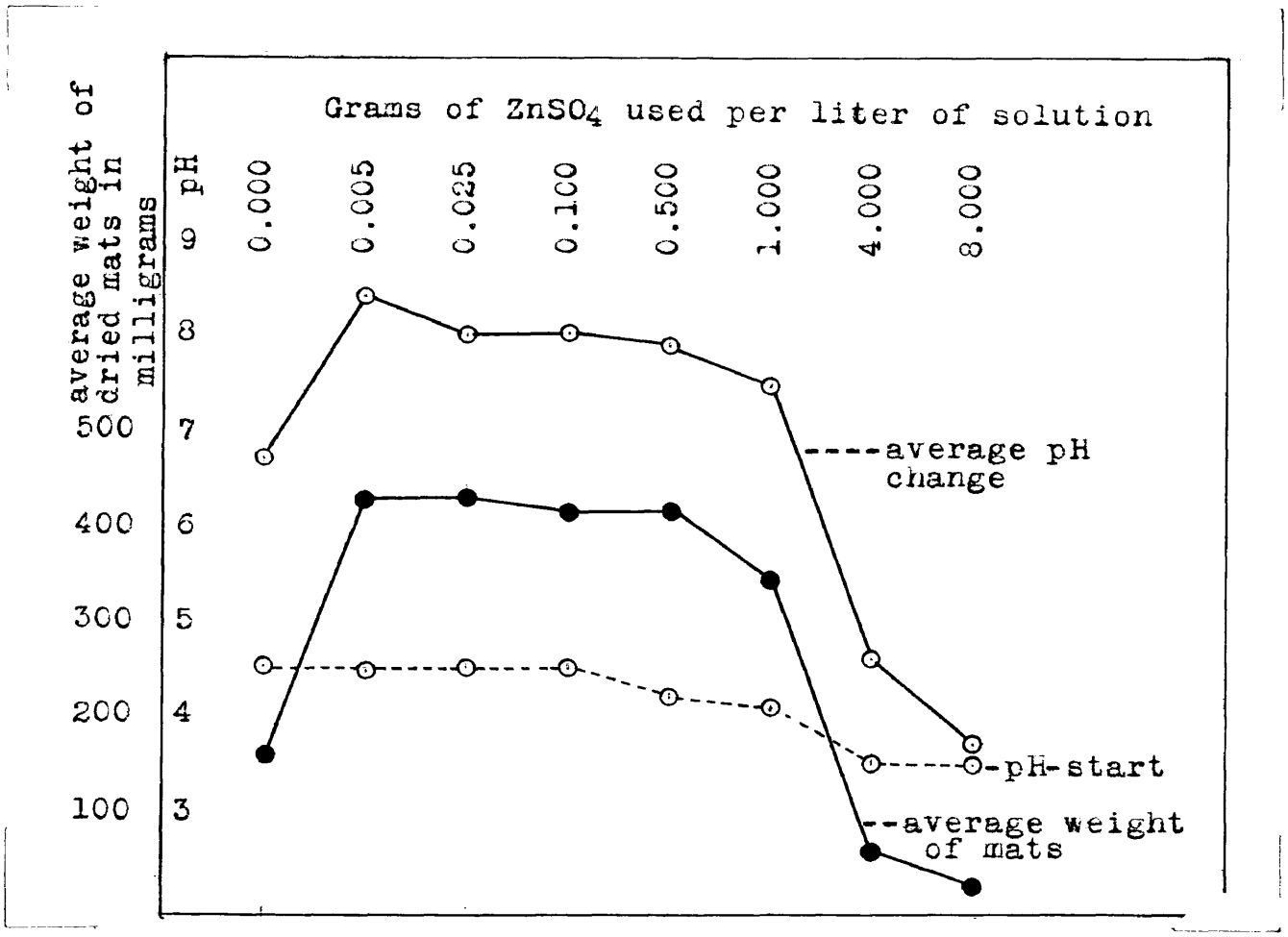

Fig. 13. The effect of different concentrations of zinc sulphate on the average weight of mycelial mats and the pH changes in the culture medium produced by Fusarium radicicola.

of the media containing 4.0 and 8.0 gms./L. were identical (3.6) hence it may be suggested that growth was restricted in the latter concentration either by the titrable acidity or the specific action of zinc rather than the concentration of the hydrogen ions. In this type of culture medium this organism tends to decrease the acidity with the greater decrease in the presence of zinc when supplied in non toxic amounts. The production of alkaline substances in the culture medium is not considered as being due directly to zinc but as the result of a normal growth of the fungus and autolytic processes.

In all the experiments involving a large number of cultures in the 125 cc. Pyrex Erlenmeyer flasks, incubator space was not available so as to use a uniform incubation temperature. Under such conditions results obtained one month may be quite difficult to exactly duplicate a month later. The differences in temperature usually result in variable growth rates. In these experiments, where definite comparisons were desired, all the necessary cultures were innoculated on the same day and stored side by side on one of the laboratory tables at room temperature, hence daily variations in temperature were common to all cultures involved. The one day old cultures of the fungi used to secure the data presented in Table 20 are shown in Figure 1. In this experiment 128 individual cultures were involved. A slight change in the environmental conditions was common to each contrasting culture series.

Previous investigators have often attempted to compare the relative nutritive value of different sources of nitrogen. An experiment was conducted to obtain a comparison of the growth responses of F. radicicola 701 to ammonium nitrate and urea when supplied in amounts equivalent on the basis of total nitrogen, in cultures with and without zinc. Two 15 individual culture series were prepared using 4.506 grams per liter of ammonium nitrate, and to one of these series zinc was supplied at the rate of 50 mm. per liter. The dry weights of the mycelial mats and the corresponding pH of the culture media for five harvests covering a period of 19 days are given in Table 22. Urea, according to these weights, is a poor source of nitrogen in the absence of zinc, but with zinc it supports a much more rapid and greater growth than ammonium

Table 22. The effect of zinc on the relative value of Urea and ammonium nitrate as nitrogen sources for Fusarium radicicola.

Age of cultures at harvest	Nitrogen sources							
	Urea 4.290 grams/L.				Ammonium nitrate 4.506 grams/L.			
	minus zinc		plus zinc		minus zinc		plus zinc	
	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	Ph of fil.
6 days	56	7.2	187	6.2	141	4.0	157	2.6
	66	7.2	164	6.2	142	4.0	156	2.6
	54	7.2	184	6.2	143	4.0	152	2.6
	59	7.2	178	6.2	142	4.0	155	2.6
10 days	52	7.0	512	8.4	234	4.0	258	3.6
	65	7.0	534	8.6	256	4.0	227	3.6
	63	7.0	516	8.6	219	4.0	255	3.6
	60	7.0	521	8.5	236	4.0	247	3.6
14 days	118	7.2	455	8.6	329	4.4	428	4.2
	131	7.0	478	8.8	307	4.4	433	4.2
	143	7.0	481	8.8	313	4.4	429	4.2
	131	7.0	471	8.8	313	4.4	429	4.2
16 days	151	6.8	441	8.6	362	4.6	410	5.2
	161	7.0	468	8.6	322	4.6	415	5.2
	148	6.8	440	8.6	346	4.6	426	5.2
	153	6.9	450	8.6	343	4.6	417	5.2
19 days	218	6.6	430	8.4	409	4.6	379	6.8
	199	6.6	420	8.4	410	4.6	386	6.8
	211	6.6	412	8.4	395	4.6	415	7.2
	209	6.6	421	8.4	405	4.6	393	6.9

($MgSO_4$-2, KH_2PO_4-2, Sucrose, C. P. Merk, -32, Ferric tartrate-trace)
(60 individual cultures-40 cc. of soln. in 125 cc. Pyrex flasks).

nitrate under the same conditions. It seems probable that the relative nutritive value of other nitrogen sources might vary in a corresponding manner due to the presence or absence of zinc. Zinc in effective amounts may be present in one compound and not in another so as to give misleading results regarding their actual nutritive value. Hence, it would seem advisable to include sufficient amounts of these elements which are so effective in small amounts in order to mask responses due to possible impurities in the different compounds to be tested.

Some of the above experiments have proven that a single salt may be varied over quite a range without materially influencing the dry weight yield if the concentration of the other nutrient components is kept constant. A study has been made of the growth responses of Fusarium eumartii to a series of different total salt concentrations. In the experiments dealing with variations of single salts, check series were not used such that would show the effect the added zinc had on the amount of the salt required to give the highest yield. These experiments did not prove the presence or absence of traces of zinc as impurities. If zinc is present in effective amounts as an impurity, an increase in the concentration of non toxic salts should be followed by an increase in yield throughout the series until the zinc requirement had been satisfied. There should be less increase in growth in the high salt concentrations than in the low total salt series brought about by the addition of zinc. In the present experiment five different total concentration series with and without zinc were prepared as outlined in Table 23. In each series the carbon source was not varied and in the test series zinc sulphate was supplied at the rate of 50 mm. per liter of medium. Triplicate cultures were prepared for each series thus giving a total of thirty individual cultures for the entire experiment.

Table 23. Five nutrient solutions having a different total salt concentration. (Grams per liter of solution).

Nutrients	Solution number				
	1	2	3	4	5
Sucrose	50.0 gms.	50.0 gms.	50.0 gms.	50.0 gms.	50.0 gms.
KNO_3	6.0 "	4.0 "	2.0 "	1.0 "	0.5 "
$MgSO_4$	6.0 "	4.0 "	2.0 "	1.0 "	0.5 "
KH_2PO_4	6.0 "	4.0 "	2.0 "	1.0 "	0.5 "
pH of series	4.2 "	4.3 "	4.4 "	4.5 "	4.6 "

After growing at room temperature for eleven days one culture was removed from each series for harvest. It is admitted that results based on single culture harvests are questionable from the standpoint of statistics, however, the culture to be harvested was selected from a series of three cultures which, as near as the eye could detect, were identical. The dry weights of these cultures given in Table 24 do not show inconsistent variations. The remaining two cultures of each series were harvested at the end of a sixteen day period at which time the zinc cultures had apparently ceased to show an increase in growth and noticeable autolysis had not begun. From the dry weight of the mycelial mats thus obtained it is evident under the conditions of the experiment that an increase in the total salt concentration resulted in an increased yield in the presence or absence of zinc. There is no evidence to show any increases in growth directly due to a possible increase in the concentration of zinc as an impurity in the salts. The numerical ratio of the dry weight of the mycelial mats from the plus zinc cultures to corresponding weights from the check cultures varies directly with the total salt concentration. (These ratios are calculated from dry weights of sixteen day old cultures. Average weight of series E plus zinc (154) divided by the average weight of series E minus zinc (64) gives a ratio of 2.40-1). If there was an effective increase of zinc as an impurity these ratios should vary indirectly with the total salt concentration. In the plus zinc series it is evident that 4.0 grams of each salt were as effective as 6.0 grams of each salt since there was not a corresponding increase in the dry weights of the mycelial mats. In this case the carbon supply and growing space were limiting factors. A

Table 24. Some growth responses of Fusarium eumartii 528 to five variations in the total salt concentration in the presence and absence of added zinc sulphate.

Culture series	soln. number	gms. each salt per liter	Original soln. pH	Age of cultures when harvested: 11 days dry wts. mgs.	11 days pH of fil.	11 days Estim. amt. of reduc. sugar	16 days dry wts. mgs.	16 days pH of fil.	16 days Estim. amt. of reduc. sugar	Ratio of dry wts. of mats of plus zinc cultures to dry wts. of mats of minus zinc cultures ---- Calculations based on 16-day old cultures
Minus zinc series	1	6.0	4.2	165	4.8	5	195 194 194	5.0	5	
	2	4.0	4.3	100	4.8	4	157 146 151	4.8	4	
	3	2.0	4.4	84	4.6	3	109 110 109	4.6	2.5	
	4	1.0	4.5	59	4.4	2	80 83 81	4.6	2	
	5	0.5	4.6	49	4.2	2	61 67 64	4.4	2	
Plus zinc series	1	6.0	4.2	473	6.4	4.5	662 659 660	6.8	1	3.39-1
	2	4.0	4.3	362	6.2	3	670 667 668	7.4	1	4.42-1
	3	2.0	4.4	297	5.8	2.5	423 418 420	6.4	2	3.85-1
	4	1.0	4.5	200	5.2	2	278 277 277	5.6	2	3.42-1
	5	0.5	4.6	147	4.8	2	155 153 154	5.0	2	2.40-1

comparison of these growth responses are presented in graphic form in Figure 14.

The amounts of reducing sugars present in the filtrates of each culture series after each harvest were estimated by the use of Fehlings Solutions. Aliquot portions of the filtrates were placed in small bore test tubes and treated with the Fehlings

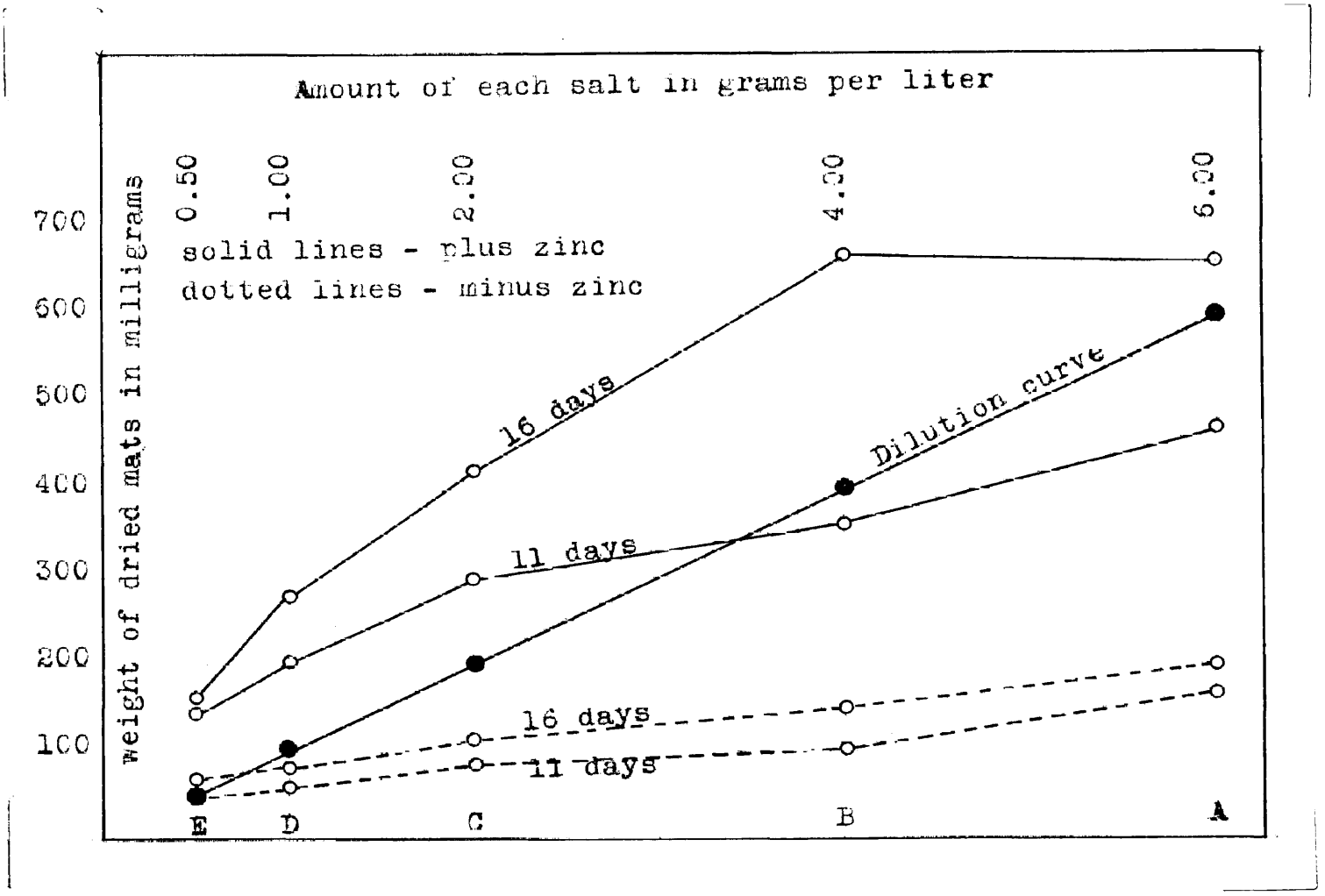

Fig. 14. The effect of the total salt concentration with and without the addition of zinc on the dry weight of mycelial mats produced by Fusarium eumartii. (See Table 24).

Solutions. The reduced copper was allowed to settle to the bottom of the tubes and the amount estimated. The results of these tests are included in Table 24. These tests show that the minus zinc cultures contained a little more reducing sugars than the plus zinc cultures and that the hydrolysis of cane sugar was almost directly proportional to the total salt concentration at the eleven day harvest. In the filtrates from the sixteen day harvest the amount of reducing sugars in the minus zinc cultures was the same as at the eleven day harvest. The filtrates from the plus zinc cultures containing two or more grams of each salt per liter showed a marked decrease in the reducing sugars. It is evident, therefore,

that the presence of reducing sugars is not an important factor in the growth acceleration due to zinc. It seems logical to assume that zinc does not function directly in the formation of reducing sugars.

Strains of Aspergillus niger have been used almost exclusively by previous investigators in their studies of growth stimulation. It seemed worthwhile to compare the responses of a Fusarium with one of the cultured strains of Aspergillus niger. It was possible to secure a strain of Aspergillus niger which was a direct culture descendent of Aspergillus niger 4247w used by Steinbery (47). A series of cultures were prepared from the same stock solutions. To one half of these cultures zinc sulphate was added at the rate of 50 mm. per liter. Fusarium radicicola 701 and Aspergillus niger 4247w were used as the test organisms in the plus and minus zinc cultures.

A difference between the check and test cultures of both fungi was soon apparent. After four days, the A. 4247w test cultures (plus zinc) showed more growth than the checks. When five days old the check cultures had a darker and more abundant spore crop than the zinc cultures. After eight days the surface mats in the test cultures began to fold in several places and sink below the surface of the liquid forming bag-like projections in the mats. In the check cultures only a thin surface mat was formed.

There was little differentiation between the check and test cultures of F. radicicola at the end of the four day period. In each flask there was a colony on the surface about the size of a penny, while there was an abundance of submerged growth which later

appeared on the surface. After five days these surface colonies in the test cultures had begun to curl and fold downwards from the edges, while the colonies in the check cultures remained smooth and thin. A yellow-brown coloration appeared in the zinc cultures and increased in intensity as the cultures aged. The liquid medium in the check cultures was always clear. Slimy masses of spores appeared in both test and check cultures.

Some measurements were made on the spores from the test and check eleven day old cultures of F. radicicola. There was a marked difference in the appearance of these spores. The spores from the less mature check cultures were completely filled with dense protoplasm so that no septation was visible. The ends of these spores were not sharp pointed, a result of high turgitity. After treatment with 95% alcohol and a sodium chloride solution these spores were injured and plasmolized to such an extent that the septa became plainly visible and the spore ends became pointed. The spores from the zinc cultures contained only a thin film of protoplasm around the walls, the three septa stood out clearly and the ends were sharp pointed giving a typical fusiform appearance. From the summary of these measurements given in Table 25, it is evident that somewhat longer spores were present in the check cultures. The arithmatical mean length of the spores in the test and check cultures respectively were 5.06 and 5.43 ocular divisions. (One ocular division is equal to 7.47 microns). It seems logical to assume that this slight difference in spore length is largely a question of maturity rather than a direct effect of the zinc.

Table 25. Length measurements of spores of Fusarium radicicola from plus and minus zinc cultures after eleven days growth.

Frequency (100 spores)	Classes (length in occular divisions)								
	2.0 to 2.5	3.0 to 3.5	3.5 to 4.0	4.0 to 4.5	4.5 to 5.0	5.0 to 5.5	5.5 to 6.0	6.0 to 6.5	6.5 to 7.0
plus zinc cultures	2	1	3	5	15	28	33	12	1
minus zinc cultures	4	0	1	2	5	20	32	22	14

Arithmatical mean length-plus Z cult. --5.06 occular division
" " " minus Z " --5.43 " "

Bean plants about ten inches in height were cut and placed in filtrates from the eleven and fifteen day old cultures of both fungi. In three to four hours the plants wilted in the filtrates from the check cultures of both organisms, while wilting did not occur in less than twelve hours inthe filtrates from the plus zinc cultures. Some plants were placed in tap water and used as checks in which case wilting was not pronounced until after twenty-four hours. The slight difference in the culture age did not cause a noticeable difference in wilting the bean plants. Although this experiment was not extensive enough to draw definite conclusions it suggests some interesting possibilities for similar studies. Different types of media have been used by other investigators to study the effect of metabolic products and changes in the liquid medium of several fungi in relation to the wilting phenomenon. Their studies have not involved a careful comparison of the effects of using media which permitted a relatively good vegetative growth of the fungus in contrast to those permitting a poor vegetative growth of the fungus in contrast to those permitting a poor vegetative growth. The exact nature of the substances which cause

wilting is not known but their quanitative production may be more or less inversely proportional to the amount of vegetative growth occuring in the medium. Herrick and May (17) have found that a medium which gives a relatively poor vegetative growth is most efficient for the production of bluconic acid by some Penicillia. Similar results were obtained by Currie (13) in the production of citric and oxalic acids by Aspergillus niger. The results presented in Table 27 show that the addition of zinc tends to decrease the amount of acids produced, which is closely correlated with the total growth of the fungus. It is possible that the substances which cause wilting of the higher plants both in nature and when cut and pused in experiments similar to the one mentioned above, are similar to the organic acids in their production. Such parasites as the Fusaria seldom, if ever, find conditions in the host plant as favorable for vegetative growth as in the common nutrient solutions. Hence, it may be suggested that relatively larger amounts of toxic substances would be produced in the host plants than in the synthetic nutrient solutions permitting a luxuriant growth. It would be an interesting problem to make comparative studies of the production of substances which will cause wilting by both parasitic and non-parasitic fungi in media which supported variable vegetative growth. A knowledge of the factors influencing the production of such toxic substances may lead to a clearer understanding of the nature of disease resistance and susceptibility.

The dry weights of the fungus mats in Table 26 show that the Aspergillus and Fusarium are quite similar in their responses to the presence of zinc, differing somewhat in the growth rate and maximum yield. In the check cultures the growth rates and dry

Table 26. Weight of dried mycelial mats and pH changes in the filtrates of cultures of Aspergillus niger 4247w and Fusarium radicicola 701 in a culture medium plus and minus zinc.

Age of cultures at harvest	Culture series							
	minus zinc				plus zinc			
	Aspergillus		Fusarium		Aspergillus		Fusarium	
	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.	dry wts. mgs.	pH of fil.
5 days	100	4.0	93	6.6	305	4.2	162	7.2
	93	4.0	76	6.4	272	4.0	163	7.2
	86	4.0	125	6.8	315	4.2	192	7.4
	93	4.0	98	6.6	297	4.2	172	7.3
8 days	228	3.0	177	7.2	585	3.4	520	8.0
	224	3.0	153	7.0	594	3.4	494	7.8
	214	3.0	173	7.2	599	3.4	499	8.0
	222	3.0	168	7.1	594	3.4	504	7.9
11 days	265	2.4	302	7.6	477	4.0	520	8.4
	263	2.4	320	7.6	496	4.0	505	8.4
	272	2.4	318	7.6	490	4.0	510	8.4
	267	2.4	313	7.6	488	4.0	512	8.4
15 days	325	2.2	329	7.8	472	4.2	455	8.6
	329	2.2	321	7.6	453	4.2	452	8.6
	325	2.2	336	7.8	442	4.2	439	8.6
	326	2.2	329	7.7	456	4.2	449	8.6
18 days	384	2.4	342	8.0	463	4.4	460	8.8
	361	2.4	---	---	453	4.4	439	8.8
	382	2.4	344	8.0	452	4.4	430	8.8
	376	2.4	343	8.0	456	4.4	443	8.8

($MgSO_4$-2, KH_2PO_4-2, KNO_3-2, comm. sucrose -32, $ZnSO_4$-0.050).
(Trace of ferric tartrate in all cultures)
(40 cc. of soln./culture in 125 cc. flasks. pH-4.5).

weights are almost identical. The chief differences in the responses of these organisms are the pH changes induced in the liquid medium. In general one tends to decrease the acidity while the other increases the acidity. It is interesting to note that Aspergillus niger 4247w produced a greater acidity in the minus cultures than in the plus zinc cultures, and that Fusarium radicicola responded

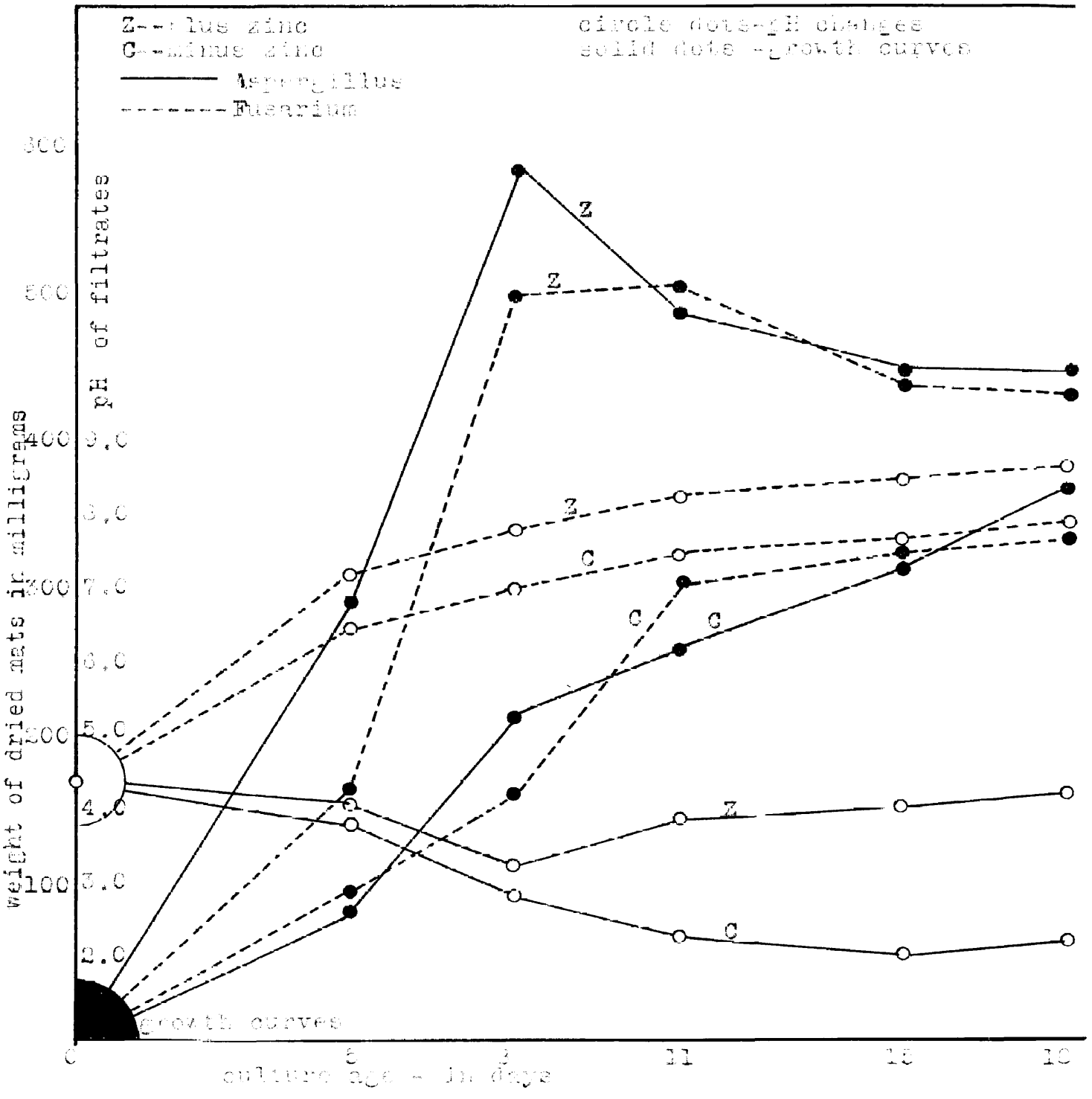

Fig. 15. Growth curves and pH changes produced by *Aspergillus niger* WAW and *Fusarium radicicola* Vol. 1tu are without the addition of zinc sulphate to the culture medium.

to the zinc in a medium which was usually toxic to plants. This relation of pH changes to vegetative growth is illustrated in Figure 15. These facts do not seem to agree with Steinberg's

conception of the action of zinc. He assumes that the zinc sulphate upon hydrolysis increases the acidity of the medium thus forming a condition more favorable to growth. The results of this experiment do not support his hypothesis in regard to either organism. It is more logical to consider these pH relationships as the result of growth rather than the cause of growth acceleration.

The difference in the pH of the filtrates from the plus and minus zinc cultures of Aspergillus niger 4247w reported in Table 26 suggested similar and probably even greater differences in the amounts of titratable acids present. To determine the effect of increased vegetative growth due to the addition of zinc on the amounts of titratable acid present the following experiment was arranged. Two series of cultures were started, to one series zinc was added at the time of preparation, while in the other series zinc was not added at the beginning of the experiment. Cultures were harvested from the plus and minus zinc series at six day intervals during a culture period of eighteen days. At the time of the first harvest (6-days) zinc sulphate was added to three of the original minus zinc cultures which in turn were harvested six days later. Again at the second harvest (all cultures 12 days old) zinc was added to three more of the cultures which had grown for twelve days without zinc. The harvest data given in Table 27 includes the dry weight of the mycelial mats, the pH of the filtrates, and the amount of a weak sodium hydroxide solution required to titrate to a standard color, using Brom cresol purple as the indicator, one fourth of the filtrate from the individual cultures.

The effect of the added zinc, on the weight of the dried mycelial mats and the amount of titratable acid present is presented

Table 27. The effects of adding zinc at time of innoculation and later when cultures were 6 and 12 days old, on the dry weight of the mycelial mats, pH, and total acidity of culture medium produced by Aspergillus niger 4247w.

Time of zinc addition	Age of cultures when harvested								
	6 days			12 days			18 days		
	dry wts. mgs.	cc. of NaOH used	pH of fil.	dry wts. mgs.	cc. of NaOH used	pH of fil.	dry wts. mgs.	cc. of NaOH used	pH of fil.
checks	509	2.8	3.8	656	11.2	2.2	869	17.0	2.0
no	487	2.8	4.0	637	11.4	2.2	875	16.5	2.2
zinc at	517	2.8	3.8	662	11.5	2.2	893	16.4	2.2
start	504	2.8	3.9	652	11.4	2.2	879	16.6	2.2
	1011	2.7	3.8	1101	8.3	2.2	1213	2.4	3.8
plus	980	2.7	3.8	1146	8.1	2.2	1147	5.1	3.0
zinc at	996	2.7	3.8	1098	8.2	2.2			
start	999	2.7	3.8	1115	8.3	2.2	1180	3.7	3.4
zinc added to cultures when				1038	7.0	2.6	975	11.2	2.6
6 and 12 days old and each				1036	7.0	2.6	1008	11.3	2.6
series harvested six days				1050	7.0	2.6	998	10.0	2.8
later.				1045	7.0	2.6	994	10.8	2.7

($MgSO_4$-2, KH_2PO_4-2, KNO_3-4, sucrose 50, $znSO_4$-0.050).
(100 cc. of solution per 250 cc. culture flask).
(One-fourth of filtrate used in each titration).

graphically in Figure 16. In the six day old cultures there is little difference between the pH and titratable acid present in the plus and minus zinc cultures, however, this difference becomes more marked at the subsequent harvests. The total acidity of the zinc stimulated cultures becomes less than in the minus zinc cultures. The addition of zinc to six and twelve day old cultures (originally minus zinc) resulted in an increased vegetative growth and a corresponding decrease in the total acidity of the culture medium.

The pH changes of the filtrates listed in Table 27 check closely with those presented in Table 26. From a general comparison of the data in these two tables it is evident that in the latter experiment the culture growth rate was somewhat slower. This difference is due to temperature, the former experiment was per-

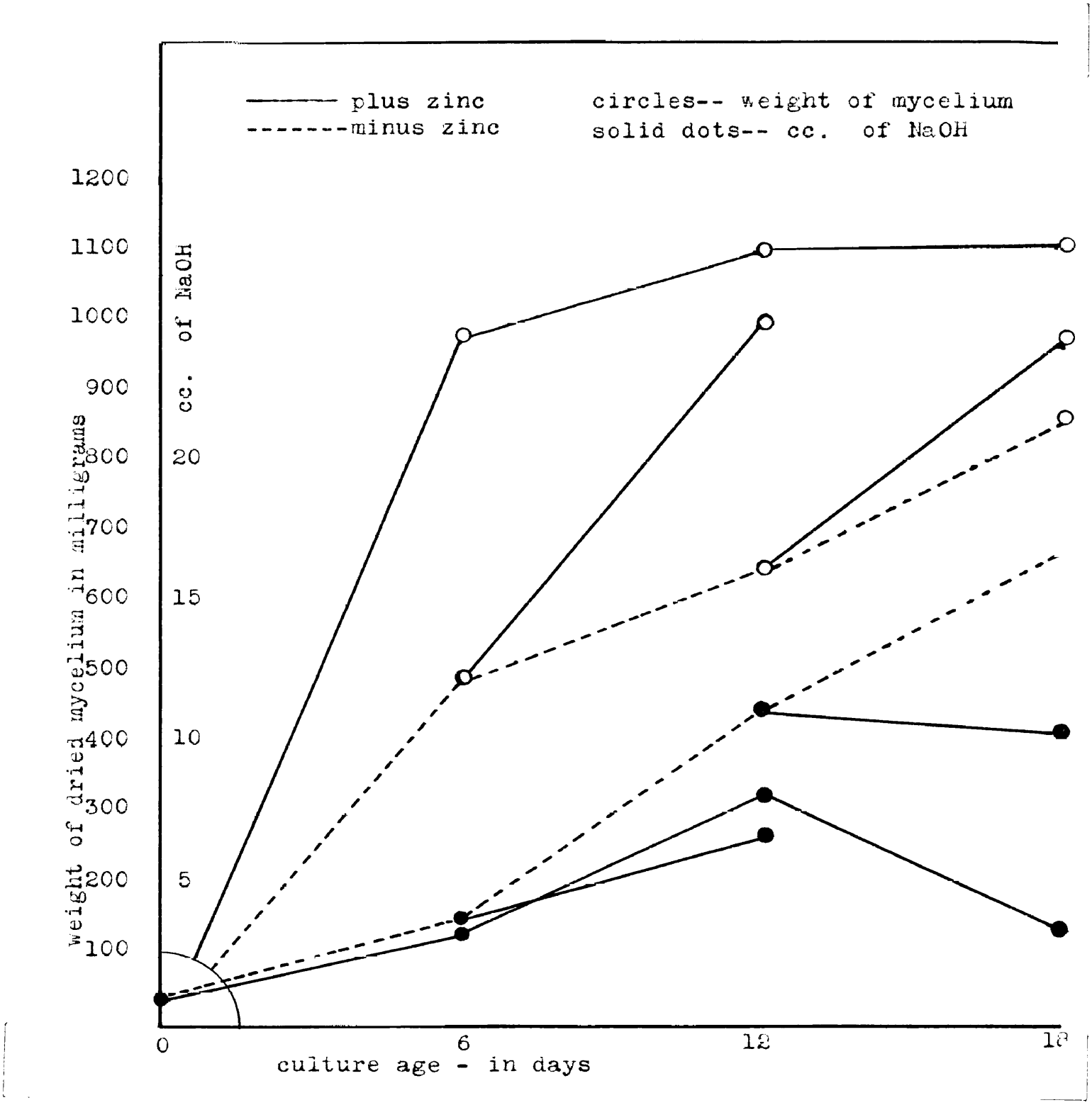

Fig. 1c. The effect of zinc on the weight of the dried mats and the total acidity produced by Aspergillus niger 4x47w.

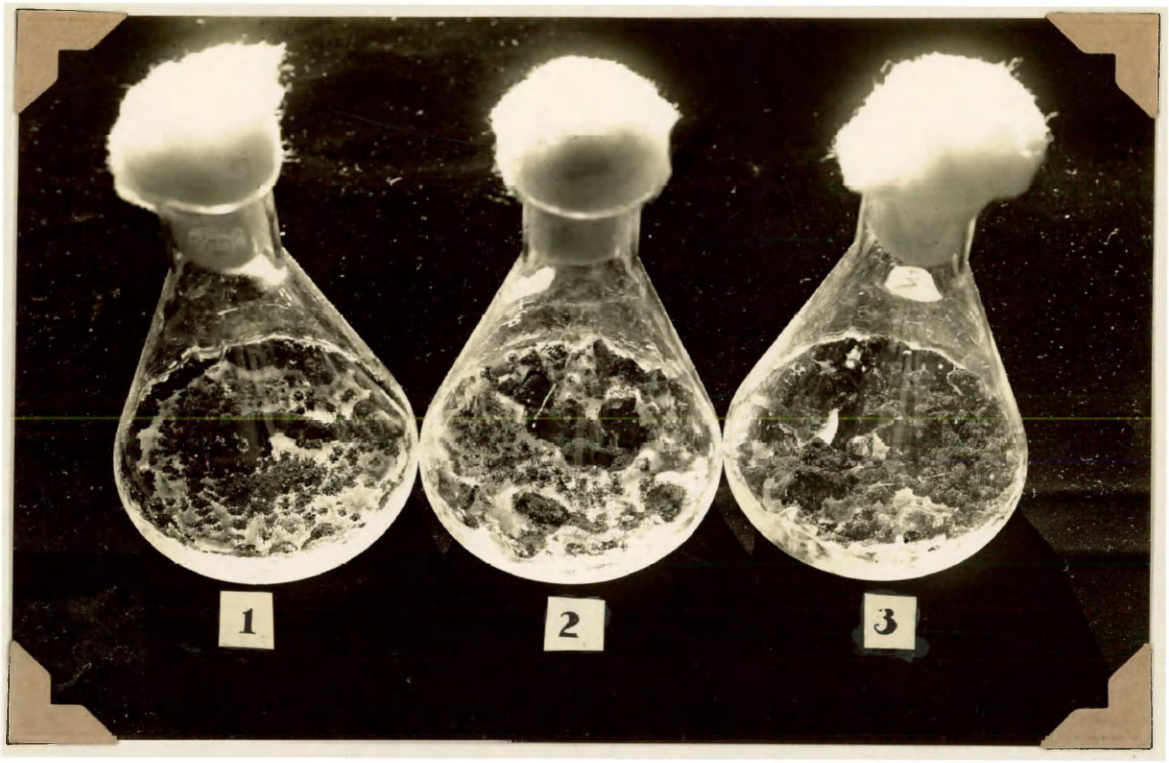

Fig. 17. Cultures of Aspergillus niger 4247w, twelve days old. 1. - Original minus zinc culture. 2. - Culture treated with zinc, when six days old. 3. - Original plus zinc culture.

formed in June, 1928, while the latter was carried out in February, 1929.

The original cultures plus and minus zinc showed the characteristic differences in spore production and thickness of mats. The addition of zinc to the six day old minus zinc cultures resulted in a marked stimulation of growth during the following six days. (See Figure 17). The addition of zinc to the twelve day old cultures did not greatly influence the general appearance of the cultures. It was interested to note that the original minus zinc cultures were able to make a slow yet steady increase in the size and thickness of the surface mats, probably due to the impurity of the original nutrients.

GENERAL DISCUSSION OF RESULTS.

A nutrient solution which is suitable for good growth of all the fungi studied must contain proper carbon and nitrogen sources. Magnesium sulphate, monopotassium acid phosphate, and zinc sulphate can be used successfully to supply the other growth essentials. Other elements, if needed in small amounts seem to be present in sufficient quantities as impurities to give a good vegetative growth. The concentration of the obvious components of the nutrient solution can be varied greatly without corresponding growth responses. The fact that such variation is possible seems to be the reason for the heterogenous list of so-called standard nutrient solutions. Within wide limits the osmotic pressure of the culture solution did not exert a toxic action on the organisms studied.

The minimum amount required of any component of the nutrient solution must be defined in terms of the culture form and the concentration of the other nutrient components. For instance, different amounts of potassium nitrate would be required in the utilization of ten and fifty grams of glucose per liter. Variations in the concentration of the carbon source in a suitable medium usually result in corresponding variations in the dry weight of the mycelial mats. The minimum amounts of monopotassium acid phosphate and magnesium sulphate required to give a yield which larger amounts of these salts would not materially increase does not closely agree with the amounts in many commonly used solutions. The result of these studies show that gram per gram about two times more magnesium sulphate is required than potassium

acid phosphate. The addition of zinc to the medium is advised whenever measurements are made on the growth response of a fungus, to variations in the concentration of a single component, or to different components, so as to mask the effect of traces of zinc that might be present as an impurity.

The role played by the elements which are needed or are effective in small amounts is not clearly understood. The data presented in the above tables show that all the organisms except the white spore strain of Aspergillus responded to the addition of zinc sulphate to the culture medium. The culture medium was not carefully purified (by chemical precipitation, recrystallization or redistillation) to remove possible traces of effective impurities. Even though some of the organisms were able to make a fairly good growth when zinc was not added it does not follow that zinc is an unessential element. The results obtained by Roberg (44) and others seems to justify the conclusion that zinc is an essential element the same as the more obvious elements, carbon, sulphur, phosphorus, etc. The fact that all the organisms did not respond equally to the added zinc does not necessarily mean that zinc is unessential as different organisms may require different amounts of zinc. The amount of zinc or other elements as impurities in the chemicals used in the nutrient solution may have been nearly sufficient to satisfy the needs under such experimental conditions. Hence, when the culture medium is not free from such elements as boron, manganese or zinc, the addition of such elements may be ineffective, or result largely in growth acceleration or stimulation.

The slight increase in the acidity of the culture medium brought about by the addition of zinc sulphate does not seem to be a satisfactory explanation of the manner in which zinc influences the growth responses. The addition of fifty milligrams of zinc sulphate to one liter of culture solution did not give an increase in the H-ion concentration that could be detected by colorimetric measurements. The data reported in Tables 20, 26, and 27 do not verify Steinberg's suggestion that an increase in the acidity is associated with growth acceleration. Fusarium radicicola was able to make a quicker and greater total growth when zinc was present even though the medium was becoming less acid. Aspergillus niger 4247w never produced a greater acidity in the plus zinc cultures than in the minus zinc cultures. In the case of Aspergillus fuscus 3534c the medium to which zinc was added rapidly became alkaline while in the minus zinc medium the active acidity was increased.

In general the effective action of zinc may be extracellular or intracellular. Javillier (18) showed that Aspergillus niger absorbed zinc from the culture medium and fixed it within the cell even in greater amounts than was necessary for the maximum growth under the experimental conditions. The secretion of organic acids by some fungi may be an asset in disolving certain substrata but it does not follow that that acid secretion is advantageous under all conditions. The presence of zinc within the cell in some manner influences this acid secretion. If the amount of acid secreted by Aspergillus niger was directly proportional to the amount of growth then far more acid should be present in the plus zinc cultures. Assuming that the dry weight of the mats and

number of vegetative cells are closely correlated, it then follows that each cell in the minus zinc culture was secreting at least twice as much acid as a similar cell in the plus zinc culture. Either less acid was formed in the cells containing zinc and as a result less secreted, or approximately equal amounts were formed in both plus and minus zinc cells but the presence of zinc in some manner made possible the direct utilization of the acids before they could all be secreted. Ono (35) and Richards (42) believed that most of these acids were utilized directly in the presence of zinc thus decreasing the amount of acid secreted out in the medium. The data in Table 27 show that acid formation occured in cultures of Aspergillus niger 4247w with or without the addition of zinc.

If this fungus is able to utilize the acids before they can be entirely secreted then there should be a more efficient utilization of the carbohydrates supplied and a corresponding decrease in the total acidity of the culture medium. Kinoshita (22) showed that the production of Cojic acid by Aspergillus oryzae was increased when the supply of nitrogen was limited or when the nitrogen source was difficult to utilize. This increase in the acid secretion may be due to a retardation of the synthesizing processes caused by a deficient nitrogen supply. In this case the acids could not be used in the synthetic processes, hence were secreted. The exact nature and sequence of the phenomena involved in cell growth is not known. However, it seems logical to assume that; first, the food materials enter the cells through a complex membrance; second, these nutrients, once within the cell, undergo some transformations in the production of energy and in the formation of certain building units which are finally utilized in

the synthesis of protoplasm. It is difficult to say just where and how zinc or other chemical units function in the growth processes. The writer is inclined to consider zinc as an essential element which functions in the synthesizing processes within the cell. This concept receives some support from the fact that a deficiency of an obviously essential element such as nitrogen has the same effect as an insufficient supply of zinc in that acid secretion continues even though an increase in dry weight is greatly retarded or in some cases inhibited. Moreover, the permeability of the cell membranes seems to be practically the same in the culture with or without the added zinc. The carbon sources were able to enter the cells, as apparently were the other food materials. The acids in the case of certain Aspergilli were able to go out through the membrances in the presence or the absence of the added zinc. The Fusaria, in general, do not secrete acid, probably due to the absence of certain enzymes but the addition of zinc usually resulted in an acceleration of growth and a more efficient utilization of the available nutrients in the production of dry weight.

There was no abnormal increase in respiration according to Watterson (48) in the presence of zinc. She found the ratio of carbon dioxide to dry weight produced to be practically the same in the plus and minus zinc cultures. These results indicate a normal metabolism in the presence of zinc. There is a quanitative relationship between the amount of zinc supplied and the dry weight of the mycelium according to Steinbery (47). Brenchley and Warrington (8) found a similar relationship in the boron requirements of Vicia faba. The boron was absorbed and in some

way removed from action, a constant supply thus being necessary. These results suggest that boron and zinc do not act as ordinary catalysts.

In view of the present knowledge, the writer is unable to name the exact role played by many elements which are effective in minute quantities. However, he is inclined to consider those which act similar to zinc as true essential elements which function in the synthesizing processes of the living cell, and forming a more or less permanent part of some molecular structure.

SUMMARY.

1. A large number of experiments are recorded in which pure cultures of different fungus genera were grown in liquid media to determine certain growth responses to some essential and some so-called stimulating elements.

2. In a favorable nutrient solution the amount of vegetative growth varies almost directly with the concentration of such carbon sources as glucose and sucrose.

3. Several nitrogen compounds can be used as suitable sources of nitrogen. The amount of nitrogen actually required depends upon the concentration of the other nutrients. The nitrogen sources play an important role in the acidity changes in the culture medium. In the presence of nitrate salts of alkali metals fungi generally decrease the acidity of the culture medium, while the reverse is true in the presence of ammonium nitrate, probably due to the different rates of ionic absorption. The use of ammonium nitrate with the nitrate salts of the alkali metals retards the development of extreme acid or alkaline conditions in

the culture medium. However, those fungi which excrete organic acids will increase the acidity of the medium regardless of the nitrogen source.

4. The minimum amounts of magnesium sulphate and mono potassium acid phosphate necessary to give maximum growth was determined under certain experimental conditions. Fusarium radicicola 701 and Aspergillus niger 4247w have practically the same mono potassium acid phosphate requirement. The addition of more than the required amount of magnesium sulphate to the medium resulted in growth acceleration but gave only slight increases in the dry weight yield. Large amounts of the mono potassium acid phosphate exert a buffer action in the culture medium.

5. A nutrient solution of approximately the following composition has few objectionable features for cultures in 125 cc. Erlenmeyer flasks containing 40.0 cc. of solution.

Potassium nitrate,.....	KNO_3	5.000	grams
Magnesium sulphate	$MgSO_4.7H_2O$ ..	2.000	"
Mono potassium acid phosphate..	KH_2PO_4	2.000	"
Glucose or sucrose		40.000	"
Zinc sulphate, $ZnSO_4$		0.050	"

Distilled water to make volume of one liter.

The addition of other elements may influence the growth responses. The extent of such responses will depend upon the purity of the nutrient components and the organism employed.

6. More accurate results can be obtained with several harvests during a culture period than with a single harvest of comparatively young cultures, due to differences in the growth rate and the occurence of autolytic processes.

7. Under the experimental conditions, the fungi used were only slightly benefitted by the addition of minute quantities of boric acid.

8. An increase in growth of Fusarium radicicola 701 was obtained in test tube cultures with ammonium nitrate as the nitrogen source by the addition of approximately 0.500 grams of boric acid per liter.

9. Careful studies showed that boric acid tolerance varied with the organisms and the composition of the culture medium.

10. The addition of manganese to the culture medium resulted in an acceleration of growth and a slightly greater dry weight of mycelium. The response to manganese was less than to zinc.

11. All the Fusaria cultured showed definite growth responses to the addition of zinc to the culture medium. The extent of such responses varied somewhat with the different organisms.

12. Zinc is considered as an essential element which functions in anabolic metabolism.

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