INFLUENCE OF POTASSIUM NITRATE ON NODULE FORMATION AND NITROGEN FIXATION BY CLOVER[1]

E. W. Hopkins, P. W. Wilson, and W. H. Peterson

Introduction

The unique position which the Leguminosae occupy in the plant world has made them particularly inviting subjects for investigation. The specialized structure arising from association with benevolent symbionts enables this group of plants to develop independently of the supply of nitrogen present in the soil. Not only do leguminous plants with associated bacteria require no added fixed nitrogen, but also the peculiar fact was long ago observed that nitrogenous compounds adversely affect this so-called symbiotic relation. Under these conditions there may be present large numbers of effective strains of the nodule bacteria, and yet the plant appears to resist their invasion or to make little use of its nodules. Thus it would seem that if both sources of nitrogen are available the plant prefers to feed upon fixed nitrogen in the soil. This subject has been investigated repeatedly, and several hypotheses have been proposed to explain this peculiar condition. However, the investigations have considered chiefly the effect of nitrate treatment on nodule formation, since that is the form in which plants usually take up their nitrogen, and because of its wide occurrence in soil. A second important form of soil nitrogen, *viz.*, ammonia, has been given far less attention; while organic nitrogen sources have been almost entirely neglected.

The major part of the work that has been done in this field has dealt with the effect of sources of nitrogen on the number of nodules. Inquiries that deal with the effect on the nitrogen fixation process are rare and often open to criticism. Thus the use of open pots for growing the plants is not satisfactory since growth of algae and other microörganisms would account for part of the nitrate that disappears. Obviously plants grown under bacteriologically controlled conditions are desirable if a satisfactory nitrogen balance is to be made. To grow plants free from contaminating organisms, it is necessary to adopt precautions that result in smaller plants than those grown in open pots. This leads to much smaller nitrogen uptake and the nitrogen balances are more likely to be affected by analytical errors. In spite of these objections it appeared desirable to investigate the formation of nodules and nitrogen fixation with plants grown under controlled conditions.

[1] Herman Frasch Foundation Research in Agricultural Chemistry, Paper no. 28. Contribution from the Departments of Agricultural Bacteriology and Agricultural Chemistry, University of Wisconsin.

A second point that does not seem to have received sufficient consideration is the question whether the effects noted are due to the concentration of nitrogen at any one time, or to the total nitrogen added. It is possible that different results would be obtained if the nitrogen were added periodically rather than at one time.

RAUTENBERG and KÜHN (**11, 12**) appear to have been the first to observe that vetch (*Vicia faba*) formed no root nodules in solutions containing ammonia or nitrate nitrogen, while plants grown in nitrogen-free solutions possessed abundant nodules. DE VRIES (**13**) noted that red clover plants grown in nitrogen-rich solutions developed only a few nodules, as compared with plants without access to combined nitrogen. FRANK (**5**) found that horse-manure extract prevented formation of nodules by peas grown in a soil which had been ignited.

Historical review

It is not possible to give here a detailed review of the numerous papers which discuss the effect of nitrate treatment on nodule formation. A summary of these papers is given in the monograph by FRED, BALDWIN, and McCOY (**6**). However, certain papers may be reviewed here, as they offer details not given in this monograph. FLAMAND (**4**) has made an extensive study of the concentrations of nitrate which inhibit nodule formation. Plants were grown in SACH's solution with various nitrates added. With peas (***Pisum sativum***), KNO_3, $NaNO_3$, and NH_4NO_3 were effective in dilutions of 1/10,000 in preventing nodule formation, while with $Ca(NO_3)_2$ a 1/2000 dilution was required. Vetch (***Vicia narbonensis***) exhibited no nodules in $NaNO_3$ 1/2000; KNO_3 1/10,000; $Ca(NO_3)_2$ less than 1/10,000; and NH_4NO_3 1/20,000. Horse bean (***Faba equina***) was somewhat less sensitive to some nitrates and more sensitive to others; KNO_3 1/10,000; $Ca(NO_3)_2$ 1/20,000; $NaNO_3$ 1/2000. $(NH_4)_2SO_4$ inhibited nodule formation on ***Pisum sativum*** and ***Faba equina*** at a dilution of 1/10,000 and on ***Vica narbonensis*** at 1/20,000.

WILSON (**14**) has studied the effect upon soybeans of many alkali and metal nitrates. An amount of these nitrates which had been found to have no injurious effect on the plant was added to 208 gm. of soil. The nitrates used were as follows: $Ca(NO_3)_2$, KNO_3, NH_4NO_3, $Mg(NO_3)_2$, $NaNO_3$, $Al(NO_3)_3$, $Ba(NO_3)_2$, $Fe(NO_3)_3$, $Pb(NO_3)_2$, $Ce(NO_3)_4$, $Sr(NO_3)_2$, $LiNO_3$, $UO_2(NO_3)_2$, 0.1 gm. added per 208 gm. of soil; $Cd(NO_3)_2$, $Ni(NO_3)_2$, 0.02 gm. per 208 gm. of soil; and $Zn(NO_3)_2$ and HNO_3, 0.05 gm. per 208 gm. of soil. $Al(NO_3)_3$ and HNO_3 had no effect upon the number of nodules, but all other nitrates exerted a marked depressing result. Of the ammonium salts tested, the carbonate was without effect, the phosphate and aluminum ammonium sulphate were stimulating, while all others markedly decreased nodule production on soybeans.

ALBRECHT (1) found that the application of $NaNO_3$ corresponding to 10, 50 and 150 lb. of nitrate per acre did not appreciably decrease nodule formation by cowpeas, and that soybeans were not affected by amounts less than 150 lb. per acre.

GIÖBEL (8) has considered in some detail the effect of $NaNO_3$ upon soybeans and alfalfa. It was found that the gradual addition of the equivalent of 500 to 2600 lb. per acre of $NaNO_3$ to pot cultures of alfalfa was without result on nodule formation. When, however, the nitrate was all added at the beginning, 1200 to 1600 lb. per acre decreased the number of nodules to one fourth of normal. Amounts up to 1000 lb. of $NaNO_3$ were not effective. In another experiment soybeans were allowed to form nodules, and then $NaNO_3$ equivalent to 100 to 2100 lb. per acre was added gradually. The number of nodules was not consistently depressed, although the series containing about 300 lb. of $NaNO_3$ showed a decrease in weight of nodules. When the nitrate was added at the start, both the numbers and weights of nodules were decreased if more than 200 lb. of $NaNO_3$ per acre were applied. NH_4NO_3 was found to be twice as active as $NaNO_3$ in its effect upon the number of nodules. Generally, 100 to 200 lb. per acre of $NaNO_3$ greatly benefited the plant and stimulated nodule formation. GIÖBEL made nitrogen balances to determine whether nitrogen was fixed in the presence of the nitrate. He calculated the nitrogen fixed, by the difference between the nitrogen content of the tops of inoculated and uninoculated plants. Although the data show some fixation even when there was an excess of nitrogen added to the soil, GIÖBEL evidently did not consider this as significant, since he concludes: "Absorption of nitrates and fixation of atmospheric nitrogen by inoculated cultures took place simultaneously when the plants did not receive their full need of nitrogen from the soil. When this was the case, however, scarcely any fixation of nitrogen took place. In general the absorption of nitrates resulted in correspondingly smaller amounts of nitrogen being fixed. In other words the process of nitrate absorption dominated the process of nitrogen fixation." GIÖBEL's conclusions are probably sound, but the data offered are not without serious objections. In the first place he used an indirect method for estimation of nitrogen fixed, and in the second place, as he says, not all of the nitrate nitrogen that disappeared in his cultures could be accounted for in the nitrogen in the tops. This difference was ascribed to nitrate used for root development and *microbial assimilation*. In one experiment he added the nitrate in one-fifth portions at intervals distributed over the growth period and compared the results with those obtained with cultures in which all of the nitrate was added at the start.

He states that no differences could be observed in nodule counts or dry weight of plants because of this difference in treatment.

OHKAWARA (**10**) grew lupine and serradella in small pots of sand. Different solutions containing 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 per cent. of nitrate were added in the proportion of 80 cc. of solution to 400 gm. of sand. The 0.2 and 0.5 per cent. KNO_3 solution and 0.5 per cent. $NaNO_3$ solution prevented the formation of nodules on the plants. 0.2 and 0.5 per cent. $Ca(NO_3)_2$ solution inhibited nodule production on serradella, and the 0.5 per cent. on the lupines. The solutions exhibited no effect or were slightly stimulating from 0.0 to 0.02 per cent., but all higher concentrations exerted a depressing action on nodules. $(NH_4)_2SO_4$ solutions in the same concentrations as the nitrates were also tested. The 0.05 per cent. solution decreased nodule formation, and the 0.1 per cent. solutions were found to be effective in preventing nodules.

In summary, the quantities of alkaline earth nitrates and ammonium salts which are required to affect nodule formation may be briefly presented as follows. In water cultures, concentrations of 1/4000 to 1/200,000 of nitrate nitrogen have been reported to prevent nodules from forming. Ammonium salts have been found effective in concentrations of 1/2000 to 1/100,000. For field experiments, 100 to 300 lb. of nitrate per acre depress or inhibit the formation of nodules, although some workers report that 1000 lb. are necessary. Small amounts of nitrate, however, appear to favor plant growth, and sometimes to increase the number of nodules.

Experimentation

METHODS

The plant cultures were put up under aseptic conditions, and attempts were made to maintain sterility throughout the growth period. The plant used was mammoth red clover, which was set out in cotton-plugged 32-oz. round bottles containing the modified Crone's solution recommended by BRYAN (**2**). In all cases, 0.8 per cent. agar had been added to the plant culture solutions. This concentration of agar gave a gel of proper rigidity, but which was still soft enough to offer very little resistance to the plant roots. The amount of nutrient agar used in each bottle was 200 cc. in experiment I, and 250 cc. in experiment II.

The nitrate used throughout the work was KNO_3. In some cases, varying amounts of this compound were added to the agar before sterilization; in other cases a small amount of nitrate was introduced at the start, and additions were made at intervals. The cotton plug of each bottle was pierced with an 8-mm. cotton-plugged glass tube. The nitrate was added through this tube as a sterile solution with a sterile 1-cc. pipette.

The methods employed in sterilizing and planting the seeds have been described previously by HOPKINS, WILSON, and FRED (9).

For inoculation, a suspension of a pure culture of *Rh. trifolii* strain number 209 was used; this organism has been found to increase greatly the total nitrogen content of the host plant. During the period of growth in the greenhouse the plant cultures were covered with layers of absorbent cotton. These caps were sprayed with a 5 per cent. solution of sodium benzoate containing a bacteriostatic dye. This method of preventing contamination has since been replaced by the use of 10-oz. waxed drinking cups inverted over the tops of the bottles.

The total nitrogen of the plants was determined by the Davisson-Parsons procedure (FRED and WAKSMAN, 7 p. 68) which includes nitrate-nitrogen. The nitrate remaining in the agar was estimated by a modification of this method. The agar was melted and made up to 500 or 1000 cc. After cooling, 50 cc. of approximately normal potassium alum solution was added with agitation. By filtering, a solution practically free of agar was obtained. The nitrate in a suitable aliquot of this was determined by alkaline reduction with Devarda alloy and distillation into $\frac{N}{28}H_2SO_4$. The filtrates of several cultures in each series were tested qualitatively for the presence of NH_3 N and NO_2 N but none was found.

EXPERIMENT I

SERIES 1.—Potassium nitrate was added to the agar at the start in varying amounts, 1, 2, and 4 mg. of NO_3 N per 200 cc. The same quantity of nitrate as originally introduced into the agar was added at intervals of 2 weeks for a period of 10 weeks. Thus, one set of 5 bottles contained 1 mg. of NO_3 N and 5 additions of 1 mg. each were made; a second set contained 2 mg. of NO_3 N and 2 mg. were added five times; a third set contained 4 mg. of NO_3 N and 4 mg. were added five times. The inoculated controls received no nitrate.

When harvested at the end of 4 months the plants of the three sets were very similar in size and color to the controls; all of the cultures were somewhat pale. Distribution and number of nodules were determined on all of the plants; the results are given in table I. In the column headed *No. of nodules* is given the mean of each treatment with its standard deviation.

SERIES 2.—4 mg. of NO_3 N were present in the agar at the start (except in the control), and nitrate was added at the rate of 1 mg. per week for varying lengths of time. In the first set, only one addition was made; in the second, two; and so on up to the last set which received nine additions of 1 mg. each. The control is the same as that used in series 1. The plants, which were 4 months old when harvested, resembled the controls; all

TABLE I

EFFECT OF ADDITION OF POTASSIUM NITRATE PERIODICALLY ON NUMBER AND DISTRIBUTION OF NODULES

TOTAL NO_3 N ADDED	NO. OF SAMPLES	NO. OF NODULES PER 10 PLANTS	DIFFERENCE	PROBABILITY*	SIZE AND DISTRIBUTION OF NODULES
Control	8	124 ± 16	......	....	Principally on tap root, long type
Experiment I, series 1					
6 mg.	5	92 ± 5	32	0.15	Principally on tap root, both long and round
12 mg.	5	72 ± 8	52	0.04	Principally on secondary roots, mostly round and scattered
24 mg.	5	62 ± 12	62	0.02	Same as 12 mg. but smaller
Experiment I, series 2					
5 mg.	3	106 ± 7	18	0.50	Principally on secondary roots, mostly long type
6 mg.	4	96 ± 20	28	0.25	Same as 5 mg.
7 mg.	3	66 ± 20	58	0.07	Principally on secondary roots, mostly round and scattered
8 mg.	3	61 ± 9	63	0.05	Same as 7 mg.
9 mg.	2	77 ± 18	47	0.20	Same as 7 mg.
10 mg.	3	83 ± 11	41	0.20	Same as 7 mg.
11 mg.	3	70 ± 15	54	0.09	Same as 7 mg.
12 mg.	2	91 ± 9	33	0.35	Same as 7 mg.
13 mg.	3	93 ± 27	31	0.30	Same as 7 mg.

Series I. 1 mg. at start; 1, 2, and 4 mg. respectively added every 2 weeks for 10 weeks.

Series II. 4 mg. at start; 1 mg. added per week until designated quantity reached.

* Probability that observed difference could have arisen from random sampling.

attained a fair size but were pale green. The results for series 2 also are given in table I.

EXPERIMENT II

SERIES 1.—The nitrate was added in varying amounts, about 3, 5, 10, 15, 20, and 40 mg. per bottle (250 cc.) at the start of the experiment, and no further addition was made during the growth period. The plants were harvested after seven weeks and examined for size and distribution of nodules as in experiment I. In addition, nitrogen determinations were made so that a nitrogen balance could be determined. In appearance, the high nitrate cultures were somewhat paler than either the low nitrate or the controls. The remainder of the data for this series are given in tables II and IV.

TABLE II

EFFECT OF ADDITION OF POTASSIUM NITRATE AT THE START ON NUMBER AND DISTRIBUTION OF NODULES

TOTAL NO_3 N ADDED	NO. OF SAMPLES	NO. OF NODULES	DIFFERENCE	PROBABILITY	SIZE OF DISTRIBUTION OF NODULES
Control	11	73 ± 4	......	....	Principally on tap root, mostly long type
Experiment II, series 1					
3.3 mg.	4	70 ± 11	3	0.75	Principally on secondary roots, round and scattered
5.4 mg.	3	52 ± 10	21	0.04	Both tap and secondary Round and scattered
10.1 mg.	3	59 ± 10	14	0.15	Principally on tap roots Round and scattered
14.8 mg.	3	49 ± 10	24	0.02	Same as 10.1 mg. but slightly smaller
19.8 mg.	3	60 ± 9	13	0.20	Entirely on secondary roots, all round, small and scattered
39.0 mg.	3	36 ± 5	37	<0.01	Same as 19.8 mg.

SERIES 2.—Nitrate was added in the same amounts as in series 1 (3, 5, 10, 15, 10, and 40 mg.) except that its addition was extended over a period of 5 weeks. Three mg. of NO_3 N were added at the start to all cultures except the controls. The remaining nitrogen was added at weekly intervals; *e.g.*, to the 5 mg. set, 0.4 mg. per week; to the 10 mg. set, 1.4 mg. per week, and so on. The same controls were used for series 1, 2, and 3. The plants receiving nitrate were a paler green than the controls, the color and size of the plant tending to be adversely affected by the increase in nitrate. However, the differences were not so marked that they require consideration in interpreting the results. The plants were harvested after 7 weeks.

The data on the nodules are given in table III and those on nitrogen fixation in table IV.

TABLE III

EFFECT OF ADDITION OF POTASSIUM NITRATE PERIODICALLY ON NUMBER AND DISTRIBUTION OF NODULES

TOTAL NO_3 N ADDED	NO. OF SAMPLES	NO. OF NODULES	DIFFERENCE	PROBABILITY	SIZE OF DISTRIBUTION OF NODULES
Control	11	73 ± 4	......	....	Principally on tap root, mostly long type
Experiment II, series 2[1]					
3.3 mg.	5	54 ± 4	19	.01	Principally on secondary roots; both round and long, scattered
5.4 mg.	5	48 ± 8	25	<.01	Principally on secondary roots; round and scattered
10.1 mg.	5	43 ± 7	30	<.01	Same as 5.4 mg.
14.8 mg.	5	45 ± 8	28	<.01	Same as 5.4 mg. but smaller
19.8 mg.	5	22 ± 4	51	<.01	Same as 5.4 mg. but a few long type
39.0 mg.	5	27 ± 5	46	<.01	Same as 5.4 mg.
Experiment II, series 3[2]					
2.6 mg.	5	78 ± 12	5	0.60	Principally on secondary roots, both long and round, scattered
4.2 mg.	5	69 ± 12	4	0.70	Same as 2.6 mg.
7.4 mg.	5	65 ± 18	13	0.50	Principally on secondary roots, mostly round and scattered
12.2 mg.	5	54 ± 13	24	0.08	Same as 7.4 mg.
15.4 mg.	5	33 ± 3	45	<.01	Same as 7.4 mg.

[1] Series 2. 3.3 mg. NO_3 N at start. Remainder added in aliquots, weekly, for five weeks.

[2] Series 3. 2.6 mg. at start. Remainder added weekly at rate of 1.6 mg. per week.

SERIES 3.—It was intended that the additions of nitrate in this series should be in 2-mg. quantities as in series 1 and 2. However, on analysis of control cultures, *i.e.*, unplanted but receiving the same addition of NO_3 N it was found that the solution added each week contained 1.6 mg. NO_3 N instead of 2.0 mg. For this reason the total quantities of NO_3 N added in this series are somewhat less than the corresponding ones in series 1 and 2 of this experiment. In this series 2.6 mg. of NO_3 N were added to all the cultures at the start, then 1.6 mg. were added weekly until the following quantities were reached: 4.2, 7.4, 12.2, 15.4 mg. of NO_3 N. Thus only one addition was made to the 4.2 mg. set and eight additions were made to the 15.4 mg. set. At the time of harvesting (8 weeks) the plants

TABLE IV

EFFECT OF ADDITION OF POTASSIUM NITRATE UPON NITROGEN FIXATION

NO_3 N ADDED	NO. OF SAMPLES	NO_3 N IN AGAR AT END	NO_3 N USED	NITROGEN* IN SIX PLANTS	NITROGEN FIXED	PROBABILITY†
		mg.	*mg.*	*mg.*	*mg.*	
Control	11	0.0	0.0	4.1	4.06 ± 0.21	
Experiment II, series 1						
3.3	4	0.0	3.3	5.4	2.08 ± 0.55	0.04
5.4	3	0.2	5.2	6.9	1.70 ± 0.35	0.04
10.1	3	3.5	6.6	7.6	1.00 ± 0.34	0.10
14.8	3	8.3	6.5	7.5	1.00 ± 0.34	0.10
19.8	3	12.2	7.6	8.2	0.6 ± 0.53	0.35
39.0	3	31.9	7.1	6.5	–0.6 ± 0.60	0.45
Experiment II, series 2						
3.3	5	0.0	3.3	5.6	2.28 ± 0.16	<0.01
5.4	5	0.1	5.3	6.1	0.78 ± 0.25	0.04
10.1	5	3.5	6.6	7.5	0.94 ± 0.52	0.15
14.8	5	9.1	5.7	6.8	1.14 ± 0.51	0.10
19.8	5	13.8	6.0	6.2	0.20 ± 0.90	0.80
Experiment II, series 3						
2.6	5	0.0	2.6	5.8	3.20 ± 0.26	<0.01
4.2	5	0.0	4.2	6.5	2.28 ± 0.28	<0.01
7.4	5	0.4	7.0	8.0	1.06 ± 0.35	0.04
12.2	5	4.2	8.0	7.7	0.30 ± 0.20	0.20
15.4	4	9.2	6.2	7.1	0.90 ± 0.25	0.05

* Corrected for uninoculated controls.

† Probability that observed fixation could have resulted from random sampling.

receiving the higher quantities of nitrogen (12.2 and 15.4 mg.) were paler in color than the remaining sets. The data are given in tables III and IV.

Discussion

The data given in the tables are best suited for discussion as a whole rather than individually; for this reason interpretation of the results of the various experiments has been reserved for this section of the paper.

Effect of added nitrate on number of nodules

In experiment I (table I) the number of nodules on the control plants, *i.e.*, those receiving no nitrate, was extremely variable; it will be noticed that the standard deviation of the mean is about 13 per cent. of the latter. Unpublished experiments in which we have made a study of the distribution curve of nodule formation in agar under the same conditions as were used in these experiments have indicated that the standard deviation of the mean should be about 5 per cent. of the mean. Because of the high variance in this experiment, significant decrease in the number of nodules in those plants receiving nitrate is rather difficult to demonstrate. The observed differences were tested by the "Student" method as modified by Fisher (3). Our experience has indicated that nodule formation satisfies the fundamental assumption of this method, *viz.*, that the estimated standard deviation is independent of the mean. The 4th column of the table gives the probability that the observed difference could have arisen from sampling. This probability is taken from a table which is entered by calculation of the statistic t:

$$t = \frac{X_1 - X_2}{s}\sqrt{\frac{n_1 n_2}{n_1 + n_2}}$$

$$s = \sqrt{\frac{\Sigma\,(X_1 - \overline{X}_1)^2 + \Sigma\,(X_2 - \overline{X}_2)^2}{n_1 + n_2 - 2}}$$

Where $\overline{X}_1$ is mean of n_1 observations (X_1), $\overline{X}_2$ is mean of n_2 observations (X_2); the table is entered at $n = n_1 + n_2 - 2$. A consideration of the formula shows that if the variance $\left[\frac{\Sigma\,(X_1 - \overline{X}_1)^2}{n_1}\right]$ of the controls is extremely high this will lead to large values of s independent of the variance in the treated samples. This large value of s will decrease the value of t which leads to a high probability that the difference arose from random sampling. For this reason it seems that any probability in this series of the order of 0.05 very likely indicates a significant decrease in the number of nodules. In series 1 the set in which 12 mg. were added at the rate of 2 mg. per week, and the set in which 24 mg. were added at the rate of 4 mg. per week, clearly decreased the number of nodules. In series 2, many of the treated samples have a high variance combined with a small number of samples; this leads to erratic results. Those samples that received 8 mg. of NO_3 N showed significant decreases in the number of nodules but the others did not.

In experiment II, the variance of the controls was much less and very similar to that found in previous experiments. On the other hand, the treated samples very often have extremely large values for the variance. This might be expected, since the effect of nitrate appears to be due to the disturbance of a delicate equilibrium of a nutritional character between plant and bacteria, and wide variations in results of the same treatment are not surprising. Thus in series 1 in which the nitrate is added at the start, it appears that the 5.4 mg., 14.8 mg., and 39.0 mg. sets show a decrease in the number of nodules whereas the 10.1 mg. and 19.8 mg. sets do not. On examination of the original data for these two sets, it was observed that two of the three samples taken showed a significant decrease in the number of nodules formed but the third sample was not affected. This led to a large variance and consequently an apparent high probability that the observed difference arose from sampling. It is likely with larger numbers of samples that this erratic response would be eliminated to a certain extent. In series 2 (table III) in which the nitrate was added over a period of 5 weeks, all the sets exhibited less variability in response to the treatment. In every case, even the 3.3 mg. treatment, there was a significant decrease in the number of nodules formed in the presence of the nitrate. In series 3, wide variation among the replicates in a given treatment was again noticed. For example, the number of nodules per six plants in this series were: 2.6 mg., 36, 101, 70, 101, 80; 4.2 mg., 116, 67, 49, 56, 58; 7.4 mg., 131, 50, 48, 45, 52; 12.2 mg., 101, 40, 59, 34, 38; 15.4 mg., 40, 35, 39, 28, 23. It will be noticed that in each set except the 15.4 mg., four of the five replicates checked very well in the response to nitrate addition but the 5th culture gave a large increase in the number of nodules which led to an increase in both mean and variance for the set. Consequently the effect on four of the cultures was obscured by the unusual behavior of a single culture. Calculation of the probabilities for this series, with the elimination of the erratic culture, gave: 2.6 mg., 0.09; 4.2 mg., 0.05; 7.4 mg., 12.2 mg., and 15.4 mg., < 0.01. Therefore it appears that in four of the cultures in all sets receiving more than 2.6 mg. of NO_3 N, addition of the nitrate decreased the number of nodules; the remaining culture showed an unexplained stimulation in nodule formation.

In general it seems that about 5 mg. of NO_3 N per 250 cc. is sufficient to cause a decrease in the nodule formation of clover plants grown in agar. There is also an indication that, as the quantity of NO_3 N is increased, there is a decrease in number of nodules formed, but this decrease is too variable to be of a linear nature. As far as could be determined, it made little difference in the response of the plant whether the addition of the nitrate was made at the start or periodically. Series 2, experiment II, indicated that addition of the nitrate over a 5 weeks' period was more effective than the other treatments, but this is without doubt partially due to the smaller variability in the data for this series as compared with the others. Finally it is to be noted that in none of our treatments was nodule formation entirely suppressed. The highest concentration used (39 mg. per 250 cc.) corresponds to a concentration of about 1.6 parts per 10,000. This exceeds the concentration that has been reported by various authors as sufficient to prevent formation of nodules. It is very likely that the quantity of nitrate nitrogen necessary for complete suppression of nodules on leguminous plants is variable, dependent on the growth of the plant, which in turn is dependent largely on experimental conditions used.

Effect of added nitrate on the size and distribution of the nodules

The use of the number of nodules as a criterion for evidence of suppression of the activity of rhizobia in leguminous plants is not entirely satisfactory. It was shown in these experiments that the variability in the response of the plants as measured by the number of nodules often obscures the interpretation of a treatment. Because of erratic individuals in a set, differences that are significant are not always apparent when a statistical test is used. Other workers encountering this have discarded the nodule count for other methods. Thus Giöbel used both fresh and dry weight of nodules as a measure of the response of soybeans to the addition of nitrates. He found that in many cases no significant decrease in the numbers of nodules could be discerned but that the weight of nodules was inversely proportional to the quantity of nitrate nitrogen added. Furthermore, there appeared to be a sharp correlation between mass of nodules and nitrogen fixed. While in our experiments it was not possible to weigh the nodules, observations on the size and distribution were made. As shown in tables I, II, and III, the effects of nitrate on size and distribution are extremely consistent. The nodules on the controls were located principally on the tap root, often near the crown; the majority of them were of the long finger-like type. The addition of small amounts of NO_3 N altered this. More of the nodules were found on the secondary roots and the round type of nodule was in evidence. As the quantity of NO_3 N added was increased, the nodules were found almost exclusively on the secondary roots and became progressively smaller and more scattered. This effect on size and distribution of nodules appeared to be fairly independent of the method used for adding the nitrate. However, it does appear that about

3 mg. per 250 cc. at the start is necessary to cause the distribution to change from the tap root to the secondary roots, since in series 1, experiment I, many of the nodules in the 6-mg. set were of the long type and on the tap root. In this set there was only 1 mg. of NO_3 N at the start and 1 mg. was added weekly. In the other series at least 3 mg. were present at the start and in all cases both the 3- and 5-mg. set showed the nodules distributed principally on the secondary roots. From these data it is apparent that observation of the size and distribution of the nodule is a decidedly more consistent method of measuring the effect of nitrate on inoculated plants than is the counting method, even though the results can be expressed only in a qualitative manner.

Effect of added nitrate on nitrogen fixation by the plants

The nitrogen balance for experiment II is given in table IV. These data show that even the smallest quantity of NO_3 N added was effective in decreasing the amount of nitrogen fixed by the association of organism and plant. However, in the presence of nitrate the total quantity of nitrogen found in the plant was increased so that a slight fixation did occur. For example, fixation took place in the 5-mg. set in every series, although it appeared from the controls that about 4.0 mg. were sufficient to meet the needs of the plants grown under these conditions. This suggests that the presence of the nitrate stimulates the plant to a certain extent and a better growth results. It is probable that this stimulation is due to the beneficial effect on the plant of a source of nitrogen during the period of nitrogen starvation, *i.e.*, before nitrogen fixation begins. Finally, it should be noted that in every case in which there is present an excess of nitrate there was no significant fixation. In the border line cases, *viz.*, about 10 mg., there often appears to be a slight gain (order of 1 mg.), but when the errors attendant on the nitrate determination in the agar are taken into consideration, this fixation cannot be considered significant. The results for the 15.4-mg. set in series 3 appear to indicate a slight fixation in this set. This might have been because the small quantity added each week (1.6 mg.) was insufficient for the needs of the plant in the early stages. However, since no fixation was observed in the 12.2-mg. set in this series, it is more likely that apparent fixation was obtained because of errors in analysis of the nitrate left in the agar.

From these data it is concluded that the addition of NO_3 N to inoculated plants does not prevent nodule formation by the bacteria but it does delay establishment of the bacteria in the host plant and modify their behavior toward the latter. Even the addition of quantities of NO_3 N that are much in excess of the requirements of the plants does not entirely prevent the formation of nodules; however, these nodules do not develop normally and appear to act parasitically on the plant. A very delicate equilibrium must exist between the needs of the plants for nitrogen and the fixation process; in all cultures which were not supplied with sufficient nitrogen to meet the needs of the plant, fixation occurred, but as soon as an excess of nitrogen was added, the fixation process appeared to be entirely suppressed in spite of the fact that nodules were present. These effects were independent of whether the nitrate was added at the start or periodically to the plant culture. In the latter case, the addition of NO_3 N at the rate of 2 mg. per week was evidently sufficient to keep a supply available to the plant, if this addition was continued throughout the growth period.

Summary

1. The addition of nitrate nitrogen to clover plants grown in agar generally resulted in a decrease in the number of nodules formed when the concentration exceeded 2 to 3 parts per 100,000. However, the response of the plant to nitrate addition was erratic, and significant decreases were not always noted. Whenever decreases were observed, the effect appeared to be independent of whether the nitrate was added at the start or periodically. However, the most consistent depression of nodule formation was noted in the series in which the nitrate was added over a period of 5 weeks. Complete prevention of nodule formation was not effected by even the highest concentration of NO_3 N used, *viz.*, 1.6 parts per 10,000.

2. The distribution and size of the nodules were markedly affected by all the concentrations used. Addition of even small quantities of nitrate brought about formation of scattered nodules, largely of the round type, on the secondary roots; whereas normally the nodules are mostly of the long type and are found on the tap root near the crown. The size of the nodules was decreased as the quantity of nitrate was increased. These results were independent of the method of adding the nitrate provided a certain minimum quantity of nitrogen was available at the start.

3. All concentrations of nitrate nitrogen used markedly decreased the fixation of free nitrogen. If the quantities of NO_3 N added were insufficient for the needs of the plants, the deficit was made up by fixation of atmospheric nitrogen. However, as soon as an excess of nitrogen was provided, the fixation process appeared to be completely suppressed, even though nodules were present.

University of Wisconsin,
Madison, Wisconsin.

LITERATURE CITED

1. Albrecht, W. A. Symbiotic nitrogen fixation as influenced by the nitrogen in the soil. Soil Sci. 9: 275–328. 1920.

2. Bryan, O. C. Effect of different reactions on the growth and nodule formation of soybeans. Soil Sci. **13**: 271–287. 1922.

3. Fisher, R. A. Statistical methods for research workers. 3rd ed. London, 1930.

4. Flamand, H. De l'influence de la nutrition sur le développement des nodosités des Legumineuses. L'Ingén. Agr. Gembloux **14**: 755–765. 1904.

5. Frank, B. Über die Parasiten in der Wurzelanschwellungen der Papilionaceen. Bot. Ztg. **37**: 377–388, 393–400. 1879.

6. Fred, E. B., Baldwin, I. L., and McCoy, E. Root nodule bacteria and leguminous plants. Univ. Wisconsin Studies in Science no. 5, 1932.

7. Fred, E. B., and Waksman, S. A. Laboratory manual of general microbiology. New York, 1928.

8. Giöbel, G. The relation of the soil nitrogen to nodule development and fixation of nitrogen by certain legumes. New Jersey Agr. Exp. Sta. Bull. 436. 1926.

9. Hopkins, E. W., Wilson, P. W., and Fred, E. B. A method for the growth of leguminous plants under bacteriologically controlled conditions. Jour. Amer. Soc. Agron. **23**: 32–40. 1931.

10. Ohkawara, S. Influence of nitrates and sulfates on the nodule bacteria and nodule formation of genge, lupine, and serradella. Proc. and Papers, Internat. Cong. Soil. Sci. (Washington, 1927) **3**: 172–184. 1928.

11. Rautenberg, F., and Kühn, G. Vegetationsversuche im Sommer 1863. Landw. Vers.-Sta. **6**: 355–359. 1864.

12. ————, ————. Vegetationsversuche im Sommer 1863. Jour. Landw. **12**: 107–140. 1864.

13. de Vries, H. Beiträge zur speziellen Physiologie Landwirtschaftlicher Kulturpflanzen. Landw. Jahrb. **6**: 465–514, 893–956. 1877.

14. Wilson, J. K. Physiological studies of *Bacillus radicicola* of soybeans (*Soja max* Piper), and of factors influencing nodule production. New York (Cornell) Agr. Exp. Sta. Bull. 386. 459–507. 1917.