INCUBATION STUDIES WITH SOIL FUNGI.¹

BY S. A. WAKSMAN, *Research Assistant, Agricultural Experiment Station*, and R. C. COOK, *Research Fellow, Rutgers College*.

INTRODUCTION.

Recent investigations in the field of microbiology have shown in a measure the importance of recognizing fungi as one of the factors in soil fertility. In studying the complex problem of microörganic activities, too little attention has been directed to the possible influence of fungus forms. In fact, many of the chemical changes taking place constantly in soils have been considered almost wholly the result of bacterial life, while now we know that a large number of fungi are capable of working in some directions quite as efficiently as bacteria. Whether or not fungi have the capacity to fix nitrogen or to nitrify, cannot be definitely settled as yet, but many of them do have the power to ammonify. Their importance is thus further emphasized by the fact that they occur in such large numbers in cultivated and uncultivated soils.

Since the morphology and life history of fungi are much more complex than those of bacteria, we have reason to suspect that the development of different stages in the life of these organisms may simultaneously affect their powers to decompose organic matter; whereas the very short life cycle of bacteria renders their functions in the soil a more or less continuous process, the fact that fungi have a much longer life cycle would no doubt affect their relation to the fertility of the soil differently at successive stages of growth.

In order to learn something of the factors involved in this phase of biological activities, the present investigations were begun.

EXPERIMENTAL.

The character of this study was such that in the very beginning the usual methods of working with soil fungi were questioned. In general, these methods were essentially the ones used in work with bacteria, and the adaptability was thereby merely assumed. It was therefore planned to carry out a few preliminary experiments in an effort to determine two important elements: first, moisture requirements; and second, the proper incubation period.

The soil used throughout was a Sassafras gravelly loam, dried blood and cottonseed meal serving as ammoniates. Three organisms were employed as follows: *Mucor plumbeus*, *Penicillium sp.*² and *Monilia sito-*

¹ Received for publication September 15, 1915.

² This organism belongs to a group of soil *Penicillia* which are at present under investigation.

phila. These were isolated and purified on the fungi medium noted below.¹ Subcultures were made in liquid media of the same composition and incubated at 25° C. until spores were produced. One-cubic centimeter portions of these cultures were used for inoculation.

SERIES I.

Moisture Relationship and Incubation Period.

In this series four different amounts of moisture were tried. The organic matter, in quantities containing 100 mg. of nitrogen, was added to fifty-gram portions of soil and thoroughly mixed. The physical optimum of the mixtures was then determined (16 per cent resulting for the soil with dried blood and 17 per cent for the soil with cottonseed meal). The moisture was varied and added as noted below; 1 c.c. in excess being added to all to allow for loss during sterilization which was accomplished at 15 pounds pressure for 15 minutes. The soil portions were properly inoculated and subsequently incubated at 22° C. These were divided into three sets, a set being distilled for ammonia after six, twelve, and eighteen days respectively. *Mucor plumbeus* and *Penicillium sp.* were used for inoculation.

TABLE I.
AMMONIFICATION BY MUCOR PLUMBEUS AND PENICILLIUM SP. IN 6 DAYS.

Moisture added	Organic comp.	Check		Mucor plumbeus			Penicillium sp.		
		Ammonia N found		Ammonia N found			Ammonia N found		
		mg. N	Ave'ge	mg. N	Ave'ge	Inc. over check	mg. N	Ave'ge	Inc. over check
¼ optimum	100 mg. N. in Dried Blood	2.2		3.20			5.10		
		2.4	2.30	4.40	3.80	1.50	6.50	5.80	3.50
		2.2		5.80			8.65		
½ "	"	2.2	2.20	8.10	6.95	4.75	8.30	8.48	6.28
		2.3		14.65			10.20		
1 "	"	2.2	2.25	13.45	14.05	11.80	9.50	9.85	7.60
		2.3		12.55			9.30		
2 "	"	2.3	2.30	12.85	12.70	10.40	10.30	9.80	7.50
		2.6		8.65			3.75		
¼ optimum	100 mg. N. in Ctn Sd Meal	2.6	2.60	7.55	8.10	5.50	3.60	3.68	1.08
		2.3		12.60			3.40		
½ "	"	2.4	2.35	12.80	12.70	10.35	3.40	3.40	1.05
		2.4		13.75			3.15		
1 "	"	2.5	2.45	13.60	13.68	11.23	3.15	3.15	.70
		2.3		9.80			3.15		
2 "	"	2.3	2.30	11.60	10.70	8.40	3.45	3.30	1.00

¹ Medium No. II as described in "The relation of parasitic fungi to the contents of the cells of the host plants," Cook, M. T., and Taulenhaus, M. S., Del. Agr. Exp. Stat., Bul. 91, p. 11.

Table I shows that the amount of ammonia accumulation in six days by *Mucor plumbeus* from both dried blood and cottonseed meal increases from one-fourth optimum to optimum moisture and then slightly decreases. Essentially the same conditions are true with *Penicillium sp.* except that with cottonseed meal the ammonia accumulation was so little that differentiation was difficult.

TABLE II.
AMMONIFICATION BY MUCOR PLUMBEUS AND PENICILLIUM SP. IN 12 DAYS.

Moisture	Organic comp.	Check		Mucor plumbeus			Penicillium sp.		
		Ammonia N found		Ammonia N found			Ammonia N found		
		mg. N	Ave'ge	mg. N	Ave'ge	Inc. over check	mg. N	Ave'ge	Inc. over check
$\frac{1}{4}$ optimum	100 mg. N. in Dried Blood	2.3		6.60			13.15		
		2.4	2.35	6.50	6.55	4.30	16.50	14.83	12.48
		2.1		10.00			23.00		
$\frac{1}{2}$ "	"	2.8	2.45	14.00	12.00	9.55	26.20	24.60	22.15
		2.1		19.80			26.20		
1 "	"	2.6	2.35	19.70	19.75	17.15	25.10	25.65	23.30
		2.1		20.40			24.05		
2 "	"	2.2	2.15	lost	20.40	18.25	20.40	22.23	20.08
		2.4		17.50			8.00		
$\frac{1}{4}$ optimum	100 mg. N. in Ctn Sd Meal	2.8	2.60	15.70	16.60	14.00	8.80	8.40	5.80
		2.3		20.80			7.50		
$\frac{1}{2}$ "	"	3.3	2.80	21.00	20.90	18.10	12.25	9.88	7.08
		2.4		22.55			12.50		
1 "	"	2.8	2.60	21.15	21.85	19.25	lost	12.50	9.90
		2.2		18.60			12.60		
2 "	"	2.9	2.55	23.10	20.85	17.95	12.00	12.30	9.40

In Table II we find that in twelve days also the ammonia accumulation goes hand in hand with the increase in moisture up to optimum, beyond which it decreases.

The eighteen-day period again shows that the optimum physical moisture corresponds closely to the optimum moisture conditions for the fungi, except in the case of the cottonseed meal, where one-half optimum gave better results.

The fact that moisture variation has not seemed to alter appreciably the behavior of the organisms at different periods, permits us to see at once the effect of time of incubation upon ammonia accumulation.

With the *Mucor* the increase from the sixth to the twelfth day was marked, while no appreciable advantage was gained by incubating eighteen days. However, with the *Penicillium*, in eighteen days there was still a large increase over that obtained at twelve days. But if we turn to Table III we find that with cottonseed meal less ammonia was noted at optimum than at one-half optimum, a point which might indicate that some other

process had started, thus consuming the ammonia where there was sufficient moisture. This fact in itself would tend to warrant the choice of a twelve-day period. One can easily see how the length of incubation might alter the relationship between organisms in ammonification studies.

TABLE III.
AMMONIFICATION BY *MUCOR PLUMBEUS* AND *PENICILLIUM* SP. IN 18 DAYS.

Moisture	Organic comp.	Check		Mucor plumbeus			Penicillium sp.		
		Ammonia N found		Ammonia N found			Ammonia N found		
		mg. N	Ave'ge	mg. N	Ave'ge	Inc. over check	mg. N	Ave'ge	Inc. over check
$\frac{1}{4}$ optimum	100 mg. N. in Dried Blood	1.9		4.40			20.4		
		1.8	1.85	4.20	4.30	2.45	21.8	21.10	19.25
		1.9		13.15			31.1		
$\frac{1}{2}$ "	"	2.2	2.05	14.05	13.60	11.55	30.2	30.65	28.60
		1.8		21.90			36.3		
		1.8	1.80	22.90	22.40	20.60	35.7	36.00	34.20
1 "	"	1.8		21.00			32.7		
		1.6	1.70	21.70	21.35	19.65	30.3	31.50	29.80
		1.9		20.45			12.6		
$\frac{1}{4}$ optimum	100 mg. N. in Cot's'd Meal	1.6	1.75	19.25	18.85	18.10	19.9	16.25	14.50
		1.9		24.50			19.2		
		2.1	2.00	23.70	24.10	22.10	19.9	19.55	17.55
$\frac{1}{2}$ "	"	2.1		22.00			17.4		
		1.8	1.95	23.50	22.75	20.80	16.9	17.15	15.20
		2.1		20.60			15.5		
2 "	"	lost	2.10	20.40	20.50	18.40	16.8	16.15	14.05

In the work of McLean and Wilson,² where fungi were compared as to their capacity to ammonify, a seven-day period was employed. Had a longer period been chosen, which would have given the slower ammonifiers time to develop, no doubt different results would have been obtained. The *Aspergillaceae* group was found in their work to be composed of weak ammonifiers, and the *Mucors* of strong ammonifiers. It is interesting to note and compare the results obtained in the above tables with those in the work referred to. In ammonia accumulation the *Mucor* led by a large margin the *Penicillium*, a member of the *Aspergillaceae* group, with both dried blood and cottonseed meal for the six-day period. However, in twelve days the *Penicillium* with dried blood surpassed the *Mucor*, and nearly equalled it with cottonseed meal, holding this relationship through the eighteenth day. It appears that with slow growing organisms a longer period of incubation is necessary to secure truly characteristic activity.

In an effort to determine whether or not the biological stage of the fungi bears any relationship to their capacity to accumulate ammonia, the preliminary experiments served as a guide to the following plan.

² McLean and Wilson, Ammonification Studies with Soil Fungi. N. J. Agr. Exp. Sta., Bulletin 270, 39 p., 1 pl.

SERIES II.

The Biological Stage of Fungi as Affecting Ammonification.

The organisms, soil, and organic matter were the same as noted above. One hundred grams of soil were placed in Erlenmeyer flasks of 250 c.c. capacity, thoroughly mixed with the organic matter in portions containing 155 mg. of nitrogen, and brought to optimum moisture conditions. These were then plugged and sterilized as before, after which proper inoculation was made. One cubic centimeter of the inoculum contained spores as follows: *Mucor*, about 1,000,000; *Penicillium*, 100,000-200,000; *Monilia*, 3,000,00-4,000,000. Incubation was effected at about 27° C. Daily determinations of ammonia were made in duplicate for each organism with both organic materials. The development of the organisms was watched daily as well.

TABLE IV.

DAILY ACCUMULATION OF AMMONIA IN THE SOIL BY MUCOR PLUMBEUS.

Period of Incubat'n Days	Dried Blood				Cottonseed Meal			
	Ammonia Nitrogen found				Ammonia Nitrogen found			
	mg. N.	Average	Inc. over check	Daily gain	mg. N.	Average	Inc. over check	Daily gain
1	2.35	2.50	0.07	0.07	4.55	4.26	0.07	0.07
	2.64				3.97			
	3.53				8.82			
2	3.82	3.68	1.25	1.18	8.65	8.74	4.55	4.48
	4.41				12.05			
3	4.85	4.63	2.20	.95	10.44	11.25	7.06	2.51
	5.00				14.70			
4	5.12	5.06	2.63	.43	13.26	13.98	9.79	2.73
	6.47				31.31			
5	6.47	6.47	4.04	1.41	26.75	29.03	24.84	15.05
	6.76				34.10			
6	7.20	6.98	4.55	.51	31.05	32.58	28.39	3.55
	8.06				38.96			
7	8.23	8.15	5.72	1.17	37.53	38.30	34.11	5.72
	8.23				41.31			
8	8.53	8.38	5.95	.23	39.10	40.21	36.02	1.91
	9.56				39.84			
9	12.64	11.10	8.67	2.72	40.87	40.36	36.17	.15
	10.58				41.60			
10	11.17	10.88	8.45	-0.22	40.86	41.23	37.04	.87
	11.32				42.92			
11	10.88	11.10	8.67	0.22	40.72	41.82	37.63	.59
	11.61				42.78			
12	11.73	11.17	8.74	0.07	45.42	44.10	39.91	2.28
	2.44				4.26			
Check	2.42	2.43			4.12	4.19		

In the column of daily gains of ammonia by *Mucor plumbeus*, one sees that the strongest activity occurred about every other day; and from actual observation of the growth of the organism, this seemed to coincide with the times immediately following the periods of active spore formation.

TABLE V.

DAILY ACCUMULATION OF AMMONIA IN THE SOIL BY *MONILIA SITOPHILA*.

Period of Incubat'n Days	Dried Blood				Cottonseed Meal			
	Ammonia Nitrogen found				Ammonia Nitrogen found			
	mg. N.	Average	Inc. over check	Daily gain	mg. N.	Average	Inc. over check	Daily gain
1	2.94				4.85			
	3.09	3.02	0.59	0.59	5.88	5.37	1.18	1.18
	4.50				33.96			
2	4.70	4.60	2.17	1.58	36.30	35.13	30.94	29.75
	13.38				44.10			
3	13.52	13.45	11.02	8.85	43.66	43.88	39.69	8.75
	20.00				50.86			
4	20.58	20.29	17.86	6.84	51.16	51.01	46.82	7.13
	23.08				51.30			
5	23.52	23.30	20.87	3.01	52.77	52.04	47.85	1.03
	26.17				53.65			
6	26.31	26.24	23.81	2.94	53.94	53.80	49.61	1.76
	27.05				56.30			
7	29.20	28.13	25.70	1.89	53.80	55.05	50.86	1.25
	32.78				57.18			
8	32.49	32.64	30.21	4.51	55.86	56.52	52.33	1.47
	34.69				57.92			
9	34.25	34.47	32.04	1.83	58.95	58.44	54.25	1.92
	39.39				59.68			
10	lost	39.39	36.96	4.92	61.25	60.47	56.28	2.03
	39.54				59.53			
11	40.13	39.84	37.41	.45	60.86	60.20	56.01	—0.27
	41.01				60.71			
12	41.90	41.46	39.03	1.64	59.09	59.90	55.71	—0.30
	2.44				4.26			
Check	2.42	2.43			4.12	4.19		

Monilia sitophila, known to be a very strong ammonifier, showed in this series the accumulation of large quantities of ammonia even in the first days of incubation. With dried blood, about half of the total amount registered for twelve days was accumulated in four days, while with cottonseed meal about 80 per cent was accumulated in the same length of time. Or, in other words, at least 15 per cent of the total nitrogen in dried blood and 30 per cent of that in cottonseed meal, had been ammonified in four days. Spores were abundant here in the early stages of growth, particularly about the second and third days, the greater number occurring earlier in the cottonseed meal cultures. The correlation between sporulation and ammonia accumulation seems again to hold true.

Turning to Table VI, one finds that very little ammonia accumulated within the first six days by *Penicillium sp.*, and only on the seventh day was a marked increase in ammonia noticed. Up until the sixth and seventh days there was no notable sporulation, but principally development of mycelium. Extensive sporulation was observed beginning at

this period and about every two or three days thereafter. The figures in this table after the six-day period show a similar variation in the amount of ammonia accumulated.

TABLE VI.

DAILY ACCUMULATION OF AMMONIA IN THE SOIL BY *PENICILLIUM* SP.

Period of Incubat'n Days	Dried Blood				Cottonseed Meal			
	Ammonia Nitrogen found				Ammonia Nitrogen found			
	mg. N.	Average	Inc. over check	Daily gain	mg. N.	Average	Inc. over check	Daily gain
1	2.77				4.23			
	2.69	2.73	0.33	0.33	4.07	4.15	—0.04	—0.04
	2.36				4.06			
2	2.48	2.42	0.02	—0.31	4.06	4.06	—0.13	—0.09
	3.20				4.50			
3	2.91	3.06	0.66	0.64	4.22	4.36	0.17	0.30
	4.05				4.89			
4	3.72	3.89	1.49	0.83	5.12	5.01	0.82	0.65
	4.66				6.55			
5	4.95	4.82	2.42	0.93	5.96	6.26	2.07	1.25
	4.80				7.72			
6	5.39	5.10	2.70	0.28	7.72	7.72	3.53	1.46
	7.94				15.73			
7	6.76	7.35	4.95	2.25	16.61	16.17	11.98	8.45
	8.08				18.41			
8	9.42	8.75	6.35	1.40	18.86	18.64	14.45	2.47
	10.88				25.73			
9	9.26	10.07	7.67	1.32	26.90	26.32	22.13	7.68
	11.83				26.46			
10	12.20	12.02	9.62	1.95	27.56	27.01	22.82	0.69
	15.88				36.82			
11	16.76	16.32	13.92	4.30	34.54	35.68	31.49	8.67
	15.88				37.90			
12	17.40	16.64	14.24	.32	38.80	38.35	34.16	2.67
	19.55				41.60			
13	19.25	19.40	17.00	2.76	41.44	41.52	37.33	3.17
	22.05				43.12			
14	23.50	22.78	20.38	3.38	46.60	44.86	40.67	3.34
	27.20				48.95			
15	28.66	27.93	25.53	5.15	47.33	48.14	43.95	3.28
	2.40				4.26			
Check	2.40	2.40			4.12	4.19		

The curves give a graphical expression to the foregoing statements and show to some extent the behavior of the individual organisms accentuating the variability in daily gains of ammonia. Attention is called to the fact that in general there is a period of low activity in the beginning, followed usually by a period of maximum activity, and later by one of lesser and varying activity. The intensity of the period of maximum activity has its reaction in the subsequent growth of the organism, as is to be expected, when there is a point of very large ammonia accumulation. The later development is more uniform, but where there is no such point of extreme activity the periods of extensive ammonification alter-

Figure 1.—Daily Ammonia Accumulation by (1) *Mucor plumbeus*, (2) *Monilia sitophila*, and (3) *Penicillium sp.*

nate with comparatively inactive ones. Each organism seems to have its own peculiarities in this respect. The application becomes of importance in considering data secured with different fungi. For instance, two or more organisms compared may give on one day a certain relationship to each other, while on another day this relationship may not exist, depending upon the respective sporulation periods.

It must not be understood, however, that ammonia accumulation depends upon sporulation; rather, it seems to follow it closely. The maximum gain in ammonia coincides with the germination of the spores and the subsequent development of the mycelium; while the minimum gain occurs when the organism prepares itself to produce new spores. While spore production may be a more or less continuous process, there are, however, well defined periods when it is the predominant activity of the organism.

A good analogy is found in the growth of legumes like the clovers. Nitrogen is fixed during the period of active growth of the plant, the fixation ceasing almost entirely when seed formation begins. In a like manner most of the ammonia seems to be accumulated when the spores of the fungus germinate to form new mycelium, this process being appreciably hindered at the time at which the organism forms its sporophores immediately preceding the complete development of the corresponding spores.

The three fungi studied represent three very important groups of soil organisms, and their behavior should therefore be indicative of the activities of those groups of which they are members. *Monilia sitophila* represents the Moniliaceae, which are strong ammonifiers, accumulating most of their ammonia in a very short period of time. This is undoubtedly due to their early and extensive spore formation. In contrast to the Moniliaceae, the Aspergillaceae, of which the *Penicillium sp.* is a member, are weak ammonifiers in short incubation periods; but if the period of incubation is sufficiently long, large quantities of ammonia will be accumulated. *Mucor plumbeus*, representing a third important group of soil organisms, places itself intermediate between the two above.

Taken collectively, the experiments show that there is an important connection between the biological stage of a fungus and its capacity to decompose organic matter as indicated by ammonia accumulation.

SUMMARY.

The results of these experiments indicate that:

1. Optimum moisture conditions for ammonia accumulation by fungi lie near the physical optimum.
2. The proper incubation period depends entirely upon the organism.
3. A twelve-day incubation period is preferable to a shorter one for practical work.

4. A correlation exists between the biological stage of the organism and the periods of ammonia accumulation; the largest amount seems to accompany the periods of spore germination and the smallest amount the time preparatory to actual spore formation.

5. *Monilia sitophila* shows the largest ammonia accumulation within the first three or four days; *Penicillium sp.*, between ten and fifteen days; and *Mucor plumbeus*, between six and ten days. These periods correspond to those of active spore formation for the respective organisms.