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INFLUENCE OF NITRATES ON NITROGEN-ASSIMILATING BACTERIA

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INFLUENCE OF NITRATES ON NITROGEN-ASSIMILATING BACTERIA[1]

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INTRODUCTION

RELATION OF NITRATES TO VARIOUS FORMS OF PLANT LIFE

The importance of nitrogen to plant life can not be overestimated. It is one of several elements essential to plant growth, one, moreover, which is apt to be deficient in arable soils. These facts are well brought out by the almost innumerable investigations which have been made concerning the source of nitrogen for plants.

The influence of nitrate nitrogen on various plants has been the controlling idea in many of these experiments. Very little attention has been placed on the effect of nitrates on the lower plants, especially the bacteria. Because of the relation that exists between higher plants and bacteria it seems advisable to consider the effect of nitrates on the soil bacteria. Indeed, progress in the knowledge of nitrogenous fertilizers depends on a study of the effect of the fertilizer on the soil organisms as well as on the higher plants. The action of fertilizers on the different groups of soil organisms, the relation of these organisms to higher plants, and the separation of the important from the unimportant groups are some of the factors involved in the problem of soil fertility.

REVIEW OF LITERATURE

The relation of nitrates to the germination of seeds has been studied by De Chalmot (*11*)[3], who found that corn germinated in solutions containing nitrate was more robust than corn germinated under similiar conditions without nitrate. He also noted that if too concentrated solutions of nitrate were used germination was retarded rather than hastened. The presence of nitrate also increased the amount of albuminous material in the seed.

The direct influence of nitrate nitrogen on the growing plant is too well known to justify any lengthy discussion here. Jost (*26, p. 134*) gives the results of experiments made by Boussingault, who grew the sunflower (*Helianthus argophyllus*) in sand with and without nitrate.

[1] Major portion of a paper submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in bacteriology in the Graduate School of the University of Wisconsin, December, 1916.

[2] The writer wishes to acknowledge his appreciation of the suggestions and criticisms obtained throughout the progress of this work from Prof. E. B. Fred and E. G. Hastings, of the University of Wisconsin.

[3] Reference is made by number (italic) to "Literature cited," pp. 227–230.

Journal of Agricultural Research,
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During the three months' growth of the plants 1.40 gm. of potassium nitrate were added. At the end of the period the dry weight of the plant supplied with nitrate was nearly 60 times greater than that of the plant where no nitrate was added. The relation between the growth of nonleguminous plants and the amount of nitrate nitrogen supplied is shown in a very striking manner in the following table taken from Hellriegel and Wilfarth (*21, p. 53–54*):

Nitrogen as Ca $(NO_3)_2$ added to pots, gm	None	0.056	0.112	0.168	0.224	0.336
Dry weight of oats (grain and straw), gm	0.3605 .4191	5.9024 5.8510 5.2867	10.9814 10.9413	15.9974	21.2732 21.4409	30.1750

But little work has been done on the direct influence of nitrates on the development of the Eumycetes. Some investigations have been made as to the ability of certain fungi to assimilate nitrate nitrogen directly. Ritter (*42*) studied many species and found that some forms would assimilate nitrate directly, while others reduced it to nitrite and ammonia. He found some forms which failed to grow on media containing nitrate. Kossowicz (*28*) found that various fungi utilized nitrates and that nitrite and ammonia were produced.

Münter (*36*) studied the influence of inorganic salts on the growth of various Actinomycetes. He found that potassium and sodium nitrates in quantities equivalent to 5 per cent permitted good growth of the organisms but retarded spore formation. Calcium, barium, and strontium nitrates in small quantities affected some species but not others. Small quantities of these nitrates did not affect growth to any extent, but larger quantities were detrimental to growth and spore formation. Silver nitrate in all amounts studied almost entirely prohibited growth.

Nitrates appear to exert some influence on the yeasts. Drabble and Scott (*13*) studied the effect of sodium nitrate on these organisms. They found that the greatest reproduction took place in solutions containing 0.2 gram-molecule of the nitrate. Increasing amounts of the salt led to a decrease in reproductive activity until with 0.7 gram-molecule present no reproduction took place. From their results it is evident that small quantities of nitrate stimulated reproduction, whereas larger amounts proved detrimental. Kayser (*27*) studied the effect of manganese nitrate on yeasts. He found that the amount which produced the maximum increase in the alcoholic fermentation of sugar varied with the strain of yeast employed. He likewise found that manganese nitrate produced greater increase than did the same quantity of potassium nitrate. Fernbach and Lanzenberg (*14*) concluded that nitrates hindered the rapidity of cell multiplication of yeasts but greatly accelerated the action of the zymase. More alcohol was formed in the presence than in the absence of nitrate. According to Kossowicz (*28*), nitrates are not a suitable source of nitrogen for yeasts.

The direct influence of nitrates on bacteria has been studied to a limited extent. The influence of various nitrates on soil bacteria has been studied by Greaves (*19*). He added sodium, potassium, calcium, magnesium, manganous and ferric nitrates to soil in varying quantities. The amount added to the soil was such that in each case equivalent quantities of the anion (NO_3) in the various forms were added. The effect of these salts on the bacteria was determined by using ammonification as an index of the bacterial activity. He found that sodium-potassium, manganous and ferric nitrates in small amounts, approximately 0.97 to 5.5 mgm. of nitrate in 100 gm. of soil, slightly stimulated ammonification. Greater concentrations of these salts proved toxic as evidenced by a decrease in the amount of ammonia formed. Sodium nitrate was much more beneficial to ammonification than potassium nitrate. From his results as a whole Greaves concludes that it is the electronegative ion which stimulates bacterial activity. Calcium and magnesium nitrates proved toxic in all concentrations studied.

However, a majority of the investigations have been directed toward a determination of the effect of the bacteria on the nitrates. But little work appears to have been done on the direct action of nitrates on bacteria. Pfeffer (*38, p. 351*) cites some experiments showing the repellant action of potassium nitrate toward certain bacteria. *Spirillum undula* was repelled by a solution of potassium nitrate having an osmotic concentration equivalent to 0.5 to 1.0 per cent. With *Spirillum volutans* a much higher concentration was necessary to bring about the same reaction. It was found that different organisms required different quantities of the same nitrate to repel them.

It can be readily seen that by far the greatest amount of work on the relation of nitrates to plant growth has been done in the realm of the higher plants. Obviously further investigations should be made in respect to the effect of nitrates on the lower forms of plant life, especially the bacteria. In this paper an attempt is made to set forth the results secured in a study of the influence which nitrates exert on certain groups of soil bacteria, including not only their reproduction but also some of their physiological properties.

EXPERIMENTAL WORK

OUTLINE OF PROBLEM

The results of much careful experimentation show that nitrate nitrogen is most readily assimilated by higher plants. As a rule it seems to stimulate the plant to increased activity. In some cases this is undoubtedly due to increased nutrition, while in others it is a result of nuclear stimulation with a consequent cell multiplication. No sharp line can be drawn between these two effects. Probably one overlaps the other, and the increased growth of the organism can be attributed to a combination of the two actions.

From a practical standpoint the relation of nitrates to the nitrogen-assimilating organisms of the soil is of importance. Hence, it was arranged to study the effect of nitrates on soil bacteria, especially those forms concerned with the fixation of atmospheric nitrogen. The work naturally falls into two rather distinct lines of investigation. First, the influence of nitrates on Azotobacter was determined. Here studies were made of the effect of nitrates on the growth of the organism in soil and also the effect of these salts on the nitrogen-fixing property of these bacteria. The action of Azotobacter on nitrates in solution, the relation of nitrates to pigment production and to the formation of volutin bodies were studied. Second, the influence of nitrates on the growth of *Bacillus radicicola* in soil was studied. The action of *B. radicicola* on nitrates in solution and the possible nitrogen-assimilating properties of the legume in the presence of nitrates were investigated. Also the influence of nitrates on gum production was determined. The latter part of the investigations included a study of the relation of nitrates to nodule formation on alfalfa.

METHODS USED IN EXPERIMENTS

Nitrates were determined by the reduction method with Devarda's alloy and also by the phenolsulphonic acid (colorimetric) method.

The total nitrogen content of all samples was determined by the modified Kjeldahl method with sulphuric acid, salycilic acid, sodium thiosulphate, and copper sulphate. Where nitrate nitrogen was present, 50 c. c. of concentrated sulphuric-salycilic acid (25 c. c. of concentrated acid plus 25 c. c. of distilled water) were added to the cultures slowly and with constant stirring. This acid was allowed to react for a few days, after which the usual procedure was carried out. Digestion was continued for five to six hours subsequent to the clarification of the liquid.

The amount of ammonia was determined by distillation with steam in the presence of magnesium oxid.

Nitrites (qualitative test) were tested for with Trommsdorf's reagent.

In all distillations *N/14* acid and alkali were used.

In the preparation of agar cultures of alfalfa seedlings the seeds were treated with a 0.25 per cent solution of mercuric chlorid and rinsed in sterile distilled water. Three bacteria-free seeds were transferred to the surface of soft mannit agar (0.7 per cent agar) in each tube.

The nitrates were added in solution to all cultures. Gram-molecular quantities of potassium, sodium, calcium, and ammoniun nitrates (Merck's) were weighed into sterile distilled water. These solutions were prepared in such a manner that 5 c. c. contained 450 mgm. of nitrate. In all nitrate solutions the nitrate radical, or anion, was present in the same quantities, while the cation, or metal, was present in varying quantities, depending upon the particular salt.

Plate counts of all soil cultures were made by weighing 20 gm. (dry weight) of the soil into a 200-c. c. water blank. From this suspension all subsequent dilutions were made. Mannit agar [1] was used for the plate counts in the cultures of Azotobacter and *B. radicicola*. Duplicate plates were made for each dilution poured.

SOIL USED

Only one type of soil was employed, Miami silt loam obtained from the Experiment Station farm. No chemical analyses of the soil were made other than an estimation of its organic matter content, which was approximately 2.75 per cent. The soil was neutral in reaction and its nitrate content was approximately 1.5 mgm. of nitrogen as nitrate in 100 gm. of the dry soil.

ISOLATION OF AZOTOBACTER AND BACILLUS RADICICOLA

AZOTOBACTER.—(1) Strain A was isolated from a silt loam soil. This strain grew well on mannit agar, but produced no pigment after three weeks' growth. (2) Strain B was isolated from a sandy loam soil. This strain grew equally well on mannit agar and produced a brownish black pigment within one week's growth. Both strains assimilated practically the same amount of atmospheric nitrogen under laboratory conditions.

BACILLUS RADICICOLA.—A stock laboratory culture of *B. radicicola* was replated twice before taking the final culture. The nodule producing power of the organism was determined by inoculating bacteria-free alfalfa seedlings (in soft agar). After sufficient incubation nodules were produced in abundance.

INFLUENCE OF NITRATES ON AZOTOBACTER

INFLUENCE OF NITRATES ON THE GROWTH AND REPRODUCTION OF AZOTOBACTER IN STERILIZED SOIL

What effect do nitrates have on pure cultures of Azotobacter in sterilized soil? Do these salts cause a decrease in the numbers of the organisms? Do they cause an increase in numbers? Or do they exert no particular influence one way or the other? It is difficult to believe that the latter could be true, inasmuch as nitrates have such a profound effect on higher forms of plant life. Such readily soluble and assimilable substances as nitrates could hardly remain without affecting either an increase or a decrease in the number of organisms existing in their presence.

With the idea of determining what effect nitrates might have on Azotobacter when grown in sterilized soil, the following experiments were planned. In this work both strains of the Azotobacter (described on

[1] FRED, E. B. A LABORATORY MANUAL OF SOIL BACTERIOLOGY. p. 108. Philadelphia and London, 1916.

p. 187) were employed and conditions governing the preparation and incubation of the cultures were similar in the case of each strain. The only variation was the periods used in incubating the cultures. Counts were made after one and two weeks' incubation with strain A and after one, two, and three weeks with strain B.

TABLE I.—*Influence of potassium nitrate on the growth of Azotobacter (strain A) in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.				
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*
1	0	15,600	825,000	100	315,000	100
2	0	15,600	935,000		360,000	
3	10	15,600	1,500,000	170	1,175,000	348
4	10	15,600	.....		.....	
5	25	15,600	4,200,000	523	12,350,000	3,418
6	25	15,600	5,000,000		10,750,000	
7	50	15,600	20,400,000	2,233	27,750,000	8,210
8	50	15,600	18,900,000		.....	
9	100	15,600	11,000,000	1,295	9,000,000	2,685
10	100	15,600	11,820,000		9,150,000	
11	150	15,000	.....	179	25,000	12
12	150	15,600	1,575,000		55,000	
13	200	15,600	225,000	27	0	0
14	200	15,600	250,000		0	
15	300	15,600	0	0	0	0
16	300	15,600	0		0	

TABLE II.—*Influence of sodium nitrate on the growth of Azotobacter (strain A) in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.				
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*
1	0	13,800	310,000	100	425,000	100
2	0	13,800	225,000		490,000	
3	10	13,800	575,000	188	875,000	191
4	10	13,800	430,000		.....	
5	25	13,800	2,850,000	1,615	2,250,000	492
6	25	13,800	5,800,000		.....	
7	50	13,800	15,200,000	5,217	15,500,000	3,150
8	50	13,800	12,750,000		13,300,000	
9	100	13,800	17,750,000	6,335	9,850,000	2,800
10	100	13,800	16,200,000		15,750,000	
11	150	13,800	550,000	177	690,000	117
12	150	13,800	400,000		375,000	
13	200	13,800	0	0	0	0
14	200	13,800	0		0	
15	300	13,800	0	0	0	0
16	300	13,800	0		0	

TABLE III.—*Influence of calcium nitrate on the growth of Azotobacter (strain A) in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.				
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*
1	0	10,000	260,000	100	310,000	100
2	0	10,000	330,000		260,000	
3	10	10,000	5,800,000	1,966	975,000	362
4	10	10,000	.....		1,090,000	
5	25	10,000	10,700,000	3,440	9,200,000	3,122
6	25	10,000	9,600,000		8,600,000	
7	50	10,000	13,250,000	4,213	13,200,000	4,526
8	50	10,000	11,600,000		12,600,000	
9	100	10,000	6,600,000	2,144	8,750,000	2,938
10	100	10,000	6,050,000		8,000,000	
11	150	10,000	3,500,000	1,254	2,000,000	763
12	150	10,000	3,900,000		2,350,000	
13	200	10,000	0	0	0	0
14	200	10,000	0		0	
15	300	10,000	0	0	0	0
16	300	10,000	0		0	

One hundred and fifty gm. of soil (dry weight) were weighed into 500-c. c. Erlenmeyer flasks and the nitrates added in solution, as indicated in the following tables. At the same time 1 per cent of mannit was added in solution and the moisture content was raised to approximately 18 per cent. The flasks were allowed to remain at room temperature for one day, when the contents were thoroughly mixed. The flasks and contents were then sterilized at 15 pounds' pressure for three hours. Upon cooling they were inoculated with 5 c. c. of a suspension of the organisms in sterile distilled water. The cultures were incubated at 28° C. and counts made at the intervals already indicated. Mannit agar was used in pouring the plates. Each number in the following tables represents an average of duplicate plates. Tables I, II, and III show the results of the work with strain A and Tables V, VI, and VII the results with strain B.

It will be seen at a glance that all three nitrates exerted an enormous influence on the growth of the Azotobacter. The smallest concentration did not appear to exert much influence either in increasing or decreasing the number of Azotobacter. There was a slight gain, but it was not so marked as that brought about by higher concentrations of nitrates. When 25, 50, and 100 mgm. of nitrate were present in 100 gm. of soil, very large increases were obtained in practically all instances. In one instance sodium nitrate caused the greatest relative gain, but the most consistent increase was produced by calcium nitrate. Beginning with 150 mgm. the number of Azotobacter began to decrease. This decrease was especially noticeable in the cultures containing potassium and sodium nitrates. At the end of the first week, Azotobacter organisms

were still found in the potassium-nitrate cultures where 200 mgm. were present. However, at the end of the second week the organisms were dead. The same concentration of sodium and calcium nitrates proved even more toxic. No evidences were secured, indicating that these organisms can resist concentrations in excess of 300 mgm. of nitrate per 100 gm. of soil.

The question may be raised in regard to the influence of sterilization on the nitrate present in the soil, Does the prolonged heating in the presence of soil organic matter reduce the nitrate? In order to study this point, a few cultures were prepared similar to those already described. They were subjected to sterilization under pressure of 15 pounds for two, three, and five hours. Nitrate determinations at the end of these periods failed to show any reduction. In the presence of 1 per cent of mannit the nitrate content remained unchanged during sterilization.

From these results it is evident that small amounts of nitrate up to 150 mgm. of nitrate in 100 gm. of soil greatly increased the reproduction of Azotobacter. In regard to the toxicity of higher concentrations, sodium nitrate appeared to exert the greatest influence in this direction, followed by calcium and potassium nitrates in the order named. The results of the experiment are recorded in Table IV.

TABLE IV.—*Influence of ammonium nitrate on the growth of Azotobacter (strain A) in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.				
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*
1	0	18, 500	1, 400, 000	100	975, 000	100
2	0	18, 500	1, 050, 000		1, 100, 000	
3	25	18, 500	5, 600, 000	427	5, 000, 000	430
4	25	18, 500	4, 900, 000		3, 900, 000	
5	100	18, 500	2, 900, 000	223	3, 950, 000	388
6	100	18, 500	2, 600, 000		4, 100, 000	
7	200	18, 500	1, 100, 000	84	875, 000	86
8	200	18, 500	950, 000		915, 000	

That the nitrate radical and not the combined metal was the causal agent in the increase in the number of Azotobacter was indicated from the results of the next test. Here ammonium nitrate was used.

It will be seen from the data of this experiment that ammonium nitrate caused an increase in the number of Azotobacter when present in small amounts. However, the increase in the presence of ammonium nitrate was less marked than when equal quantities of the other nitrates were used. Since the experiments with ammonium nitrate were not made at the same time as the preceding experiments (discussed on pp. 189–190), it is possible that conditions varied sufficiently to account for the less pronounced results. When 200 mgm. of nitrate were present in 100 gm. of

soil the number of Azotobacter showed a decrease. Apparently ammonium nitrate is more toxic than potassium, sodium, and calcium nitrate. However, the main point at issue seems fairly well established—namely, that the increase in the number of Azotobacter is caused by the nitrate radical and not by the combined metal.

TABLE V.—*Influence of potassium nitrate on the growth of Azotobacter (strain B) in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.						
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.	After 3 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*		*Per ct.*
1....	0	12,600	235,000	100	112,500	100	116,000	100
2....	0	12,600	.....		110,500		117,000	
3....	10	12,600	3,750,000	1,510	2,100,000	1,950	875,000	916
4....	10	12,600	3,300,000		2,250,000		1,260,000	
5....	25	12,600	5,750,000	2,436	1,575,000	1,581	1,700,000	1,300
6....	25	12,600	5,700,000		1,950,000		1,325,000	
7....	50	12,600	3,100,000	1,340	3,250,000	3,655	3,525,000	2,783
8....	50	12,600	3,200,000		4,900,000		2,960,000	
9....	100	12,600	3,200,000	1,320	4,000,000	3,363	2,500,000	2,317
10....	100	12,600	3,000,000		3,500,000		2,900,000	
11....	150	12,600	2,100,000	851	2,000,000	1,838	1,500,000	1,502
12....	150	12,600	1,900,000		2,100,000		2,000,000	
13....	200	12,600	875,000	373	800,000	695	650,000	580
14....	200	12,600	880,000		750,000		700,000	
15....	300	12,600	0	0	0	0	0	0
16....	300	12,600	0		0		0	

TABLE VI.—*Influence of sodium nitrate on the growth of Azotobacter (strain B) in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.						
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.	After 3 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*		*Per ct.*
1....	0	15,600	158,000	100	110,500	100	112,500	100
2....	0	15,600	149,000		126,000		115,000	
3....	10	15,600	1,250,000	727	1,750,000	1,310	5,000,000	5,097
4....	10	15,600	990,000		1,350,000		6,600,000	
5....	25	15,600	1,765,000	1,165	6,600,000	5,029	9,150,000	7,161
6....	25	15,600	1,825,000		5,300,000		7,150,000	
7....	50	15,600	1,875,000	1,338	2,025,000	2,141	15,950,000	13,423
8....	50	15,600	2,250,000		3,040,000		14,600,000	
9....	100	15,600	2,200,000	1,350	2,775,000	2,525	5,800,000	4,860
10....	100	15,600	1,950,000		3,200,000		5,250,000	
11....	150	15,600	165,000	108	530,000	556	3,100,000	2,573
12....	150	15,600	170,000		785,000		2,750,000	
13....	200	15,600	0	0	0	0	0	0
14....	200	15,600	0		0		0	
15....	300	15,600	0	0	0	0	0	0
16....	300	15,600	0		0		0	

TABLE VII.—*Influence of calcium nitrate on the growth of Azotobacter (strain B) in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.						
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.	After 3 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*		*Per ct.*
1....	0	22,000	905,000	100	1,475,000	100	1,130,000	100
2....	0	22,000	860,000		1,460,000		1,157,500	
3....	10	22,000	23,200,000	2,423	28,000,000	2,181	34,050,000	3,002
4....	10	22,000	19,600,000		36,000,000		34,600,000	
5....	25	22,000	17,200,000	2,084	52,000,000	3,255	29,750,000	2,273
6....	25	22,000	19,600,000		43,500,000		22,250,000	
7....	50	22,000	11,800,000	1,461	22,500,000	1,448	30,400,000	2,633
8....	50	22,000	14,000,000		20,000,000		29,850,000	
9....	100	22,000	7,500,000	1,053	12,000,000	818	21,750,000	1,780
10....	100	22,000	11,000,000		.....		18,950,000	
11....	150	22,000	2,550,000	342	5,300,000	402	4,800,000	420
12....	150	22,000	3,500,000		6,500,000		.....	
13....	200	22,000	107,500	11	2,750,000	203	130,000	11
14....	200	22,000	87,500		3,225,000		120,000	
15....	300	22,000	0	0	0	0	0	0
16....	300	22,000	0		0		0	

A glance at the figures of Tables V, VI, and VII shows that the smallest concentration of nitrate used produced a much more marked relative increase in numbers with strain B than it did with strain A. On the other hand, the greater resistance of this strain to the higher nitrate concentrations is clearly evident. In the potassium- and calcium-nitrate cultures the organisms were present in an active state where the nitrate was added in amounts equivalent to 200 mgm. of nitrate in 100 gm. of soil. However, this same concentration of sodium nitrate prevented the development of the Azotobacter. The first five concentrations of all three nitrates caused a very large increase in the number of Azotobacter when compared with control cultures where no nitrate was added. In one instance an enormous increase was noted after three weeks' incubation in the presence of 50 mgm. of nitrate as sodium nitrate. This increase far excelled that noted with other concentrations of the same salt. The writer can offer no conjecture as to this occurrence.

Similar results were obtained by the writer in 1914 (*23*) with a strain of Azotobacter isolated from a silt loam soil at the Pennsylvania Experiment Station. It was found that soil and liquid cultures containing small amounts of potassium, sodium, and calcium nitrates caused an increase in the number of Azotobacter in pure culture compared with control cultures containing no nitrate. An increasing concentration of the nitrates continued favorable to the growth of the organism up to a certain limit, but higher concentrations retarded its growth. Finally a nitrate concentration was attained at which Azotobacter growth altogether ceased.

The results of the study of nitrates and their influence on Azotobacter in sterilized soil show very clearly that small amounts of nitrate cause a great increase in the number of Azotobacter cells. Higher concentrations are not so favorable to the growth of the organisms, and the highest concentrations studied prevented the development of the Azotobacter in sterilized soil.

From a study of the results of these experiments, it seems that the increase in number of Azotobacter in the presence of small amounts of nitrate is a direct result of nuclear stimulation. Later studies to be cited (*pp. 205–208*) show that nitrates exerted considerable influence on the internal structure of the Azotobacter cell. It appears reasonable to expect that the nitrate affected the nuclear structure in such a manner that an increase in cell multiplication resulted. It seems probable that the action of nitrate as a simple nutrient would be shown by a slower increase in cell multiplication.

INFLUENCE OF NITRATES ON THE FIXATION OF NITROGEN BY AZOTOBACTER

It has been shown in the preceding paragraphs that the presence of small quantities of nitrate in sterilized soil bring about a large increase in the number of Azotobacter. This increase was noted in the case of both strains of Azotobacter. It would be of interest to know whether the increase in bacterial numbers was accompanied by a corresponding increase in the amount of nitrogen assimilated.

The results secured by a few investigators indicate that in the presence of combined nitrogen as nitrates the nonsymbiotic nitrogen-fixing organisms will not fix atmospheric nitrogen. Stoklasa (*44, p. 492–503*) studied the influence of Azotobacter on sodium nitrate in aerobic and anaerobic liquid cultures. He found only a small gain in organic nitrogen and from these results he concluded that in the presence of nitrates Azotobacter could not assimilate atmospheric nitrogen. It has been shown by Hanzawa (*20*) that in a liquid culture containing 12 mgm. of nitrate (from potassium nitrate) in 100 c. c. of medium, a mixed culture of Azotobacter fixed 5.25 mgm. of nitrogen. Under the same conditions with 60 mgm. of nitrate present in 100 c. c. of medium he found but 5.35 mgm. of nitrogen fixed. He concluded that nitrates remained, as far as small quantities were concerned, almost without influence on the amount of atmospheric nitrogen fixed by Azotobacter.

Some studies have been carried on with respect to the influence of nitrates on the nonsymbiotic anaerobic nitrogen-assimilating organism, *Clostridium* spp. Bredemann (*9*) showed that ammonium nitrate in solution caused a decrease in the amount of nitrogen fixed by species of Clostridium. Pringsheim (*40*) grew cultures of *C. americanum* in solutions containing potassium nitrate. He found that in the presence of available energy the organism fixed some nitrogen when nitrate was

present but to a less extent than did control cultures containing no nitrate.

From these results it appears that nitrates do not stimulate the nitrogen-assimilation of the nonsymbiotic nitrogen-fixing bacteria.

Inasmuch as nitrates in small amounts caused such an increase in the number of Azotobacter in sterilized soil, it was thought advisable to determine just what influence these salts exert on nitrogen fixation by Azotobacter. Accordingly, experiments were carried out with Azotobacter on agar films, in soil cultures and in solution.

AGAR-FILM CULTURES.—In this work both strains of the Azotobacter were used. One hundred c. c. of mannit agar were placed in liter Erlenmeyer flasks and nitrates of potassium, sodium and calcium added in varying quantities. The flasks and contents were sterilized at 10 pounds' pressure for 25 minutes, cooled, and inoculated with 10 c. c. of a suspension of the organism in sterile distilled water. The flasks were incubated at 28° C. for three weeks. The weight of both inoculated and uninoculated flasks was maintained throughout the experiment by the addition of sterile distilled water. At the end of the incubation period total nitrogen analyses were made. Because of the high nitrate content dilute sulphuric-salycilic acid was added slowly and carefully to prevent loss of nitrogen by the evolution of gaseous oxids of nitrogen. The acid was allowed to react for a few days before continuing the total nitrogen determination. The results of the experiments are presented in Tables VIII and IX.

TABLE VIII.—*Influence of nitrates on the fixation of nitrogen by Azotobacter (strain A) on agar films*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Nitrogen contained in 100 c. c. of medium.				Nitrogen fixed.
		Inoculated.		Uninoculated.		
		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	0	13.00	12.80	4.1	4.05	8.75
2	0	12.70		4.0		
3	0	12.60		4.1		
4	50 mgm. of NO_3 potassium nitrate	18.50	18.45	7.00	7.10	11.35
5	.....do	18.40		7.20		
6	100 mgm. of NO_3 potassium nitrate	27.60	27.70	16.80	16.25	11.45
7	.....do	27.75		15.70		
8	50 mgm. of NO_3 sodium nitrate	18.65	18.50	7.50	7.4	11.10
9	.....do	18.30		7.30		
10	100 mgm. of NO_3 sodium nitrate	27.00	27.35	15.00	15.1	12.25
11	.....do	27.65		15.20		
12	50 mgm. of NO_3 calcium nitrate	13.75	13.75	8.00	8.25	5.50
13	.....do	13.70		8.50		
14	100 mgm. of NO_3 calcium nitrate	18.80	18.95	14.50	14.40	4.55
15	.....do	19.15		14.30		

TABLE IX.—*Influence of nitrates on the fixation of nitrogen by Azotobacter (strain B) on agar films*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Nitrogen contained in 100 c. c. of medium.				Nitrogen fixed.
		Inoculated.		Uninoculated.		
		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	0	15. 50	15. 60	6. 50	6. 40	9. 20
2	0	15. 70		6. 30		
3	0	15. 60		6. 40		
4	75 mgm. of NO_3 as potassium nitrate	25. 20	25. 30	13. 00	13. 85	11. 45
5	do	25. 40		14. 70		
6	150 mgm. of NO_3 as potassium nitrate	36. 40	36. 65	24. 00	23. 60	13. 05
7	do	36. 90		23. 20		
8	75 mgm. of NO_3 as sodium nitrate	25. 60	25. 65	12. 80	13. 00	12. 65
9	do	25. 70		13. 20		
10	150 mgm. of NO_3 as sodium nitrate	37. 60	37. 40	26. 20	25. 80	11. 60
11	do	37. 20		25. 40		
12	75 mgm. of NO_3 as calcium nitrate	20. 10	19. 85	12. 00	12. 35	7. 50
13	do	19. 60		12. 70		
14	150 mgm. of NO_3 as calcium nitrate	32. 80	33. 05	24. 50	24. 85	8. 20
15	do	33. 30		25. 20		

A glance at the results (Tables VIII and IX) shows that an increase in nitrogen fixation occurred where potassium and sodium nitrates were present, whereas a marked decrease in the total nitrogen content was observed where calcium nitrate was used. Whether the calcium itself is detrimental to an increase in organic nitrogen or whether it is the combination of calcium with nitrate can not be stated. It is significant, however, that this decrease in fixation of nitrogen was noted throughout all the experiments where calcium nitrate was employed. It is very evident that calcium nitrate exerts some detrimental effect on the nitrogen assimilating properties of the organism.

There seems to be but a slight difference in the nitrogen-fixing ability of the two strains studied. In the absence of nitrates the amount fixed varies but little. Also in the presence of potassium and sodium nitrates the relative increase in amount of nitrogen fixed remains about the same. Calcium nitrate offers an exception where it is employed. The detrimental effect seems to be more marked in the case of strain A than with strain B. Strain A under normal conditions fixed slightly less nitrogen than strain B, so it may be possible that this strain is weaker.

The formation of pigment by the Azotobacter in the presence of the nitrates is of interest. Strain A normally produced no pigment by the end of three weeks' incubation. But when grown on the agar films in the presence of nitrate a most marked pigment production appeared. This pigment was especially noticeable in the presence of the calcium

salt. Since strain B normally produces a good pigment, the influence of nitrate on this strain was not very marked. The relation of nitrates to pigment formation will be taken up later (pp. 203–205).

From the results of the experiments with agar films containing various amounts of nitrate, it seems apparent that potassium and sodium nitrates in amounts of 50 and 100 mgm. of nitrate in 100 c. c. of medium cause a small increase in the amount of nitrogen fixed. However, this increase in fixation is not at all parallel with the increase in number of Azotobacter caused by nitrates in sterilized soil.

It may be concluded that an increase in the number of Azotobacter in sterilized soil as a result of nitrate stimulation does not mean a corresponding increase in nitrogen fixation on agar films.

SOIL CULTURES.—The conditions obtaining in these experiments were strictly comparable with those heretofore cited dealing with the influence of nitrates on Azotobacter in sterilized soil (pp. 187–193).

The fixation of nitrogen was studied in pure culture in sterilized soil and in unsterilized soil. One hundred and fifty gm. of soil (dry weight) were weighed into 1-liter Erlenmeyer flasks, nitrates were added in varying amounts from 10 to 200 mgm., and 1 per cent of mannit was also added. Triplicate flasks were prepared for each amount of nitrate studied. The moisture content was raised to approximately 18 per cent and the flasks allowed to remain at room temperature for one day. The contents were then thoroughly mixed and a fine crumb structure produced. The flasks for the experiments with pure cultures in sterilized soil were immediately sterilized at 15 pounds' pressure for three hours. After cooling, two of each set were inoculated with 5 c. c. of a suspension of Azotobacter (strain A) in sterile distilled water. The remaining flask of each set was not inoculated, but was incubated at 28° C. with the inoculated flasks. The moisture lost by evaporation was replaced from time to time by the addition of sterile distilled water. At the end of the incubation period the soil was removed and spread out in thin layers and allowed to dry. It was then thoroughly ground in a porcelain-ball mill for one hour. At the end of this time all of the soil passed through a 100-mesh sieve.

Soil cultures used in the study of the effect of nitrates on nitrogen fixation in unsterilized soil were prepared in a similar manner, except that the flasks were not sterilized. Previous to incubation a small inoculum of Azotobacter (strain A) was added to insure the presence of the nitrogen-fixing organism in the soil cultures. The proper moisture content was maintained in the same manner as in the case of the pure cultures in sterilized soil and the incubation period was the same for both. The results are given in Tables X, XI, XII, and XIII.

TABLE X.—*Influence of sodium nitrate on the fixation of nitrogen by Azotobacter in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Total nitrogen in 100 gm. of dry soil.				Nitrogen fixed in 100 gm. of dry soil.
		Inoculated.		Uninoculated.		
		Found.	Average.	Found.	Average.	
	Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	0	135.0	133.7	132.0	131.5	2.7
1	0	134.0		131.5		
1	0	132.0		131.0		
2	0	133.0	135.0	.....	.....	3.5
2	0	137.0		.....	.....	
2	0	.....		.....	.....	
3	10	137.0	136.6	134.0	133.7	2.9
3	10	136.0		133.5		
3	10	137.0		134.0		
4	10	136.5	137.0	.....	.....	3.3
4	10	137.5		.....	.....	
4	10	137.0		.....	.....	
5	50	149.0	149.0	140.0	138.5	10.5
5	50	149.0		137.0		
5	50	149.0		138.5		
6	50	148.5	149.2	.....	.....	10.7
6	50	149.5		.....	.....	
6	50	149.5		.....	.....	
7	150	163.0	162.3	152.0	151.5	10.8
7	150	162.0		150.0		
7	150	162.0		152.5		
8	150	162.5	162.5	.....	.....	11.0
8	150	163.0		.....	.....	
8	150	162.0		.....	.....	

TABLE XI.—*Influence of sodium nitrate on the fixation of nitrogen by Azotobacter in unsterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Total nitrogen in 100 gm. of dry soil.				Nitrogen fixed in 100 gm. of dry soil.
		Inoculated.		Uninoculated.		
		Found.	Average.	Found.	Average.	
	Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	0	132.0	134.0	130.0	131.8	2.2
1	0	135.0		133.5		
1	0	135.0		132.0		
2	0	132.0	133.3	.....	.....	1.5
2	0	134.0		.....	.....	
2	0	134.0		.....	.....	
3	10	137.5	137.8	134.0	133.3	4.5
3	10	138.8		133.0		
3	10	138.8		133.0		
4	10	137.5	137.7	.....	.....	4.4
4	10	137.5		.....	.....	
4	10	138.0		.....	.....	
5	50	150.0	150.3	140.0	140.8	9.5
5	50	151.0		140.5		
5	50	150.0		142.0		
6	50	149.0	149.7	.....	.....	8.9
6	50	149.5		.....	.....	
6	50	150.5		.....	.....	
7	150	169.0	168.0	148.0	151.8	16.2
7	150	167.0		154.0		
7	150	168.0		153.5		
8	150	167.5	168.0	.....	.....	16.2
8	150	168.0		.....	.....	
8	150	168.5		.....	.....	

TABLE XII.—*Influence of calcium nitrate on the fixation of nitrogen by Azotobacter in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Total nitrogen in 100 gm. of dry soil.				Nitrogen fixed in 100 gm. of dry soil.
		Inoculated.		Uninoculated.		
		Found.	Average.	Found.	Average.	
	Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	0	133.0	133.3	131.0	131.3	2.0
1	0	133.6		131.0		
1	0	133.3		132.0		
2	0	133.0	133.5	.....	.....	2.2
2	0	134.2		.....	.....	
2	0	133.4		.....	.....	
3	10	137.0	136.8	135.0	134.7	2.1
3	10	137.0		134.0		
3	10	136.5		135.0		
4	10	136.5	137.0	.....	.....	2.3
4	10	137.0		.....	.....	
4	10	137.5		.....	.....	
5	50	148.0	148.5	140.5	140.7	7.8
5	50	148.5		141.0		
5	50	149.0		140.5		
6	50	148.5	148.5	.....	.....	7.8
6	50	149.0		.....	.....	
6	50	148.0		.....	.....	
7	200	173.0	173.7	163.0	163.8	9.9
7	200	173.0		164.0		
7	200	174.0		164.5		
8	200	173.5	173.5	.....	.....	9.7
8	200	173.0		.....	.....	
8	200	174.0		.....	.....	

TABLE XIII.—*Influence of calcium nitrate on the fixation of nitrogen by Azotobacter in unsterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Total nitrogen in 100 gm. of dry soil.				Nitrogen fixed in 100 gm. of dry soil.
		Inoculated.		Uninoculated.		
		Found.	Average.	Found.	Average.	
	Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	0	134.5	135.7	134.0	133.2	2.5
1	0	136.0		133.5		
1	0	136.5		132.0		
2	0	135.0	135.3	.....	.....	2.1
2	0	135.5		.....	.....	
2	0	135.5		.....	.....	
3	10	138.5	138.5	133.5	133.2	5.3
3	10	138.0		133.0		
3	10	139.0		133.0		
4	10	138.0	138.0	.....	.....	4.8
4	10	137.5		.....	.....	
4	10	138.5		.....	.....	
5	50	151.5	151.5	140.5	141.0	10.5
5	50	152.0		141.0		
5	50	151.0		141.5		
6	50	150.0	150.8	.....	.....	9.8
6	50	151.5		.....	.....	
6	50	151.0		.....	.....	
7	200	177.0	177.0	164.0	164.3	12.7
7	200	178.0		165.0		
7	200	176.0		164.0		
8	200	176.5	177.2	.....	.....	12.9
8	200	177.0		.....	.....	
8	200	178.0		.....	.....	

It will be seen at a glance that a greater relative increase in nitrogen fixation in the presence of nitrates occurred in the soil cultures than on the agar films. But in the latter instance the amount of nitrogen assimilated in the absence of mistakes is far in excess of that assimilated in the soil cultures under similar conditions. The amount of nitrogen fixed in the soil cultures is surprisingly low, but as relative increases or decreases are desired this does not materially influence the results.

The influence of sodium nitrate on the fixation of nitrogen by pure cultures of Azotobacter in sterilized and unsterilized soil is brought out very clearly in the figures of Tables X and XI. In both cases, where no nitrate was added, an equal fixation of nitrogen occurred. Where 10 mgm. of nitrate were added to 100 gm. of soil, slightly more nitrogen was assimilated in the unsterilized soil than in sterilized. The reverse seemed to be true when 50 mgm. of nitrate were added. But in the presence of 150 mgm. of nitrate, the fixation by the pure culture in sterilized soil did not increase materially in comparison with that which occurred in the 50 mgm. of nitrate concentration. Evidently the maximum fixation under these conditions had been reached. The gain in the unsterilized soil at the highest concentration of nitrate studied almost doubled the amount fixed in the pure culture. It appears evident that the presence of sodium nitrate causes a greater fixation of nitrogen in unsterilized soil than it does under similar conditions in sterilized soil inoculated with Azotobacter.

In the case of calcium nitrate, somewhat comparable results were obtained. The fixation where no nitrate was added was equivalent to that obtained in the controls for the sodium nitrate. Where nitrate was added in amounts equal to 10 mgm. of nitrate in 100 gm. of soil, an increased fixation was obtained in the unsterilized soil, but practically no increase occured in the pure culture in sterilized soil. Fifty mgm. of nitrate in 100 gm. of soil produced an increase in fixation. In the highest concentration of calcium nitrate the difference in nitrogen fixed between the pure culture in sterilized soil and unsterilized soil was not so great as in the case where sodium nitrate was used.

In the sterilized soil where the two nitrates were present in equal amounts it can be seen that more fixation took place in the presence of sodium nitrate. The difference is not marked, but it exists nevertheless. It will be remembered that calcium nitrate had a detrimental effect on nitrogen fixation by Azotobacter on agar films. However, in soil cultures this same nitrate stimulated Azotobacter to an increased assimilation of nitrogen. This difference is not suprising as it has been shown repeatedly that bacterial activities in soil and in artificial cultures are not always comparable.

From the results of the experiments performed with reference to the influence of nitrates in soil on the fixation of nitrogen therein, it appears

evident that in pure cultures both sodium and calcium nitrates in the amounts studied produced an increase in the amount of nitrogen fixed. The sodium salt stimulated this process to a slightly greater extent than did the calcium salt. In unsterilized soil nitrates exerted the same action but to a more marked extent. The amount of nitrogen fixed under these conditions was generally in excess of that fixed under similar conditions in sterilized soil inoculated with a pure culture of Azotobacter.

Such large relative increases in total nitrogen in the soil in the presence of nitrates would not normally take place under field conditions for here no accumulations of nitrate occur in quantities sufficiently large enough to influence this process.

Summing up all the experiments performed in relation to the influence of nitrates on the fixation of atmospheric nitrogen by Azotobacter, it appears that the increase in total nitrogen in the presence of these salts is by no means comparable to the increase in the number of organisms in sterilized soil under the same conditions. An increase in the number of Azotobacter does not mean a parallel increase in the amount of nitrogen fixed.

INFLUENCE OF AZOTOBACTER ON NITRATES IN SOLUTION

Attention has been thus far directed toward the influence exerted by nitrates on the growth and nitrogen-assimilating power of Azotobacter. The following points are now to be considered: Do the nitrogen-fixing bacteria reduce nitrates to nitrites and ammonia? Is there an increase or decrease in the amount of organic nitrogen as a result of the presence of nitrate in the medium?

Beijerinck and Van Delden (*5*) found that *Azotobacter chroococcum* reduced nitrate directly to ammonia. Stoklasa (*44, p. 492-503*) studied the changes in a nutrient solution containing 0.2 per cent of sodium nitrate inoculated with Azotobacter. He found under anærobic conditions that the nitrate was largely reduced to nitrite and ammonia and that a very small amount of organic nitrogen was formed. Under ærobic conditions there was more nitrite formed than under anærobic conditions and very little ammonia or oganic nitrogen. He concluded, therefore, that Azotobacter did not fix atmospheric nitrogen in the presence of nitrates.

The following experiments were performed in an endeavor to answer the questions raised in the initial paragraph of this section. To Erlenmeyer flasks of 500-c. c. capacity, containing 100-c. c. portions of mannit solution, sodium and ammonium nitrates were added in amounts equivalent to 150 mgm. of nitrate in 100 c. c. of the solution. Nine flasks were prepared for each nitrate and the same number for the controls containing no nitrate. The flasks and contents were sterilized at 10 pounds pressure for 30 minutes. After cooling, six of each set were inoculated, three

with strain A and three with strain B, and the remaining three were left uninoculated to serve as controls. The flasks were incubated at 28° C. for 21 days. The total weight was maintained throughout the experiment by the addition of sterile distilled water from time to time. At the end of three weeks the contents of each set of triplicate flasks were poured together and 50-c. c. samples drawn for analysis. Nitrate ammonia and total nitrogen were determined as given under "Methods." The results are shown in Tables XIV, XV, and XVI.

TABLE XIV.—*Influence of Azotobacter on nitrates in solution, giving the quantity of nitrate lost*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Nitrate in 100 c. c. of medium.									
		Strain A.					Strain B.				
		Inoculated.		Uninoculated.		Nitrate lost.	Inoculated.		Uninoculated.		Nitrate lost.
		Found.	Average.	Found.	Average.		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1–9	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1–9	0	.00		.00			.00		.00		
10–18	150 gm. of NO_3 as sodium nitrate	80.9	80.75	150.4	150.8	[a]—70.05	105.6	105.4	150.4	150.8	[b]—45.40
10–18	do	80.6		151.3			105.2		151.3		
19–27	150 mgm. of NO_3 as ammonium nitrate	100.3	101.2	149.6	149.8	[a]—48.60	131.1	130.9	149.6	149.8	[c]—18.90
19–27	do	102.1		150.0			130.7		150.0		

[a] Strong NO_2 reaction. [b] Medium NO_2 reaction. [c] Slight NO_2 reaction.

TABLE XV.—*Influence of Azotobacter on nitrates in solution, giving the quantity of ammonia produced*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Nitrogen as ammonia in 100 c. c. of medium.									
		Strain A.					Strain B.				
		Inoculated.		Uninoculated.		Ammonia produced.	Inoculated.		Uninoculated.		Ammonia produced.
		Found.	Average.	Found.	Average.		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1–9	0	0.20	0.15	0.00	0.00	0.15	0.00	0.10	0.00	0.00	0.10
1–9	0	.10		.00			.20		.00		
10–18	150 mgm. of NO_3 as sodium nitrate	2.00	1.90	—.10	.05	1.85	2.20	2.30	—.10	.05	2.25
10–18	do	1.80		.20			2.40		.20		
19–27	150 mgm. of NO_3 as ammonium nitrate	13.90	13.97	13.90	13.90	.07	13.80	13.80	13.90	13.90	.10
19–27	do	13.95		13.90			13.80		13.90		

TABLE XVI.—*Influence of Azotobacter on nitrates in solution, giving the quantity of nitrogen fixed*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Total nitrogen in 100 c. c. of medium.									
		Strain A.					Strain B.				
		Inoculated.		Uninoculated.		Nitrogen fixed.	Inoculated.		Uninoculated.		Nitrogen fixed.
		Found.	Average.	Found.	Average.		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1-9	0	5.00	5.00	2.60	2.65	2.35	5.00	5.05	2.60	2.65	2.40
1-9	0	5.00		2.70			5.10		2.70		
10-18	150 mgm. of NO_3 as sodium nitrate	22.50	22.55	14.00	14.10	8.45	28.00	27.90	14.00	14.10	13.80
10-18	do	22.60		14.20			27.80		14.20		
19-27	150 mgm. of NO_3 as ammonium nitrate	47.00	46.95	43.20	43.05	3.90	48.10	48.15	43.20	43.05	5.10
19-27	do	46.90		42.90			48.20		42.90		

Table XIV showing the effect on the total nitrate content will be discussed first. Strain A differed widely from strain B in its ability to reduce nitrates. It will be noted that strain A reduces nitrate more readily than strain B in the presence of both sodium and ammonium nitrate. In order to determine the nature of the reduction of the nitrates, qualitative and quantitative tests were made. The reduction of nitrates by Azotobacter takes place with the formation of nitrites as shown in Table XIV. Strain A effected a strong reduction of nitrate to nitrite with both sodium and ammonium nitrate. Strain B also reduced nitrate to nitrite, but to a lesser degree than did strain A.

An inspection of the data in Table XV indicates that the reduction of nitrates ceased with the formation of nitrite, since no appreciable amounts of ammonia were produced by either strain of Azotobacter.

In regard to the fixation of atmospheric nitrogen by these strains of Azotobacter, it was found that nitrogen was assimilated both in the presence and absence of nitrate. In the presence of nitrate there was a large increase in the total organic nitrogen. Sodium nitrate stimulated both strains, although strain B fixed the larger amount. Similar results were obtained when the fixation of nitrogen on agar films was studied. In the presence of ammonium nitrate the amount of nitrogen fixed was considerably decreased, but the amount fixed was in excess of the control cultures containing no nitrate. It seems evident that sodium and ammonium nitrate in the amounts studied did not prevent the fixation of atmospheric nitrogen. In fact, the presence of these salts seemed to stimulate the process.

Under aerobic conditions both strains of Azotobacter studied caused a reduction in the total amount of nitrate present in the solution. This reduction may be accounted for in two ways: (1) The reduction of nitrate to nitrite and (2) the assimilation of nitrate by the organisms. Practically no ammonia was formed under the conditions of these experiments. These results agree with those of Stoklasa. However, in con-

trast to the work of Stoklasa, both strains of Azotobacter assimilated more atmospheric nitrogen in the presence of nitrates in solution than in the absence of these salts.

INFLUENCE OF NITRATES ON THE PRODUCTION OF PIGMENT BY AZOTOBACTER

It has already been noted in the experiments dealing with the effect of nitrates on the fixation of atmospheric nitrogen on agar films that nitrates favor pigment production. This was true in the case of both strains of the Azotobacter.

Moreover, it has been observed by other investigators that Azotobacter when grown in the presence of nitrate will produce a darker pigment than when grown in its absence. Beijerinck (*4, p. 575*) states that Azotobacter in pure culture will form a dark-brown pigment in the presence of glucose and a small amount of nitrate. Sackett (*43*) found that nitrate caused an increase in pigment production by Azotobacter. In media without the nitrate the pigment formation was materially decreased and in some cases practically eliminated. He also noted that the amount of nitrate present has a direct influence on the intensity of the pigment formation. He found that when sodium nitrate was added to a suitable medium to give a content of 0.0, 0.01, 0.03, 0.05, 0.08, 0.1, 0.3, and 0.5 per cent, with glucose used as the source of energy, the organisms produced pigment. Streak inoculations were made, and after 14 days' incubation he found that the maximum of color was obtained at 0.05 to 0.08 per cent and that greater concentrations did not increase the intensity of the brown-black pigment. From his results it is evident that sodium nitrate caused an increase in pigment formation by azotobacter.

In order to determine the possible effect of potassium, sodium, and calcium nitrate on pigment formation with strains A and B, the following experiment was performed.

Under normal conditions on mannit agar free from nitrate strain A produced little or no pigment even after three weeks' growth. At the end of this time dirty-yellow streaks occurred throughout the growth, but no brown pigment was produced. However, with strain B at the end of two or three weeks a decided brown to brown-black pigment was produced in the absence of nitrate.

Agar slope cultures containing increasing amounts of potassium, sodium, and calcium nitrate, as indicated in Table XVII, were prepared. These were inoculated with both strains of Azotobacter and incubated at 28° C. for 10 days. Daily observations were made for first evidences of pigment formation. In some of the cultures of strain A growing on media containing calcium nitrate this pigmentation was observed as early as 48 hours subsequent to inoculation. The following day pigmentation developed in strain B. The cultures on the potassium and sodium-nitrate media began to show evidence of pigmentation in four to six days. The final results, obtained after 10 days' incubation, are found in Table XVII.

TABLE XVII.—*Influence of nitrates on the formation of pigment in Azotobacter*

Culture No.	Treatment (Nitrate in 100 c. c. of medium).	Relative increase in pigment formation.					
		Strain A.			Strain B.		
		Potassium nitrate.	Sodium nitrate.	Calcium nitrate.	Potassium nitrate.	Sodium nitrate.	Calcium nitrate.
	Mgm.						
1	0	None	None	None	Medium brown	Medium brown	Medium brown.
2	10	Very light brown	Very light brown	Very light brown	do	do	Dark brown.
3	25	Light brown	do	Light brown	do	do	Medium brown.
4	50	do	Light brown	Medium brown	do	do	Do.
5	100	Medium brown	do	do	do	do	Do.
6	150	do	Medium brown	Dark brown	do	do	Dark brown.
7	200	do	do	do	do	Dark brown	Brown-black.
8	300	do	do	Brown-black	Dark brown	do	Do.

A general idea may be gained from Table XVII concerning the relative increase in pigment formation in the presence of the nitrates. A study of the table gives a fair idea of the relative differences in pigment production.

Very interesting results were obtained with strain A. It will be seen from Table XVII that no pigment was produced in the control culture after 10 days, while in the presence of nitrates pigmentation was noted. The intensity of the pigment varied with the increase of nitrate up to 150 mgm. Beyond 150 mgm. there was no increase. Potassium and sodium nitrate did not exert such a decided influence on pigment production as calcium nitrate. The latter salt produced an intense dark-brown to brownish-black pigment.

In the case of strain B the influence of nitrate was not so pronounced since this strain normally produced considerable pigment in the absence of nitrates. Potassium and sodium nitrate caused a slight increase in pigment formation. Here, again, the calcium salt brought about most pronounced increase. However, the relative increase in pigment formation in strain B was not so pronounced as in strain A.

Where the nitrate was present, a much more spreading growth was obtained. A heavy bacterial growth accumulated at the base of the slope except in the two cultures in which the highest concentrations were used. In the latter instances the accumulation was less than those in cultures growing on media containing no nitrate. Although the original inoculation could not be made absolutely uniform, so far as number of organisms was concerned; nevertheless it was evident that on those slopes containing 10, 25, 50, and 100 mgm. of nitrate in 100 c. c. of the medium a much more abundant growth was obtained than on those slopes free from nitrate. Here, again, it is seen, in a rough, comparative way, that the smaller amounts of nitrates caused an increase in the number of Azotobacter.

The results of this work on pigment production are quite in accord with those of Sackett. Potassium, sodium, and especially calcium, nitrates in varying amounts increase pigment formation by Azotobacter with an increase in nitrate concentration. This effect is especially marked in strain A, which under normal conditions does not produce any pigment.

INFLUENCE OF NITRATES ON THE FORMATION OF VOLUTIN BODIES IN AZOTOBACTER

The presence of volutin bodies, or metachromatic granules, in Azotobacter has been shown by Bonazzi (7). These substances, according to Meyer (*34, p. 238*), are reserve food materials other than fat droplets, glycogen, and similar substances reacting with iodin stain which occur in the cytoplasm of the cells of various bacteria. With Millon's reagent they give no reaction. He believes that these bodies are composed of nucleic-acid compounds, but are not nuclear proteids.

In connection with the foregoing investigations concerning the influence of nitrates on pigment formation by Azotobacter, it was thought that some results of cytological interest might be obtained in regard to the effect of varying amounts of nitrates on the volutin bodies.

Slope cultures of mannit agar were prepared containing the different nitrates as indicated in Table XVIII. These slopes were inoculated with both strains of Azotobacter and incubated at 28° C. for 10 days. At the end of this time each culture was stained and examined microscopically. The following method was used for demonstrating the presence of the volutin bodies. The organisms to be examined were air dried on a glass slide and then fixed in the flame of a Bunsen burner. The preparation was then flooded with a 1 to 10 aqueous solution of methylene blue (Merck's) prepared by adding 10 c. c. of a saturated aqueous solution of methylene blue to 90 c. c. of distilled water. The stain was washed off after five minutes with a 1 per cent solution of sulphuric acid and immediately rinsed in distilled water. The preparation was dried and examined with the oil-immersion objective. The volutin bodies appeared within the cytoplasm as very dark blue dots, the outline of the cell wall was a lighter blue, while the cell net work was stained a very light blue.

Guignard's stain [1] was also used to demonstrate the presence of the volutin bodies. Fresh smears on a glass slide were fixed over 10 per cent osmic acid for three minutes. The preparation was then air-dried and fixed to the slide by rapidly passing the latter a few times through a Bunsen burner. The preparation was covered with the stain which was allowed to react for five minutes. The stain was then washed off with distilled water, dried, and examined with the oil-immersion objective. The outline of the cell as well as the net work within-was stained light purple. The granules within the cytoplasm were a reddish purple. The results are given in Table XVIII.

TABLE XVIII.—*Influence of nitrates on the formation of volutin bodies in Azotobacter in 10 days*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Strain A.			Strain B.		
		Potassium nitrate.	Sodium nitrate.	Calcium nitrate.	Potassium nitrate.	Sodium nitrate.	Calcium nitrate.
	Mgm.						
1	0	Present.[a]	Doubtful.	Doubtful.	Present[a]	Doubtful.	Present.[a]
2	10	do.[a]	Present[a]	Present[a]	do.[a]	Present[a]	Do.[a]
3	25	do.[a]	do.[a]	do.[a]	do.[a]	do.[b]	Do.[a]
4	50	do.[a]	do.[b]	Doubtful.	do.[a]	do.[a]	Do.[a]
5	100	do.[a]	do.[b]	Present[a]	do.[b]	do.[b]	Do.[a]
6	150	do.[a]	do.[b]	do.[a]	do.[b]	do.[b]	Do.[b]
7	200	do.[b]	do.[b]	do.[b]	do.[b]	do.[b]	Do.[a]
8	300	do.[b]	do.[b]	do.[a]	do.[b]	do.[b]	Do.[b]

[a] Representing an approximate average of two volutin bodies per cell.
[b] Representing an approximate average of four volutin bodies per cell.
[1] Guignard'sstain. Fifty c. c. of 2 per cent fuchsin in 1 per cent acetic acid; 40 c. c. of 0.2 per cent methyl green in 1 per cent acetic acid; 1 c. c. of glacial-acetic acid. Distilled water was used in making the 1 per cent acetic-acid solution.

It will be seen that all three nitrates exerted considerable influence on the formation of volutin bodies. Not only was the number of bodies increased, but also the size. The relative increase in size of the granules was much more marked than was the numerical increase. In Azotobacter cells grown on mannit agar containing no nitrate the number of volutin bodies in each cell averaged about two; in the presence of nitrate four to five volutin granules were found. The greatest increase in number, as well as size, occurred where the nitrate concentration was highest. With both strains sodium nitrate apparently caused the greatest increase. This was true in the lower as well as in the higher concentrations. The volutin bodies in strain B seemed to respond to the presence of nitrates more noticeably than did those of strain A, especially in the presence of potassium nitrate. It is evident that nitrates of potassium, sodium, and calcium cause an increase in the number and size of volutin bodies in Azotobacter cells.

Do these salts tend to hasten the appearance of these bodies, or do they at first retard their development? The following experiment was carried out in an endeavor to determine this point. Only sodium nitrate was used, since this particular salt proved most beneficial to the formation of volutin bodies. Agar slopes were prepared containing the different amounts of nitrate as indicated in Table XIX. The cultures were incubated at 28° C. and examined daily for the presence of volutin bodies. The methylene blue—1 per cent sulphuric acid—method of staining was employed. The results of the experiment are given in Table XIX.

TABLE XIX.—*Influence of sodium nitrate on the rate of formation of volutin bodies in Azotobacter*

Time.	Nitrate in 100 c. c. of medium.							
	Strain A.				Strain B.			
	0 Mgm.	25 Mgm.	100 Mgm.	300 Mgm.	0 Mgm.	25 Mgm.	100 Mgm.	300 Mgm.
Day.								
1	Absent...	Absent...	Doubtful.	Doubtful.	Absent...	Absent...	Doubtful.	Dbtful.
2	Present[a].	Present[a].	Present[a].	...do.[b] ...	...do.....	Present[a].	...do.[b] ...	Do.[b].
3	...do.[a]....	...do.[a]....	...do.[a]....	...do.[b] ...	Present[a].	...do.[b] ...	...do.[b] ...	Do.[b].
4	...do.[a]....	...do.[a]....	...do.[b] ...	...do.[b] ...	...do.[a]....	...do.[b] ...	...do.[b] ...	Do.[b].

[a] Representing an approximate average of two volutin bodies per cell.
[b] Representing an approximate average of four volutin bodies per cell.

A study of Table XIX shows that it is rather doubtful whether the nitrate present tended to hasten the appearance of the volutin bodies. No convincing evidence has been presented for or against this statement. No granules were seen in the first day's growth of strain A, although the next day they were present in all four cultures. In strain B more convincing proof is furnished that the sodium nitrate hastened the appearance of these reserve food substances. The volutin bodies were not present in the control and lowest nitrate concentration cultures the first day, but they were very noticeable in the culture containing the highest concentration of nitrate and doubtful in the remaining one. On the second day volutin bodies were present in all cultures grown on

nitrate media, while the control culture was still free from them. The third day showed the presence of volutin bodies in all four cultures. Strain B offers the better proof that sodium nitrate tends to hasten the appearance of volutin bodies in the cells of Azotobacter. Further experiments were not made in an endeavor to determine what influence nitrates might have on the cytology of the Azotobacter cell. The brief studies reported here were made in connection with the pigment formation experiments, but do not bear any particular relation to them. The increase in number and size of volutin bodies may bear some relation to the increased amount of nitrogen fixed or assimilated by Azotobacter in the presence of nitrates.

INFLUENCE OF NITRATES ON BACILLUS RADICICOLA

INFLUENCE OF NITRATES ON THE GROWTH AND REPRODUCTION OF BACILLUS RADICICOLA IN STERILIZED SOIL

One hundred and fifty gm. (dry weight) of the soil were weighed into 500-c. c. Erlenmeyer flasks and the nitrates added as indicated in Tables XX–XXII. Duplicate cultures for each amount of nitrate were prepared. One per cent of mannit (in 5 c. c. of distilled water) was also added. The flasks were kept at room temperature for one day and the contents then thoroughly mixed. The flasks were sterilized at 15 pounds' pressure for three hours. Upon cooling they were inoculated with 5 c. c. of a suspension of *Bacillus radicicola* in sterile distilled water. The number of bacteria in the inoculum was determined. The moisture content was then approximately 18 to 20 per cent. The flasks were incubated at 28° to 30° C. and mannit-agar plates poured at the end of one and two weeks. The results of these experiments are given in Tables XX, XXI, and XXII, in which each figure represents an average of duplicate plates.

TABLE XX.—*Influence of potassium nitrate on Bacillus radicicola in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.				
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*
1	0	10,670	680,000	100	8,015,000	100
2	0	10,670	825,000		7,000,000	
3	10	10,670	2,195,000	372	14,600,000	171
4	10	10,670	3,410,000		11,050,000	
5	25	10,670	3,900,000	517	15,400,000	201
6	25	10,670	3,885,000		14,800,000	
7	50	10,670	1,555,000	208	11,500,000	173
8	50	10,670	1,585,000		14,400,000	
9	100	10,670	445,000	51	2,680,000	40
10	100	10,670	320,000		3,290,000	
11	150	10,670	375,000	46	560,000	8.9
12	150	10,670	330,000		790,000	
13	200	10,670	135,000	20	90,000	1.2
14	200	10,670	170,000		(a)	
15	300	10,670	45,000	6.3	25,000	.36
16	300	10,670	50,000		30,000	

a Contamination.

TABLE XXI.—*Influence of sodium nitrate on Bacillus radicicola in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.				
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*
1	0	15, 500	1, 500, 000	100	6, 750, 000	100
2	0	15, 500	1, 250, 000		5, 950, 000	
3	10	15, 500	2, 560, 000	201	10, 000, 000	177
4	10	15, 500	3, 000, 000		12, 500, 000	
5	25	15, 500	6, 150, 000	418	14, 650, 000	240
6	25	15, 500	5, 375, 000		15, 700, 000	
7	50	15, 500	4, 850, 000	378	(a)	134
8	50	15, 500	5, 570, 000		8, 500, 000	
9	100	15, 500	2, 000, 000	140	1, 520, 000	25
10	100	15, 500	1, 850, 000		1, 650, 000	
11	150	15, 500	1, 060, 000	69	850, 000	14
12	150	15, 500	835, 000		940, 000	
13	200	15, 500	760, 000	54	500, 000	8. 8
14	200	15, 500	725, 000		620, 000	
15	300	15, 500	250, 000	22	150, 000	2. 8
16	300	15, 500	365, 000		210, 000	

a Contamination.

TABLE XXII.—*Influence of calcium nitrate on Bacillus radicicola in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.				
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*
1	0	10, 000	960, 000	100	4, 675, 000	100
2	0	10, 000	850, 000		4, 590, 000	
3	10	10, 000	3, 650, 000	419	6, 000, 000	124
4	10	10, 000	3, 940, 000		5, 450, 000	
5	25	10, 000	5, 500, 000	674	10, 650, 000	274
6	25	10, 000	6, 700, 000		14, 700, 000	
7	50	10, 000	4, 000, 000	414	9, 350, 000	195
8	50	10, 000	3, 500, 000		8, 670, 000	
9	100	10, 000	1, 200, 000	180	1, 500, 000	35
10	100	10, 000	2, 050, 000		1, 750, 000	
11	150	10, 000	865, 000	106	765, 000	17
12	150	10, 000	1, 050, 000		800, 000	
13	200	10, 000	375, 000	35	350, 000	7. 0
14	200	10, 000	260, 000		300, 000	
15	300	10, 000	35, 000	4. 5	25, 000	. 70
16	300	10, 000	47, 000		40, 000	

An inspection of all three tables reveals two marked differences from the results obtained in similar work with Azotobacter. First, it will be noted that nitrates do not appear to exert such a marked stimulating effect with *B. radicicola* as with Azotobacter. The numerical increase due to the presence of the nitrate is clearly shown in the percentage columns. Second, it will be noted that *B. radicicola* does not seem to be so sensitive to higher concentrations of nitrates as does Azotobacter. In all instances at concentrations equivalent to 300 mgm. of nitrate in

100 gm. of soil the legume organisms were still alive, although present in numbers far below those of the control cultures. In all Azotobacter cultures no organisms survived this concentration.

No one nitrate produced an excessive stimulation in comparison with the others. The calcium salt present as 150 mgm. of nitrate in 100 gm. of soil at the end of the first week gave the greatest stimulation for concentrations of that amount. However, at the end of the second week this concentration had caused a marked decrease in the number of organisms. In the case of all three nitrates the concentration representing 25 mgm. of nitrate in 100 gm. of soil produced the greatest stimulation. This resulting stimulation also held true throughout the second week. The decrease in number below those of the control cultures, due to increasing concentrations of nitrate, began first in the presence of potassium nitrate at 100 mgm. of nitrate per 100 gm. of soil, then with sodium nitrate at 150 mgm., and lastly with calcium nitrate at 200 mgm. But the number of organisms present in the soil cultures containing sodium nitrate in amounts equivalent to 100 mgm. and calcium nitrate at 100 mgm. at the end of the second week was below those of the control cultures.

It therefore appears from these results that small amounts of potassium, sodium, and calcium nitrate stimulate the reproductive activity of *B. radicicola*. Concentrations of nitrates greater than those amounts which produced maximum stimulation cause a decrease in the number of organisms. The highest concentration of nitrate studied did not entirely prevent the growth of the bacteria, but it reduced the number of organisms far below those contained in control cultures where no nitrates were added.

Ammonium nitrate was also employed. The soil cultures were prepared as already described and inoculated with *B. radicicola*. The cultures were incubated at 28° to 30° C. and counts were made at the end of one and two weeks' time. The results of the study with ammonium nitrate are given in Table XXIII.

TABLE XXIII.—*Influence of ammonium nitrate on Bacillus radicicola in sterilized soil*

Culture No.	Treatment (nitrate in 100 gm. of dry soil).	Number of organisms in 1 gm. of dry soil.				
		At beginning.	After 1 week.	Relative.	After 2 weeks.	Relative.
	Mgm.			*Per cent.*		*Per cent.*
1	0	10,000	850,000	100	1,365,000	100
2	0	10,000	765,000		1,400,000	
3	25	10,000	2,500,000	343	5,060,000	338
4	25	10,000	3,050,000		4,320,000	
5	100	10,000	1,350,000	148	1,030,000	71
6	100	10,000	1,050,000		950,000	
7	200	10,000	700,000	84	635,000	45
8	200	10,000	655,000		605,000	

From the results as a whole it appears that it is the nitrate radical and not the combined salt which causes the increase in the number of *B. radicicola* when small amounts of nitrates are present. A stimulation occurred, resulting in an increase in number which is quite comparable to that obtained with potassium, sodium, and calcium nitrates. The highest concentration of ammonium nitrate used did not appear to have such an inhibiting effect as did the corresponding concentrations of the three other salts.

Throughout the work with *B. radicicola* in sterilized soil comparatively low numbers of these organisms were found. Whether or not this depression was due to toxic substances formed as a result of sterilization can not be stated. If this decrease in numbers as a result of the presence of toxic substances is true, it is very evident that the detrimental effect had not become materially lessened at the end of the incubation period. However, in any event the validity of the outcome is not impaired, since comparative and not absolute data are of importance and since in all probability the same conditions obtained throughout the cultures.

It seems certain from the results of these studies on the effect of potassium, sodium, calcium, and ammonium nitrates on the growth of *B. radicicola* in sterilized soil that small amounts of nitrate stimulate the growth of the organisms. It is also shown that *B. radicicola* is much more resistant than Azotobacter to higher concentrations of potassium, sodium, calcium, and ammonium nitrates.

INFLUENCE OF BACILLUS RADICICOLA ON NITRATES IN SOLUTION

The series of soil culture experiments just discussed served to give an idea concerning the effect of nitrates on the legume organism. It was found that in small amounts nitrates stimulated the bacteria to increased reproduction. But no study was made as to the effect of *Bacillus radicicola* on the nitrate. Does the organism break up the nitrate, reducing it to nitrite or ammonia? Does it cause any loss in nitrate when grown in a solution containing that salt? Beijerinck (*2*, *p.* *762*) as a result of physiological experiments with *B. radicicola*, states that the organism does not reduce nitrate. Prucha (*41*) also states that *B. radicicola* does not reduce nitrates. However, Zipfel (*49*) found that *B. radicicola* will reduce nitrates to nitrites but not to ammonia.

The following experiments, somewhat similar to those already cited in relation to Azotobacter, were carried out in an endeavor to answer these questions.

To twenty 500-c. c. Erlenmeyer flasks containing 200 c. c. of mannit solution, potassium, sodium, calcium, and ammonium nitrates were added as indicated in Tables XXIV, XXV, and XXVI. Quadruplicate flasks were prepared for each concentration of nitrate and for the control cultures without nitrate. The flasks and contents were sterilized at

10 pounds' pressure for 30 minutes. After cooling, two of each set of four flasks were inoculated with 5 c. c. of a suspension of *B. radicicola* in sterile distilled water. The remaining two flasks of each set (uninoculated) served as controls. The flasks were incubated at 28° C. for 21 days. The total weight of the flasks was maintained throughout the incubation period by the addition of sterile distilled water from time to time. At the expiration of the period of incubation the nitrate, ammonia, and total nitrogen contents were determined as given under "Methods used in experiments." The contents of the duplicate inoculated flasks were poured together and 50 c. c. samples drawn for analysis. The same procedure was followed in the case of the uninoculated flasks. The results are given in Tables XXIV, XXV, and XXVI.

TABLE XXIV.—*Influence of Bacillus radicicola on nitrates in solution giving the quantity of nitrate lost*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Nitrate in 100 c. c. of medium.				Nitrate lost.
		Uninoculated.		Inoculated.		
		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	None	0. 00	0. 00	0. 00	0. 00	0. 00
2	.....do	. 00		. 00		
3	150 mgm. of NO_3 as potassium nitrate	151. 4	151. 2	117. 0	117. 0	−34. 2
4	....do	151. 0		117. 0		
5	150 mgm. of NO_3 as sodium nitrate	148. 8	148. 8	114. 4	114. 2	−34. 6
6	.....do	148. 8		114. 0		
7	150 mgm. of NO_3 as calcium nitrate	154. 8	155. 2	76. 6	76. 7	−78. 5
8	.....do	155. 6		76. 8		
9	150 mgm. of NO_3 as ammonium nitrate	151. 4	151. 5	142. 6	142. 6	− 8. 9
10	.....do	151. 6		142. 6		

TABLE XXV.—*Influence of Bacillus radicicola on nitrates in solution giving the quantity of nitrogen as ammonia formed*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Nitrogen as ammonia in 100 c. c. of medium.				Nitrogen as ammonia formed.
		Uninoculated.		Inoculated.		
		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	None	0. 20	0. 15	0. 10	0. 15	0. 00
2	.....do	. 10		. 20		
3	150 mgm. of NO_3 as potassium nitrate	. 10	. 15	. 00	. 05	−. 10
4	.....do	. 20		. 10		
5	150 mgm. of NO_3 as sodium nitrate	. 20	. 20	. 20	. 25	+. 05
6	.....do	. 20		. 30		
7	150 mgm. of NO_3 as calcium nitrate	. 40	. 35	. 30	. 20	−. 15
8	.....do	. 30		. 10		
9	150 mgm. of NO_3 as ammonium nitrate	13. 90	13. 92	13. 80	13. 82	+. 10
10	.....do	13. 95		13. 85		

TABLE XXVI.—*Influence of Bacillus radicicola on nitrates in solution giving the quantity of nitrogen fixed*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Total nitrogen in 100 c. c. of medium.				Nitrogen fixed.
		Uninoculated.		Inoculated.		
		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	None	2. 40	2. 45	3. 30	3. 40	0. 95
2	.....do	2. 50		3. 50		
3	150 mgm. of NO_3 potassium nitrate	18. 00	17. 95	18. 70	18. 85	. 90
4	.....do	17. 90		19. 00		
5	150 mgm. of NO_3 sodium nitrate	16. 80	16. 90	19. 30	19. 25	2. 35
6	.....do	17. 00		19. 20		
7	150 mgm. of NO_3 calcium nitrate	14. 00	13. 90	14. 60	14. 65	. 75
8	.....do	13. 80		14. 70		
9	150 mgm. of NO_3 ammonium nitrate	40. 50	40. 85	41. 30	41. 50	. 65
10	.....do	41. 20		41. 70		

The data in Table XXIV show that a rather large reduction in the total nitrate content took place. This reduction varied rather markedly among the four different nitrates studied. The greatest reduction occurred where calcium nitrate was used. Potassium and sodium were next in order; the loss was almost the same for both salts. Ammonium nitrate was last with but a very small comparative reduction in total nitrate.

The question arises as to whether the nitrate is reduced to nitrite, ammonia, or elemental nitrogen or whether the reduction in amount is due to a natural assimilation of the nitrate by the organisms. The first possibility was precluded when qualitative tests for nitrites were made and none found. Table XXV reveals the fact that no ammonia was produced. Table XXVI shows no loss in total nitrogen. Therefore it seems obvious that reduction in total amount of nitrate present is brought about by the assimilation of those compounds by the organisms.

An inspection of Table XXVI, which gives the results of the total nitrogen determinations, shows that a slight fixation of atmospheric nitrogen took place. This fixation is entirely possible, as will be shown later when the influence of nitrates on the fixation of nitrogen is taken up. In the presence of potassium, sodium, and ammonium nitrates the amount of nitrogen assimilated is somewhat decreased. But in the case of sodium nitrate a large increase in the amount of total nitrogen seems to have taken place. This is interesting in the light of results to be presented later.

From the results of the work on the effect of *B. radicicola* on nitrates it may be concluded that the organisms do not reduce the nitrates to nitrite or ammonia or elemental nitrogen under aerobic conditions.

INFLUENCE OF NITRATES ON THE FIXATION OF ATMOSPHERIC NITROGEN BY BACILLUS RADICICOLA

The ability of *B. radicicola* to fix atmospheric nitrogen in the absence of the host plant has been studied by numerous investigators. From the results of their work it seems fairly probable that the legume organism can fix nitrogen to a slight extent when growing in a nonsymbiotic state. Beijerinck (*3*) was one of the earliest to make a study of the possible fixation of atmospheric nitrogen by *B. radicicola* under these conditions. He found that a small quantity, 0.91 to 1.82 mgm. of nitrogen was fixed per 100 c. c. of the medium. Prasmowski (*39, p. 55*) and Berthelot (*6*) concluded as a result of their experiments that when the organism was grown outside the host plant the gain in nitrogen was small. The greatest gain in nitrogen was found by Mazé (*32*) who reported an increase of 23.4 mgm. of nitrogen per 100 c. c. of the medium in 16 days. Lewis and Nicholson (*30*) found by incubating the cultures for a considerable length of time that a large increase in fixation occurred. Bottomley (*8*) found that a pure culture of *B. radicicola* fixed approximately 1 mgm. of nitrogen in 15 days. Fred (*17*) made a study of the possible fixation of nitrogen by the legume organism and found that it fixed approximately 1.2 mgm. of nitrogen in 100 c. c. of the medium. He found that on agar films a greater fixation occurred than when the organisms were grown in a liquid medium.

A few investigators, however, found that no increase in nitrogen occurred when *B. radicicola* was grown outside the host plant. Frank (*16*) states that in a nitrogen-free medium the legume organisms did not fix enough nitrogen to be accurately measured. Immendorf (*25*) also found no increase in nitrogen when pure cultures of *B. radicicola* were grown in soil containing a nitrogen-free solution.

It will be seen that the majority of investigators, especially the more recent ones, found that a slight amount of atmospheric nitrogen was fixed or assimilated by *B. radicicola* when grown outside the host plant and on a medium suitable for its development.

It has already been shown that nitrates cause an increase in the number of *B. radicicola* when grown in pure culture in sterilized soil. Does such an increase in the number of organisms necessarily mean an increased fixation of nitrogen? Three experiments using agar films were carried out in order to determine this point. Erlenmeyer flasks of 1-liter capacity containing 100 c. c. of mannit agar were used. Before the medium solidified, the nitrates were added in the proportions indicated in Table XXVII. Six flasks for each different quantity of nitrate were prepared, except in one case, as shown in Experiment II. The flasks were plugged with nonabsorbent cotton and sterilized at 10 pounds' pressure for 30 minutes. After cooling, three of each set were inoculated with 5 c. c. of a suspension of *B. radicicola* in sterile distilled water. The organisms had been growing on mannit agar at 28° C. for six days. The flasks in Experiments I and III (Table XXVII) were incubated at

28° C. for three weeks and those in Experiment II for two weeks. The moisture lost by evaporation in both inoculated and uninoculated flasks was replaced from time to time by the addition of sterile distilled water. At the expiration of the incubation period the total nitrogen was determined as given under "Methods used in experiments." The results of the experiments are given in Table XXVII.

An inspection of the data reveals the fact that *B. radicicola* in pure culture fixed a small amount of nitrogen when growing in a nonsymbiotic state with no nitrate present. In the presence of nitrates there was an increased fixation. Although the increase in total nitrogen is small, because of the number of determinations made, it may be considered as positive. The potassium and sodium salts seemed to be more effective than the calcium nitrate, with one exception (Table XXVII, Experiment I). It will be remembered that the latter salt appeared to depress nitrogen fixation by Azotobacter and the two former somewhat to favor it (p. 194–195).

TABLE XXVII.—*Influence of nitrates on the fixation of nitrogen by Bacillus radicicola, giving the increase in nitrogen*

EXPERIMENT I

Culture No.	Treatment (nitrate in 100 c. c. of medium.)	Total nitrogen in 100 c. c. of medium.				Nitrogen increase.
		Uninoculated.		Inoculated.		
		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	None	4.5	4.45	4.7	4.60	0.15
2	do	4.4		4.6		
3	do	4.4		4.5		
4	75 mgm. of NO_3 as sodium nitrate	8.7	8.70	11.9	11.75	3.05
5	do	8.7		11.8		
6	do	8.6		11.6		
7	150 mgm. of NO_3 as sodium nitrate	12.5	12.60	14.9	14.70	2.10
8	do	12.7		14.6		
9	do	.....		14.7		
10	75 mgm. of NO_3 as calcium nitrate	8.8	8.90	12.3	12.40	3.50
11	do	8.9		12.8		
12	do	9.0		12.1		
13	150 mgm. of NO_3 as calcium nitrate	13.3	13.20	14.5	14.10	0.90
14	do	13.1		14.0		
15	do	13.2		13.8		

EXPERIMENT II

1	None	4.90	4.90	5.10	5.075	0.175
2	do	4.90		5.05		
3	75 mgm. of NO_3 as sodium nitrate	8.70	8.60	9.20	9.50	0.90
4	do	8.50		9.80		
5	150 mgm. of NO_3 as sodium nitrate	13.30	13.15	14.20	14.35	1.20
6	do	13.00		14.50		
7	75 mgm. of NO_3 as calcium nitrate	11.15	11.125	11.60	11.65	0.525
8	do	11.10		11.70		
9	150 mgm. of NO_3 as calcium nitrate	14.70	14.70	15.40	15.25	0.550
10	do	(a)		15.10		

a Lost by breakage during sterilization.

TABLE XXVII.—*Influence of nitrates on the fixation of nitrogen by Bacillus radicicola, giving the increase in nitrogen*—Continued

EXPERIMENT III

Culture No.	Treatment (nitrate in 100 c. c. of medium):	Total nitrogen in 100 c. c. of medium.				Nitrogen increase.
		Uninoculated.		Inoculated.		
		Found.	Average.	Found.	Average.	
		Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
1	None	5. 10		5. 50		
2	.....do	5. 10	5. 07	5. 40	5. 50	0. 43
3	.....do	5. 00		5. 45		
4	75 mgm. of NO_3 as potassium nitrate	9. 35		10. 85		
5	.....do	9. 50	9. 37	10. 90	10. 90	1. 53
6	.....do	9. 25		10. 95		
7	150 mgm. of NO_3 as potassium nitrate	14. 50		15. 65		
8	.....do	14. 20	14. 28	15. 30	15. 45	1. 17
9	.....do	14. 15		15. 40		
10	75 mgm. of NO_3 as sodium nitrate	8. 50		9. 85		
11	.....do	8. 30	8. 38	9. 90	9. 83	1. 45
12	.....do	8. 35		9. 70		
13	150 mgm. of NO_3 as sodium nitrate	12. 35		12. 95		
14	.....do	12. 40	12. 33	13. 10	13. 03	0. 70
15	.....do	12. 20		13. 05		
16	75 mgm. of NO_3 as calcium nitrate	8. 95		9. 85		
17	.....do	9. 10	9. 01	9. 90	9. 93	0. 92
18	.....do	9. 00		10. 05		
19	150 mgm. of NO_3 as calcium nitrate	13. 90		14. 40		
20	.....do	13. 80	13. 80	14. 50	14. 42	0. 62
21	.....do	13. 70		14. 35		

It has been shown that, when nitrates are added in varying quantities to sterilized soil, the number of *B. radicicola* are increased. Provided the the organism can fix a small amount of nitrogen in the absence of nitrate nitrogen, is it not possible that this increase in nitrogen fixation may be due merely to the increase in the number of cells? It seems that this is true according to the results in Table XXVII. It appears probable that the increase in nitrogen fixed in the presence of nitrates is very likely because of an increase in the number of bacterial cells and not to any physiological change brought about in the organism itself.

There was a marked increase in bacterial growth on the media containing the nitrate compared with the same media free from nitrate. The growth on the latter medium exhibited a normal, whitish watery appearance, characteristic of this organism. On the cultures containing nitrates a much more profuse growth occurred. In many instances a pinkish tint was observed. This pigment production was especially marked in the case of the culture containing the sodium salt. After the first experiment had been completed, it was thought that possibly this pigmentation was due to an impurity in the culture. Therefore the two remaining experiments were made, using a subculture from the original.

This culture was plated three times, each plating being made from a well-isolated colony. The final subculture was taken from a similar well-isolated colony. However, pigment formation in the presence of nitrate persisted in the two final experiments, showing clearly that some reaction took place between the nitrate and the organism grown on the medium. It is of interest to note that the pigment formation in the presence of nitrate was observed in later work where the influence of nitrates on nodule formation was investigated. Prucha (*41*) found that on agar slopes of medium containing 0.5 per cent of potassium or calcium nitrate, the growth of *B. radicicola* became opaque and that an iridescent tint was produced.

Although the results of these experiments may vary somewhat among themselves, taken as a whole it appears evident that B. *radicicola* may fix a small amount of atmospheric nitrogen when grown without the host plant and on a suitable medium. The addition of various amounts of nitrates as indicated increased somewhat the amount of nitrogen assimilated by *B. radicicola*.

INFLUENCE OF NITRATES ON THE PRODUCTION OF GUM BY BACILLUS RADICICOLA

Since nitrates, especially in smaller amounts, cause an increase in the number of *B. radicicola* in pure culture, it was thought advisable to determine what influence these salts have on the production of gum. In culture media favorable to the growth of *B. radicicola* these bacteria will produce a gelatinous substance which is readily precipitated with 95 per cent alcohol or acetone. Upon the addition of either of these precipitants a fairly heavy, water-white, frothy gelatinous mass is formed which soon rises to the surface of the liquid. Upon standing, this mass contracts somewhat, and portions of it may fall to the bottom of the liquid from which it has been precipitated.

Chemical analyses, according to Buchanan (*10*), have shown that this gum is a carbohydrate. Upon hydrolysis with 2 per cent sulphuric acid and 15 pounds' pressure for one hour, Fehling's solution is reduced, showing the presence of a sugar. The gum does not give proteid reactions with the Millon, biuret, or xanthoproteic tests. Hence, the gum is not protein in character; nor does it contain nitrogen in the combined form. Clearly it is a nonnitrogenous body.

In the experiment undertaken to determine whether nitrates influence the formation of gum only relative differences are noted. No attempt was made to obtain quantitative results.

Erlenmeyer flasks of 1-liter capacity containing 200 c. c. of mannit solution were used. The cultures contained various quantities of nitrate as indicated in Table XXVIII. Triplicate flasks for each amount of nitrate were prepared. In this table these three flasks are represented as "a," "b," and "c." After sterilization at 15 pounds' pressure for 25 minutes the flasks were cooled and inoculated with 5 c. c. of a suspension

of *B. radicicola* in sterile distilled water. The cultures were then incubated at room temperature (approximately 25° C.) for eight weeks.

At the expiration of the incubation period the contents of the flasks were poured into hydrometer cylinders of equal depth and diameter. One hundred and fifty c. c. of acetone were added to precipitate the gum. After careful shaking, the cylinders were covered with inverted petri dishes to prevent evaporation. At the end of 24 hours the amount of gum precipitated was observed. The relative amounts are recorded in Table XXVIII.

TABLE XXVIII.—*Influence of nitrates on the production of gum by Bacillus radicicola*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Relative production of gum—precipitated by acetone.		
		Flask a.	Flask b.	Flask c.
1	None	Large	Large	Large.
2	75 mgm. of NO_3 as potassium nitrate	Very large.	Very large.	Very large.
3	450 mgm. of NO_3 as potassium nitrate.	Large	Large	Large.
4	75 mgm. of NO_3 as sodium nitrate	Very large.	...do.	Very large.
5	450 mgm. of NO_3 as sodium nitrate	Large	...do.	Large.
6	75 mgm. of NO_3 as calcium nitrate	...do.	...do.	Do.
7	450 mgm. of NO_3 as calcium nitrate.	...do.	Considerable.	Considerable.

From the results it is certain that the nitrates, especially in the smaller of the two concentrates, caused a very considerable increase in the amount of gum produced by *B. radicicola*. The nitrates of potassium and sodium caused a production of more gum than did the calcium salt. It will be remembered that in the experiments where the influence of nitrates on the fixation of atmospheric nitrogen by *B. radicicola* was studied, less nitrogen was fixed in the presence of calcium nitrate than in the presence of the other two salts. Here again the greater stimulative action of potassium and sodium nitrates is emphasized.

Buchanan in his investigations on the formation of gum by *B. radicicola* has found that varying amounts of potassium nitrate in a 2 per cent saccharose solution or in a 2 per cent saccharose-clover-extract solution caused a slight increase in growth and in gum production.

It seems probable that the increased gum production in the nitrate cultures is caused not only by an increase in bacterial cells but also perhaps by an increased stimulation in the formation of gum by the cells themselves. The relative increase in the amount of gum produced in the presence of nitrates seems to be greater than the actual increase in number of organisms brought about by the stimulating effect of the nitrate. In the latter instance this stimulating effect has been determined in soil cultures only and so a fair basis of comparison can not be

found. Had the influence of nitrates on the growth and reproduction of *B. radicicola* been determined in liquid culture, as well as in soil cultures, then a comparison could have been made. Furthermore, the divergencies in the time element, eight weeks' incubation in the liquid cultures and three weeks in the soil cultures, are such as to render futile any attempt at correlation. It may be that the large formation of gum was due to the prolonged incubation. A shorter period of three weeks undoubtedly would show a relatively smaller amount of gum produced as a result of the presence of the nitrate.

However, from the results of the experiment it is certain that potassium, sodium, and calcium nitrate influence the formation of gum by *B. radicicola*. The three nitrates studied caused a large increase in the amount of gum obtained by precipitation with acetone. Calcium nitrate caused the least stimulation, but the difference was not large.

INFLUENCE OF NITRATES ON NODULE FORMATION

The results of numerous investigations have shown that nitrates retard and oftentimes entirely prevent the formation of nodules on leguminous plants when grown in soil or liquid cultures. Vines (*45*), working with the horse bean, found that the use of large amounts of nitrate in the form of potassium nitrate retarded nodule formation. He concluded that a decrease in the amount of nitrates meant an increase in the number of nodules. Woods (*48*) found that leguminous plants assimilated more nitrogen when they were grown in the absence of potassium and calcium nitrate than when thus supplied. His results seem to indicate that nodule development was retarded somewhat by these salts. Similar results were obtained by Frank (*16*). Nobbe and Richter (*37*) in 1902 grew soybeans in a rich garden soil and found upon inoculation that a gain of 74.7 per cent of nitrogen occurred. However, upon the addition of nitrates this gain was considerably reduced, the extent of the reduction corresponding to the amount of nitrate added. About this same time, Wohltmann and Bergené (*47*) using many different types of soils, found that nodules were not formed on the roots of peas when ammonium nitrate was added. Creydt (*12*) found that sodium nitrate retarded nodule formation on yellow lupines when these legumes were grown in soil. Fred and Graul (*18*) found that very small amounts of nitrates did not appreciably decrease nodule formation, but that larger amounts proved detrimental and finally prohibited entirely the development of nodules.

The presence of nitrates in culture solutions has also been found to reduce and oftentimes to inhibit the formation of nodules on leguminous plants. Marchal (*31*) concluded that alkaline nitrates in concentrations of 1 to 10,000 in liquid cultures prevented the formation of nodules on peas. Flamand (*15*) grew vetch and beans in a nutrient solution and

found that nitrates in the following amounts prevented nodule formations: potassium nitrate, 1 to 10,000, sodium nitrate 1 to 2,000, ammonium nitrate 1 to 2,000, and calcium nitrate 1 to 2,000 and 1 to 10.000. Hiltner's (*24*) experiments showed that 5 mgm. of nitrogen as potassium nitrate per liter prevented nodule formation on peas.

In contrast to these experiments Bässler (*1*) claimed that results obtained from his work indicated that no effect was noticed by adding nitrates to lupines growing in quartz sand.

The question naturally arises whether this condition is due to the weakening of the organism brought about by growth in a nitrated environment and to a consequent impairment or entire loss of its infecting power, or whether it is caused by some interreaction between the salt and the plant root, tending to increase the latter's resistance to the attack of this particular organism.

INFLUENCE OF NITRATES ON THE INFECTING POWER OF BACILLUS RADICICOLA

Some investigations have been carried out to determine what effect nitrates have on the legume organisms themselves. Wilson (*46*) showed that although nitrates inhibit the formation of nodules, the organisms capable of producing nodules did not lose their vitality or nodule-producing power when grown in the presence of nitrates. The results of Prucha (*41*) are in accord with those of Wilson. He found that *B. radicicola* does not seem to lose its infecting power when grown on media containing nitrate. During the course of his work he found that potassium and sodium nitrates inhibited the formation of nodules. Further evidence that the organisms appear to retain their vitality in the presence of nitrates is produced by the results of Mazé (*33, p. 15–17*), who showed that legume bacteria were able to fix a slight amount of nitrogen when grown in a soil extract solution containing 1 per cent sodium nitrate. Herke (*22*) states that potassium nitrate favors the growth of nodule bacteria.

However, other investigators state that nitrates have a harmful effect on *B. radicicola*. Laurent (*29, p. 134*) found that legume organisms failed to grow in a pea or lupine decoction containing nitrate in the form of potassium and sodium salts in amounts equivalent to 1 to 500 and 1 to 1,000. Moore (*35*) in his experiments demonstrated that nitrates at 1 to 10,000 were sufficient to prevent nodule formation. He states that *B. radicicola* loses its power of infection when grown in a medium containing nitrates.

From the results cited it can be seen that there is some disagreement as to the influence exerted by nitrates on *B. radicicola*. In some cases the organism seems to retain its vitality in the presence of nitrates, while in others it appears to have become weakened. It must be ad-

mitted, however, that the evidence seems to favor the former contention—namely, that nitrates do not cause the bacteria to lose their nodule-producing power.

In order to determine whether or not nitrates weaken these organisms, the following experiments were made: Slopes of mannit agar (in test tubes) containing various amounts of sodium and calcium nitrates as indicated in Table XXIX were inoculated with *B. radicicola.* These cultures were incubated at 28° C. for one week, when transfers were made to fresh nitrate media and incubated at 28° C. for another week. At the expiration of this time, three 4-day-old seedlings of alfalfa were inoculated with three drops of a suspension of the organism in 5 c. c. of sterile distilled water. The same slope cultures were incubated at 28° C. and used for all subsequent inoculations in this experiment. The inoculated seedlings were placed in the greenhouse under cheesecloth covering. The temperature here during the daytime averaged approximately 30° C. The seedlings were examined for the first appearance of nodules and in no case did they appear before 18 to 20 days. A total count of nodules on all plants was made at the end of 45 days. Three subsequent inoculations were made under the same conditions. In this way organisms in contact with nitrate for varying lengths of time could be used. The results of the inoculation experiments are given in Table XXIX.

TABLE XXIX.—*Influence of nitrates on the infecting power of Bacillus radicicola*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Number of nodules after 45 days.			
		Inoculated June 3.	Inoculated June 15.	Inoculated July 11.	Inoculated July 17.
1	None	5	4	7	3
2	15 mgm. of NO_3 as sodium nitrate	5	4	5	5
3	37 mgm. of NO_3 as sodium nitrate	7	6	15	5
4	75 mgm. of NO_3 as sodium nitrate	4	5	3	11
5	150 mgm. of NO_3 as sodium nitrate	5	8	4	8
6	225 mgm. of NO_3 as sodium nitrate	6	5	5	7
7	450 mgm. of NO_3 as sodium nitrate	2	4	6	3
8	None	7	8	7	8
9	15 mgm. of NO_3 as calcium nitrate	4	4	9	4
10	37 mgm. of NO_3 as calcium nitrate	9	4	8	6
11	75 mgm. of NO_3 as calcium nitrate	5	5	11	8
12	150 mgm. of NO_3 as calcium nitrate	7	5	9	9
13	225 mgm. of NO_3 as calcium nitrate	5	7	4	3
14	450 mgm. of NO_3 as calcium nitrate	6	8	6	3
15	Uninoculated	0	0	0	0
16	.....do	0	0	0	0

From the results given in Table XXIX it is very evident that under the conditions of the experiment the legume organisms did not lose their power of producing nodules when grown on a medium containing

varying amounts of sodium and calcium nitrates. The numbers of nodules produced on the alfalfa plants by organisms grown on media containing nitrate do not vary widely from those on the plants inoculated with organisms grown on media containing no nitrate. Not only did the organisms fail to lose their nodule-producing power, but from all appearances their infecting power did not seem to be materially weakened.

It therefore seems apparent that an explanation for the failure of nodules to develop on leguminous plants in the presence of nitrates is not found in the theory that the organisms producing these nodules are weakened when grown in the presence of nitrates.

INFLUENCE OF NITRATES ON ALFALFA ROOTS AND NODULE FORMATION

The next step taken would naturally be in the direction of a study of the influence of the nitrates on the plant roots themselves in order to determine whether or not they thus are made more resistant to the attack of these organisms.

A review of the literature shows that almost nothing has been done touching this phase of the question. Wilson (*46*), studying the effect of certain salts on nodule production, states that possibly the salt has some effect on the root, making it resistant to the attack of the organism. Mazé (*33, p. 15–17*), who also concluded that nitrates did not cause *B. radicicola* to lose its infecting power, says that nodules do not develop on roots of legumes when nitrates are present because the carbohydrate in the roots is changed into protein material by the absorption of the nitrate.

Alfalfa seedlings (*Medicago sativa*) growing in soft agar containing potassium, sodium, and calcium nitrates, as indicated in Table XXX, were used in this study. Quadruplicate tubes were prepared for each amount of nitrate. The higher concentrations of the nitrate were not used, since it was found that germination and subsequent growth were considerably impaired in the presence of such large amounts. The tubes with the mannit agar and nitrate were sterilized at 15 pounds' pressure for 30 minutes. These were cooled and sterilized alfalfa seeds planted as given under "Methods used in experiments." The tubes were then placed in the greenhouse under cheesecloth covering and the seeds allowed to germinate. Germination took place in all instances, although it was retarded somewhat by the presence of the nitrate. At the end of five days the first tube of each set was inoculated with three drops of a suspension of *B. radicicola* in sterile distilled water. Subsequent inoculations were made as indicated in Table XXX. These were made at different intervals in order to allow the roots of the seedlings to remain for a longer time in contact with the media. It was hoped that in this way an idea might be obtained as to the time when the root first became resistant. The results are given in Table XXX.

TABLE XXX.—*Influence of nitrates on alfalfa roots and nodule formation*

Culture No.	Treatment (nitrate in 100 c. c. of medium).	Total number of nodules in each tube of seedlings inoculated after—			
		5 days' growth.	10 days' growth.	18 days' growth.	22 days' growth.
1	None	3	3	5	4
2	10 mgm. of NO_3 as potassium nitrate	0	1	2	0
3	25 mgm. of NO_3 as potassium nitrate	0	0	0	0
4	50 mgm. of NO_3 as potassium nitrate	0	0	0	(a)
5	100 mgm. of NO_3 as potassium nitrate	0	0	0	0
6	150 mgm. of NO_3 as potassium nitrate	0	0	(b)	0
7	10 mgm. of NO_3 as sodium nitrate	0	1	3	2
8	25 mgm. of NO_3 as sodium nitrate	0	0	0	0
9	50 mgm. of NO_3 as sodium nitrate	0	0	0	0
10	100 mgm. of NO_3 as sodium nitrate	(b)	0	0	0
11	150 mgm. of NO_3 as sodium nitrate	(b)	0	0	0
12	10 mgm. of NO_3 as calcium nitrate	1	3	1	0
13	25 mgm. of NO_3 as calcium nitrate	0	0	0	0
14	50 mgm. of NO_3 as calcium nitrate	0	0	0	0
15	100 mgm. of NO_3 as calcium nitrate	0	(b)	0	0
16	150 mgm. of NO_3 as calcium nitrate	(b)	0	0	0

[a] Fungus contamination. [b] Plant died after few days' growth.

It will be seen that in a few instances where a high concentration of nitrates occurred the development of the seedlings subsequent to germination ceased. This condition may have been due to too high a concentration of soluble salts or to inferior seed. However, losses were not sufficiently serious materially to affect the outcome of the experiment.

In all cases the seedlings grown in agar without nitrate produced nodules when inoculated with *B. radicicola*. A few nodules appeared on seedlings in cultures containing the lowest concentration of all three nitrates. The number of nodules in these cases was less than in the control cultures. No nodules whatever developed in any concentration above 10 mgm. of nitrate in 100 c. c. of medium. Under normal conditions in test-tube cultures the nodules make their appearance at about 18 to 20 days after inoculation. The incubation of all cultures was extended 40 days after inoculation in order to make certain that no further nodule development would take place.

The nonproduction of nodules was not due to the failure of the inoculum. In all cases an excellent inoculum growth was obtained, especially in the case where nitrate was present in the medium. Indeed, it was so luxuriant that in many cases the organism grew in considerable quantity far down into the root zone. In many cases where nitrates were present the same pink coloration was produced that was discussed under another caption, on page 216.

As has been already stated, seedlings of varying ages were inoculated for the reason that it was thought that a more or less prolonged contact of the roots with the nitrate in the medium might serve as an index to

the time in the growth of the seedling when permanent resistance to attack of the organisms was established. The results obtained do not seem to indicate that seedling roots 18 to 20 days' old are any more resistant to the attack of the organisms than are those that are younger. Evidently if any reaction takes place between the nitrate and the plant root it occurs during the very early stages in the development of the plant.

These results seem to point to the conclusions that the nonformation of nodules in the presence of nitrates is due not to a weakening of the vitality of the organism, but to some reaction between the plant root and nitrate. One naturally queries whether the plant root cells are made more resistant to the bacteria seeking to gain entrance there or whether the naturally occurring carbohydrate food supply to be used by the organisms after gaining entrance is diminished by its conversion into protein material by the absorption of nitrate? Further studies were not made in an endeavor to solve this question.

INFLUENCE OF NITRATES IN SOIL ON ALFALFA NODULES AND ON THE REFORMATION OF NODULES

Additional studies were made with nitrates in relation to their influence on nodules already formed and on the redevelopment of nodules once removed from alfalfa plants. The experiments were carried out in an endeavor to determine whether nitrates prevented an increase in the number of nodules on plants possessing nodules and whether they prevented the reformation of nodules when removed. Experiments revealed clearly that removed nodules were replaced by new ones provided the plant was carefully replaced in the soil (soil with normal low nitrate content) and the proper amount of moisture maintained.

In these experiments 1-gallon earthenware jars were used. These were filled to within an inch of the top with 1,800 gm. of soil of a low nitrate content. Different amounts of the nitrates to be studied were added in the quantities indicated in Table XXXI. Concentrations of 100 and 300 mgm. of nitrate in 100 gm. of soil were also used, but the transplanted alfalfa seedlings were unable to withstand such excessive concentration, with the result that all died within a week or ten days after transplanting. Quadruplicate pots were prepared for each concentration of nitrate. The nitrates in solution were mixed with the proper amount of distilled water which, when added to the pots, brought the moisture content to approximately 20 per cent. The pots were then allowed to remain undisturbed for one day at room temperature to allow the water containing the nitrate to become well diffused throughout the soil mass. Into two pots of each set were transplanted young alfalfa plants from which the nodules had been removed. The two remaining pots contained transplanted alfalfa plants with the nodules left on and their location noted. The plants used in this experiment

were removed from an alfalfa plot, the soil of which was a sandy loam. Previous to transplanting the roots of the young plants were carefully washed in running water and immediately transplanted. The pots were kept well watered, and after two or three days they were removed to the greenhouse. Here they were watered when necessary. Transplantations were made on the 27th of June and the experiment terminated on the 3d of August. The plants were removed from the pots, the roots carefully washed and examined for the presence of nodules. The results are presented in Table XXXI.

TABLE XXXI.—*Influence of nitrates in soil on alfalfa nodules and on the reformation of nodules*

Pot No.	Nitrate in 100 gm. of dry soil.	Treatment of nodules.	Number of nodules—	
			At beginning.	At end.
A 1	None	Removed	0	3
A 2	do	do	0	4
A 3	do	Not removed	4	8
A 4	do	do	3	7
B 1	25 mgm. of NO_3 as potassium nitrate	Removed	0	0
B 2	do	do	0	(a)
B 3	do	Not removed	4	3
B 4	do	do	8	5
C 1	50 mgm. of NO_3 as potassium nitrate	Removed	0	0
C 2	do	do	0	0
C 3	do	Not removed	4	2
C 4	do	do	1	1
D 1	25 mgm. of NO_3 as sodium nitrate	Removed	0	0
D 2	do	do	0	0
D 3	do	Not removed	4	2
D 4	do	do	5	1
E 1	50 mgm. of NO_3 as sodium nitrate*b*	Removed	0	0
E 2	do	do	0	(a)
E 3	do	Not removed	2	1
E 4	do	do	6	3
F 1	25 mgm. of NO_3 as calcium nitrate	Removed	0	0
F 2	do	do	0	0
F 3	do	Not removed	2	2
F 4	do	do	4	3
G 1	50 mgm. of NO_3 as calcium nitrate	Removed	0	0
G 2	do	do	0	0
G 3	do	Not removed	4	3
G 4	do	do	2	1

[a] Plants died.

It will be seen in the control pots, where no nitrate was present (except the small amount normally present in the soil at the beginning of the experiment), that if the nodules were removed, new ones formed. The location of the nodules before their removal was noted, and the new ones were found to occupy the same place. However, when nitrates were added to the soil no new nodules were formed. This statement holds true for both concentrations of all three salts in all experiments.

Some interesting results were obtained where the nodules were not removed. In the control pots an increase in nodule formation took place. It can not be stated definitely whether the new nodules appeared as a result of inoculation from the soil or whether the organisms had already gained entrance to the roots before the plants were removed from the field soil previous to transplanting. Nevertheless, it is shown that the number of nodules increased as compared with the number present at the time of transplanting. But where nitrates were added a reduction in number occurred rather regularly throughout all the pots. In two instances the number remained constant, in 10 it was reduced, and in none was it increased. The calcium salt appeared to effect the least reduction in number of nodules. Conclusions concerning the comparative influence of the three salts in this regard can not be drawn because of the small number of determinations made. It is sufficient to note that nitrates present in amounts equal to 25 and 50 mgm. of nitrate in 100 gm. of soil did not permit an increase in number of nodules, but rather caused a decrease.

The conclusions drawn from the experiments relative to the influence of nitrates on nodule formation are: (a) the presence of nitrates is detrimental to the formation of nodules by alfalfa; (b) the nonformation of nodules is not due to a weakening of *B. radicicola* when grown in the presence of nitrates; (c) some reaction takes place between the nitrates and the plant root, thus preventing nodule formation; (d) nitrates in the soil prevent the re-formation of nodules once removed and also cause a decrease in the number of those already present.

SUMMARY

(1) Small quantities of potassium, sodium, and calcium nitrates caused a great increase in the number of Azotobacter in sterilized soil. Ammonium nitrate in the same quantities caused a less marked increase. Higher concentrations were not so favorable to the growth of the organisms.

(2) Potassium and sodium nitrates in the concentrations studied caused an increase in the amount of nitrogen assimilated by Azotobacter on agar films. Calcium nitrate in the same amounts brought about a decrease in the amount of nitrogen fixed to a point even below that representing the amount assimilated in the absence of nitrates. In soil cultures nitrates of sodium and calcium caused an increase in total nitrogen, which was more marked in the unsterilized cultures than in those cultures sterilized and inoculated with a pure culture of Azotobacter. However, the increase in total nitrogen is not commensurate with the increase in the number of Azotobacter noted under the same conditions.

(3) Under aerobic conditions Azotobacter in liquid cultures reduced nitrate to nitrite, but not to ammonia. More atmospheric nitrogen was assimilated in the presence of nitrate than in the absence of this salt.

(4) Pigmentation occurred when potassium and sodium nitrates, and especially calcium nitrate, were used with Azotobacter, the coloration increasing with the concentration of the salt. This effect was more marked in Azotobacter strains which produce little or no pigment in the absence of nitrates.

(5) All three nitrates studied caused an increase in the number and size of volutin bodies in Azotobacter cells. From all appearances these salts also tended to hasten the development of these bodies.

(6) The number of *Bacillus radicicola* in sterilized soil was increased by the addition of small quantities of potassium, sodium, ammonium, and calcium nitrates. This increase was not so marked as in the Azotobacter cultures. *B. radicicola* appeared to be much more resistant to higher concentrations of nitrates than Azotobacter.

(7) *B. radicicola* under aerobic conditions did not reduce nitrates in solution to nitrite, ammonia, or elemental nitrogen. The presence of nitrates did not materially influence the small amount of atmospheric nitrogen fixed under these conditions.

(8) When grown on agar films, *B. radicicola* fixed a small amount of nitrogen, varying from 0.15 to 0.43 mgm. of nitrogen in 100 c. c. of the medium. The addition of various amounts of potassium, sodium, and calcium nitrates increased to a slight extent the amount of nitrogen assimilated.

(9) In liquid cultures all three nitrates caused a large increase in the amount of gum obtained by precipitation with acetone.

(10) The presence of large amounts of potassium, sodium, and calcium nitrates proved detrimental to the formation of nodules on alfalfa. *B. radicicola* did not appear to lose its infecting power when grown on media containing varying amounts of sodium and calcium nitrates. Alfalfa seedlings grown in the presence of large amounts of nitrate did not produce nodules when inoculated with a viable culture of *B. radicicola*. Nitrates in soil cultures prevented the re-formation of nodules once removed and also caused a decrease in the number of nodules already present.

LITERATURE CITED

(1) Bässler, P.
1895. Sandkulturversuche über die Stickstoffassimilation der gelben Lupine im sterilisierten und geimpften Boden bei dargebot wechselnder mengen von salpetersauren salzen. *In* Jahresber. Agr. Chem., n. F., Bd. 18 (Jahrg. 38), p. 131.

(2) Beijerinck, M. W.
1888. Die Bacterien der Papilionaceen-Knollchen. *In* Bot. Ztg., Jahrg. 46, No. 48, p. 758–771. (Continued article.)

(3) ———
1891. Over ophooping van atmospherische stickstof in culturen van bacillus radicicola. *In* Verslag. en Meded. K. Akad. Wetensch. [Amsterdam], r. 3, deel 8, p. 460–475.

(4) Beijerinck, M. W.
1901. ueber oligonitrophile mikroben. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 7, No. 16, p. 561–582, 1 pl.

(5) ——— and Delden, A. van.
1902. ueber die assimilation des freien stickstoffs durch bakterien. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 9, no. 1/2, p. 3–43.

(6) Berthelot, M. P. E.
1893. recherches nouvelles sur les microrganismes fixateurs de l'azote. *In* Compt. Rend. Acad. Sci. [Paris], t. 116, no. 17, p. 842-849.

(7) Bonazzi, Augusto.
1915. cytological studies of azotobacter chroococcum. *In* Jour. Agr. Research, v. 4, no. 3, p. 225–239. Literature cited, p. 238–239.

(8) Bottomley, W. B.
1909. some effects of nitrogen-fixing bacteria on the growth of non-leguminous plants. *In* Proc. Roy. Soc. London, s. B, v. 81, no. 548, p. 287–289.

(9) Bredemann, G.
1909. bacillus amylobacter a. m. et bredemann in morphologischer, physiologischer und systematischer beziehung. *In* Centbl. Bakt [etc.], Abt. 2, Bd. 23, no. 14/20, p. 385–568, 13 fig., 2 pl. Literaturverzeichnis, p. 559–566.

(10) Buchanan, R. E.
1909. the gum produced by bacilus radicicola. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 22, No. 11/13, p. 371–396. Citations, p. 395–396.

(11) Chalmot, G. de
1894. the influence of nitrates on germinating seeds. *In* Agr. Science, v. 8, no. 10/11, p. 463–465.

(12) Creydt, Bodo.
1915. untersuchungen über die kalkempfindlichkeit der lupine und ihre bekämpfung. *In* Jour. Landw., Bd. 63, Heft 2, p. 125–191, 6 pl.

(13) Drabble, Eric, and Scott, Daisy G.
1907. on the effect of acids, alkalis, and neutral salts on the fermentative activity and on the rate of multiplication of yeast cells. *In* Biochem. Jour., v. 2, no. 7/8, p. 340–349, 1 fig. Literature p. 349.

(14) Fernbach, A., and Lanzenberg, A.
1910. de l'action des nitrates dans la fermentation alcoolique. *In* Compt. Rend. Acad. Sci. [Paris], t. 151, no. 17, p. 726–729.

(15) Flamand, Henri.
1905. über den einfluss der ernährung auf die entwicklung der knöllchen der leguminosen. *In* Centbl. Agr. Chem., Jahrg. 34, Heft 11, p. 738–740.

(16) Frank, B.
1892. die assimilation freien stickstoffs bei den pflanzen in ihrer abhängigkeit von species, von ernährungsverhältnissen und von bodenarten. *In* Landw. Jahrb., Bd. 21, p. 1–44.

(17) Fred, E. B.
1913. a physiological study of the legume bacteria. *In* Va. Agr. Exp. Sta., Ann. Rpt., 1911/12, p. 145–173, fig. 34. Literature, p. 172–173.

(18) ——— and Graul, E. J.
1916. the effect of soluble nitrogenous salts on nodule formation. *In* Jour. Amer. Soc. Agron., v. 8, no. 5, p. 316–328. Literature cited, p. 327–328.

(19) GREAVES, J. E.
1916. THE INFLUENCE OF SALTS ON THE BACTERIAL ACTIVITIES OF THE SOIL. *In* Soil Science, v. 2, no. 5, p. 443–480, 4 fig.

(20) HANZAWA, J.
1914. EINIGE BEOBACHTUNGEN ÜBER STICKSTOFF-BINDUNG DURCH AZOTOBACTER IN STICKSTOFFARMEN UND IN STICKSTOFFREICHEN SUBSTRATEN. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 41, No. 18/23, p. 573–576.

(21) HELLRIEGEL, H., and WILFARTH, H.
1888. UNTERSUCHUNGEN ÜBER DIE STICKSTOFFNAHRUNG DER GRAMINEEN UND LEGUMINOSEN. *In* Ztschr. Ver. Rübenzuckerindus., Beilageheft, Nov., 234 p., 6 pl.

(22) HERKE, S.
1913. CONTRIBUTIONS ON NITROGEN FIXATION AND NUTRITION OF BACILLUS RADICICOLA AND ON BACTERIAL TESTS OF NITRAGIN AND AZOTOGEN. (Abstract.) *In* Exp. Sta. Rec., v. 29, no. 8, p. 733. (Original article in Kisérlet. Közlem., v. 16, no. 3, p. 311–322, 1913. Not seen.)

(23) HILLS, T. L.
THE INFLUENCE OF NITRATES ON THE GROWTH OF AZOTOBACTER. Unpublished. Offered for publication in the Ann. Rept. of the Pa. Agr. Exp. Sta.

(24) HILTNER, L.
1900. UEBER DIE URSACHEN, WELCHE DIE GRÖSSE, ZAHL, STELLUNG UND WIRKUNG DER WURZELKNÖLLCHEN DER LEGUMINOSEN BEDINGEN. *In* Arb. K. Gsndhtsamt., Biol. Abt., Bd. 1, Heft 2, p. 177–222, pl. 3.

(25) IMMENDORFF, H.
1892. BEITRÄGE ZUR LÖSUNG DER "STICKSTOFFRAGE." *In* Landw. Jahrb. Bd. 21, p. 281–339.

(26) JOST, Ludwig.
1907. LECTURES ON PLANT PHYSIOLOGY. Translated by R. J. H. Gibson. 464 p., 172 fig. Oxford. Bibliography at the end of each lecture.

(27) KAYSER, E.
1910. INFLUENCE DES NITRATES SUR LES FERMENTS ALCOOLIQUES. *In* Compt. Rend. Acad. Sci. [Paris], t. 151, no. 19, p. 816–817.

(28) KOSSOWICZ, ALEXANDER.
1914. ÜBER DAS VERHALTEN VON HEFEN UND SCHIMMELPILZEN ZU NITRATEN. In Biochem. Ztschr., Bd. 67, Heft 4/5, p. 400–419.

(29) LAURENT, Emilé.
1891. RECHERCHES SUR LES NODOSITÉS RADICALES DES LÉGUMINEUSES. *In* Ann. Inst. Pasteur, année 5, p. 105–139, 3 fig.

(30) LEWIS, L. L., and NICHOLSON, J. F.
1905. SOIL INOCULATION. TUBERCLE-FORMING BACTERIA OF LEGUMES. Okla. Agr. Exp. Sta. Bul. 68, 30 p., 8 fig.

(31) MARCHAL, Emilé
1901. INFLUENCE DES SELS MINÉRAUX NUTRITIFS SUR LA PRODUCTION DES NODOSITÉS CHEZ LE POIS. *In* Compt. Rend. Acad. Sci. [Paris], t. 133, no. 24, p. 1032–1033.

(32) MAZÉ, P.
1897. FIXATION DE L'AZOTE LIBRE PAR LE BACILLE DES NODOSITÉS DES LÉGUMINEUSES. *In* Ann. Inst. Pasteur, année 11, no. 1, p. 44–54.

(33) ———
1898. LES MICROBES DES NODOSITÉS DES LÉGUMINEUSES. *In* Ann. Inst. Pasteur, ann. 12, no. 1, p. 1–25, 1 fig.

(34) MEYER, Arthur.
1912. DIE ZELLE DER BAKTERIEN. 285 p., 34 fig., 1 col. pl. Jena. Literatur, p. 267–282.

(35) MOORE, G. T.
1905. SOIL INOCULATION FOR LEGUMES; WITH REPORTS UPON THE SUCCESSFUL USE OF ARTIFICIAL CULTURES BY PRACTICAL FARMERS. U. S. Dept. Agr. Bur. Plant Indus. Bul. 71, 72 p., 10 pl.

(36) MÜNTER, F.
1916. ÜBER DEN EINFLUSS ANORGANISCHER SALZE AUF DAS WACHSTUM DER ACTINOMYCETEN. III. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 44, No. 24/25, p. 673–695, 9 fig.

(37) NOBBE, F., and RICHTER, L.
1902. ÜBER DEN EINFLUSS DES NITRATSTICKSTOFFS UND DER HUMUSSUBSTANZEN AUF DEN IMPFUNGSERFOLG BEI LEGUMINOSEN. *In* Landw. Vers. Stat., Bd. 56, Heft 5/6, p. 441–448.

(38) PFEFFER, W.
1906. THE PHYSIOLOGY OF PLANTS. ed. 2. Translated by A. J. Ewart. v. 3. Oxford.

(39) PRAZMOWSKI, Adam.
1891. DIE WURZELKNÖLLCHEN DER ERBSE: II. TEIL. DIE BIOLOGISCHE BEDEUTUNG DER WURZELKNÖLLCHEN. *In* Landw. Vers. Stat., Bd. 38, p. 5–56.

(40) PRINGSHEIM, Hans.
1914. ZUR STICKSTOFFASSIMILATION IN GEGENWART VON SALPETER. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 40, no. 1/8, p. 21–23.

(41) PRUCHA, M. J.
1915. PHYSIOLOGICAL STUDIES OF BACILLUS RADICICOLA OF CANADA FIELD PEA. N. Y. Cornell Agr. Exp. Sta. Mem. 5, 83 p. Bibliography, p. 79–83.

(42) RITTER, G.
1909. AMMONIAK UND NITRATE ALS STICKSTOFFQUELLE FÜR SCHIMMELPILZE. *In* Ber. Deut. Bot. Gesell., Bd. 27, Heft 10, p. 582–588.

(43) SACKETT, W. G.
1915. THE PIGMENT OF AZOTOBACTER CHROOCOCCUM. *In* Proc. 35th Ann. Meeting Soc. Prom. Agri. Sci., 1914, p. 80–88, 2 col. pl.

(44) STOKLASA, Julius.
1908. BEITRAG ZUR KENNTNIS DER CHEMISCHEN VORGANGE BEI DER ASSIMILATION DES ELEMENTAREN STICKSTOFFS DURCH AZOTOBACTER UND RADIOBACTER. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 21, no. 15/16, p. 484–511.

(45) VINES, S. H.
1888. ON THE RELATION BETWEEN THE FORMATION OF TUBERCLES ON THE ROOTS OF LEGUMINOSAE AND THE PRESENCE OF NITROGEN IN THE SOIL. *In* Ann. Bot., v. 2, no. 7, p. 386–389.

(46) WILSON, J. K.
1915. PHYSIOLOGICAL STUDIES OF BACILLUS RADICICOLA OF SOY BEAN. (Abstract.) *In* Science, n.s., v. 41, no. 1048, p. 180.

(47) WOHLTMANN, Ferdinand, and BERGENÉ, ———
1902. DIE KNÖLLCHEN-BAKTERIEN IN IHRER ABHÄNGIGKEIT VON BODEN UND DÜNGUNG. *In* Jour. Landw., Bd. 50, Heft 4, p. 377–395.

(48) WOODS, C. D.
1892. THE ACQUISITION OF ATMOSPHERIC NITROGEN BY GROWING PLANTS. *In* Conn. Storrs Agr. Exp. Sta., 4th Ann. Rpt., 1891, p. 17–28.

(49) ZIPFEL, Hugo.
1911. BEITRÄGE ZUR MORPHOLOGIE UND BIOLOGIE DER KNÖLLCHENBAKTERIEN DER LEGUMINOSEN. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 32, No. 3/5, p. 97–137. Literature, p. 136–137.

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