BIOLOGICAL AND ENVIRONMENTAL REGULATION OF NITRATE REDUCTASE IN NAVY BEANS, PHASEOLUS VULGARIS L.

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BIOLOGICAL AND ENVIRONMENTAL REGULATION OF NITRATE REDUCTASE IN NAVY BEANS, PHASEOLUS VULGARIS L.

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ABSTRACT

BIOLOGICAL AND ENVIRONMENTAL REGULATION OF NITRATE REDUCTASE IN NAVY BEANS, PHASEOLUS VULGARIS L.

By

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Experiments were conducted in the field, under controlled conditions in growth chambers, and in the greenhouse to study the environmental and biological factors affecting nitrate reductase in navy beans (Phaseolus vulgaris L.).

These factors included plant part studies, stage of development, variety, availability of nitrate, soil water stress, and other chemical substances, particularly simazine and chlorate. The role of nitrate as an inducing factor was studied. The possible biological interaction between enzymatic assimilation of nitrate and atmospheric nitrogen fixation was investigated.

The distribution of enzyme activity, nitrate and protein varied greatly in different parts of the plant. The leaves showed higher enzyme activity than did the root, nodules, stems, and petioles at the same physiological age.

The enzyme could not be detected in leaf tissues of bean plants grown in sterile cultures with ammonium sulphate in the absence of nitrate. Plants starved of nitrate for short

periods lost enzyme activity which was restored in intact tissues upon resupplying nitrate to the root medium.

The capacity of intact tissues to respond to the inducer varied with their age and position on the plant. Progressive increases in nitrate reductase activity and protein content occurred as time passed during the induction studies. Lag periods in response to nitrate were observed with tissues from plants previously deprived of nitrate. The results are interpreted as evidence for induced enzyme formation in vivo in response to the substrate. Induction occurred most readily in actively-growing tissues.

Studies with cultures of excised roots confirmed the presence of an endogenous nitrate reductase system within the root as well as in nodules. The results indicated that the expanding leaf was the main center of reduction of incoming nitrate. The assays suggest that the enzyme is most active as the leaf was expanding or just as a leaf was fully expanded. Thereafter, enzyme activity fell sharply, although small amounts of activity was detected until a leaf became senescent. These patterns were observed under varied environmental conditions. The extent to which these organs function was apparently influenced by environmental factors, particularly the level of nitrate, available moisture and simazine or salts such as chlorate in the rooting medium.

The nitrate concentration in sap released from the roots was dependent upon the nitrate concentration in the rooting medium.

Significant differences in enzyme activity between inbred lines were evident. The repeatability of the results of nitrate reductase activity on a seasonal mean basis suggested that both genetic and environmental control were involved.

The changes in nitrate reductase activity were not closely related to the total soluble protein content in tissues. However, young tissues had higher nitrate reductase activity and total protein than old ones.

During the period of induction, the increase in activity of the enzyme greatly exceeded corresponding changes observed in soluble protein content in leaf assays.

Varietal differences in nitrate reductase activity and nitrate uptake by roots of plants of the same age were established. The variety with high activity utilized more nitrate than the variety with a low level of the enzyme.

The adverse conditions brought about by drought or a high level of simazine in the root medium caused a reduction in enzyme activity levels accompanied by an accumulation of nitrate. The data suggested that simazine acts as a growth factor at sublethal and very low concentrations. Low concentrations of sodium-azide, potassium-cyanide and potassium chlorate resulted in a reduction of enzyme activity *in vitro*.

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INTRODUCTION

The plant as a functioning organism cannot be separated from its environment since it does not possess within itself all necessary conditions for its existence. It is constantly under the influence of its environment, which shapes and modifies plant development, giving substance to the genetically controlled characteristics and processes.

We may view the bean plant and its environment as a system for the production of plant protein. Within this context, our basic interest lies in a metabolic input system that might under various circumstances be found limiting with respect to yield of seeds or protein. We believe it might be informative as a first step to observe causes of and extent of variation in at least one major input system in beans, and especially among genotypes that differ with respect to some of the obvious growth patterns.

One of several possible major input systems--the reduced nitrogen system--has been chosen for consideration in this thesis. The system is not independent of other metabolic processes, but it was not possible to observe all systems simultaneously. Furthermore, only the primary enzymatic step wherein nitrogen as nitrate (NO_3^-) is reduced to nitrate (NO_2^-) will be considered, since this has been held to be rate-limiting in the process of reduced-nitrogen input.

LITERATURE REVIEW

Pathway of Nitrate Reduction

It is well established that nitrate is a major source of the nitrogen absorbed from soil and utilized by green plants. This process of nitrate reduction is called nitrate assimilation or assimilatory nitrate reduction. The reduction and assimilation of nitrate belongs to the class of fundamental biochemical reactions in the plant. Hageman et al (19) stated that a metabolic pathway was suggested by Mayer and Schultz in 1894 to be:

$$NO_3^- \longrightarrow NO_2^- \longrightarrow H_2N_2O_2 \longrightarrow HONH_2 \longrightarrow NH_3$$

and by Kessler (24) in a more general form as:

$$HNO_3 + 8H \longrightarrow NH_3 + 3H_2O$$

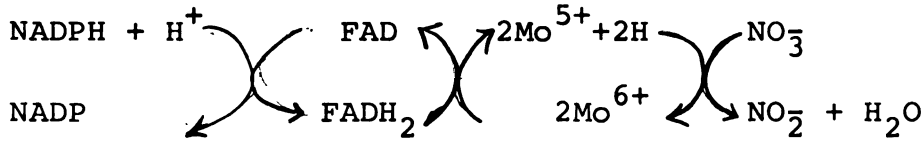
Kessler also reported that the transformation of nitrogen from the oxidation state +5 to -3 thus requires eight atoms of hydrogen or eight electrons which must be supplied by other metabolic processes of the cell. Fewson and Nicholas (13) gave the following sequence of reactions based upon biochemical studies with plants from several phyla:

$$HNO_3 \longrightarrow HNO_2 \longrightarrow NO \longrightarrow (NOH) \longrightarrow NH_2OH$$

The large amount of earlier, mainly physiological, work in this field has been reviewed by Burstron (5). In the course of the past fifteen years, the problem of nitrate reduction has again received much attention. The results of work in several laboratories has been summarized in a number of reviews (4, 30, 32, 36, 47). In most cases enzymology has received the primary attention.

The enzyme responsible for initiating this conversion, nitrate reduction, may exist in different forms, in association with different hydrogen donors, cofactors, and enzymatic reaction sequences depending upon the condition of growth of the organism (33). This enzyme was first obtained by Evans and Nason (9) from the fungus Neurospora. It is a metallo-flavo-protein requiring FAD, molybdenum, and active sulfhydryl groups, with $NADPH_2$ serving as a hydrogen donor (9, 34).

During nitrate reduction, the hydrogen goes from $NADPH_2$ via FAD and Mo to nitrate. It involves a change of oxidation state of the molybdenum between +5 and +6 (35). Kessler (24) described the reaction by the following scheme:



Nitrate reductase has been shown to be present in fungi (34, 44), bacteria (5), and higher plants (6, 9, 14, 18, 20, 43, 47, 50). In some cases $NADPH_2$ acts as the hydrogen donor; in others, $NADH_2$ was effective or even essential.

Light intensity and temperature also influence nitrate reductase activity (50). The reduction of nitrate and its assimilation to ammonia in higher plants depends on several factors including light (6, 30, 50), mineral elements (5, 6, 34), and the source of nitrogen supply (6, 20, 47).

Plant Development and Nitrate Reductase Activity

Candela et al (6) found that net nitrate reductase activity was maximal in mature leaves of cauliflower and was markedly lower in both senescent and rapidly expanding young leaves. Also, they found that extracts from leaves possessed greater net activity than those from stems or petioles, and the activity in the roots was extremely low. The specific activity per mg protein was much greater in petioles than in leaves or stems. Hageman and Flesher (18) reported that the decrease in nitrate reductase with increase in age of corn plants grown in vermiculite was observed repeatedly under varied environmental conditions. Younes (5) reported that nitrate reductase activity was maximal in rapidly expanding bean leaves, distinctly lower in the mature

leaves, and extremely low in senescent leaves under varied environmental conditions. He also reported that at any given stage of growth, bean leaves had higher activity than stems or petioles. The pattern for nitrate reductase, (NR), activity throughout the growing season of beans was also established. Wallace and Pate (47) reported that assays for nitrate reductase in field pea plants suggest that the enzyme is most active just as a leaf is fully expanded. Thereafter enzyme activity falls sharply, although small amounts of active enzyme may be recovered until a leaf becomes senescent.

Nitrate Reductase Activity and Protein Levels

Candela, et al (6) reported that changes in nitrate reductase activity were not closely related to the total soluble protein content of the tissues. In contrast, Hageman and Flesher (18) reported that, in general, there was a positive correlation between the growth (fresh wt.) or protein content of the corn plant and nitrate reductase activity. However, in a later paper, Schrader, et al (41) reported that no correlation exists between the seasonal mean of nitrate reductase and the seasonal mean of water soluble protein. Hewitt and Alfridi (20) showed that an increase in the activity of the enzyme greatly exceeded corresponding changes, if any, observed in soluble protein

content in small fragments of leaves excised from cauliflower, and mustard plants.

Varietal Differences in Nitrate Reductase Activity

Schrader, et al (41) studied nitrate reductase activity, NR, levels in four corn hybrids. They reported that no significant differences were observed between the four hybrids with respect to yields. However, the hybrids, $Hy_2 \times Oh_7$, and Illinois 1996, were shown to have a significantly higher NR level than $WF_9 \times C_{103}$. The hybrids in decreasing order with respect to nitrate reductase activity were: $H_{y2} \times Oh_7$, Illinois 1996, $WF_9 \times Oh_7$, and $WF_9 \times C_{103}$. This order agrees with established agronomic yield performance.

Schrader et al (41) reported that two groups of inbred corn ranking "high" and "low" with respect to seasonal mean nitrate reductase activity were selected and used to obtain F_1 hybrids representing the following categories: "high x high", "high x low", and "low x low".

The level of nitrate reductase activity in the F_1 hybrid appeared to differ from category to category. In the "high x high" category, the level of nitrate reductase activity of the hybrids of all three sets of materials was lower than the mean of the respective parental inbreds. In the "high x low" category, the hybrids were essentially intermediate in nitrate reductase activity with respect to their

parental inbreds and lower in all cases than the higher inbred parent. Two inbreds of the "low x low" category exhibited heterosis for this character. That is, the level of nitrate reductase activity of the hybrids was significantly higher than the level of their higher inbred parent. Other hybrids of this category were not significantly different in NR activity than the mean of their respective inbred parents or the higher inbred.

Nitrate Reductase Activity and Water Change

Maltas and Pauli (29) reported that in young corn plants, NR activity decreased sharply with short exposure to stress before changes in water status became evident. Decreased nitrate reductase activity was reflected in accumulation of nitrate. However, this phenomenon may account for the various observations in which in one case a plant may show better utilization of ammonium nitrogen than nitrate nitrogen.

Nitrate Reductase Activity and the Effects of Stimulators and Inhibitors

The herbicide 2-chloro-4, 6 bis (ethyl-amino)-s-triazine (simazine) has gained widespread use for weed control in corn (Zea mays L.). Several reports indicate that under some conditions, simazine has stimulated the growth of gramineous

cultures (17); (27); (37, 38, 39). Simazine in the nutrient solution culture has been reported to cause an increase in nitrogen uptake by young corn plants (DeVries (8); Ries and Gast (37); Tweedy and Ries (46)). DeVries (8) reported that simazine caused a reduction in the dry weight of tops and roots of corn. Cultures receiving 15 lb/A simazine contained 0.94 mg total nitrogen per pot, compared to 0.71 mg in the control. Tweedy and Ries (46) reported on corn grown at different temperatures using two forms of nitrogen. Simazine at 0.08 ppm in the nutrient solution caused an increase in dry weight and total nitrogen at the lower temperatures when nitrate-nitrogen, instead of ammonium nitrogen, was used in the nutrient solution.

Fink and Fletchall (15) reported that corn treated with 0, 2.5, 5, and 10 lb/A of atrazine and simazine and harvested 5 weeks after planting resulted in a significant reduction in dry matter. Reductions were more pronounced on corn treated with simazine than with atrazine and the herbicides caused an increase in percent nitrate in forage approximately 5 weeks after the treatment.

Audus (2) reported that Exer (12) and Moreland et al (31) have reported that simazine inhibited the photochemical activity of isolated chloroplasts. The simazine inhibited the Hill reaction. The evidence presented by Exer indicated

that the inhibited reaction involved the photochemical reduction of NAD. Interference with CO_2 fixation by Coleus blumei treated with simazine was shown by the blocking of starch production in light.

Roth (40) reported that simazine inhibited photosynthesis in Elodea and had a stimulating effect on the respiration of this plant. A simazine concentration of 0.25 ppm in contact with the roots of 14-day old bean plants for 40 hours resulted in a 30% reduction in 14_{CO2} fixation by the leaves. At 1 ppm and above, CO_2 fixation was almost completely blocked as reported by Ashton et al (1). One hour exposure of the roots to trietazine, simeton and simazine, resulted in an initial increase in CO_2 fixation.

MATERIALS AND METHODS

Experimental Plant Materials in the Field

Most of the work reported in this thesis has been done on plants grown under controlled conditions in a growth chamber or greenhouse; however, in order to extend the scope some work was undertaken on field-grown plants.

Six inbred lines of the navy bean, Phaseolus vulgaris L., three determinate types and three indeterminate types, were planted at the Michigan State University farm on June 7, 1965. These six lines differed in maturity, yield, and some morphological characteristics. All lines were in the sixth selfed generation.

The experimental plan involved three replications in a randomized block design. The plots consisted of three 12-foot rows, 32 inches apart with 3 inches between the plants. The seeds were treated just before planting with a combination of fungicide and insecticide. Fertilizer in the amount of 255 pounds of 5-20-20 plus two percent manganese and one percent zinc was side-dressed at planting time. The plants were thinned approximately a week after emergence to get as uniform a stand as possible. Data concerning reproductive structures were taken on 15 plants randomly chosen and marked in each plot of each of the three replications.

Young fully expanded leaves were selected and combined from plants of each replication as one sample for immediate analysis for nitrate reductase activity and protein.

Sap Collection Method

This study was conducted in sand culture in a growth chamber. The plants were grown under constant temperature (72 $\pm$ 2 F), and a 13 hour photoperiod. Six hours before sap collection, the plants were exposed to light and a temperature of 45 F. Four hours later all the leaves were excised and the cut surfaces of petioles were covered with vaseline. With a sharp blade, the stem was decapitated in a sloping cut 6 inches above the sand surface, then the terminal one-inch length was inserted into a plastic test tube. The top of the tube was covered with parafilm paper to minimize evaporation.

After all plants had been decapitated, the sand was saturated with the proper nutrient treatment. Then the decapitated stems were exposed to constant temperature (72 F.) and constant light.

Immediately after 24 hours of collection, the sap was either analyzed immediately, the usual case, or when necessary, stored at -10C. prior to analysis.

Nitrate Reductase Determination in Roots and Nodules

Washed roots or nodules were separately ground in a chilled mortar and pestle in four volumes of cold 0.4 molar phosphate, pH 7.8, 0.005 M EDTA, and 0.001 M glutathione, and the resulting homogenate was separated from the cell debris by passing it through fine glass wool.

The root or nodule homogenate was centrifuged 10 minutes at 500 G to remove cell debris. The activity of nitrate reductase in the homogenate was determined by the same assay used for leaves (9).

Soil Moisture Level Experiment

The experiment was carried out in a warm greenhouse (approximately 25 F.). The twelve-inch diameter plastic lined pots containing a 1:1 mixture (v/v) of organic soil and sand (to improve aeration of the soil) were sterilized. Moisture blocks were placed two inches beneath the surface and above the bottom of the pot. The percentage of available water above the wilting point was measured by a meter purchased from the N-B Moisture Meter Company. The average of the two moisture blocks was considered the mean moisture level for a given treatment. After seed germination on Kimpack paper, four germinated seeds of line 6 with a radicle length of about 1/4 inch were transferred to each pot and evenly distributed.

Fifty mg of nitrate-nitrogen per plant were added in an equivalent amount and at an even distance from the plants. Six 12-inch pots containing a total of 24 plants represented a given soil moisture level treatment.

All pots under the treatments were saturated with Hoagland nutrient solution (22) without nitrogen. The plants were then treated as follows:

Treatment I; watered immediately after transferring the germinated seeds, and not watered for 20 days before sampling.

Treatment II; watered immediately after transferring the germinated seeds, then 2 weeks later and not watered for 6 days before sampling.

Treatment III; watered immediately after transferring the germinated seeds, every 4th day and left 3 days without irrigation before sampling.

Treatment IV; watered immediately after transferring the germinated seeds, daily thereafter, and left one day without irrigation before sampling.

The soil surface was covered with plastic to prevent surface evaporation. Twenty days after transferring the germinated seeds, the leaves were sampled and assayed for nitrate reductase and nitrate level. Data on plant height, fresh weight and dry weight were obtained.

Experimental Procedure for Studying Nitrate Uptake in Culture Solution

Beans, (Phaseolus vulgaris L.) vars. Algarrobo and Michelite, were germinated on Kimpack paper. Seven days after seeding, when the primary (juvenile) leaves started growing, uniform plants were selected and transferred to black painted glass containers of one liter capacity, covered with aluminum foil, and containing a full Hoagland solution (22) in which ferric citrate was added as an iron source. The solution was changed at the end of the third day, and was continuously aerated. Each container included 900 ml of the nutrient solution, two plants, and one air-stone. The containers were covered with parafilm paper to prevent evaporation. The experimental design was a randomized block. The experiment comprised eight replicates (containers), each containing two plants of the same variety. The experiment lasted for six days. The volume remaining in each container was measured on the third day and at the end of the sixth day. Nitrate concentration was measured in the bulked solution left for each variety using the method of Lowe and Hamilton (28). The volume taken in by the 16 plants of each variety was then calculated: (original conc of NO_3^-/ml x original volume in all containers)--(final conc of NO_3^- in the solution left in the containers x volume left in all containers. The uptake of solution as well as nitrate per plant per day was then calculated. The total dry weight per plant was measured.

Extraction of the Enzyme

It is general practice to include cysteine in the extracting medium to prevent oxidation of the sulfhydryl groups of the reductase enzyme.

The extraction procedure used was as follows:

The plant part to be assayed was removed from six to eight plants and composited to form a sample from each treatment. The sample was immersed immediately in cold (0-2 C) deionized water and carried to the laboratory. The sample was blotted dry, weighed, cut into small pieces, and blended in an Omni-mixer at maximum speed for five minutes at 1-2 C. The grinding medium was 0.1 M Tris, 0.01 M cysteine, and 0.0003 M EDTA at a pH of 7.5 (adjusted with HCl). Four ml of cold (1 C) grinding medium was added for each gram of tissue. The homogenate was pressed through cheesecloth and centrifuged for 15 minutes at 20,000 G. The supernatant liquid was decanted through glass wool and assayed. The homogenates and extracts were kept cold at (1-3 C) throughout. The assay was completed within two hours after sampling.

Assays: The modified procedure was based on that originally used for soybeans by Evans and Nason (9). The assay mixture consisted of 0.8 m. of 0.1 potassium phosphate buffer, 0.2 ml of 0.1 M KNO_3, 0.5 ml of 1.36×10^{-3} M DPNH, and 0.5

ml enzyme extract. The final volume was 2.0 ml. The assay was initiated by addition of first, DPNH and immediately thereafter the enzyme extract. The mixture was incubated at 28 C for 20 minutes and the reaction was stopped by adding 1.0 ml of 1 percent (W/V) sulphanilamide in 1.5 NHCl followed immediately by 1 ml of an aqueous solution of 0.02 percent (W/V) N - (1-naphthyl)-ethylene-diamine hydrochloride (Hageman and Flesher (18). The color was allowed to develop for 10 minutes before centrifuging at 1500 G for 5 minutes to remove the turbidity. The absorbency of the colored product was estimated at 540 mμ using a Unicam SP 600 spectrophotometer. A reagent blank (enzyme omitted) and a reaction blank (NADH omitted) were included with each assay. Enzyme extracts that were heated for 5 minutes in boiling water were inactive.

Nitrate Determination

The method used was based on a simple procedure which utilized soybean nodule bacteroids for reduction of nitrate to nitrite and its subsequent quantitative determination. The bacteroids possess an active nitrate reductase and utilize an exogenous supply of succinate as an electron donor. This is a modified procedure based upon that of Lowe and Hamilton (28).

Culture of soybeans: Soybeans, Glycine Max L. Merr. var. Lee, were grown in a warm greenhouse (30-35 C.) in 12-inch pots containing a 1:1 mixture (v/v) of organic soil and sand. After 3 days of seed germination on Kimpack paper, the radicles were inoculated with an extract of nodules of the same soybean variety previously grown. After a few minutes, newly germinated seeds were transferred to the pots. The plants were watered for the first two weeks from the bottom of the pots by placing them in trays in which a nitrogen-deficient nutrient solution was maintained to a depth of about one inch. The nutrient solution used included nutrient elements as in a micro Hoagland solution (22) minus nitrogen.

Collection of Bacteroids

Five to seven weeks after seeding, nodules were removed from the roots and immediately placed in cooled, distilled water. They were washed repeatedly in cold distilled water, blotted dry, and macerated with a cold mortar and glass pestle in 0.1 M potassium succinate at pH 6.8 (5 ml per gram of nodules). The resulting slurry was squeezed by hand through two layers of cheesecloth and then the liquid portions were centrifuged at 500 G for 7 minutes in a refrigerated centrifuge at -2 C. Sediments containing the bacteroids were resuspended in the original volume of 0.1 M potassium succinate

at pH 6.8 with a stirring rod. After recentrifuging, the sediments were resuspended with the aid of a homogenizer in the original volume of 0.1 M potassium succinate at pH 6.8. The suspension was placed in small test tubes fitted with rubber stoppers through which was inserted a glass capillary tube to facilitate driving all the air from the inside and then was covered with parafilm. This stock suspension of bacteroids was stored in a freezer until a few hours before use when it was replaced in a refrigerator at 0.1 C to melt the frozen stock in preparation for use. This stock suspension was tested and found to be usable for 3-4 weeks if the conditions previously mentioned were maintained.

Assay for Nitrate

A modified assay based on the procedure reported by Lowe and Hamilton (28) was used.

Sample preparation: Nitrate was tested in tissue homogenate extract in the same manner used for nitrate reductase activity, or on sap exudate as mentioned earlier.

Incubation media was as follows:

0.6 ml of 0.1 M potassium succinate buffer at pH 6.8
0.2 ml deionized water
0.2 ml nodule extract
0.1 ml of the sample to be tested (plant extract or sap).

The above were mixed in the same order and incubated at 35 C for 30 minutes.

At the end of the incubation period, the reaction was stopped by adding 1.0 ml of each of the diazo-coupling reagents--1% (w/v) sulfanilamide in 1.5 N HCl--and followed immediately by 1 ml of 0.02% (w/v) N-(1-napthyl)-ethylene-diamine hydrochloride. The absorbence in a 1-cm light path was determined after 10 minutes, using a spectrophotometer at a wavelength of 540 mμ. A duplicate reaction mixture stopped at zero time was used as a reference solution.

Assay for Total Protein in Plant Extract

An adaptation of the biuret method to the estimation of total protein, separated by Kingsley's (25) modification of Howe's procedure (but at a 1 in 4 dilution) was used.

Reagents

Biuret reagent: 1.50 g of cupric sulfate ($CuSO_4 \cdot 5H_2O$) and 6.0 g of sodium potassium-tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$) were weighed and transferred to a dry 1 liter volumetric flask, and dissolved in about 500 ml of water. With constant swirling, 300 ml of 10 percent sodium hydroxide (prepared from stock, carbonate-free, 65 to 75 percent sodium hydroxide solution) were added, made to volume with water, mixed, and stored in a paraffin-lined bottle. This reagent will keep indefinitely but discard if, as a result of concentration or faulty preparation, it shows signs of depositing any black or reddish precipitate.

Procedure

Two test tubes were marked as blank (B) and total protein (T) for a given sample to be tested.

Into tube B, 2.0 ml of 22.6 percent sodium sulfate solution was pipetted.

This "blank" served for the total protein analysis being carried on at any one time.

Into a centrifuge tube, pipette 0.5 ml of plant extract used for enzyme assay and add 9.5 ml of 22.6 percent sodium sulfate. The tube was stoppered and mixed thoroughly by inversion (not by shaking). At once 2.0 ml of the mixture was transferred to tube T. Then into each of the T and B tubes, 8.0 ml of biuret reagent were added and mixed thoroughly by stirring. This mixture was allowed to stand for 30 minutes at room temperature (20-25 C). Using a spectrophotometer transmitting maximally at 540 mμ adjust it to 100 percent transmission with the blank. Replacing B tube with T tube and the percentage transmission (or optical density) of each was recorded.

The concentration of total protein was obtained by reference to the calibration curves using casein as a standard.

Protein Determination in Seeds
Using the U D Y Analyzer

Five hundred mg of seeds were ground into fine powder and placed in the reaction tube. Then 50.0 ml of the reagent dye solution were mixed with the sample. Stopper tightly and clamp the reaction tube securely in the react-R-Mill. Reaction lasted 3 minutes. Filter disc was placed into the screw cap of the filtering bottle, and the reaction tube was emptied into the filtering bottle. The color was left to develop for 10 minutes from the end of the reaction period. Screw on the cap and proceed to filter at least 10 drops with light squeeze pressure. The percentage transmission on the meter was recorded. From the bean standard conversion table the percentage of protein in the seeds was obtained.

RESULTS AND DISCUSSION

Sites of Nitrate Reductase Activity in Different Parts of Bean Plants

Whether nitrate assimilation in a bean plant will follow a specific pathway with a distinct pattern, will depend first upon whether the required enzyme(s), nitrate reductase, is present. Sites of reduction capacity should be recognized before consideration is given to factors controlling enzyme activity. In previous work (50) it was indicated that the leaf is an active site of nitrate reduction in beans, and it was suggested that further work would be required to test for the enzyme in different parts of the plant at different stages of growth.

The phase dealing with roots, stems, petioles, and leaves has been done on uninoculated plants grown in sand culture under greenhouse conditions. Plants were watered daily with Hoagland solution (22). Nodules were collected from five-week old plants grown in the field. The indicated age of roots and nodules presented in Table 1 is approximate. Furthermore, it is assumed to be a combination of different ages (different stages of growth).

Table 1. Nitrate reductase activity in different parts of different ages in bean plants (inbred line #6)

Age of tissue (days)	µg NO_2 20 min/g fr wt			
	Lateral Roots	Stems and Petioles	Leaves	Nodules
10	2.33	0.84	11.24	3.45
20	1.25	0.45	5.95	3.60
34	0.00	0.00	0.80	--

The level of enzyme activity reached its maximum in young expanding leaves while the lowest activity was in the stems and petioles. It is expected, then, that almost all nitrate-nitrogen absorbed by the roots will be transported as free nitrate with the young leaves becoming the main site for reduction. An appreciable amount of activity was present in nodules of early age and in the smaller young roots. The presence of the enzyme in nodules where symbiotic atmospheric nitrogen fixation takes place might suggest that nitrate reductase has something to do with the regulation of symbiotic nitrogen fixation, since it was established that application of nitrogen fertilizers inhibits symbiotic nitrogen fixation. Further work will be required to clarify whether the enzyme is present in the nodule tissues or in the bacterial cells.

Enzyme activity decreased as tissue aged, regardless of the plant part involved. No adequate explanation can be given for the continuous decline in activity with increasing age. This may be due to "spontaneous" inactivation of the enzyme when the substrate was lacking or perhaps to a metabolic destruction, since the loss or decrease in activity occurred in leaves of the same plant at different stages of growth. Preliminary assays for nitrate under conditions suitable for normal bean growth indicated that aged leaves have less nitrate than younger ones.

Table 2. Nitrate reductase activity and nitrate (NO_3^-) - N level in different parts of the navy bean in inbred line #6

	Amount/g fr wt	
Sampled material at 4 weeks of age	N R A µg NO_2^-/20 min	N O_3^- µg NO_3^-
Stems and petioles from the entire plant	0.26	85.60
Leaves from the entire plant	9.85	12.20

It is clear from the data in Table 2 that the leaves are the principal site of nitrate reduction, while the stems and petioles have a very small amount. The low reduction in stems and petioles might not have resulted, from lack of substrate

(NO_3^-), since these tissues contain higher nitrate concentration than leaves of the same physiological age. Other regulatory factors are apparently involved. However, low activity in the stems and petioles might be accounted for in other ways. The first is that the enzyme substrate (NO_3^-) is transported mainly in the vascular tissues, and does not reach the site of synthesis of the enzyme quickly and easily. The cell membrane may present a permeability barrier to the substrate. A second possibility is that the stem and petiole are composed mainly of vascular tissues, with only a minor fraction of cells capable of being induced by nitrate to form the enzyme.

Table 3. Nitrate reductase activity and total protein in different parts of Algarrobo and Michelite varieties

Parts at 5 leaf stage		Amount/g fr wt		
		Nitrate Reductase Activity µg NO_2/20 min	Total Protein mg	Specific Activity
Algarrobo	leaves	4.75	24.5	0.194
	stems	0.45	13.0	0.034
Michelite	leaves	6.95	25.1	0.277
	stems	0.50	13.4	0.037

This last point might be supported by the fact that the specific activity of the enzyme is lower in stems and petioles (0.037 unit) than in leaves (0.277 units), of the same physiological age, (Table 3). What has been said about the substrate could possibly apply to the enzyme co-factors (e.g. $NADH_2$, molybdenum, etc.).

A third possibility is that each of the plant organs might possess a different nitrate reductase system i.e. different enzymes of different functional capacity.

This last point will only be clear after making comparative studies on the structural and functional properties of the pure enzyme from different parts of the bean plant.

Nitrate Reductase Activity in Inbred Lines Grown in the Field

Six of the twelve inbred lines tested for nitrate reductase activity in 1964 were again tested in the field in 1965. The level of enzyme activity in recently, fully-expanded bean leaves was sampled and assayed.

Enzyme activity fluctuated during the period of sampling. There was a continuous decrease in activity with little fluctuation as the plant advanced in maturity. This pattern of enzyme activity in 1965 agreed with that of the 1964 assays.

Table 4. Seasonal mean* of nitrate reductase activity and total protein in recent, fully-expanded leaves grown in the field, 1965

Inbred Line	Amount/g fr wt		
	Nitrate Reductase activity (μg NO_2^-/20 min)	Total Protein* (mg)	Specific Activity
#1	2.05	35.4	0.058
#6	2.60	39.9	0.065
#7	2.73	38.5	0.071
#8	2.84	40.9	0.069
#10	6.25	38.7	0.161
#11	7.74	41.1	0.188
Bottom leaves mature #11	0.30	35.4	0.008
Rapidly expanding leaves #11	22.20	43.7	0.508

*Average of 14 sampling days during the growing season

The 1965 seasonal mean values for total and specific activity of nitrate reductase and total protein content of the six inbred lines are presented in Table 4. A wide difference (more than three-fold) in nitrate reductase activity existed between lines. No meaningful correlation between nitrate reductase activity and total protein content was evident.

Seasonal changes in nitrate reductase (Figure 1) indicate the presence of characteristic patterns in different aged leaves of inbred lines. The leaves of different ages of line #11 show a corresponding change exists between specific activity and activity per gram of fresh weight.

Seasonal mean values were used for preliminary comparisons between lines in order to remove certain obvious differences in rate of development and physiological age at different sampling dates, since enzyme activity and protein content vary with physiological age.

The rapidly expanding leaves had higher nitrate reductase activity, protein content, and specific activity of the enzyme than either recently fully expanded or mature (near senescent) leaves. The latter had the lowest content of total protein, and lowest specific enzyme activity.

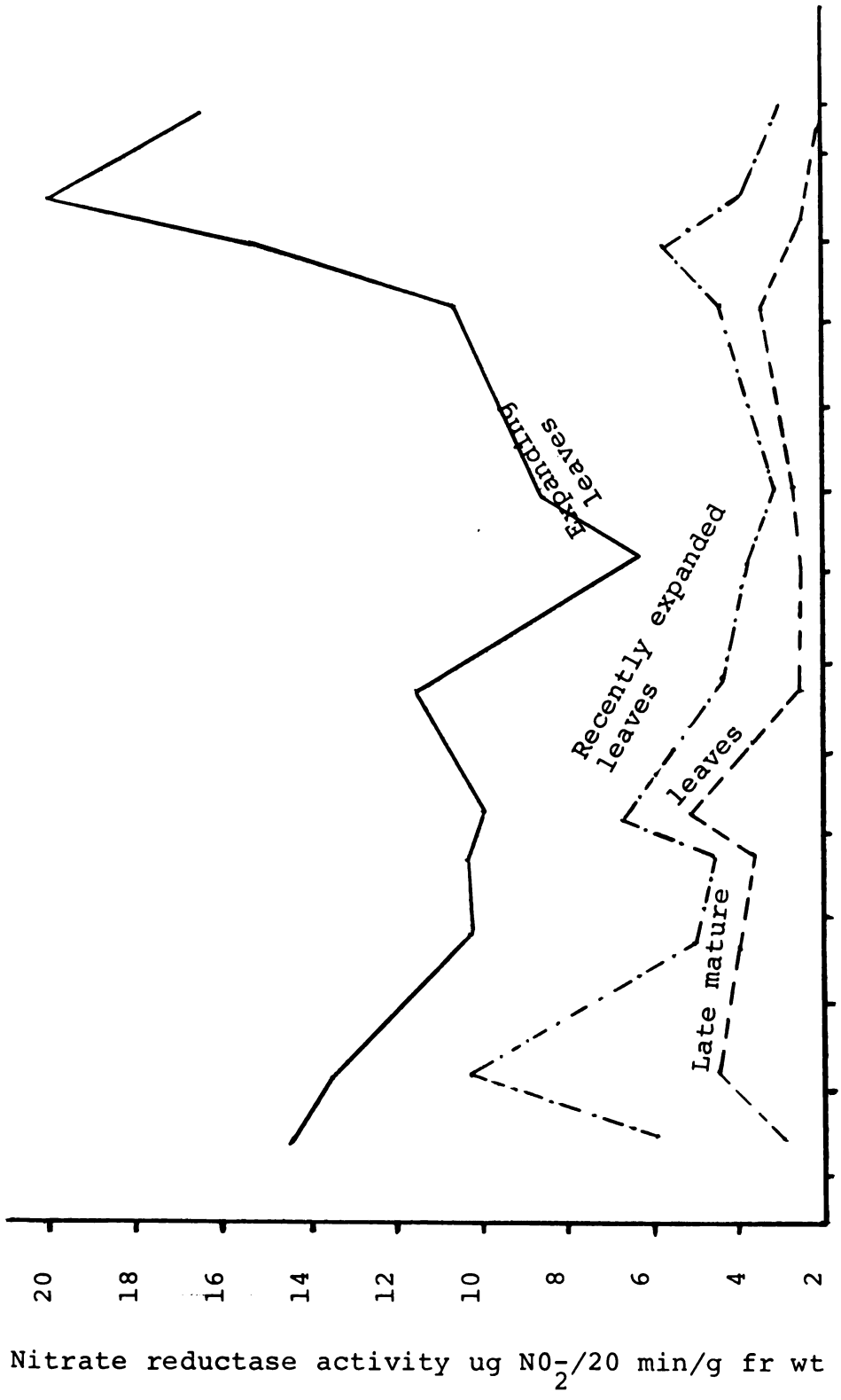


Figure 1. Nitrate reductase activity in bean leaves at different stages of growth and development under field conditions (inbred line #11)

Highly significant differences in total protein content were found between certain inbred lines, Table 4, with line #11 showing higher protein content than lines #1 or #7. No further significant differences in protein content were noted.

With the exception of line #10, the data of 1965 substantiates the data of 1964, Table 5. The repeatability of the data suggests some measure of genetic control in regulating the enzyme.

Table 5. Seasonal mean* of nitrate reductase activity in recent fully-expanded leaves in plants grown in the field

Year	µg NO_2/20 min/g fr wt					
	Line #1	Line #6	Line #7	Line #8	Line #10	Line #11
NRA 1964	1.56	2.80	2.09	3.15	3.93	9.04
1965	2.05	2.60	2.73	2.84	6.25	7.74

*Average of 12 sampling days during the growing season.

Changes in the seasonal mean of nitrate reductase activity from year to year suggest a significant environmental influence. It is also very important to notice that the activity varies greatly with respect to tissue age. Therefore, standardization of the sampling technique was very

important in studying enzyme activity. Large number of samplings representing different stages of metabolic activity and nutritional need of the plant during its growth and reproductive development were also necessary to obtain meaningful data.

Nitrate Reductase Activity in Different Inbred Lines in the Greenhouse

Three inbred lines of beans were evaluated under greenhouse conditions and ranked with respect to levels of nitrate reductase activity in each leaf according to its position. It is clear from the data in Table 6 that a marked (four-fold) difference in enzyme level existed among these inbred lines. The mean levels of nitrate reductase activity in all leaves of the plant of line #8 showed 9.75 units of activity whereas line #12 showed only 2.20 at the same physiological age. Line #4 showed an intermediate activity of 3.80 units. It is also interesting that nitrate reducing capacity was maximal in young meristematic tissues (youngest leaves) and decreased as the tissue aged.

Enzyme activity per unit fresh weight of leaf tends to be reduced once a leaf reached its full size (the 3rd leaf in line #4 and the 2nd in lines #8 and #12). In consequence, the largest enzymatic product was always obtained from the youngest (expanding) leaf. Top leaves always gave a greater

Table 6. Nitrate reductase activity level in bean leaves grown in sand culture in the greenhouse at about 70 F.

Plant material at 4 weeks of age	μg NO_2^-/ 20 min/g fr wt		
	Line #4	Line #8	Line #12
Primary leaf (mature)	0.125	0.20	0.05
1st trifoliate	1.500	6.00	0.50
2nd trifoliate	3.500	7.00	1.50
3rd trifoliate	4.750	13.00	4.25
4th trifoliate (rapidly expanding)	9.250	21.75	4.75
Average of all trifoliates on the plant	4.75	12.00	2.50
Average of all leaves on the plant	3.80	9.75	2.20

enzyme activity than basal leaves. Active enzyme could still be detected in the primary leaf of a five-leaf plant.

The differences among lines in rate of change of enzyme activity between the youngest leaf and the next younger one might be due to differences in the rate of development and physiological stage at the time of sampling.

Nitrate Reductase Activity and Total Protein Levels in Inbred Lines and Their Parents

Algarrobo, a broad leaf variety, and Michelite, a narrow one, were crossed, then during the segregating generations, plants were selected for different levels of yield components. The selections had been inbred for five generations. Twelve of these lines and the original parents were chosen to be evaluated for nitrate reductase activity in leaves during the growing season.

Plants were grown on acid-washed sand in 10-inch sterilized pots with six cultures of each variety, each culture containing four plants. The plants were watered daily with Hoagland nutrient solution. A composite of the four youngest trifoliates from one of the four plants in each of six cultures were assayed for enzyme activity. Three replicates were run from each extract from each composite. Nine assays were conducted during the growing period. The seasonal mean of enzyme activity, total soluble protein, and specific enzyme activity are shown in Table 7.

The data showed striking differences in enzyme activity between Algarrobo with 2.0 units and Michelite with 8.1 units. There was no significant correlation between the enzyme activity and leaf protein when line 614 is compared with line 636. None of the lines tested fell below the low ancestral parent (Algarrobo) in the seasonal mean activity and most were intermediate.

Table 7. Seasonal mean* of nitrate reductase activity levels in young fully-expanded leaves of twelve inbred lines and their parents.

		amount/g fr wt		
		Nitrate reductase activity	Total protein	Specific activity
		µg NO_2^-/ 20 min	mg	
Algarrobo		2.00	26.1	.086
Michelite		8.10	26.7	.303
Pods per plant High	Line 602	6.30	25.7	.249
	Line 603	4.70	25.5	.184
Low	Line 609	4.90	25.5	.192
	Line 610	5.95	27.4	.217
Seeds per pod High	Line 613	5.70	26.5	.213
	Line 614	8.25	27.3	.302
Low	Line 621	6.10	26.1	.233
	Line 622	5.75	27.1	.206
Seed weight High	Line 625	10.50	27.9	.376
	Line 626	7.85	27.6	.284
Low	Line 633	4.40	24.7	.178
	Line 636	5.50	27.7	.198

*Average of 9 sampling days

The high specific activity in lines 625 and 626 (both belong to high seed wt category); might suggest a correlation. Algarrobo is a big seeded variety, having a low specific activity (.086), while line 625 has a high specific activity (.376) and belongs to a high seed weight category. This emphasizes the necessity of broadening the test before drawing any conclusions. However, a correlation of seed protein and leaf protein together with the enzyme activity might be speculated between total leaf protein and nitrate reductase activity during the sampling period. No significant correlation between enzyme activity and protein levels in the leaf has been found in any line, e.g. line 625 (r) = .377 and (r) for 626 = (-.241), for the nine sampling dates involving 27 observations for each.

Even line 602, which had a high seasonal mean of nitrate reductase activity, showed no significant correlation between leaf protein and enzyme activity during the sampling period, r = (-.234). The seasonal mean of enzyme activity does not correlate with total protein. This might suggest that enzyme activity was influenced more by environmental factors than was protein. Low leaf protein in some cases may be the result of translocation of protein to other organs such as pods or seeds.

However, these data again demonstrate differences in levels of the seasonal mean of enzyme activity. This suggests genetic control, not for formation of the enzyme, but for factors which regulate its activity.

Uptake and Assimilation of Nitrate by Uninoculated Seedlings Grown in a Nitrate-Nitrogen-Free Medium

Time course of detection of nitrate reductase in roots and shoots:

Adaptive formation of nitrate reductase in shoots of seedlings of beans was studied following application of nitrate to uninoculated, four-leaf seedlings. The enzyme assays were conducted on shoot tissues represented by the youngest three trifoliates on the main stems. Plants were assayed immediately before treatment and then at various times after exposure of their roots to nitrate. Results are shown in Table 8.

With respect to morphological observations, the plants that received a continuous supply of nitrate were more vigorous, and darker green than those deficient in nitrate. The data in Table 8 indicate that plants grown in the absence of nitrate had an enzyme activity at the end of the starvation period of 0.54 units, whereas plants grown on a continuous supply of nitrate had an activity of 10.85 units. These plants also contained 20.9 and 29.5 total protein units, respectively.

Table 8. Influence of time and nitrate application on the nitrate reductase activity in nitrate deficient bean plants

Inbred line #6	Hours after receiving NO_3			
Amount/g fr wt	0	8	30	Continuous
NRA (µg NO_2^-/20 min/ g fr wt)	0.54	7.47	15.11	10.85
Total protein (mg/g fr wt)	20.90	23.00	27.00	29.50
Specific activity	0.026	0.325	0.559	0.367

Eight hours following nitrate introduction, an increase was obtained in total protein of 2.1 units. The increase in specific activity significantly exceeded protein synthesis, and indicated that protein synthesis might be involved in the enzyme induction as a result of either activation or specific synthesis. After 30 hours an increase in total enzyme activity of 14.57 units and 6.9 units in protein resulted in an increase in specific activity of 0.523 units.

However, after 30 hours of nitrate introduction to the root medium, the total protein content in treated plants was 27.0 units, while in plants receiving a continuous supply of nitrate, it was 29.5 units indicating a slow recovery in protein synthesis after the starvation period.

Induction studies described in Table 8 were limited to young seedlings relying on the nitrogen reserve in their cotyledons. Plants were germinated on Kimpack paper and then transferred after two weeks to a culture nutrient solution aerated with a continuous flow of air through airstone. The nutrient solution contained 180 ppm of nitrate. The cotyledons were removed 24 hours before analysis. When the cotyledons were removed, extracts from untreated seedlings failed to exhibit nitrate reductase activity. Enzymatic activity was detectable in young leaves after a lag period of about one hour (Table 9).

Table 9. Induction of nitrate reductase in the leaves of four-leaf, nitrate deficient seedlings of navy beans (line #6) following exposure of their roots to nitrate

Hours after treatment	μg NO_2^-/20 min/g fr wt									
	0	1/2	1-1/2	2	3	4	5	6	10	43
Treated with NO_3^-	0.00	0.00	1.10	3.14	7.90	11.05	9.42	10.64	8.25	9.75
Untreated (petioles removed)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

There was a rapid and steady increase in the enzyme activity in the first four hours immediately after adding nitrate, then fluctuation became evident as time passed, as shown in Table 8. By removing the cotyledons and lengthening the starvation period, symptoms of nitrogen deficiency were sharply increased in stunted chlorotic, untreated plants.

The increase in the enzymatic activity as shown in Table 8 after adding nitrate, from 0.54 to 15.11, is attributable to induction. The amount of activity present in the untreated plants may have been due to the nitrate in the cotyledons, or that stored in the stems and petioles. A primary problem is whether this considerable increase in specific activity corresponds to the synthesis of entirely "new" enzyme molecules, or to the activation or conversion of pre-existing protein precursors.

The increase in enzyme activity greatly exceeded corresponding changes in total protein content. While the enzyme activity increased from 0.54 to 15.11 units after 30 hours of nitrate treatment, protein increased from 20.9 to 27.0 units and the specific activity, in turn, increased from 0.026 units to 0.559 units. However, study of several other induced systems (23) has confirmed that the inducer brings about the complete de novo synthesis of enzyme molecules which are new by their specific structure and by the origin of their elements.

Adaptive Formation of Nitrate Reductase in Leaves Following Exposure of Effectively Nodulated Plants to Nitrate

In this experiment the nodulated plants were grown in the absence of combined nitrogen in the root medium. At 12 hours on the day of assay, selected seedlings were placed under continuous artificial light and constant temperature (72 F.). The roots received nitrate in a complete Hoagland solution. Before and after nitrate treatment, the leaves of 15 representative plants were harvested at each assay time, and pooled according to their position on the shoot. (The leaves were numbered in sequence from the base of the shoot).

Assay was conducted on the leaflets and the petioles, stipules, and tendrils of each leaf discarded. A study was made of enzymatic induction in primary and second trifoliate leaves.

The enzymatic activities of the extracts of primary and second trifoliate leaves were recorded over a five-week period of growth. The results clearly demonstrate that each leaf seems to pass through a stage in which adaptive formation of the enzyme occurs readily to one where it becomes increasingly difficult to demonstrate induction (Table 10). The highest activity is in the younger leaves. Induction becomes increasingly more difficult to demonstrate in advanced mature leaves. At about six or seven weeks of age only a

Table 10. Adaptive form of nitrate reductase activity with respect to induction and stage of development of primary and second trifoliate leaves of line #6

Leaf Age in Days	N R* in Primary Leaves	N R* in 2nd Trifoliate
7	0.05	0.15
8 →	0.04	0.06 ←
9	6.40	8.50
10	14.80	16.00
12	16.42	18.50
20	11.35	13.00
25	7.42	9.40
32	0.94	3.00
36	0.45	1.90
39	0.00	0.70
42	0.00	0.00

NO_3^- added twelve hours before next assay

NO_3^- added in sequence 12 hours before next assay

* µg NO_2^- produced in 20 min/gm fr wt

small amount of activity, if any, is detectable. In any case, the data demonstrate the time-course of response (induction) of nitrate reductase to nitrate.

The groups of bean seedlings, growing either in water, ammonia as the nitrogen source or nutrient media lacking nitrate, did not show any nitrate reductase activity, indicating that these seedlings did not contain the active form of the enzyme. The enzyme only appeared after nitrates had been introduced into the medium. Seedlings, when transferred again into a buffer solution lacking nitrate lose nitrate reductase activity.

Effect of Nitrate Supply on its Transfer from Root to Shoot in Uninoculated Seedlings

Uninoculated, nitrate-starved seedlings in the four-leaf stage were treated with nitrate representing 10.0 and 60.0 mg per liter. The plants were grown in sand culture and the sand was saturated with nutrient once every 12 hours. The plants had been grown under continuous light and constant temperature (70 ± 2 F) in a growth chamber for two weeks after emergence. Sixteen plants selected for each treatment were decapitated and all the leaves were removed (i.e. two hours after supplying the nitrate). The exudate from the stem was collected every 12 hours from all the plants and pooled for a given treatment. In each case the exudate was collected

for a period of 12 hours and was held at -10 C until a 24-hour collection was combined as one sample for nitrate analysis.

From the data of Table 11, the level of nitrate in the sap was higher for plants treated with 60.0 mg NO_3^--N per liter in the nutrient solution. This indicated that the absorption of a nutrient (NO_3^-) element to a great extent depends on the supply.

Table 11. Effect of level of nitrate in the nutrient on the rate of its release from the roots of decapitated seedlings of nitrate deficient plants - line #6

mg NO_3^-/liter	PPM NO_3^- in the sap		
	1st day	2nd day	7th day
10	80.00	140.00	60.10
60	145.00	460.00	110.00

The amount (or concentration) of nitrate in the exudate builds up for at least the first 48 hours and then declines, as the seedling responds to decapitation and loss of photosynthetic area. There was a drastic reduction in volume of sap and in nitrate concentration on the 7th day of collection.

Varietal Differences in Nitrate Reductase and Nitrate Uptake in Nutrient Culture Solution in the Greenhouse

Uninoculated bean seeds of Algarrobo and Michelite were germinated on Kimpack paper. Seven days after seeding, when the primary (juvenile) leaves started growing, uniform plants were selected and transferred to darkened glass containers, (covered with aluminum foil), of one liter capacity. Each container included two plants (of one variety), and were aerated with one air-stone with a reasonably uniform continuous air flow. The experiment was conducted for a period of six days. The nutrient solution was changed every three days. Nitrate concentration was measured in the bulked solution left for each variety using the method of Lowe and Hamilton (28).

The uptake of solution and nitrate per plant per day was then calculated. The leaves from eight plants were removed and composited to form a sample for measurement of nitrate reductase activity, Table 12.

Algarrobo is a bean variety produced and grown in Columbia, South America, and Michelite is a variety once widely grown in Michigan.

The results emphasize the marked differences in the amount of uptake of water and the nitrate ion. While Algarrobo used 20.1 ml of the nutrient solution, (presumably mainly

water) per plant per 24 hours, the Michelite variety used 8.4 ml per plant.

Table 12. Varietal differences in nitrate reductase and nitrate uptake in beans grown in the greenhouse

Variety	Amount per plant			N R
	Ml solution absorbed/ 24 hrs	µg NO_3^-/ml of nutrient solution absorbed	µg NO_3^- used per 24 hours	µg NO_2^-/ 20 min/ g fr wt
Algarrobo	20.1	4.5	90.5	3.9
Michelite	8.4	14.7	123.5	9.9

Algarrobo absorbed 4.5 µ gm nitrate per ml solution used while Michelite absorbed 14.7. This might be a result of more transpiration from Algarrobo because of its broader leaves.

The amount of nitrate used per day per plant was significantly different between the two varieties. Algarrobo used 90.5 µgm while Michelite used 123.5.

Albarrobo also had a lower nitrate reductase activity than did Michelite. The rate of nitrate uptake did not correspond well with the rate of nitrate reductase activity. It might be expected that the faster expansion in the size of the broad leaves of Algarrobo, in comparison with the narrow

Michelite leaves, resulted in a dilution of the enzyme as the leaf grew. If this is the case, the rate of enzyme activity partially depends on the rate of leaf growth. Thus developing an index compounding the age of the leaf with its size might be more indicative of enzyme activity.

Biological Interaction Between Nitrogen Fixation and Nitrate Assimilation in Legumes

The importance of legume plants in relation to soil fertility depends on whether they effectively fix nitrogen. The importance of having appropriate rhizobia in the soil is seen particularly when cultivated legumes are introduced into areas from which they are normally absent, or into freshly reclaimed land, or areas devastated by fire, etc., for then nodulation fails to occur due to lack of effective rhizobia. Also, nitrate-nitrogen inhibits nitrogen fixation when present in appreciable amounts.

This experiment was carried out in a warm greenhouse (70-80 F). The twelve-inch pots containing acid-washed sand were washed with deionized water and then steam sterilized. One-half of the seeds of the Saginaw and Sanilac varieties were surface sterilized in 1% borax solution then washed several times in deionized water. Four seeds were planted in each pot. The remaining half of the seeds were inoculated with Rhizobium phaseoli, and then planted, four in each pot.

This experiment comprised five replicates (pots) each containing four plants of the same variety. All the plants were watered with nutrient solution lacking nitrate for the first two weeks from sowing to allow the Rhizobia to start effective nodulation. Then the plants were watered with a Hoagland solution modified to include nitrate at one-third the normal level.

During the vegetative stage and early flowering, the youngest three trifoliates were sampled for nitrate reductase activity. The results are shown in Table 13.

Table 13. Nitrate reductase activity* in the top three trifoliates of bean plants grown in the greenhouse

Treatment	11/10/67	12/27/67	12/30/67	1/5/68	1/14/68	Seasonal mean of NRA*	% of protein in seed
Saginaw inoculated	19.49	24.24	9.30	6.35	7.35	13.37	20.1
uninoculated	20.00	27.76	11.12	8.11	15.29	16.28	19.95
Sanilac inoculated	6.76	4.41	9.64	5.76	7.76	6.87	22.95
uninoculated	6.97	10.24	10.88	7.53	14.00	9.92	22.75

* $\mu g\ NO_2^-/20$ min/g fr wt

The data showed that a reduction in enzyme activity in the leaves of the inoculated plants occurred in relation to those uninoculated. There was no significant difference in assay on the first sampling date. This is probably a result of ineffective nodulation at two weeks of age. The rest of the assays showed a significant reduction in enzyme activity in inoculated plants. The reduction in enzyme activity in nodulated plants might be a result of interaction between symbiotic nitrogen fixation and enzymatic nitrate assimilation. At the present, the nature or cause of the postulated interaction is not known; possibly certain amino acids produced via a symbiotic nitrogen fixation pathway inhibit the enzyme.

Filner (14) has demonstrated that certain amino acids act as co-repressors of nitrate reductase induction in tobacco callus tissue. Furthermore, one might speculate that as a result of inoculation and nodulation, an inhibition of nitrate uptake or transport occurs or inhibition is a result of intermediary substances containing nitrogen produced via the symbiotic nitrogen fixation pathway. As mentioned earlier, the existence of nitrate reductase in nodules might suggest this kind of interaction.

The data showed no significant differences in total protein nitrogen in the beans from the inoculated and uninoculated plants. This was the case for both varieties.

Effect of Soil Moisture Level on Nitrate Assimilation

Little is known concerning the effects of water shortage on the metabolism of nitrate. It is generally believed the hydrolytic reactions predominate and synthetic reactions are depressed by water shortage, but the evidence is contradictory. Interpretation of the effects of water-deficit on changes in metabolism in vascular plants is complicated by the fact that water deficits are rarely measured in the particular tissues being investigated and by indirect effects arising from variations in stomatal opening, leaf temperatures, transport and mineral uptake.

Some of these complications could be studied on excised roots, tissue slices of water plants or unicellular organisms. But findings obtained with these tissues or organisms must eventually be tested on vascular plants.

A study was conducted to show the relationship between water level in soil and nitrate reduction capacity in bean plants. Bean plants of inbred line #6 were grown in soil (1:1 sand to organic matter) in 12-inch plastic lined pots. The plants were grown in a greenhouse at approximately 25 F. The soil surface was covered with plastic to prevent surface evaporation. At no time during the growing period did the plants in any treatment wilt. At the end of the 20th day after transferring in soil the moisture level of the soil was

determined as percent of available moisture above the wilting point in each of the treatments. Immediately plants were assayed for nitrate and nitrate reductase. Data on plant growth were recorded. The results are shown in Table 14. The data show that bean plants responded to differences in soil moisture regimes (levels) with respect to nitrate reductase activity. A depression occurred in nitrate reduction capacity over the range of available soil moisture.

It is difficult at present to evaluate whether the decreases in growth rate were caused directly by the increasing water stress or indirectly by decreasing nitrogen, or lack of availability or mobility of molybdenum.

The result of this experiment, however, clearly demonstrates that moderate soil moisture stress caused a considerable decrease in vegetative growth and fresh weight production. The data also showed that nitrate accumulation accompanied the reduction in soil moisture. One could speculate that the decrease in enzymatic activity might be due to depressed substrate transport, the nitrate ion being unable to reach the enzyme site quickly or easily; or that the cell membrane under conditions of water stress might present a permeability barrier to the substrate as in the case where nitrate accumulates. A further possibility is an inactivation of the enzyme or reduction in its synthesis brought about by lack of availability or mobility of molybdenum

as a result of water stress. Since nitrate reduction *in vivo* is not an independent system, still other factors might be involved.

Table 14. Response of bean plants to change in soil moisture levels and its effect on nitrate reductase activity and nitrate in leaves

Treatment	Percent of Available Soil Moisture	Amount/g fr wt		Plant height inches	Fr wt g	
		NRA µg NO_2^-/20 min.	NO_3^- µg NO_2/ 30 min		Top	Root
I	10	0.1	86.0	5.0	1.5	0.2
II	26	1.9	76.5	8.0	3.5	0.4
III	45	5.2	59.5	14.0	9.0	0.9
IV	95	8.6	30.0	19.5	15.0	1.2

In another experiment conducted in a growth chamber where relative humidity was kept high ($90 \pm 4\%$) with 14 hours of light and a 10-hour-dark photo period at 75 F, there was less accumulation of nitrate under drought stress. This might be due to the high relative humidity causing reduced transpiration which, in turn, might have enabled the plant to efficiently reduce nitrate available under conditions of low soil moisture, optimum temperature, and light intensity. A second possibility is that the relatively high humidity indirectly resulted in maintaining the cell turgidity and

preventing denaturation of some proteins that might include the enzyme, nitrate reductase. This, in turn, might have maintained the enzymatic activity at a reasonable rate and prevented nitrate accumulation under the adverse conditions brought about by drought. Under field conditions in dry areas, relative humidity is generally low, thus nitrate may accumulate. This point should be distinguished from situations such as under cloudy or rainy days wherein nitrate tends to accumulate; this effect can be attributed to temperature or light intensity which significantly affects the nitrate reduction system in beans as indicated by previous work (50).

Chemical Toxicity and Adaptation of Plants to Chemical Application

It is assumed that the study of the toxic effect of herbicides, other chemicals, or even salts on plants will lead to a better understanding of plant tolerance to adverse conditions.

A. Effect of simazine levels on nitrate reductase activity and nitrate levels in leaves:

These studies were conducted in pot experiments in the greenhouse. Each pot contained four uninoculated plants, two of the Saginaw variety and two of the Sanilac variety. The plants were grown on acid-washed sand and watered daily with Hoagland solution, except that the nitrate-nitrogen level was at one-fourth strength.

When the plants were seven days of age, the pots were distributed in a randomized block with five pots representing each treatment level. Precautions had been taken before spraying simazine on the beans to prevent any falling on the sand on which the plants were growing. Seven days after treatment the youngest four trifoliate leaves were collected and combined as one sample for measurement of nitrate reductase activity and nitrate level. The results are shown in Table 15.

Table 15. Effect of simazine levels on nitrate reductase activity and nitrate levels

Simazine ppm	Amount/g fr wt			
	Saginaw		Sanilac	
	NRA µg NO_2^-/ 20 min	NO_3^- µg	NRA µg NO_2^-/ 20 min	NO_3^- µg
Control	8.2	58.8	13.6	63.8
0.05	10.6	66.0	16.2	70.8
0.2	5.8	67.2	13.3	75.6
1.0	5.0	91.7	12.3	85.6
2.0	5.3	91.1	13.0	98.6
5.0	4.8	93.2	7.3	94.1

The spraying of the beans with 0.05 ppm simazine resulted in a significant increase in enzyme activity in both varieties. However, a reduction of 2.2% and 29.75% in nitrate

reductase activity, respectively, occurred in Sanilac and Saginaw beans with the 0.2 ppm treatment. Saginaw was less tolerant to simazine up to 2.0 ppm than Sanilac when nitrate reductase activity was taken as a parameter.

Nitrate reductase was inhibited 41.46% at 5.0 ppm in Saginaw while Sanilac was inhibited 48.2%. Both varieties showed nitrate accumulation under all simazine treatments in comparison with the control. Nitrate concentration increased by 18.5% and 47.5% in the leaves of the Sanilac variety at the 0.2 and 5 ppm simazine levels, respectively, whereas an increase over control of 14.2% and 58.5% occurred in the Saginaw leaves.

The adverse levels of the herbicidal treatment, therefore, caused inhibition of nitrate reductase and accumulation of its substrate (NO_3^-).

From the data in Table 15, simazine might affect beans in two ways. Under sublethal concentrations and at a very low level, 0.05 ppm, it enhanced nitrate reductase as well as nitrate uptake. This might be a result of changing permeability to nitrate, within the range of concentration favoring induction where the nitrate was not in the proper concentration before treatment. Under high concentrations of simazine, (1.0 to 5.0 ppm), it is possible that disturbances occurred in nitrate-nitrogen assimilation and resulted in an accumulation of intermediary substances containing nitrogen which may be toxic to the plant.

The dependence of metabolic processes on individual enzymes or enzyme systems suggests that changes in metabolic responses resulting from the use of herbicides may stem from altered enzymatic activity (Table 15).

Enzymatic response to a particular herbicide may differ between species or even varieties. The response may even differ between organs of the same plant.

Exer (12) and Moreland et al (31) have reported that simazine inhibited the photochemical activity of isolated chloroplasts and specifically inhibited the Hill reaction. The evidence presented by Exer indicated that the inhibited reaction involved the photochemical reduction of nicotinamide adenine dinucleotide (NAD). Since the reduced NAD compound is a co-factor required for nitrate reductase, this might be a major indirect factor in simazine inhibition of nitrate assimilation. There is no lack of nitrate-nitrogen (NO_3^-) since nitrate accumulated in leaves accompanied by inhibition in nitrate reductase activity in simazine-treated plants (Table 15).

B. Time course of nitrate reductase activity in plants grown on soil treated with simazine:

Experiments were carried out in a warm greenhouse (70-80 F). The twelve-inch pots containing a 1:1 mixture (v/v) of organic soil and sand were sterilized. Each pot contained 2.5 kilograms of soil after being treated with the

proper concentration of simazine. In each pot, six seeds, three of each variety, were sown and thinned to four uniform seedlings after emergence. Each simazine level (evenly distributed in soil) was represented by eight pots with a total of 16 plants. The plants were watered daily with Hoagland solution except that the nitrate-nitrogen level was one-fourth of recommended strength. Leaves were assayed for nitrate reductase three times in the first seven weeks after emergence.

Table 16. Time course and effect of simazine in root medium on nitrate reductase activity in Sanilac navy beans

Simazine levels in soil ppm	µg NO_2^-/20 min/g fr wt		
	Days after emergence		
	10	30	45
Control	9.1	8.5	6.9
0.2	9.3	9.5	10.6
1.0	9.5	9.8	10.5
2.0	11.8	10.0	6.0
5.0	18.1	9.3	4.2
10.0	18.5	5.1	2.4

The results clearly demonstrate that simazine, directly or indirectly, caused stimulative as well as inhibitive effects on nitrate reductase activity. Ten days after emergence, an increase of 29, 91, and 103% in nitrate reductase activity occurred in plants grown on soil treated with simazine at the

rate of 2.0, 5.0, and 10.0 ppm, respectively. At 30 days after emergence, plants grown on soil treated with 2.0 and 5.0 levels of simazine had activities over control of 17.6 and 9.4%, respectively, while a reduction of 39.9% in enzyme activity occurred in plants under 10.0 ppm of simazine. While a progressive reduction in enzyme activity occurred in plants grown on the highest two levels of simazine, an increase in the enzyme activity progressively continued in plants grown on soil treated with the two lowest level of simazine (0.2 and 1.0 ppm). It seems that as the plant grew, and the root system spread, it absorbed more simazine which gradually caused increased inhibition in nitrate reductase activity.

The increase in enzyme activity might be due to a change in permeability of the root system or a change in the mobility of the nitrate ion within the root system. A similar pattern in nitrate reductase activity to that in Sanilac has been demonstrated in the Saginaw variety -- stimulation under 0.2 ppm simazine and inhibition under 2.0, 5.0, and 10.0 ppm. The data showed that Saginaw was more sensitive to simazine than Sanilac.

Exposure of bean seedlings of both varieties for a few weeks to a 10.0 ppm simazine level caused a stimulative effect, then presumably as the root expanded, longer exposure drastically reduced bean growth and drastically inhibited nitrate reductase activity and caused nitrate accumulation.

C. In Vitro Studies

Extracts from expanding leaves of the bean plants were assayed for the activity of nitrate reductase in the presence of some chemical compounds at the indicated concentrations, (Table 17).

Table 17. Effect of chemicals on nitrate reductase activity in vitro

Chemical Concentration $5x10^{-5}M$	Percent inhibition over control after 30 min.
Na-azide	85
K-CN	93
K-Chlorate	76
Hydroxylamine	00

Azides and cyanides are known to inhibit cytochrome oxidase in the respiratory chain. This might suggest the interdependence of the two systems probably through the co-enzyme(s).

GENERAL DISCUSSION

The soluble NADH requiring reductase described here for navy beans is similar to the enzyme system originally described by Evans and Nason in 1953. This enzyme apparently constitutes the major or only nitrate reductase in different parts of the plant. However, since the enzyme activity is generally low in all parts of bean plants except the leaves this discussion will mainly deal with the enzyme in leaves.

The data showed that the leaf was at its peak of nitrate reduction effectiveness just before it completed its most rapid expansion. The remainder of its normal existence was a deteriorative time, with gradually lowering efficiency of nitrate reduction potentiality until the leaf was shed from the plant. The decline in bean leaves started soon after a leaf reaches full size. A similar pattern in photosynthetic activity was reported in bean leaves by Leopold in 1964. He indicated that with increasing age, a bean leaf became progressively less effective as an assimilatory organ. In Table 6 declines in nitrate reduction potential with age have been justified for successive leaves from the top. Similar declines in photosynthesis with age have been reported by Sestak and Catsky (42). Since, according

to Zucker and Stinson (52), the chloroplasts contain a large portion of the protein in green leaves, it is possible that the degradation of chlorophyll and of protein may be structurally related. Bottger and Wollgiehn (3), reported the decline in protein-nitrogen resulted from a decline in RNA. From this scattered evidence on changes associated with senescence of leaves, it is clear that senescence involves a loss of assimilative powers and catabolism of protein and RNA.

With these facts in mind, no adequate explanation could be given to explain why nitrate reductase activity declines as leaf age increases. However, one could speculate that this might be a direct reflection of decreased rates of turnover of protein in the aging tissues, or it might result from situations where the active sites on the enzyme protein are progressively blocked by inhibitors.

There was no apparent correlation within inbred lines between the seasonal mean level of nitrate reductase activity and seasonal mean content of total protein. There were significant differences in nitrate reductase activity levels between inbred lines and varieties in beans. Comparable results were obtained in 1964 and 1965 when nitrate reductase activity was expressed on a per unit of fresh weight basis,

with the exception of one line. These results might suggest that the initial ranking of inbreds was valid.

Whether the differences in level of activity are due to a more efficient enzyme (qualitative) or to greater amounts of enzyme (quantitative) cannot be assessed at this time. Only after complete extraction and purification of the enzyme from these lines could the test for either qualitative or quantitative effects be conclusive.

The association between the seasonal mean of nitrate reductase and agronomic character is exemplified in yield components, and is not clear at this point. However, hybridization and selection in segregating generations might provide an approach to selection of inbred bean lines that have a specific metabolic constitution, e.g. high levels of nitrate reductase. Thus a line or variety having a high input of reduced nitrogen does not necessarily specify a high yield or protein potential.

In view of the present knowledge (Crick, (7)) of the role of DNA in protein (enzyme synthesis), there can be no doubt that the two classes of inbreds (high and low nitrate reductase) are divergent, at least in certain aspects, in DNA constitution (Schrader et al, 41). Since it is now accepted that the mechanism of gene action is at the biochemical level through the control of protein synthesis, it becomes

possible to estimate the diversity of portions of the genetic materials by measurement of the amount or activity of enzymes in various inbreds. Whether the level of nitrate reductase activity is directly (differences in that portion of DNA that codes for nitrate reductase) or indirectly (mutigenic which invokes metabolic interaction) dependent upon these genetic differences, (speculated by Schrader et al (41)) cannot be specified at this time.

Folkes and Sims (1964) reported that growth rates of yeast were more closely related to glutamic-alanine transaminase activity than nitrate reductase activity. Therefore, even if nitrate reductase activity is the rate regulating step, (Hageman et al, (19)) for the input of reduced nitrogen, it may not be an index of growth. Assuming that the end-product of nitrate reduction (ammonia) is toxic, any excess ammonia formed in the inbreds with high nitrate reductase activity could be detoxified and shunted into amides or similar nitrogen reserves. Presumably, plants with high nitrate-reducing capacity and reserves should have an inherent advantage under conditions imposing temporary stress on nitrogen supply, assuming water supply is adequate.

The difficulty in this type of projection lies in the extreme complexity of the interacting metabolic systems under the range of environmental conditions usually encountered in the field.

The high reproducibility of the results, as indicated by close agreement between the nitrate reductase ranking in 1964 and that in 1965, is encouraging for future studies on the mode of inheritance of this character. Only one marked deviation, in six tested, was noted between the performance of inbred lines tested in 1964 and that of the same lines in 1965.

Studies of enzymatic induction in navy beans, confirm the induction-mechanism, and adaptive formation of the enzyme occurs in the actively-expanding leaves. In these young tissues there is likely to be a high rate of turnover of protein. The fact that induction can be demonstrated in relatively old leaves would indicate that the apparatus for synthesizing or activating the enzyme is retained until quite late in the life of the leaf. Similar results have been recorded for a variety of other plants (Hageman and Flesher, (18); Hewitt and Nicholas (21); Wallace and Pate, (47)).

Levels of nitrate in sap released from the root system, were higher in the plants grown on high levels of nitrate (60 mg/l) than those grown at lower levels (10 mg/l). This might be a result of partial reduction of nitrate in roots, exhaustion from the root medium under a low level, or the absorbing areas in roots were not saturated with nitrate in the rooting zone under the low nitrate level. However, from relatively long-term studies and the exudate analysis, the

capacity of uptake in roots may eventually decline, probably through a progressive decrease of assimilate(s) needed from leaves and shoots, or a decline in root growth.

The data on nitrate reductase activity levels and nitrate uptake emphasize the marked differences in the uptake amount of water and nitrate between bean varieties (Algarrobo, Michelite). The variety with a high level of nitrate reductase activity has a high capacity for absorbing nitrate.

The decrease in nitrate reductase activity in effectively nodulated plants rather than in the uninoculated plants raises several questions. Does the nodulated root system result in a decrease in uptake or transport of nitrate? Or does the system produce nitrogenous substances that cause some kind of feedback inhibition or repression of the enzyme, nitrate reductase as suggested by Filner (14).

The nitrate assimilation in green plants is commonly limited by nitrate application, varietal differences, and biological interactions. Furthermore, adverse conditions brought on by drought or chemical applications might limit nitrate assimilation in navy beans. As the moisture progressively decreased in the rooting medium, nitrate reductase activity progressively decreased in the leaves. The significant decrease in enzyme activity under 50% of available moisture seems to indicate that enzyme activity is influenced

by changes in stress too small to be measured by relative turgidity, or by factor(s) other than moisture (Mattus and Pauli, 29). Nitrate content progressively accumulated as water stress increased. Results of nitrate determinations reflect net accumulation over reduction, thus, actual uptake and transport of nitrate probably was greater than indicated. Decrease of nitrate reductase activity caused less reduction of nitrate. The accumulation of nitrate probably affected the balance in nitrogen compounds in plants grown under drought conditions.

Molybdenum has been reported to be a metal co-factor constituent of the nitrate reductase enzyme. Thus moisture stress may influence nitrate reduction by limiting molybdenum uptake or availability. Water stress might reduce the net rate of incorporation of nitrogen into water soluble protein (Mattas and Pauli, 29). Also, in their work as stress became increasingly severe, relatively less nitrogen was incorporated into non-soluble protein-nitrogen. Results obtained by West (49) are of particular interest. With corn, increases in ribosomal RNA and decreases in polysomes were found with moisture stress. Thus, moisture stress may influence enzyme synthesis, including nitrate reductase.

From Table 15 it appears that simazine might affect beans in two ways. Under sublethal concentrations at a very low level, .05 ppm, it enhanced nitrate reductase as well as

nitrate uptake. This might be a result of changing permeability to nitrate, within the range of concentration favoring induction where the nitrate was not in the proper concentration before treatment. Under high concentrations of simazine, (1.0 to 10. ppm) it is possible that disturbances occurred in nitrate-nitrogen assimilation and resulted in an accumulation of intermediary substances containing nitrogen which are toxic to the plant.

The dependence of metabolic processes on individual enzymes or enzyme systems suggest that changes in metabolic responses resulting from the use of herbicides may stem from altered enzymatic activity. This is clear in the effect of simazine on nitrate reductase activity.

Enzymatic response to a particular herbicide may differ between species or even varieties where Saginaw was less tolerant to simazine than Sanilac. The response may even differ between organs of the same plant.

Exer (12) and Moreland et al (31) reported that simazine inhibited the photochemical activity of isolated chloroplasts and specifically inhibited the Hill reaction. The evidence presented by Exer indicated that the inhibited reaction involved the photochemical reduction of NAD. Since the reduced NAD compound is a co-factor required for nitrate reductase, this might be a major indirect factor in simazine inhibition of nitrate assimilation.

There is no lack of nitrate-nitrogen (NO_3^-) since nitrate accumulated in leaves accompanied by inhibition in nitrate reductase activity in simazine-treated plants.

However, under high simazine level in the rooting medium (10. mg/Kg soil) once the root system was established by penetrating the soil, nitrate reductase was inhibited the rest of the season and was accompanied by reduction in yield components.

The benefit which could be derived through genetic interaction with environmental factors that permits a high level of enzymatically reduced nitrogen would be an asset especially if plants were grown under conditions which would enhance the reduction and assimilation of nitrogen. Other environmental conditions can be envisioned where this higher input of reduced nitrogen might cause an unbalanced metabolic system.

The unanswered question remains: How does a plant breeder select the proper metabolic system in a bean variety for the variable environment of field conditions.

Nitrate reductase was a reasonable indicator for genetic differences under environmental conditions. The high interdependence of metabolic pathways suggest that correlating two or more key enzymes in different major pathways might be more indicative of metabolic activity in growing plants and desirable agronomic characters such as yield, or protein content.

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