AN EXTERNAL EFFECT OF INORGANIC NITROGEN ON NODULATION

by

Jack William Tanner

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Crop Physiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1962

TABLE OF CONTENTS

I. INTRODUCTION

Symbiotic nitrogen fixation is one of the most economically important and scientifically intriguing phenomena in plant science. Many research studies have attempted to determine the quantities of nitrogen fixed by nodulated legume crops and, although there has been a wide range reported in amounts of nitrogen fixed, there is general agreement that there exists an inverse relationship between nitrogen fixed and the amount of combined nitrogen in the soil. This reduction of nitrogen fixation and nodulation by combined nitrogen may be regarded as a liability, whereas it should be regarded as an asset. Legumes grown in soils low in combined nitrogen tend to fix more nitrogen, thus increasing the soil nitrogen; where soil nitrogen is higher, less fixation occurs.

The mechanism by which combined nitrogen reduces nodulation and nitrogen fixation has been the subject of many research studies. Results of most studies lend support to the idea that the effect of combined nitrogen is wrought within the plant, the end result being determined by the carbohydrate to nitrogen ratio (relationship). However, numerous results have been reported which seem to indicate that a local effect of combined nitrogen may be acting externally in the rhizosphere. This study was initiated to re-evaluate the role of combined nitrogen in reduction of nodulation and to attempt

to postulate a mechanism for a local or external effect of combined nitrogen in reducing nodulation.

II. REVIEW OF LITERATURE

Twenty years before Hellriegel and Wilfarth (10) demonstrated the relationship between nodules and nitrogen fixation, Rautenberg and Kühn (19) first observed the inhibition of nodule formation by combined nitrogen. Hallsworth (9, p. 189) emphasized the fact that both nodule formation and nitrogen fixation are adversely affected by combined nitrogen. It was extremely important, therefore, to distinguish between the two effects of combined nitrogen.

Many theories have been advanced attempting to explain the effect of combined nitrogen in nodule inhibition. Allison and Ludwig (1) in 1934 presented all of the suggested explanations at that time and discussed the merits and limitations of each. Their conclusions, based on their own studies and their review of previous work by Mazé (13) and by Weber (30) were stated as follows:

> . . . that decreased nodulation in the presence of soluble nitrogenous salts is due to inadequate carbohydrate supply in the roots.
> -
> When nitrogen is abundant, the carbohydrate synthesized is used for top growth, and little is available for the growth of roots or nodules.

This theory, with only slight modifications has been accepted by most workers engaged in this area of research. It can be convincingly demonstrated. In tests where plants were grown

under low soil nitrogen, nodulation has been greatly decreased by weekly spraying with 1/2% or 1% urea solutions (2). The theory is physiologically sound, having its basis in results obtained in growth correlation studies concerning root-top balance (12, p. 201).

However, changes in both nitrogen level and form often provide results which are difficult to interpret using the carbohydrate:nitrogen theory. The addition of low levels of nitrogen often stimulates nodulation (6). Richardson et al. (20), working at the very low levels of 0.5 and 12 ppm found a marked increase in nodulation when the ammonium ion was used, but a decrease when the nitrate ion was used on Ontario variegated alfalfa. At their highest nitrogen concentration (60 ppm) both forms of nitrogen reduced nodulation but the reduction was greater when nitrate was supplied. Gibson and Nutman (8) reported an initial delay, followed by an eventual increase in nodulation using 2.0-20 ppm nitrate or nitrite, but no delay when urea, asparagine or ammonium was used at the same levels. Their experiments were conducted in sterile mineral nutrient agar.

Nutman (15) has suggested that nodules and lateral roots arise at the same foci and that the development of a nodule subtends the development of a lateral root. The delay in nodulation by nitrate and nitrite reported by Gibson and Nutman (8) apparently resulted in more lateral root development,

which in turn resulted in more potential nodule sites. There is evidence, therefore, that even at equal concentrations of nitrogen the reduction caused by nitrate is greater than the reduction caused by ammonium. It is difficult to explain this difference if the carbohydrate:nitrogen ratio is accepted as the sole determinant affecting the degree of nodulation.

Gibson and Nutman (8) in 1960 stated that the "available carbohydrate" or "C:N" hypothesis is an inadequate explanation of nitrate inhibition of nodulation. Nutman (14, p. 135) stated further, that whatever the mechanism, a local reaction at the root surface was involved.

There is some good evidence to support a theory proposing a local or external effect of combined nitrogen. In experiments employing a divided root technique several investigators (5, 7, 31) have shown that nitrate supplied to one part of the root had no influence on infection elsewhere. Gäumann *et al.* (7) employing a split root technique obtained 50.5 nodules on the side containing 1% of the nitrogen employed in Knop's solution and 0 nodules on the side containing twice the level of nitrogen in Knop's solution. Fedorov and Kozlov (5) used similar levels and obtained approximately 400 and 0 nodules respectively. These observations appeared to be in disagreement with the "C:N ratio" theory, for if there was sufficient nitrate in the media to completely inhibit nodule formation, there should have been little carbohydrate available for

nodule growth.

Raggio et al. (18) working with excised bean roots, found that nitrate inhibited nodulation if it was supplied in the medium containing root and bacteria, but not if it was supplied through the base of the excised root. They concluded that this questions the theory of an internal effect of nitrogen and suggests an external effect. Thornton (26) observed a greatly reduced number of infection threads in root hairs when combined nitrogen was in the root medium.

Fedorov and Kozlov (5) found that if nitrate was supplied slowly to the plant at a rate equivalent to fixation, nodule number was stimulated in some species. Gibson and Nutman's (8) results, showing a delay in nodulation when nitrate or nitrite was used, but no delay when urea, asparagine, or ammonium was used, could not have resulted from differences in amounts of nitrogen used by the plants. The urea, asparagine, and ammonium were assimilated in amounts equal to or greater than the nitrate or nitrite.

Thornton (26) was able to show nitrate inhibition of root hair curling. Although root hair curling itself may or may not be an essential stage in bacterial infection of the root hair, its presence indicates that some essential process has occurred. Nutman (17) has stated that, with few exceptions, only curled root hairs become infected. Root hair curling has been shown by Thimann (25) to be caused by bacterial

production of indoleacetic acid (IAA). Production of IAA by rhizobia has been measured only in the presence of plant roots or when tryptophan has been added to the media (11). Tryptophan has been shown to be excreted in trace amounts from intact roots of Pisum sativum (21). The role of IAA in infection is not known but it probably is involved with increasing the plasticity of the root hair cell wall, thus enabling cell wall invagination and infection thread formation (17). Other investigators (11, 24) have indicated that initiation of the lateral primordium giving rise to a nodule was induced from outside by this bacteria-produced IAA.

Few attempts have been made to propose a mechanism for a local effect of combined nitrogen in inhibiting nodulation. Virtanen (29) has suggested that nitrate inhibition occurs through the formation of nitrite which reacts to form a nitrite-leghemoglobin complex. Thornton and Nicol (27) suggest that the local effect is due to nitrate inhibition of the preliminary curling reaction of root hairs. However this should be regarded as an observation rather than a mechanism, but probably merits further investigation. Thornton (26) considered that the carbohydrate level in the piliferous layer of the root was important but did not expand on this idea.

In summation, there appears to be sufficient evidence to support a theory proposing a local effect of combined nitrogen. Nutman (14) has stated that too little is known of the details

of the interrelations of carbohydrate and nitrogen metabolism and auxin activity to formulate an alternative hypothesis. Hence, at the present a mechanism for a local effect of combined nitrogen is lacking. However, as is often the case, developments in other areas of research may provide the knowledge required to propose a workable mechanism. Cheniae and Evans (3), working on the nitrate reductase enzyme system, demonstrated that R. japonicum obtained from soybean nodules contained this enzyme which reduced nitrate to nitrite. In another area of research Tonhazy and Pelczar (28) in their research in IAA oxidase demonstrated that nitrite acted catalytically to destroy IAA. This being true, it is conceivable that small amounts of nitrite could destroy relatively larger amounts of IAA.

A study of the interrelationships between plant, rhizobia, tryptophan, IAA, nitrate, and nitrite, and the overall effect of this interrelationship on root hair curling and bacterial infection, could conceivably provide sufficient information to propose a mechanism for external inhibition of nodulation.

III. METHOD OF PROCEDURE

A. General Procedure

1. Culture of Rhizobium

Four strains of Rhizobium were used in this study: R. japonicum Kirchner (strains 117 and 123, Dr. H. Johnson, U. S. D. A., Beltsville), R. meliloti Dangeard (strain Su 388, Dr. J. M. Vincent, C. S. I. R. O.), and R. trifolii Dangeard (strain 205, Dr. D. T. Parker, Ames). Three of these strains were selected because antisera for checking their purity was available. The bacteria were maintained and cultured for use on slants of the following media:

K_2HPO_4	0.5 g/l
$MgSO_4 \cdot 7H_2O$	0.2 g/l
NaCl	0.2 g/l
Mannitol	10.0 g/l
Yeast Extract	5.0 g/l
Water	1 l
Adjusted to pH	6.5 - 7.0

Pure strains of rhizobia were maintained on slants in (16 x 125 mm) screw-cap test tubes (small slants). Rhizobia grown for experimental use were grown on slants in 1/2 pint square screw-cap bottles (large slants). The large slants were

inoculated with 5 ml of 1% mannitol solution containing a suspension of cells obtained from the small slants. After inoculation the large slants were incubated at room temperature until cell growth was visible, then stored under refrigeration (5°C). Two days prior to use the large slants were again incubated at room temperature. By this method 5 ml of a very heavy suspension of bacteria could be obtained from each large slant.

2. IAA determination

Spectrophotometric: Two drops of conc. HCl were added to 5 ml of the test solution to reduce the pH and then extracted twice with equal volumes of absolute ether (analytical grade). The ether was evaporated and the residue redissolved in 3 ml of distilled water. The addition of 1.5 ml of 0.05 M $FeCl_3$ in 35% $HClO_4$ (Salkowski reagent) resulted in a red color characteristic of IAA (23). The intensity of the red color was determined by measuring the optical density at 530 mμ. The density values obtained were converted to molar concentrations from a standard curve and reported as μ moles/ml of reaction mixture unless otherwise indicated.

Chromatographic: As required, extracts of bacterial cultures were chromatographed for detection of IAA and/or tryptophan. Ascending chromatograms were made on No. 1 Whatman filter paper using the following solvent: isopropanol

- 8 N NH_4OH (4:1 V/V). Guide chromatograms of synthetic IAA and tryptophan were made for comparison of R_F values. Chromatograms were sprayed with Salkowski reagent (see before) to develop color.

3. Nitrite determination

Nitrite determinations were carried out by means of a modified Shinn (22) technique using sulfanilic acid rather than sulfanilimide. Two reagents were required for the test: 1% sulfanilic acid in 1.5 N HCl, and 0.02% N (1 naphthyl)-ethylenediamine · HCl · in water. The test was made by adding 1 ml of each reagent to a 2 ml aliquot of the solution under study. The amount of nitrite was indicated by the intensity of red color in the solution. Quantitative determination of nitrite was obtained by measuring the optical density at 525 mμ. The density values were converted to molar concentrations from a standard curve and reported as μ moles/ml of reaction mixture unless otherwise indicated.

B. Experimental Procedure

1. Hypersurface nodulation

The results of some preliminary studies, where alfalfa seedlings were grown in mineral agar media in test tubes containing various rates of nitrate, indicated that nitrate level affected frequency of hypersurface nodulation. Those nodules

borne on roots above the surface of the agar were designated as hypersurface nodules.

An experiment was designed to determine the relationship between nitrate content in the agar and occurrence of hypersurface nodulation. Ten replications of four levels of nitrate, as KNO_3, were used: 10^{-2} M, 10^{-3} M, 10^{-4} M, and 10^{-5} M. Seed of Medicago sativa var. Vernal was sterilized by soaking 5 minutes in "Chlorox" (5% sodium hypochlorite) followed by 5 rinses with ethanol and 6 rinses with sterile distilled water. In order to detect any contaminated seeds, the seeds were then placed aseptically on mineral mannitol agar media (see Culture of Rhizobium) in petri dishes, using 20 seeds per dish. When the emerging radicals were about 1/2 inch long the seed was aseptically replanted into mineral agar (see below) in sterile (16 x 150 mm) culture tubes, each tube receiving one seedling. The tubes were plugged with non-absorbent cotton to prevent contamination. Each tube was inoculated by adding 0.2 ml of a heavy suspension of bacterial strain Su 388 in sterile distilled water. This amount of inoculant resulted in approximately 10^5 cells per tube. The plants were then placed under continuous light (fluorescent, 800 fc) and nodule position and numbers were recorded daily from the 12th to the 30th day.

The mineral-agar solution on which the seedlings were grown was as follows:

K_2HPO_4	0.005 M
$MgSO_4 \cdot 7H_2O$	0.002 M
K_2SO_4	0.002 M
$FeSO_4 \cdot 7H_2O$ (Versene)	0.6 ppm
Agar	0.8 %
pH adjusted to 7.0	

2. Tryptophan exudation by soybean seedling roots

Seeds of soybean (Glycine max. Merr.) strains T202 and T201 (Dr. C. R. Weber, Ames) were sterilized by soaking in "Chlorox" for 4 minutes followed by 3 successive rinses with ethanol and 5 successive rinses with sterile distilled water. Seeds were then placed aseptically on spun glass over 5 ml of distilled water in sterile (16 x 150 mm) culture tubes. Seeds were permitted to germinate for five days after which the liquid from ten culture tubes was bulked for tryptophan assay. In selecting the tubes for assay care was taken to insure that the seedlings were developed to the same degree. One drop of liquid from each tube was placed on solid mineral nutrient agar media in a sterile petri dish in order to detect and discard any contaminated tubes. Six assays were made on each strain of soybeans. Tryptophan content was determined by using a tryptophan-requiring mutant of Lactobacillus arabinosus in the standard tryptophan bioassay described in the Difco manual (4, pp. 235-236).

3. Nitrite destruction of IAA

In an attempt to obtain more specific data on nitrite destruction of IAA an experiment was designed utilizing 5 levels of sodium nitrite and 3 levels of IAA combined in all possible combinations. The following molar concentrations were used: nitrite - 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and water; IAA - 5.7×10^{-4}, 5.7×10^{-5}, 5.7×10^{-6}, and water. Aliquots were removed from each flask immediately after mixing and at intervals of one day for 4 days. Both IAA and nitrite determinations were made on the aliquots taken.

4. Rhizobial production of IAA and nitrite

a. Rhizobial production of IAA Rhizobia were washed from a large slant and suspended in 100 ml of 1.5% mannitol solution in a 250 ml Erlenmeyer flask. Tryptophan was added to yield a final solution of 150 ml of 1% mannitol solution containing 10^{-4} M tryptophan. Dilution to 1×10^{-10} showed the solution contained approximately 10^6 cells/ml. The time of addition of the tryptophan solution to the bacterial suspension was designated as zero time. The flask was kept stationary throughout the experiment, i.e., not on a shaker. Aliquots of 7 ml of bacterial suspension were removed at designated times and centrifuged for 8 minutes at 20,000 Xg to remove cells. The concentration of IAA in the supernatant was determined colorimetrically as described elsewhere.

b. Nitrate reduction by rhizobia and IAA destruction by the nitrite produced Cells of strain 117 were washed from a large slant and suspended in 100 ml of 1.5% mannitol solution in a 250 ml Erlenmeyer flask. Nitrate (KNO_3) was added to yield a final solution of 150 ml of 1% mannitol solution containing 10^{-3} M nitrate. The time of addition of the KNO_3 to the bacterial solution was taken as zero time. The flask was kept stationary throughout the experiment. Aliquots of 7 ml of bacterial suspension were removed at regular time intervals and centrifuged for 8 minutes at 20,000 Xg to remove cells. Nitrite determinations, as described elsewhere, were made using 2 ml of the supernatant.

The supernatant remaining was used to demonstrate nitrite destruction of IAA. Two ml of supernatant from each sampling interval was added to 2 ml of IAA solution of known concentration (10^{-3} IAA). The IAA which was not destroyed by the nitrite in the supernatant was measured spectrophotometrically as described elsewhere. As nitrite concentration increased with time, IAA concentration was expected to decrease.

c. Simultaneous production of IAA and nitrite by single strains of rhizobia (i) Bacteria of strain Su 388 were washed from 5 large slants, centrifuged at 20,000 Xg and resuspended in 250 ml of 1.2% mannitol. The following reaction mixtures were set up:

tryptophan + rhizobia

potassium nitrate + rhizobia

tryptophan + potassium nitrate + rhizobia

ammonium chloride + rhizobia

tryptophan + ammonium chloride + rhizobia

The final concentrations were as follows: tryptophan, 10^{-3} M; nitrate, 10^{-2} M; ammonium, 10^{-2} M; mannitol, 1.0%; cells approximately 10^{5} per ml. Prior to adding the cells, the reaction mixtures were adjusted to pH 6.8. Aliquots were removed at specific time intervals for determination of IAA and nitrite.

(ii) This experiment was repeated using strains 117, 205, and 388.

d. Simultaneous production of IAA and nitrite by mixed strains of rhizobia (i) Previous experiments had shown that strain 117 produced relatively large amounts of nitrite from nitrate, and that strains Su 388 and 205 showed a greater tendency to convert tryptophan to IAA. A mixture of 2 strains could conceivably produce a system in which both reactions would occur. A series of experiments were instigated to test this assumption. The reaction mixtures used were the same as those used with single strains except twice the level of tryptophan was added. The 2 mixed cultures used were 117 + Su 388 and 117 + 205.

(ii) The cells of 3 large slants of strain 117 and 3

large slants of Su 388 were centrifuged at 20,000 Xg for 8 minutes and resuspended in 180 ml of 1.2% mannitol. This mixture was added to 3 Erlenmeyer flasks (125 ml) producing the three following reaction mixtures:

tryptophan + rhizobia

tryptophan + potassium nitrate + rhizobia

tryptophan + ammonium chloride + rhizobia

The final concentration of each constituent was: tryptophan, 10^{-3} M; nitrate (KNO_3), 10^{-2} M; ammonium (NH_4Cl), 10^{-2} M; and mannitol, 1%. The reaction mixture contained approximately 10^6 cells per ml. The flasks were placed on the shaker and aliquots of 7 ml were removed at intervals of 2 hours for IAA and nitrite determinations.

5. Rhizobial conversion of tryptophan to IAA in the presence of ammonium

Previous experiments had indicated that considerably less IAA was found when ammonium (NH_4Cl) was present in the reaction mixture (see Tables 3, 5, 6, and 7). In some experiments there were trace amounts of nitrite produced from the ammonium, however, in the majority of cases no nitrite was formed. For this reason, some mechanism other than nitrite destruction of IAA was believed to be acting. The possibility that ammonium might inhibit conversion of tryptophan to IAA obviously merited

some study.

Strains 117 and Su 388 were added to reaction mixtures of tryptophan, tryptophan + nitrate (KNO_3) and tryptophan + ammonium (NH_4Cl). Final concentrations of the constituents in the reaction mixtures were as follows: tryptophan, 10^{-3} M; KNO_3, 10^{-2} M; NH_4Cl, 10^{-2} M; mannitol, 1%; and cells 10^6/ml. Aliquots were removed after 24 hours and centrifuged at 20,000 Xg to remove cells. The supernatant was used for IAA and tryptophan determinations. The reaction mixture was diluted by 1/10 for tryptophan assay using L. arabinosus as described previously (see Tryptophan exudation by soybean seedling roots).

IV. RESULTS

A. Experimental Results

1. Hypersurface nodulation

The effect of nitrate concentration on hypersurface nodulation is shown in Fig. 1. These data are based on the 30-day totals. The average time required for nodule appearance after inoculation was 13.9 days for the hypersurface nodules and 18.2 days for the subsurface nodules. Because nodule formation occurred later in the agar than above it, the per cent of subsurface nodules tended to increase with time.

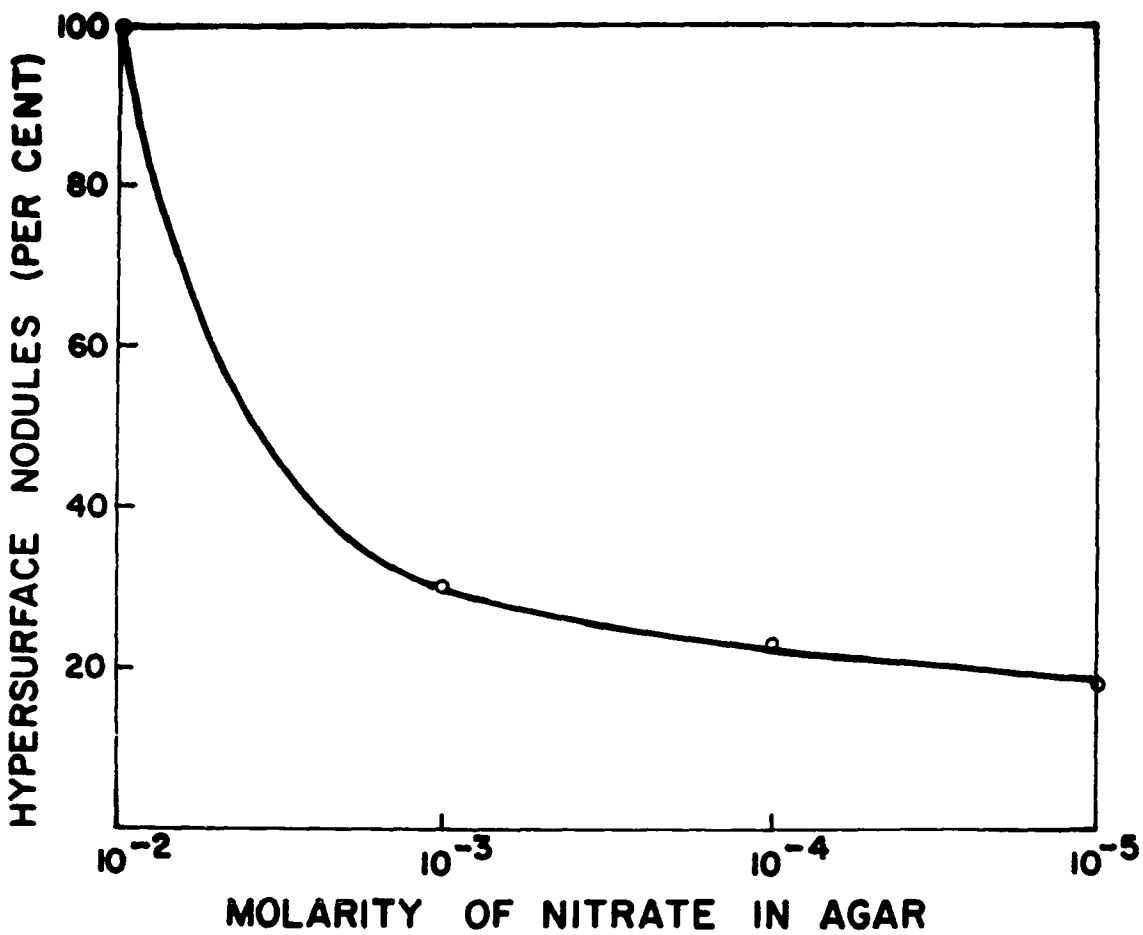
2. Tryptophan exudation by soybean seedling roots

The results of the tryptophan assay are shown in Table 1. With the exception of the higher amounts detected in strain T202 in assay no. 2 the plants were quite consistent in exuding approximately 1 µg per plant in 5 days.

Table 1. Tryptophan exuded by 5-day soybean seedlings

Soybean strain	Tryptophan per plant (mµ moles)					
	Assay no. 1		Assay no. 2		Assay no. 3	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
T201	3.75	3.65	3.75	4.15	4.25	4.50
T202	3.75	4.20	6.75	6.75	4.00	4.15

Fig. 1. Effect of nitrate level on production of hypersurface nodules



3. Nitrite destruction of IAA

The IAA at 5 x 10^{-6} M was destroyed immediately by all levels of nitrite. Concentrations of nitrite below 10^{-5} M gave no measurable destruction of IAA at the upper two levels of IAA. The concentrations of IAA after 48 hours is shown in Table 2.

Table 2. Concentrations of IAA remaining after destruction by nitrite

	IAA μ moles/ml reaction mixture					
	Sodium nitrite added (M)					
IAA added (M)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	H_2O
5.7 x 10^{-4}	.015	.045	.059	.059	.059	.059
5.7 x 10^{-5}	--	.001	.004	.006	.006	.006
5.7 x 10^{-6}	--	--	--	--	--	.001

Nitrite did not appear to act catalytically in that there was no detectable destruction of IAA after the first day. At all levels of added nitrite there was some destruction of nitrite by the high level of IAA. These observations were not in entire agreement with what might have been expected if, as reported by Tonhazy and Pelczar (28) the nitrite acted as a catalyst. However, the fact that nitrite did destroy IAA at relatively low concentrations of each was of real significance

to this study.

4. Rhizobial production of IAA and nitrite

a. Rhizobial production of IAA IAA production was illustrated using strains 117, 123, and Su 388. Because the density of cells in the reaction mixtures varied somewhat between experiments, there were some differences in IAA production between experiments. Strain 117 was very slow in producing IAA and in some experiments IAA could not be detected. Strains Su 388, 205, and 123 produced IAA quite rapidly and in relatively high concentration. Fig. 2 illustrates results which were obtained using strain Su 388.

b. Nitrate reduction by rhizobia and IAA destruction by the nitrite produced The reduction of nitrate to nitrite by rhizobia varied greatly between experiments even though great care was taken to provide uniform conditions throughout. The reason for this variability was not determined, although it appeared that cells obtained from fresh slants were more active in nitrite production. Fig. 3 shows results which were typical for these experiments. Very rapid destruction of IAA occurred even at low nitrite concentrations. There were marked differences in nitrite production between the strains used. Both strains of R. japonicum (117 and 123) caused the accumulation of nitrite in greater quantities and much less time than did the R. meliloti strain (Su 388).

Fig. 2. Tryptophan conversion to IAA by rhizobia (strain Su 388)

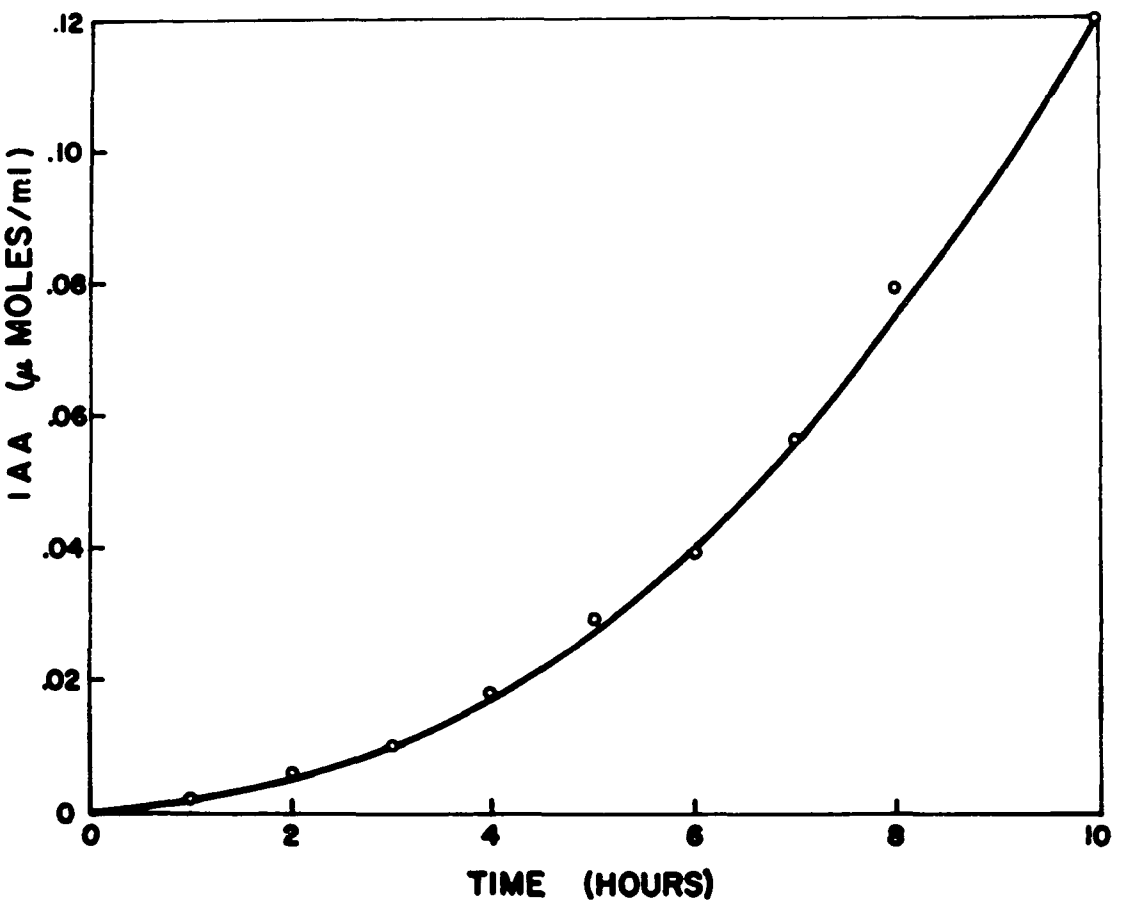
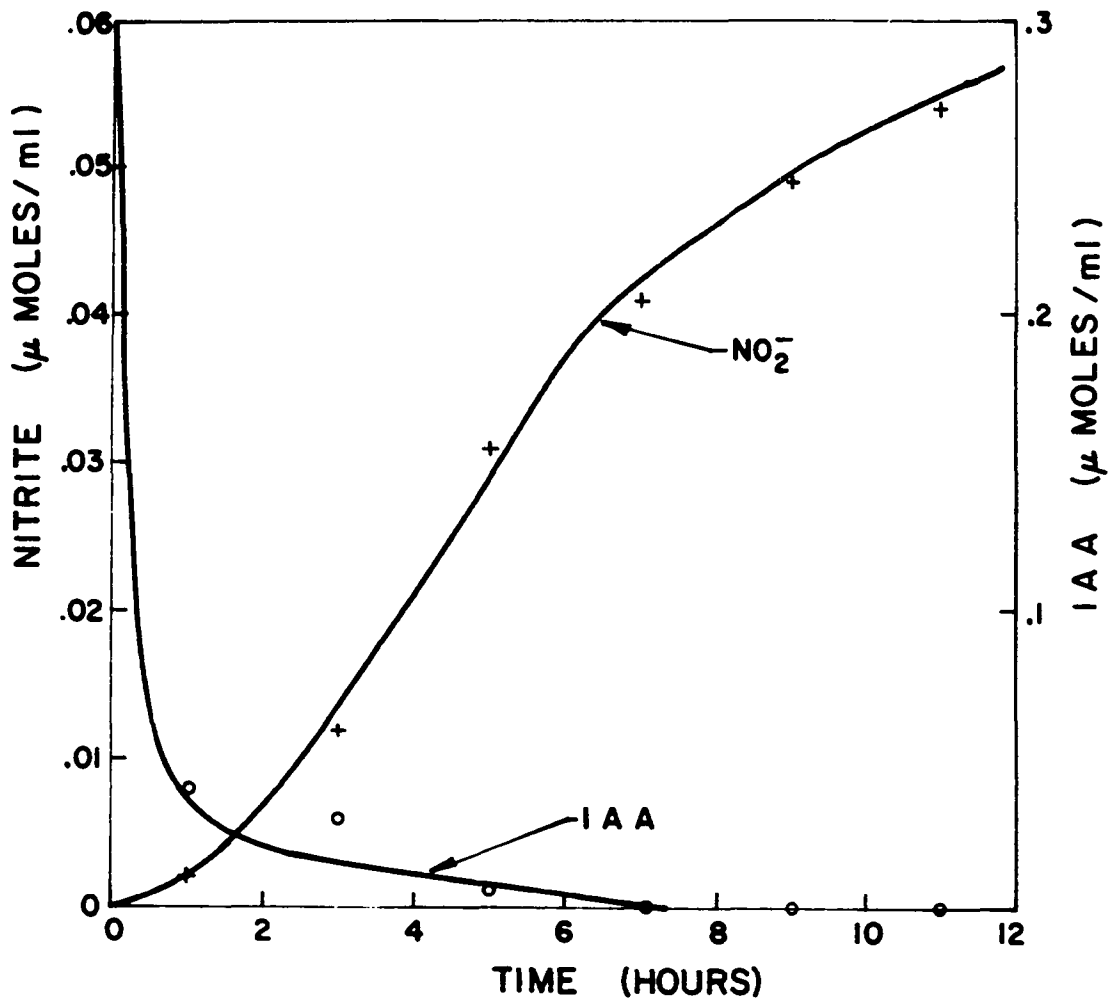

.12
.10
.08
.06
.04
.02
0
IAA (μ MOLES/ml)
0
2
4
6
8
10
TIME (HOURS)

Fig. 3. Nitrate reduction to nitrite by rhizobia (strain 117) and IAA destruction by the nitrite produced


.06
.05
.04
.03
.02
.01
0
NITRITE (μ MOLES/ml)
.3
.2
.1
IAA (μ MOLES/ml)
NO_2^-
IAA
0
2
4
6
8
10
12
TIME (HOURS)

c. Simultaneous production of IAA and nitrite by single strains of rhizobia (1) The production of IAA and nitrite is shown in Table 3. Nitrite production was very low, hence there was relatively little destruction of the IAA produced. The accumulation of IAA was affected more by ammonium than by nitrate even though there was no detectable nitrite produced from the ammonium.

Table 3. Rhizobial[a] production of IAA from tryptophan and nitrite from nitrate or ammonium

Time (hr.)	IAA (μ moles/ml) Reaction mixture			Nitrite (μ moles/ml) Reaction mixture	
	Tryptophan	Tryptophan + KNO_3	Tryptophan + NH_4Cl	KNO_3	NH_4Cl
0	.009	.007	.008	.001	none
2.0	.010	.010	.010	.002	none
4.0	.011	.011	.011	.003	none
6.5	.012	.012	.012	.004	none
8.5	.019	.017	.015	.008	none
12.0	.031	.022	.017	.024	none

[a]Strain Su 388.

(ii) There was no detectable IAA produced by strain 117 even after 20 hours. However, the production of nitrite from nitrate was very high. Table 4 shows the production of nitrite with time. Only trace amounts of nitrite were detected when ammonium was added. This was the first indication that ammonium could be oxydized to nitrite by rhizobia. Table 5 shows the IAA and nitrite production for this group of experiments.

Table 4. Rhizobial[a] production of nitrite from nitrate and ammonium

Time (hr.)	Nitrite (μ moles/ml) Reaction mixture KNO_3	NH_4Cl
0	.015	.003
1.0	.132	.003
3.0	.293	.005
4.0	.408	.006
6.0	.646	.003
7.5	.887	.003
10.0	1.054	.003
20.0	2.261	.048

[a]Strain 117.

Table 5. Rhizobial production of IAA from tryptophan and nitrite from nitrate and ammonium

Rhizobia strain	IAA (μ moles/ml) Reaction[a] mixture			Nitrite (μ moles/ml) Reaction[a] mixture	
	Tryptophan	Tryptophan + KNO_3	Tryptophan + NH_4Cl	KNO_3	NH_4Cl
388	.029	.020	.016	.022	none
388	.027	.021	.015	.012	none
117	none	none	none	1.173	.048
205	.021	.015	.010	.002	.002

[a]Reaction time - 11 hr.

d. Simultaneous production of IAA and nitrite by mixed strains of rhizobia (i) The results obtained by mixing strains showed both nitrite and IAA production and IAA destruction by nitrite. Table 6 shows a summary of these results.

Where nitrite was produced, the IAA was destroyed thereby yielding a lower IAA concentration than with no nitrate added. In most experiments where nitrite was formed in considerable amounts the addition of nitrate seemed to have a greater effect in reducing IAA concentration than did the addition of ammonium. Additions of ammonium reduced the final concentration of IAA with no or only slight production of nitrite indicating that something other than nitrite production was lowering the

Table 6. Production of IAA from tryptophan and nitrite from nitrate or ammonium by mixed strains of rhizobia

Strains	Reaction time (hr.)	IAA (μ moles/ml) Reaction mixture			Nitrite (μ moles/ml) Reaction mixture	
		Tryptophan	Tryptophan + KNO_3	Tryptophan + NH_4Cl	KNO_3	NH_4Cl
388 + 117	16	.013	.000	.007	6.324	none
388 + 117	12	.065	--	.045	.010	.001
205 + 117	12	.063	.021	.022	10.000	none
205 + 117	20	.089	.027	.017	4.000	none
388 + 117	24	.054	.015	.019	.066	none

final concentration of IAA.

(ii) The results of this experiment are presented separately because, quite by accident, they vividly illustrated nitrite destruction of IAA.

The IAA and nitrite production from tryptophan and nitrate are shown in Table 7. Up to the end of 7 hours the flasks had been kept on the shaker. The flask containing tryptophan + nitrate had shown good IAA production (although still considerably less than the tryptophan check) and only trace amounts of nitrite. After the 7-hour aliquot was taken the flasks were inadvertently left on the lab bench rather than being placed on the shaker. The 9-hour sample showed a vivid change. There was a very great increase in the amount of nitrite present, and the IAA which had been present in the 7-hour sample was greatly reduced. Furthermore the water layer remaining after the IAA had been extracted twice with ether appeared visibly orange-brown. This would approximate the tan-colored precipitate that Tonhazy and Pelczar (28) suspected to be indoleacetic aldehyde (polymerized), which was produced from nitrite destruction of IAA.

5. Rhizobial conversion of tryptophan to IAA in the presence of ammonium

The results of the IAA and tryptophan determinations are shown in Table 8. The bacteria grown on tryptophan alone and

Table 7. Rhizobial[a] production of IAA from tryptophan and nitrite from nitrate or ammonium

Time (hr.)	IAA (μ moles/ml)			Nitrite (μ moles/ml)	
		Reaction mixture		Reaction mixture	
	Tryptophan	Tryptophan + KNO_3	Tryptophan + NH_4Cl	Tryptophan + KNO_3	Tryptophan + NH_4Cl
0	.004	.004	.002	.014	none
1	.013	.012	.012	.014	"
3	.029	.028	.020	.008	"
5	.068	.055	.035	.010	"
7	.144	.103	--	.002	"
9	.148	.049	.051	.166	"

[a]Strains 117 + Su 388.

Table 8. Rhizobial[a] production of IAA from tryptophan and nitrite from nitrate and ammonium, and the residual tryptophan

Reaction mixture	IAA produced (μ moles/ml)	Nitrite produced (μ moles/ml)	Tryptophan remaining (μ moles/ml)
Tryptophan	.054	none	.093
Tryptophan + potassium nitrate	.015	.066	.096
Tryptophan + ammonium chloride	.019	none	.152

[a]Strains Su 388 + 117.

tryptophan + nitrate both used the same amount of tryptophan, however where nitrate was in the media there was less IAA due to nitrite destruction of IAA. When ammonium was in the media much less tryptophan was converted to IAA. This was borne out by the lower amount of IAA accumulated and the greater amount of tryptophan remaining.

V. DISCUSSION

It appears that adequate evidence is available in the literature to support a theory proposing a local effect of combined nitrogen in inhibition of nodulation. The observed increase in hypersurface nodulation obtained in this study, while not conclusive, lends support to such a theory.

The physiology of nodule formation, as described by Nutman (16, p. 90, 17), postulates a vital role for IAA in infection. Kefford et al. (11) suggested that the essentiality of IAA was maintained both in infection thread formation and development, and in initiation of cell division leading to nodule formation. Although the precise role of IAA has not been elucidated, its presence, coupled with its general biological importance, has involved it to the point of "near-essentiality" in bacterial infection and nodule initiation. The ideas upon which this study was conducted and the validity of resulting proposed mechanisms both are dependent upon the essentiality of IAA.

Assuming IAA to be essential for infection, it follows that substances which reduce IAA concentration below a certain critical level would reduce and/or delay infection. The duration and severity of the inhibition would be proportional to the concentration of the inhibiting material.

Tonhazy and Pelczar (28) proposed that nitrite acted

catalytically in IAA destruction. Results obtained in the present study support the observation of nitrite destruction but would question somewhat its catalytic role in that little additional destruction of IAA by nitrite occurred over time. However a mole per mole destruction of IAA by nitrite would be a conservative minimum.

The reduction of nitrate to nitrite by rhizobia appeared to vary greatly between strains. R. japonicum strains 117 and 123 consistently produced higher levels of nitrite in the reaction mixture. Similar differences between rhizobia strains with regard to tryptophan conversion to IAA were observed with strains Su 388, 205, and 123 consistently producing more IAA than 117. These differing abilities to produce IAA and nitrite merit further investigation.

The use of mixed strains resulted in production of both IAA and nitrite in amounts adequate to illustrate IAA destruction by nitrite. Mixed cultures probably approximate field conditions more closely than pure strains. Under field conditions, not only would many strains of Rhizobium be present but also many other soil micro-organisms would affect similar reactions.

Considerably less IAA was produced from tryptophan when nitrate was added to the bacterial media. The fact that the same amount of tryptophan was utilized with and without added nitrate indicated that the tryptophan conversion to IAA was

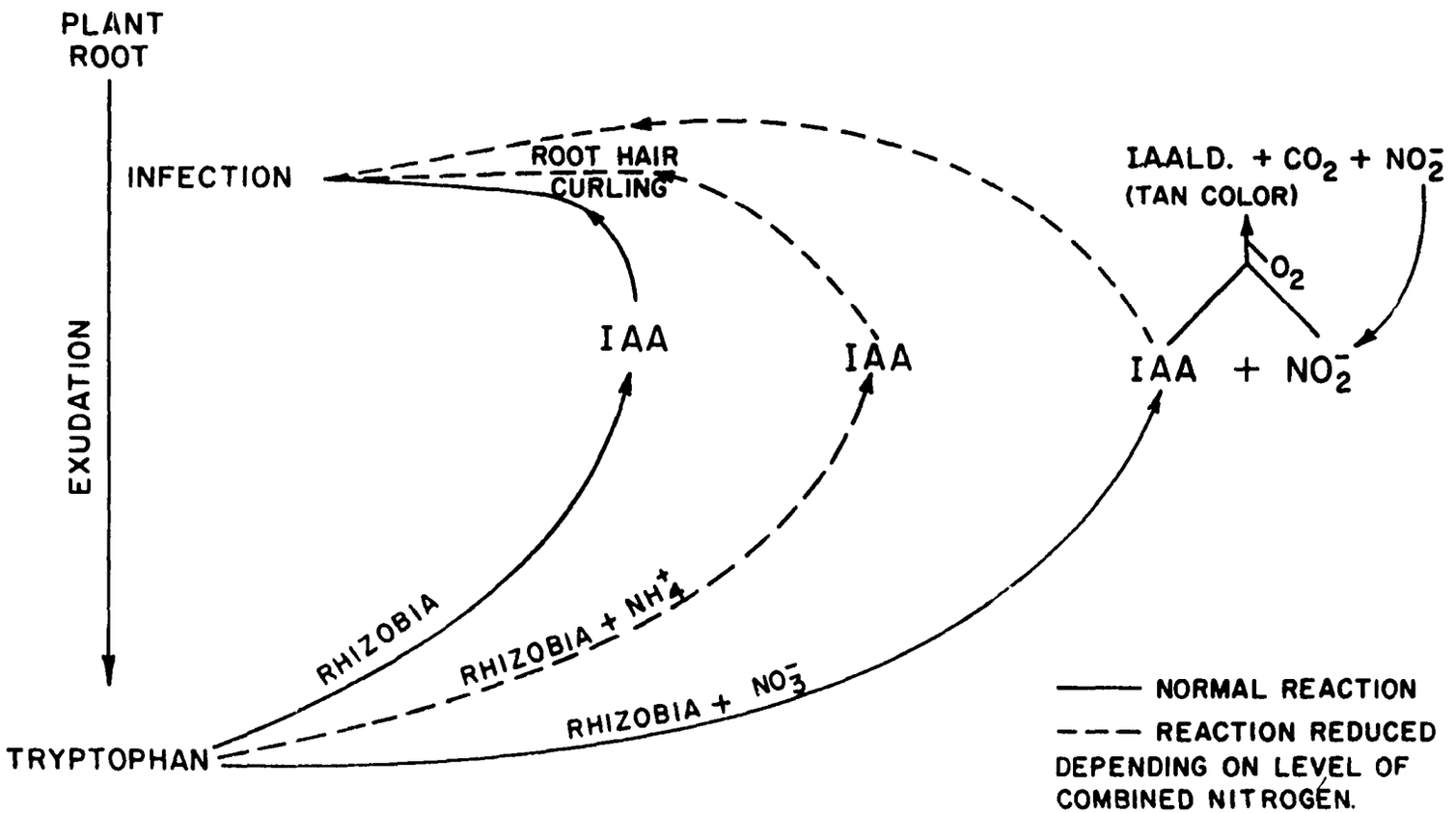
not reduced. The tan-colored precipitate present in the water fraction after IAA had been extracted with ether was evidently the same as that which Tonhazy and Pelczar (28) proposed to be indoleacetic aldehyde. It is obvious that in the presence of nitrate IAA production was not reduced, but that the observed decrease in IAA resulted from nitrite destruction of IAA.

The lower IAA level in the presence of ammonium was the result of a different mechanism. Tryptophan conversion to IAA occurred at a greatly reduced rate. This was shown by determining residual tryptophan after cells were incubated with and without ammonium in the tryptophan media. Trace amounts of nitrite were sometimes detected when ammonium was present. However it was concluded that nitrite production from ammonium did not occur in great enough quantity or frequency to be significant in IAA destruction.

Using the preceding observations, mechanisms for a local (external) effect of nitrate and ammonium were proposed. Reduced concentration of IAA is essential to both mechanisms, although the means by which this is achieved differs with nitrate and ammonium. The proposed mechanisms are represented schematically in Fig. 4.

Concentration of combined nitrogen would determine the degree of nitrite destruction of IAA (when nitrate is present) and tryptophan conversion (when ammonium is present). Complete inhibition of infection would be expected where IAA concentra-

Fig. 4. Diagram of proposed mechanisms of inorganic nitrogen inhibition of rhizobial infection


PLANT ROOT
INFECTION
ROOT HAIR CURLING
EXUDATION
IAA
IAA
IAALD. + CO_2 + NO_2^-
(TAN COLOR)
O_2
IAA + NO_2^-
RHIZOBIA
RHIZOBIA + NH_4^+
RHIZOBIA + NO_3^-
TRYPTOPHAN
NORMAL REACTION
REACTION REDUCED DEPENDING ON LEVEL OF COMBINED NITROGEN.

tion reached a certain minimum level. Insufficient research in this area has resulted in there being too little known with regard to absolute levels present or essential in the rhizosphere. Concentrations of IAA in extracts of root medium are of doubtful significance (11) and probably differ markedly from actual rhizosphere concentrations. The problem of measuring local rhizosphere levels of IAA, tryptophan, etc. represents a very challenging area for future research into the problem of bacterial infectivity. An understanding of rhizobial virulence will require a better knowledge of the interrelationship between all factors involved in infection. Strain differences in production of IAA do exist, but evaluation and significance of these differences cannot be made with the present level of knowledge of the overall infection process.

The theories proposed herein have not been proved by means of nodulation response on plants. For those experiments (see Review of Literature) where inhibition by combined nitrogen cannot be explained by the internal "C:N ratio", the proposed theories present a logical explanation. The delay in nodulation using low levels of nitrate and nitrite but not ammonium, urea, or asparagine (8) could also be explained using the proposed mechanisms. With nitrite or nitrate present, nitrite would destroy IAA until the plant assimilates the nitrite to below inhibitory levels. With ammonium, urea,

or asparagine present, IAA production would be retarded but IAA would not be destroyed. The delay would not be expected to be as long because both plant and bacteria would be utilizing the combined nitrogen and the IAA which had been produced would still be present.

The relative importance of the internal effect of nitrogen versus the external effect is difficult to assess. Under normal conditions both effects are probably operative. However the internal "C:N ratio" primarily affects nodule initiation and development; the external mechanisms postulated retard or inhibit infection. Infection must chronologically precede nodule initiation and development, hence the external mechanism could preclude the internal effect.

Because of the heterogeneous nature of the rhizosphere isolated "escapes" probably do gain entrance. In such instances, and when bacteria gain entrance through wounds, the internal "C:N ratio" would inhibit nodule initiation and development. The two theories (internal and external) would not appear to be contradictory, but instead, quite complimentary and compatible.

VI. SUMMARY

Evidence supporting an external (local) effect of combined nitrogen in nodule inhibition was discussed and additional evidence was presented.

A theory proposing that the local effect of combined nitrogen was wrought through a reduction in IAA concentration in the rhizosphere was described and supporting data presented.

Different mechanisms were suggested for nitrate reduction of IAA level and ammonium reduction of IAA level.

Nitrate nitrogen was reduced to nitrite in the presence of rhizobia. Nitrite destroyed IAA produced by the rhizobia from tryptophan.

Ammonium nitrogen, however, acted to retard the rhizobial conversion of tryptophan to IAA.

Species and strains of Rhizobium differed in their ability to reduce nitrate to nitrite and to convert tryptophan to IAA.

The proposed mechanisms were discussed with particular emphasis on their probable importance in nodulation inhibition and possible implications with regard to bacterial infectivity.

VII. LITERATURE CITED

1. Allison, F. E. and Ludwig, C. A. The cause of decreased nodule formation on legumes supplied with abundant combined nitrogen. Soil Sci. 37: 431-443. 1934.

2. Cartwright, P. M. and Snow, D. The influence of foliar applications of urea on the nodulation pattern of certain leguminous species. Ann. Bot., New Series, 26: 251-259. 1962.

3. Cheniae, G. M. and Evans, H. J. On the relation between nitrogen fixation and nodule nitrate reductase of soybean root nodules. Biochim. Biophys. Acta 26: 654-655. 1957.

4. Difco Manual. 9th ed. Difco Laboratories, Inc. Detroit, Michigan. 1953.

5. Fedorov, M. V. and Kozlov, I. V. The effects of compounds of nitrogen on the nitrogen-fixing activity of nodule bacteria in nodules of soya and pea and the inter-relationships between them and the legume plants. (Translated title) Mikrobiologiia 23: 534-543. 1954.

6. Fred, E. B. and Wilson, P. W. On photosynthesis and free nitrogen assimilation by leguminous plants. Nat. Acad. Sci., Wash. Proc. 24: 46-52. 1934.

7. Gäumann, E., Jaag, O., and Roth, S. Über einen Immunisierungsversuch mit Wurzelknöllchenkakterien bei Leguminosen. Ber. Schweiz. Bot. Ges. 55: 270-277. 1945.

8. Gibson, A. H. and Nutman, P. S. Studies on the physiology of nodule formation. VII. A reappraisal of the effect of preplanting. Ann. Bot. New Series, 24: 420-433. 1960.

9. Hallsworth, E. G. Nutritional factors affecting nodulation. In Hallsworth, E. G., ed. Nutrition of the legume. pp. 183-201. New York, New York, Academic Press, Inc. 1958.

10. Hellriegel, H. and Wilfarth, H. Untersuchungen über die Stickstoffnahrung der Gramineen und heguminosen. Beilageheft zu der Ztschr. Ver. Rübenzucker - Industrie Deutschen Reichs 234. pp. 1888. Original available but not translated; cited in Fred, E. B., Baldwin, I. L., and McCoy, E. Root nodule bacteria and leguminous plants. Univ. Wisc. Stud. Sci. No. 5: 202. 1932.

11. Kefford, N. P., Brockwell, J., and Zwar, J. A. The symbiotic synthesis of auxin by legumes and nodule bacteria and its role in nodule development. Aust. J. Biol. Sci. 13: 456-467. 1960.

12. Loomis, W. E. Growth correlation. In Loomis, W. E., ed. Growth and differentiation in plants. pp. 197-217. Ames, Iowa, Iowa State College Press. c1953.

13. Mazé, M. Les microbes des nodosités des Legumineuses. Ann. Inst. Pasteur 12: 1-25, 128-155. 1898. Original available but not translated; cited in Allison, F. E. and Ludwig, C. A. The cause of decreased nodule formation on legumes supplied with abundant combined nitrogen. Soil Sci. 37: 431-443. 1934.

14. Nutman, P. S. The influence of the legume in root-nodule symbiosis. Biol. Revs. Cambridge Phil. Soc. 31: 109-151. 1956.

15. _____. Physiological studies on nodule formation. I. The relation between nodulation and lateral root formation in red clover. Ann. Bot., Lond. 12: 81-96. 1948.

16. _____. The physiology of nodule formation. In Hallsworth, E. B., ed. Nutrition of the legume. pp. 87-107. New York, New York, Academic Press, Inc. 1958.

17. _____. The physiology of root hair infection. Unpublished paper presented at Iowa State University of Science and Technology, Ames, Iowa, Nov. 21, 1961. Mimeo. Ames, Iowa, Department of Agronomy, Iowa State University of Science and Technology. ca. 1961.

18. Raggio, M., Raggio, N., and Torrey, J. G. The nodulation of isolated leguminous roots. Am. J. Botany 44: 325-334. 1957.

19. Rautenberg, F. and Kühn, G. Vegetationsversuche im Sommer 1863. Jour. Landu. 12: 107-140. 1864. Original available but not translated; cited in Fred, E. B., Baldwin, I. L., and McCoy, E. Root nodule bacteria and leguminous plants. Univ. Wisc. Stud. Sci. No. 5: 202. 1932.

20. Richardson, D. A., Jordan, D. C., and Garrard, E. H. The influence of combined nitrogen on nodulation and nitrogen fixation by Rhizobium meliloti Dangeard. Canad. J. Plant Sci. 37: 205-214. 1957.

21. Rovira, A. D. Plant root excretions in relation to the rhizosphere effect. I. The nature of root exudate from oats and peas. Plant and Soil 7: 178-193. 1956.

22. Shinn, Marsha B. Colorimetric method for determination of nitrite. Ind. and Eng. Chem. Analy. ed. 13: 33-35. 1941.

23. Stowe, B. B. and Thimann, K. V. The paper chromatography of indole compounds and some indole-containing auxins of plant tissues. Arch. Biochem. Biophys. 51: 499-516. 1954.

24. Thimann, K. V. On the physiology of nodule formation. Nat. Acad. Sci., Wash. Proc. 22: 511-514. 1936.

25. _____. The physiology of nodule formation. Third Comm. Int. Soil Sci. Trans. Series A, 1939: 24-28. 1939.

26. Thornton, H. G. The action of sodium nitrate upon the infection of lucerne root-hairs by nodule bacteria. Roy. Soc. Proc. Series B, 119: 474-492. 1935.

27. _____ and Nicol, H. Reduction of nodule numbers and growth produced by the addition of sodium nitrate to lucerne in sand culture. J. Agric. Sci. 26: 173-188. 1938.

28. Tonhazy, N. E. and Pelczar, M. J., Jr. Oxidation of indoleacetic acid by an extracellular enzyme from Polyporus versicolor and a similar oxidation catalyzed by nitrite. Sci. 120: 141-142. 1954.

29. Virtanen, A. I. Microbiology and chemistry of symbiotic nitrogen fixation. Int. Bot. Congr. Proc., Stockholm, 1953, 7: 156-159. 1953.

30. Weber, E. Salpeterdüngung als Beeinträchtigung der Stickstoffsammlung durch Leguminosen. Centralblatt Bakteriologie II, 82: 353-359. 1930. Original available but not translated; cited in Allison, F. E. and Ludwig, C. A. The cause of decreased nodule formation on legumes supplied with abundant combined nitrogen. Soil Sci. 37: 431-443. 1934.

31. Wilson, J. K. Physiological studies of Bacillus radicicola of soybeans (Soja max Piper) and of factors influencing nodule production. New York (Ithaca) Agr. Expt. Sta. Bull. 386: 369-413. 1917.

VIII. ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. I. C. Anderson for his capable guidance during the study and during the preparation of the manuscript. He inspired, by example, and encouraged, by design, individual development of the research study; for this, I will always be grateful.

I would also thank Mrs. Geraldine Hilker for her conscientious assistance in the laboratory.

I am grateful to the Department of Health, Education, and Welfare for a research grant (no. GM09063-02) which partially supported this study.

Special gratitude is expressed to my wife, Joan, whose encouragement and understanding enabled this opportunity to become a reality.