CHANGES IN CERTAIN NITROGEN FRACTIONS AND NITRATE REDUCTASE ACTIVITY IN CROWNS OF THREE VARIETIES OF WINTER WHEAT DURING COLD HARDENING AND DEHARDENING

by

FRANK R. TOMAN

B. S., Kansas State University, 1961

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Agronomy

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1963

Approved by:

Orland W. Pauli
Major Professor

TABLE OF CONTENTS

INTRODUCTION

The past decade has brought about a better understanding of cold hardiness in plants. Since 1949, when Siminovitch and Briggs (3) showed a correlation between water soluble protein and cold hardiness in the cambial cells of black locust, extensive work in the area of nitrogenous changes with development of cold hardiness has been done. At first much of the work centered around the water soluble, non-water soluble, and water soluble non-protein nitrogen constituents of the plant. Associations between water soluble protein and cold hardiness have been reported in hard winter wheat (24), alfalfa (4) and sweetclover (4).

Recently studies have shifted to include free amino acids, protein-bound amino acids, and amides. Zech (23), reported a significant correlation between gammaamino butyric acid (a free amino acid) and cold hardening and dehardening in hard winter wheat crowns. In certain cases an association was found between the amide asparigine and cold hardiness. Pauli and Mitchell (17) reported an increase in total amide nitrogen when the leaves of Pawnee wheat plants were hardened under controlled conditions. These increases were correlated significantly at the .01 level with specific conductance, which was used to measure cold hardiness in the plant. However, no published literature was found concerning nitrogenous constituents leading to the formation of amino acids during cold hardening and dehardening. With these facts in mind, this experiment was conducted with the following objectives:

(1) To determine the relative changes in nitrate reductase activity in winter wheat crowns as they cold harden and deharden.

(2) To determine quantitatively the nitrate, nitrite and total nitrogen content of winter wheat crowns during hardening and dehardening.

(3) To compare these factors as they operate in genotypes of hard winter wheat known to vary in hardiness.

REVIEW OF LITERATURE

Nitrate nitrogen is the principal form of nitrogen absorbed by higher plants (16). It has further been established that the plant cannot utilize nitrogen in the nitrate form. The plant, however, is capable of utilizing nitrite nitrogen in the synthesis of nitrogenous plant substances such as amino acids, proteins, and amides. For some time the method by which the plant reduced nitrate to the useable form of nitrite was not understood. Early work by Eckerson (9) showed that nitrite was formed when expressed plant sap was incubated with nitrate and glucose. The experiment indicated that reduction was brought about by an enzymatic system; results were inconclusive, however, because long incubation periods were used and reduction may have resulted from contamination by microorganisms. Anderson (1) detected nitrites in the leaves and shoots of some 25 plant species. He claimed that the reduction was non-enzymatic and was dependent upon the presence of carbohydrate. Burstrom (5) reported that wheat leaves were capable of assimilating nitrates, but only in the presence of light. He found that when nitrates were absent in the leaves, all carbon dioxide from the air was assimilated to sugars. When nitrates were present, more carbon dioxide was assimilated and some of it was used to form "CN-assimilates". He also proposed that if nitrate was reduced in the roots it must be by another mechanism. Mendel and Vassar (13) conducted

experiments in tomato plants with N^{15} labeled ammonium ions and N^{15} labeled nitrate ions. A higher accumulation of N^{15} was found in the leaves and stems when nitrate was used. Greater amounts of N^{15} accumulated in the roots when ammonia was used. However, distribution was uniform throughout each particular organ. Nitrate was assimilated in both light and dark, but 50 percent more was assimilated when light was supplied. Iodoacetate was capable of blocking nitrate reduction in the dark, but had no effect under light conditions. Since iodoacetate is a respiratory inhibitor they suggested that nitrate reduction in the dark is dependent upon respiration for its source of energy while light supplies the needed energy during the day.

Proof that nitrate was reduced to nitrite by an enzymatic process came in 1952, when Evans and Nason (11) reported purification of a nitrate reductase enzyme from soybean leaves. (A reductase is defined as any enzyme that aids in the reduction of a substance.) Optimum pH of the enzyme was found at 6.0, with relatively high activity in the range of 5.5 to 7.0. The enzyme was sensitive to concentrations of nitrate and could be saturated with 2×10^{-2} M potassium nitrate. No apparent increase in activity was found when incubated with various inorganic ions. Activity was increased by additions flavine adinine dinucleotide (FAD) but very little by flavine mononucleotide (FMN), indicating that the natural prosthetic group of the enzyme was FAD. Activity was greatly inhibited by P-chloromercuribenzoate and was reversed by addition of cysteine hydrochloride. The same analysis procedure used on six other plant species gave strong evidence that a soybean nitrate reductase enzyme, or a similar enzyme, was present in these plants. Nicholas and

Nason (15) later showed that molybdenum is an essential component of the nitrate reductase enzyme system in soybeans. Candela, Fisher, and Hewitt (6) reported the presence of a nitrate reductase enzyme in cauliflower. Maximum activity of the enzyme was found in the mature leaves. The activity of the enzyme could be enhanced when leaves of deficient plants were infiltrated with molybdenum. The activity of the enzyme was greatly reduced by prolonged periods of darkness and restored when brought into light. Similar results were found when air was removed from the plants.

In 1958, Spencer (20) isolated a nitrate reductase enzyme from germinating wheat. The enzyme was soluble and highly specific for the reduced cofactor diphosphopyridine nucleotide (DPNH), in contrast with the enzyme isolated in soybeans (11) which was found to be active with both DPNH and reduced triphosphopyridine nucleotide (TPNH) cofactors. The optimum pH of the enzyme was 7.4, and activity was sharply reduced when the pH fluctuated from this level. The activity of the enzyme was greatly increased with the addition of FAD, while FMN gave no response. Inorganic phosphate was found to be essential for maximum activity of the enzyme. The enzyme was inhibited by such compounds as potassium cyanide, sodium azide, p-chloromercuribenzoate and sodium orthovanadate at relatively low concentrations. The presence of an essential heavy metal was indicated but attempts to discover the metal were unsuccessful.

Hageman and Flesher (12) reported that both light and nitrate are necessary for maximum activity of nitrate reductase in corn leaves. Leaves were shown to lose 90 percent or more of their activity when placed in the dark for 48 hours. Activity in the leaves was restored when plants were brought back to light. When the concentration of nitrate was increased in

the nutrient solution, nitrate reductase activity increased proportionately. A significant positive correlation was found between enzyme activity and water soluble protein and a negative correlation with nitrate accumulation.

MATERIALS AND METHODS

Minturki, Pawnee, and Ponca winter wheats were planted at the University Agronomy Farm September 28, 1961. Seed of all varieties was obtained from plants grown at the farm. These particular genotypes were selected because Minturki is a relatively cold hardy variety, Ponca is moderately hardy and Pawnee is intermediate in hardiness. Good stands were obtained for all varieties.

Samplings of crown tissue started November 13, 1961, and continued at approximate two-week intervals until April 5, 1962. The plants were brought from the field to the laboratory where they were washed free of soil with tap water. The crowns, defined as that portion of tissue above the roots and below the soil surface, were removed, washed with tap water and rinsed with distilled water. Excess surface water was removed by blotting between paper towels.

Hardiness Measurements

Crowns were tested for hardiness using the specific conductivity method outlined by Dexter, Tottingham, and Graber (8). Five grams of fresh crowns were placed in an uncovered Petri dish and frozen four hours at $-10^{\circ}C$ in a controlled temperature chamber. Crowns were removed, placed in 50 ml. of distilled water, and allowed to stand overnight in a chamber

maintained at 5°C. Using a Wheatstone bridge, resistance readings were made on the solution containing the exosmosed solutes from the cells damaged by freezing. Specific conductivity was calculated by dividing the cell constant of the Wheatstone bridge by the resistance reading. Results were expressed as reciprocal ohms x 10^{-6}.

Nitrate Reductase Determinations

Extraction procedure: Extraction was accomplished by a modification of the method outlined by Hageman and Flesher (12). Five grams of fresh crown material were blended in 20 ml. of blending media, composed of 0.1 M tris hydroxymethyl aminomethane, 0.01 M cysteine and 0.0003 M ethylene diamine tetraacetic acid in equal proportions. The media was adjusted to pH 7.2 with dilute hydrochloric acid. Blending was accomplished with a Servall ominimixer at 16,000 rpm for one minute, with the cup immersed in an ice bath. The blendor cup was removed, any adhering tissue pushed down into the cup, and the mixture reblended for another minute. Preliminary trials showed that a continuous blending period of two minutes or longer often left some unmacerated tissue adhering to the side of the cup. It also heated the extract sufficiently to destroy the enzyme. One minute blending periods were insufficient to completely macerate the tissue and permit complete recovery of the enzyme. The procedure used assured complete maceration of plant cells and allowed less chance for heating of the extract. The extract was filtered through a fine mesh sieve and centrifuged in a refrigerated centrifuge at 2°C. for 15 minutes at 20,000 x G. The supernatant was used for analysis. Extracts were kept at 2-3°C throughout the entire analysis.

Measurement of activity: Nitrate reductase activity was measured by further modification of the Evans and Nason method as reported by Hageman and Flesher (12). Assay mixtures for each sample were made the night before sampling. These were composed of 1 ml. of 0.1 M potassium phosphate buffer solution pH 7.2 and 0.2 ml. of 0.1 M potassium nitrate. To each assay mixture was added 0.5 ml. of 1.36×10^{-3} M reduced nicotin adenine dinucleotide (NAD·2H)[1] and 0.3 ml. of enzyme extract. The resulting mixtures were then incubated in a water bath maintained at 27°C for 45 minutes. The reaction was stopped by adding 1 ml. of 1% w/v sulfanilic acid in 1.5 N hydrochloric acid. One ml. of 0.02% ethylene diamine dihydrochloride was added and the contents mixed by inverting the tubes. Color was allowed to develop for 10 minutes and percentage transmittance was read on a Bausch and Lomb Spectronic 20 colorimeter at a wavelength of 540 mμ. Sulfanilic acid was substituted for sulfanilamide. Sulfanilic acid is more stable in solution, but requires a longer period for full color development.

Standard curves were made for nitrate reductase by substituting known concentrations of potassium nitrite for the enzyme extract. Distilled water was substituted for NAD·2H to bring the assay to 2 ml. volume. Percentage transmittances were plotted vs. concentration on semilog paper to obtain a straight line reference.

Nitrate and Nitrite Determinations

Extraction procedure: Extraction of nitrate and nitrite was the same as for nitrate reductase, except that 25 ml. of distilled water was used

---

[1] This compound is synonymous to reduced diphosphopyridine nucleotide (DPNH).

per 5 grams fresh crown material and the filtrate was centrifuged at 6,000 x G for 15 minutes.

Determinations of nitrate and nitrite were made by further modification of the Nelson, Kurtz, and Bray method as reported by Woolley, Hicks, and Hageman (22). The nitrate determination relies on the reduction of nitrate to nitrite which can be measured colorimetrically when nitrite diazotizes sulfanilic acid and couples it with 1-naphthylamine to form a red dye. Extracts were relatively high in contents of nitrate and were diluted 1:9 with water before analysis. One ml. of diluted extract was placed in a test tube to which was added 9 ml. of 20% acetic acid containing .2 ppm copper as copper sulfate. Results were variable if copper was omitted from the acetic acid. About 0.4 gram of reducing powder, consisting of 100 grams barium sulfate, 75 grams citric acid, 10 grams manganous sulfate dihydrate, 4 grams sulfanilic acid, 2 grams powdered zinc, and 2 grams of 1-naphthylamine was added. Test tubes were shaken vigorously for 15 seconds and allowed to stand for three minutes. This procedure was repeated three times. After the third shaking the mixture was centrifuged for five minutes at 6,000 x G. Percentage transmittance of the supernatant was read on a Bausch and Lomb Spectronic 20 colorimeter at a wavelength of 520 mμ. Intensity of color was reduced by the zinc in the reducing powder if excessive amounts of powder were used or if mixtures were allowed to stand too long before centrifuging. Nitrate determined by this procedure includes the nitrite present in the material. However, nitrite levels were so low in comparison to nitrate levels, that no correction was made.

Nitrite was determined by the above procedure omitting manganous

sulfate and powdered zinc from the reducing powder. Without these components nitrate was not reduced to nitrite and only the nitrite present in the original extracts was measured. Better results were obtained when copper was omitted from the acetic acid. Concentrations were low and dilution of extracts was not necessary.

Standard curves were constructed for both nitrate and nitrite. This was done by using known concentration solutions of calcium nitrate and sodium nitrite and substituting them in the above procedure for the extract. Transmission percentages were plotted in the same manner as for nitrate reductase.

Fresh Weight Determinations

Fresh weight was determined by blotting the excess water from ten crowns and weighing them. These crowns were placed in a drying oven for 18-20 hours at 70°C. and reweighed. These 10 crown samples were saved and later total nitrogen content was determined on them.

Total Nitrogen Determinations

Total nitrogen was determined by the Cunning modification of the Kjeldahl method (2) using boric acid in the recieveing flask (18).

All statistical analyses were made as outlined by Snedecor (19).

EXPERIMENTAL RESULTS AND DISCUSSION

Cold Hardiness

Results of the cold hardiness tests are shown in figure 1, and data can be found in table 1 in the appendix. In November, when the plants were

young and before they had hardened to any great extent, conductance values for the three varieties showed Minturki hardiest, Pawnee intermediate, and Ponca least hardy. Conductance values dropped by the second sampling date indicating that the plants had hardened to some extent. Due to changing conditions in environment, conductance values fluctuated during the remaining sampling dates until maximum hardiness was reached. All three varieties reached maximum hardiness on February 19, 1962 in the same order of hardiness as was shown on the first sampling date and dehardened throughout the remaining sampling dates. With a few exceptions, this ranking of hardiness was found throughout the entire sampling period. These exceptions may have been due to different hardening and dehardening rates or to sampling error. It is interesting to note that when the varietal sequence mentioned was present, the spread among conductance values was much greater than in instances where they were interchanged.

Water Content

Because water percentages varied between sampling dates and varieties, results were expressed on a dry weight basis. All results discussed in this paper are expressed in this manner. Water percentages are found in appendix table 2. Generally the water content decreased in all three varieties during the hardening stages and increased again during dehardening. With very few exceptions, Ponca had the highest content of water, Pawnee intermediate, and Minturki lowest content of water. This was true throughout the entire sampling period.

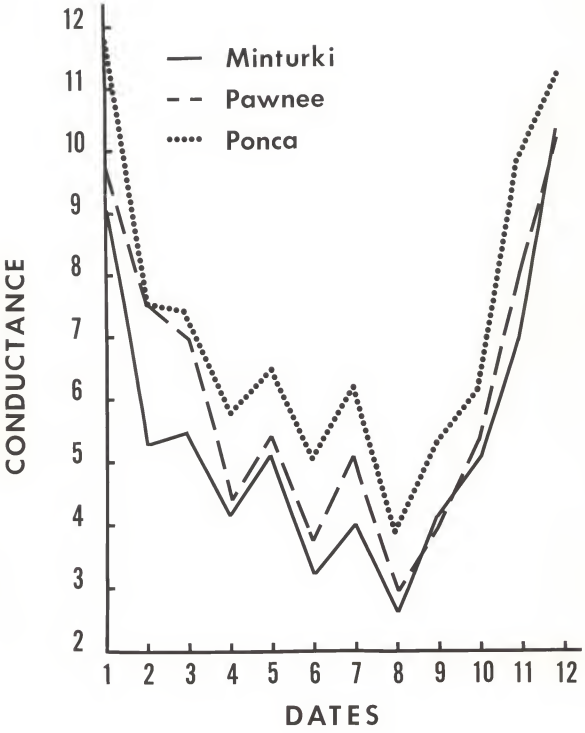

Figure 1. Specific conductance (ohms x 10^{-6}) of wheat crown tissue, Manhattan, Kansas, 1961-62.

Nitrate

Results of nitrate tests are shown in figure 2 and in appendix table 3. On November 13, 1961, concentrations of nitrate were high in plant material sampled from all three varieties. This can be attributed to high concentrations of nitrate fertilizer which were applied before planting so nitrate would be available. In the next three sampling dates from December 2 to December 28, concentrations of nitrate in the plant material were drastically reduced. On December 28, concentrations of nitrate reached a minimum in all three varieties and increased in the next two sampling dates. Levels of nitrate fluctuated during the periods of greatest hardiness and at concentrations considerably lower than those in the unhardened condition. On March 26, nitrate concentrations increased sharply in all three varieties and continued to increase through the dehardening period. Differences in concentrations of nitrate in the three varieties were found throughout the sampling period, but were not always significant. Furthermore, the varietal sequence of concentration was not always the same. Ponca generally was highest in nitrate concentration and in most cases predominantly so. On most dates Minturki was predominantly lower in nitrate content than the other two varieties. During early sampling dates, when varieties were relatively unhardy, Pawnee and Ponca were very close in concentrations; however, during the dates of generally greatest hardiness and during dehardening, concentrations in Pawnee were lower than in Ponca and usually did not differ significantly from Minturki. The predominantly higher nitrate reductase activity in Minturki, figure 5, may explain somewhat the lower nitrate concentration in Minturki than in Pawnee or Ponca. However correlation coefficients between nitrate content

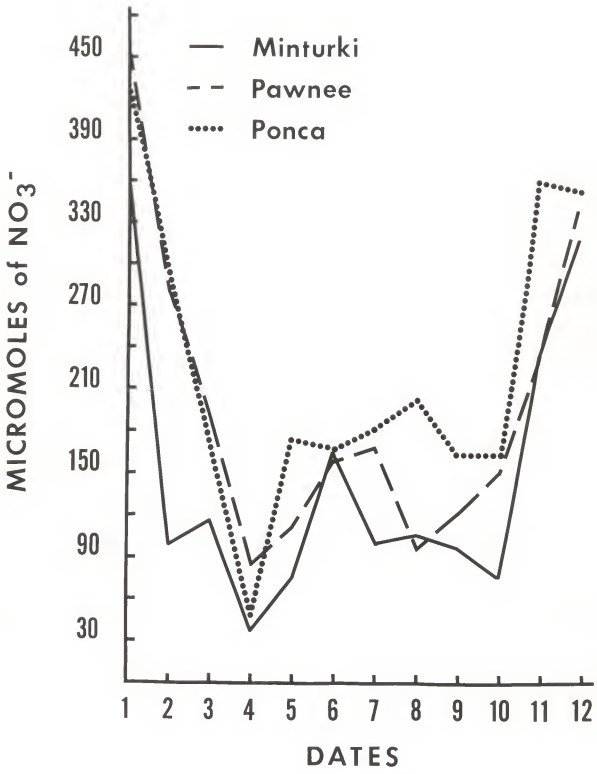

Figure 2. Nitrate content per gram dry weight of wheat crown tissue, Manhattan, Kansas, 1961-62.

and nitrate reductase activity, table 7, were non-significant in all varieties. This would indicate that changes in nitrate content were not principally associated with changes in activity of the enzyme, at least under conditions where nitrate supply was not limited.

Correlation coefficients between nitrate content and conductance are found in table 7. "R" values of .842, .903, and .848 were significant beyond the .01 level in the three varieties, Minturki, Pawnee, and Ponca, respectively. These varieties pooled together gave a coefficient of .892, also highly significant. This indicated that factors associated with decreased conductance were related to accumulation of nitrate in the crown material sampled.

Nitrite

Levels of nitrite are reported in table 4 of the appendix and shown in figure 3. No nitrite could be detected in the young unhardened plants in November, indicating that nitrite was being utilized immediately after it was formed. A sharp increase in nitrite concentration was noted at the second sampling period, particularly in Ponca and Pawnee. Nitrite concentrations continued to fluctuate greatly through the fifth sampling date, i.e., while varieties were reaching the generally hardiest period. During the hardiest periods (sampling dates 6-8), nitrite concentrations increased steadily to the time of maximum hardiness on February 19. This is the only instance when nitrite increased for more than one continuous sampling date. However, except for Ponca, nitrite concentrations were little or no higher at maximum hardiness than at certain earlier, less hardy dates. During dehardening, concentrations of nitrite fluctuated, but

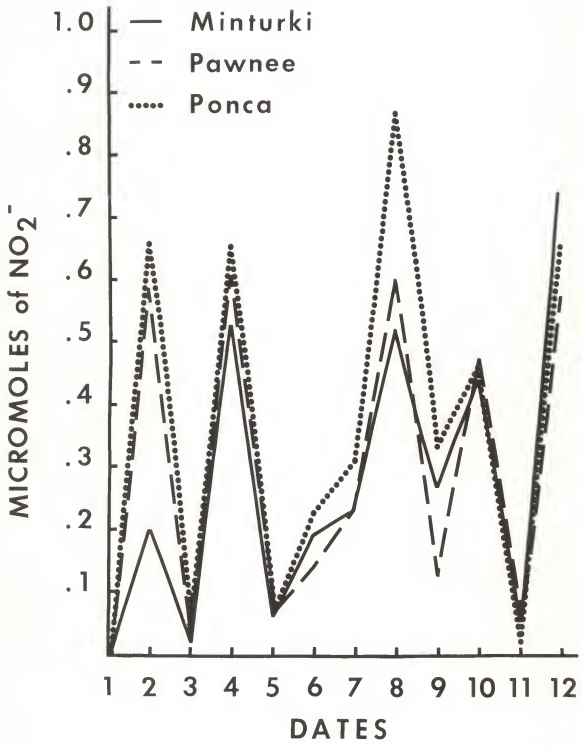

Figure 3. Nitrite content per gram dry weight of wheat crown tissue, Manhattan, Kansas, 1961-62.

tended to decrease until the last sampling date. The fairly consistent fluctuations suggest the possibility that nitrite accumulates to a certain point in the plant, probably reaching a point that is nearly toxic. By some safety mechanism the concentration apparently is reduced to a safe range. Another possibility is that the plant is able to stop or reduce the production of nitrite till enough is utilized to reduce the level.

Correlation coefficients between nitrite content and conductance are reported in table 7. "R" values of .032, -.126, -.366, and -.198 were found for Minturki, Pawnee, Ponca, and the varieties pooled, respectively. These values are non-significant and indicate that changes in nitrite accumulation in the material sampled were not associated with changes in hardiness. The negative values found in Pawnee, Ponca, and the three varieties pooled hint that nitrite accumulation may be somewhat enhanced by the low temperatures primarily responsible for increased hardiness. This is not an unreasonable possibility because at low temperatures the metabolism of the plant would be decreased. It then would seem likely that less nitrite would be utilized.

The above hypothesis is also substantiated by the general order of nitrite concentrations in the three varieties. Differences among varieties were generally not significant, but Minturki, the hardiest variety, tended to be lowest in nitrite during the hardening period. Ponca, the least hardy variety, tended to have highest levels of nitrite. These differences were most pronounced at the time of maximum hardiness when metabolic rates were probably lowest.

Total Nitrogen

Total nitrogen trends are shown in figure 4 and results can be found in table 5 of the appendix. Total nitrogen levels decreased rather sharply between the first sampling date on November 13, and the second sampling date on December 2. Nitrogen levels remained fairly constant throughout the next three sampling dates with the exception of a significant increase in Ponca between the third and fourth sampling dates. Minturki gradually increased at this time, but the differences were not significant. The level in Ponca remained high at the fifth date, but decreased sharply at the sixth sampling date. This same decline was present in Minturki, but Pawnee increased significantly at this point. Minturki and Ponca increased markedly at the seventh sampling date while Pawnee decreased rather sharply. The three varieties show a general increase in nitrogen levels in the remaining sampling dates; Ponca did decrease between sampling dates nine and ten, and Pawnee decreased between dates eleven and twelve. Neither of these changes was significant. Minturki increased consistantly through this period, but in most cases increases were not significant. Ponca increased very sharply through the last two sampling dates and ended with its nitrogen level much higher than the other two varieties. While some fluctuation in total nitrogen was noted in all varieties, Minturki and Ponca followed much the same pattern in levels of total nitrogen throughout the sampling period; Pawnee levels were more eratic, but were generally similar to the other two varieties. Total nitrogen in Ponca was always significantly higher than in Minturki and in all but four sampling dates was significantly higher than in Pawnee. Minturki concentrations remained at a fairly

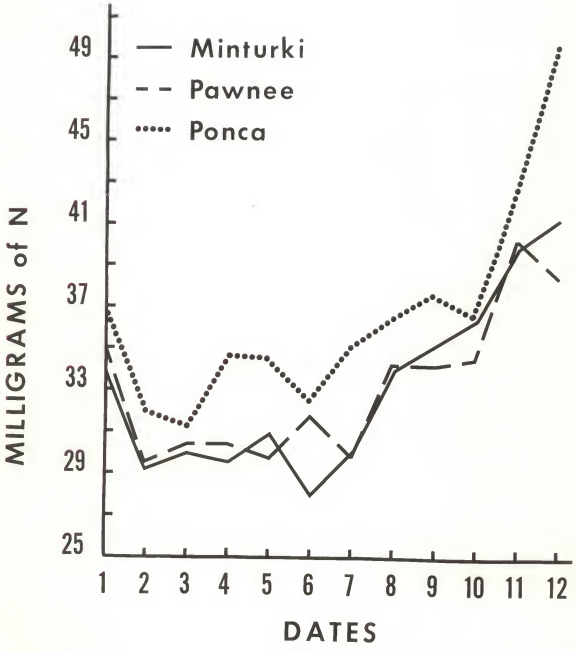

Figure 4. Total nitrogen content per gram dry weight of wheat crown tissue, Manhattan, Kansas, 1961-62.

consistent level below Ponca. Pawnee and Minturki interchanged through most of the sampling period, and differences were significant between the two on only the sixth and twelfth sampling dates.

Correlation coefficients between total nitrogen and nitrate and between nitrogen and conductance can be found in table 7. "R" values of .618, .426, and .563, were found between total nitrogen and conductance in Minturki, Pawnee, and Ponca, respectively. When the data were pooled among varieties the coefficient was .571. The only significant value was that of Minturki; however, the other values approached the value of .575 needed for significance at the .05 level. The values for Ponca and varieties pooled were particularly close to this significance level. This would indicate that there is a definite inverse relationship between total nitrogen content of the plants and hardiness, with Minturki, the hardiest variety, showing the closest relationship.

Coefficient values of .544, .395, .521, and .541 were found between total nitrogen and nitrate in Minturki, Pawnee, Ponca, and varieties pooled, respectively. None of these values are significant at the .05 level, but approach significance closely, and indicate that nitrate level probably is a factor influencing changes in total nitrogen.

Nitrate Reductase Activity

Results of the enzyme activity tests are shown in figure 5 and values can be found in appendix table 6. Nitrate reductase activity was expressed as micromoles of nitrite formed per gram dry weight, when incubated for forty-five minutes at 27°C.

In November, before hardening of the plants had occured to any great

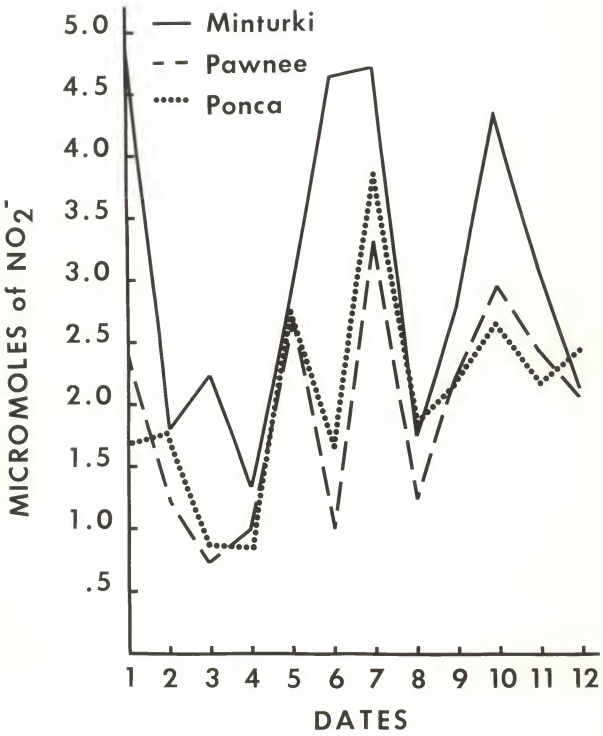

Figure 5. Nitrate reductase activity per 45 minutes incubation per gram dry weight of wheat crown tissue, Manhattan, Kansas, 1961-62.

extent, activity of the nitrate reductase enzymes in the three varieties varied significantly. Activity in Minturki was outstandingly higher than in the other two varieties, with that of Pawnee significantly higher than that of Ponca. Trends in activity of the enzymes of the three varieties varied during the next three sampling dates, but the general trend was of reduced activity. The spread in activity at these times was greatly reduced and in some instances was non-significant. The varietal sequence also changed during this time. During the period of greatest hardiness, activities fluctuated in much the same manner as for conductance, figure 1, and followed the same trends in all three varieties. Even though the activities were fluctuating through this period the overall trend was an increase in activity. During the same period the overall trend in conductance was a decrease, or an increase in hardiness of the plants. For instance, on sampling date six the conductance was lower than on date four, indicating that the plants were hardier. On sampling date six, activities were higher than they were on four. The fluctuation in enzyme activity between these dates is probably due largely to temperature fluctuations in environment, as hardiness decreased during this time also. Similar comparisons can be made between dates six and eight. The only unusual behavior during this time was in the activity of Minturki which increased significantly on sampling date six, while the other two varieties decreased significantly. Activity of the enzymes increased significantly through sampling dates nine and ten, while the plants were dehardening. While the plants continued to deharden through the last two dates, enzyme activity dropped off sharply during this time. It should be noted that in all but sampling dates eight and twelve, Minturki had the highest activity of the

three varieties. While Pawnee and Ponca interchanged in activity, Pawnee was higher than Ponca only four times and only twice was the difference significant. Activity in Ponca was higher than in Pawnee on eight sampling dates and differences were significant on six of these dates. This indicates a general sequence of Minturki, Ponca, and Pawnee in enzyme activities, not the same sequence found in conductance.

These results are similar to findings of Tysdal (21) in his work with diastatic enzymes in varieties of alfalfa plants during hardening and dehardening. He found a decrease in activity at early hardening stages, followed by an increase through the remaining stages of hardening. He detected differences in "protected enzyme activity" of varieties that varied in hardiness, hardiest varieties having greatest "protected enzyme activity".

The activity of the enzyme seems to follow three characteristic trends throughout the course of sampling: (a) a general decrease during the first few periods of hardening in the plant, (b) a general increase during that period when the plants are in the stages of maximum hardiness, and (c) a general decrease during the later stages of dehardening in the plants. Perhaps these trends can be best explained by inferring that some portion of the protein or the prosthetic group of the enzyme underwent a conditioning similar to that of the entire plant. In early stages the enzyme might have been more sensitive to low temperatures, which would explain the general decrease in activity during this time. After the conditioning period the enzyme became more adapted to low temperatures, and activity actually increased under low temperature conditions or conditions of high hardiness in the plants. The enzyme is still subject to environmental

temperature changes which would explain the fluctuating results obtained. When the activity increased during the first two stages of dehardening, it is likely that the enzyme responded in the same manner as when plants had temporarily dehardened on other sampling dates, i.e., merely a temperature response. Upon further dehardening of the plants, that portion of the enzyme capable of conditioning, changed back to its original state. The decrease in activity can be attributed to the enzymes dropping back to levels of activity similar to those on the first sampling date. These levels are probably normal for these growing conditions. Minturki however, had dropped much lower than it was at sampling date one. In view of enzyme activity trends during the sampling period, it seems reasonable to believe that the value measured for Minturki on sampling date one was too high. If this was true, then all three enzymes follow the same pattern, and the explanation given seems reasonable. It would be interesting to have known activity values previous to the first sampling date.

It should be noted that activity of the enzyme, as described in this experiment, was obviously not actual enzyme activity. It was impossible to incubate the enzyme at the same temperature as it was functioning in the plant under field conditions. A constant incubation temperature in the laboratory was used, so results could be compared. The incubation temperature was higher than temperatures to which the plants were exposed in the field. Therefore it is suggested that these results reflected the potential activity of the enzyme after extracted and brought under the laboratory conditions previously described. It must also be kept in mind that some of this variation in activity may be due to concentration of enzyme in the extract. Nason (14), states that nitrate reductase is

adaptive since it is lacking when ammonia or alanine is the sole source of nitrogen. The enzyme is produced only when appropriate substrate is furnished. Thus, concentration of the enzyme could have been a variable in this experiment. The specific proportion of variation in enzyme activity that could be attributed to enzyme concentration could not be determined from this experiment. However, it should be noted that at certain times of high activity, nitrate concentrations were low, indicating that activity of the enzyme was the factor most likely affecting the variance among values measured. This is further substantiated by lack of correlation between nitrate and nitrate reductase activity, as previously reported. If concentrations of enzyme were changing and activity remained constant, more correlation would be expected between nitrate and nitrate reductase activity. A negative correlation between nitrate and nitrate reductase activity was reported by Hageman and Flesher (12), in actively growing corn leaves.

One must remember that the above explanations are based on results from this experiment only. They cannot be substantiated by previous results and until further work can be done in this area, remain only as ideas of the experimenter.

Correlation coefficients reported in table 7 show no significant correlation between conductance and nitrate reductase activity at the .05 level. This can be attributed to the seemingly changing trends of the enzyme activity discussed earlier.

Correlation coefficients between enzyme activity and water soluble protein were computed using data for water soluble protein nitrogen from other studies conducted on this same plant material. The results are shown in table 7 of the appendix. "R" values in all varieties were non-significant

at the .05 level. These results do not correspond with Hageman and Flesher (12), who reported a significant correlation between nitrate reductase activity and water soluble protein. Their results were obtained on actively growing corn leaves.

The role light plays in nitrate reduction in plants has been investigated many times. Work with winter wheat (5), corn (12), cauliflower (6), and soybeans(14) all indicate that light is an important factor influencing nitrate reduction. Most of this work dealt with the activity of the nitrate reductase enzymes. In all cases activities of the enzymes were greatly reduced when subjected to dark conditions. In the above cases enzymes were extracted from leaves and stems of the plants. There is no doubt that light was a big factor affecting nitrate reductase activity in these cases. It should be noted that nitrate reductase has been reported in root nodules of soybeans by Evans (10). However, later Cheniae and Evans (7) were able to show that this enzyme was associated with the bacterial cells, and the root and nodule cells were free of the enzyme. This still indicates that all nitrate reducing systems do not require light for operation. Further indications of this are brought out by the present experiment, in which nitrate reductase was found to be active in crown tissue (that portion below soil level) of winter wheat plants. If light is needed for nitrate reduction in the leaves of winter wheat as Burstrom (5) reported, it would indicate that there may be two nitrate reductase enzymes in the plant.

Based on the findings in this experiment, the author feels that more work needs to be done in this area.

1. A similar experiment, in which plants are grown in controlled temperature chambers, should be conducted.

2. More concentrated work should be focused on the enzyme, to establish concentration levels in the extract and characterization of the enzyme through the hardening and dehardening stages.

Although the later proposal would require much time, money, and equipment, as well as trained personnel, I feel it would make a tremendous contribution to the overall mechanism of winter hardiness in plants.

SUMMARY

Quantitative determinations were made of changes in levels of nitrate, nitrite, total nitrogen, and relative nitrate reductase activity in field grown Minturki, Pawnee, and Ponca winter wheats, as they cold hardened and dehardened.

Cold hardiness, measured as specific conductance, fluctuated through most of the winter, but conductance values showed Minturki hardiest, Pawnee intermediate, and Ponca least hardy.

Water percentages varied throughout most of the sampling period. Percentages decreased in all three varieties during hardening and increased again when dehardening. Generally Ponca had the highest water content, Pawnee intermediate and Minturki least.

Concentrations of nitrate were high in all three varieties before the plants hardened. Concentrations were reduced when the plants hardened and remained at lower levels during the period of greatest hardiness. Generally Ponca had the highest level of nitrate and Minturki the lowest.

Correlations between nitrate content and nitrate reductase activity were non-significant. Highly significant correlations were found between

nitrate content and conductance values, indicating that factors associated with decreased conductance (increased hardiness) were related to accumulation of nitrate in the crown material sampled.

General levels of nitrite in the plants were low; however, changes in concentration could be detected. Differences among varieties were generally not significant. Consistant fluctuations in nitrite concentration suggested the possibility that nitrite accumulates to a certain point in the plant, probably reaching a point near toxic. By some mechanism the concentration apparently is reduced to a safe range.

Correlation coefficients between nitrite and conductance were non-significant.

Some fluctuation in total nitrogen was noted in all three varieties. Ponca and Minturki followed much the same pattern in nitrogen levels, with Ponca significantly higher than Minturki. Pawnee was more eratic, but followed more closely the levels of Minturki than Ponca.

A significant correlation between total nitrogen and conductance was found in Minturki. The values for Pawnee and Ponca approached significance at the .05 level very closely. This indicates a definite relationship between total nitrogen and hardiness, with the hardiest variety showing the greatest relationship.

Correlation coefficients between total nitrogen and nitrate content approached significance at the .05 level, indicating that nitrate level might influence changes in total nitrogen.

Three trends were noticed in nitrate reductase activity: (a) a general decrease during the first few periods of hardening in the plant, (b) a general increase during that period when the plants are in the

stages of maximum hardiness, and (c) a general decrease during the later stages of dehardening in the plants. These trends might be explained by inferring that some portion of the protein or the prosthetic group of the enzyme underwent a conditioning similar to that of the entire plant. The activity measured was not actual enzyme activity, and it is suggested that the results reflected potential activity of the enzyme under laboratory conditions. Some of the fluctuation in enzyme activity may have been due to changes in concentration of the enzyme in the extract.

No significant correlation between conductance and nitrate reductase activity was found at the .05 level. This is probably due to the changing trends of the enzyme.

Correlations between enzyme activity and water soluble protein nitrogen were non-significant. These results did not agree with those reported for corn leaves.

The enzyme having been extracted from below soil level indicates that light is not a requirement for all nitrate reducing systems.

ACKNOWLEDGMENTS

The author wishes to express deep appreciation to Dr. A. W. Pauli, the major professor, for his invaluable help in planning and conducting the experiment and in preparing the manuscript.

Sincere appreciation is extended to Dr. H. L. Mitchell for valuable suggestions and aid in use of materials.

LITERATURE CITED

1. Anderson, V.L.
   Some observations on the nitrate- reducing properties of plants. Ann. Bot. 38: 699-706. 1924.

2. Association of official agricultural chemists.
   Official methods of analysis. Ed. 7. Washington D. C. 1950.

3. Briggs, D. R. and Siminovitch, D.
   Studies on the chemistry of the living bark of the black locust tree in relation to frost. I. Seasonal variations in protein content. Arch. Biochem. 23: 8-17. 1949.

4. Bula, R. J. and Smith, Dale.
   Cold resistance and chemical composition in over-wintering of alfalfa, red clover, and sweetclover. Agron. J. 46: 397-401. 1954.

5. Burstrom, H.
   Photosynthesis and assimilation of nitrate by wheat leaves. Ann. Rev. Agr. Coll. Sweden II. 1-50. 1943.

6. Candella, M. I., Fisher, E. G., and Hewitt, E. J.
   Molybdenum as a plant nutrient. X. Some factors affecting the activity of nitrate reductase in cauliflower plants grown with different nitrogen sources and molybdenum levels in sand culture. Plant Physiol. 32: 280-288. 1957.

7. Cheniae, G. and Evans, H. J.
   Nitrate reductase from the nodules of leguminous plants. In. Inorganic Nitrogen Metabolism. McElroy, W. D. and Glass, B. eds. Pp. 184-188. John Hopkins Press, Baltimore. 1956.

8. Dexter, S. T., Tottingham, W. E., and Graber, L. F.
   Investigations of the hardiness of plants by measurement of electrical conductivity. Plant Physiol. 7: 63-78. 1932.

9. Eckerson, S. H.
   Protein synthesis by plants. I. Nitrate reduction. Bot. Gaz. 77: 377-390. 1924.

10. Evans, H. J.
    Diphosphopyridine nucleotide-nitrate reductase from soybean nodules. Plant Physiol. 29: 298-301. 1954.

11. Evans, H. J. and Nason, A.
    Pyridine nucleotide-nitrate reductase from extracts of higher plants. Plant Physiol. 28: 233-254. 1953.

12. Hageman, R. H. and Flesher, Donna.
    Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiol. 35: 700-708. 1960.

13. Mendel, J. L. and Vassar, D. W.
Studies on nitrate reduction in higher plants. Arch. Biochem. and Biophys. 32: 158-169. 1951.

14. Nason, A.
Enzymatic steps in assimilation of nitrate and nitrite in fungi and green plants. In. Inorganic Nitrogen Metabolism, McElroy, W. D. and Glass, B. eds. Pp. 109-136. John Hopkins Press, Baltimore. 1956.

15. Nicholas, D. J. D. and Nason, A.
Role of molybdenum as a constituent of nitrate reductase from soybean leaves. Plant Physiol. 30: 135-138. 1955.

16. Nightingale, G. T.
The nitrogen nutrition of green plants. Bot. Rev. 3: 85-174. 1937.

17. Pauli, A. W. and Mitchell, H. L.
Changes in certain nitrogenous constituents of winter wheat as related to cold hardiness. Plant Physiol. 35: 539-542. 1960.

18. Scales, F. M. and Harrison, A. P.
Boric acid modification of the Kjeldahl method for crop and soil analysis. Ind. Eng. Chem. 12: 350-352. 1920.

19. Snedecor, G. W.
Statistical Methods. Fifth edition. Ames: Iowa State University Press. 1956.

20. Spencer, D.
A DPNH-specific nitrate reductase from germinating wheat. Australian Jour. Biol. Sci. 12: 181-196. 1959.

21. Tysdal, H. M.
Determinations of hardiness in alfalfa varieties by their enzymatic responses. Jour. Agr. Research. 48: 219-240. 1934.

22. Woolley, J. T., Hicks, G. P., and Hageman, R. H.
Rapid determination of nitrate and nitrite in plant material. Jour. Agr. and Food Chem. 8: 481-482. 1960.

23. Zech, A. C.
Amino acid and amide content of winter wheat crowns as related to cold hardiness. Ph. D. Dissertation, Kansas State University. 1962.

24. Zech, A. C. and Pauli, A. W.
Cold resistance in three varieties of winter wheat as related to nitrogen fractions and total sugar. Agron. J. 52: 334-336. 1960.

APPENDIX

Table 1. Specific conductance in reciprocal ohms x 10^{-8}

Sampling Date	Minturki	Pawnee	Ponca
1. November 13	9.07	9.73	11.80
2. December 2	5.26	7.56	7.51
3. December 16	5.44	7.00	7.38
4. December 28	3.77	4.46	5.84
5. January 12	5.08	5.39	6.44
6. January 26	3.22	3.70	5.03
7. February 10	4.00	5.10	6.21
8. February 19	2.58	2.96	3.74
9. March 7	4.21	3.98	5.32
10. March 17	5.10	5.33	6.11
11. March 26	6.93	7.82	9.84
12. April 5	10.39	10.16	11.19

Table 2. Percentage of water.

Sampling Date	Minturki	Pawnee	Ponca
1. November 13	83.4	85.6	86.5
2. December 2	77.6	83.0	83.4
3. December 16	80.4	81.4	83.8
4. December 28	79.6	83.6	83.4
5. January 12	79.3	79.0	83.6
6. January 26	76.8	79.6	81.7
7. February 10	80.4	80.8	83.8
8. February 19	81.7	83.6	86.9
9. March 7	79.8	80.2	83.6
10. March 17	82.0	82.6	84.4
11. March 26	84.7	85.7	88.6
12. April 5	86.0	87.6	87.1

Table 3. Nitrate in micromoles per gram dry weight.

Sampling Date	Minturki	Pawnee	Ponca
1. November 13	356.46	453.86	426.68
2. December 2	102.22	279.86	293.71
3. December 16	116.33	186.98	181.46
4. December 28	36.52	84.51	49.08
5. January 12	76.45	109.79	176.02
6. January 26	165.42	158.28	168.58
7. February 10	100.47	167.45	178.26
8. February 19	106.39	95.29	200.80
9. March 7	97.42	121.21	160.77
10. March 17	74.47	151.27	161.90
11. March 26	234.06	228.94	360.49
12. April 5	318.41	343.54	356.76
LSD, .05 Varieties x dates = 18.05			
LSD, .01 Varieties x dates = 23.73			

Table 4. Nitrite in micromoles per gram dry weight.

Sampling Date	Minturki	Pawnee	Ponca
1. November 13	.000	.000	.000
2. December 2	.203	.588	.653
3. December 16	.021	.033	.051
4. December 28	.528	.610	.653
5. January 12	.060	.069	.064
6. January 26	.187	.142	.227
7. February 10	.233	.230	.309
8. February 19	.522	.610	.864
9. March 7	.266	.126	.330
10. March 17	.439	.478	.455
11. March 26	.055	.073	.018
12. April 5	.748	.576	.650
LSD, .05 Varieties x dates = .102			
LSD, .01 Varieties x dates = .135			

Table 5. Total nitrogen in milligrams per gram dry weight.

Sampling Date	Minturki	Pawnee	Ponca
1. November 13	34.08	34.89	36.74
2. December 2	29.08	29.34	31.80
3. December 16	29.98	30.38	31.32
4. December 28	29.64	30.36	34.50
5. January 12	30.88	29.77	34.35
6. January 26	28.02	31.83	32.34
7. February 10	30.04	29.84	35.12
8. February 19	33.98	34.15	36.45
9. March 7	35.64	34.14	37.59
10. March 17	36.29	34.52	36.54
11. March 26	39.83	40.12	42.84
12. April 5	41.16	38.44	49.62
LSD, .05 Varieties x dates = 1.96			
LSD, .01 Varieties x dates = 2.58			

Table 6. Nitrate reductase activity in micromoles of nitrite formed per 45 minutes per gram dry weight.

Sampling Date	Minturki	Pawnee	Ponca
1. November 13	4.836	2.408	1.678
2. December 2	1.810	1.244	1.777
3. December 16	2.204	.720	.904
4. December 28	1.339	.980	.862
5. January 12	2.918	2.632	2.724
6. January 26	4.629	1.013	1.678
7. February 10	4.652	3.283	3.810
8. February 19	1.747	1.224	1.826
9. March 7	2.770	2.204	2.155
10. March 17	4.343	2.935	2.599
11. March 26	3.056	2.395	2.188
12. April 5	2.102	2.056	2.428
LSD, .05 Varieties x dates = .289			
LSD, .01 Varieties x dates = .378			

Table 7. Correlation coefficients.

Correlations	Minturki	Pawnee	Ponca	Pooled
Conductance vs. Nitrate	.842**	.903**	.848**	.892**
Total Nitrogen vs. Nitrate	.544	.395	.521	.541
Conductance vs. Total Nitrogen	.618*	.426	.563	.571
Reductase vs. Nitrate	.296	.172	.098	.211
Reductase vs. Sol. Protein	.211	.000	.259	-.098
Reductase vs. Conductance	.026	.159	.001	.124
Nitrite vs. Conductance	.032	-.126	-.366	-.198

**Significant at the 1 percent level

*Significant at the 5 percent level

CHANGES IN CERTAIN NITROGEN FRACTIONS AND NITRATE REDUCTASE ACTIVITY IN CROWNS OF THREE VARIETIES OF WINTER WHEAT DURING COLD HARDENING AND DEHARDENING

by

FRANK R. TOMAN

B. S., Kansas State University, 1961

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Agronomy

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1963

Changes in the levels of nitrate, nitrite, total nitrogen and relative nitrate reductase activity were determined in field grown Minturki, Pawnee, and Ponca winter wheats as they cold hardened and dehardened.

Cold hardiness, measured by specific conductance, showed Minturki hardiest, Pawnee intermediate, and Ponca least hardy.

Water percentages fluctuated throughout most of the sampling period. All three varieties decreased in water content during the hardening process and increased when the plants dehardened. The general level of water content was highest in Ponca, intermediate in Pawnee and least in Minturki.

Nitrate concentrations were high in plant material sampled from all three varieties before they had hardened. Concentrations were drastically reduced in the next few sampling periods, and remained at lower levels throughout the hardiest period of the plants. Nitrate concentrations were generally highest in Ponca and lowest in Minturki. However, during the period of greatest hardiness Pawnee did not differ significantly from Minturki.

Correlation coefficients between nitrate content and conductance values were significant beyond the .01 level, indicating that factors associated with decreased conductance were related to accumulation of nitrate in the plants sampled. No significant correlations were found between nitrate content and nitrate reductase activity.

Nitrite levels were generally low in the crown material. Patterns of nitrite levels indicated that nitrite might accumulate to a certain point in the plant, probably reaching a level near toxic. The concentration is then apparently reduced to a safe level by some safety mechanism or stops formation of nitrite to allow the plant to utilize the excess.

No significant correlations were found between nitrite and conductance.

Fluctuations in total nitrogen levels were noted in all three varieties. The patterns of nitrogen levels were very similar in Ponca and Minturki, with the level of Ponca significantly higher than Minturki. Levels in Pawnee were more eratic, but followed more closely those of Minturki than Ponca.

A significant correlation between total nitrogen and conductance was found in Minturki. The values for Pawnee and Ponca approached significance at the .05 level. These results indicate a definite relationship between total nitrogen and hardiness, with the hardiest variety showing the greatest relationship.

Correlation coefficients between total nitrogen and nitrate were non-significant, but approached significance at the .05 level. This indicates that nitrate level might influence changes in total nitrogen.

Three trends were noted in nitrate reductase activity: (a) a general decrease during the first few periods of hardening in the plant, (b) a general increase during that period when the plants are in the stages of maximum hardiness, and (c) a general decrease during the later stages of dehardening in the plants. These trends were characteristic of all three varieties. These trends might be explained by inferring that some portion of the protein or the prosthetic group of the enzyme underwent a conditioning similar to that of the entire plant. If so, the enzyme might have been more sensitive to low temperatures in early stages; later, after conditioning, the enzyme became more adapted to low temperatures.

Because of differences between field temperatures and the laboratory incubation temperature, the activity referred to in this experiment is not

actual enzyme activity. It is suggested that the results reflected potential activity of the enzyme under laboratory conditions.

It is possible that some of the fluctuation in enzyme activity noted was due to changes in concentration of the enzyme in the extract.

Correlations between conductance and nitrate reductase activity were non-significant at the 5 percent level. This might be attributed to the changing trends of the enzyme.

No significant correlations were found between enzyme activity and water soluble protein nitrogen. These results did not agree with those reported for corn leaves.

Since the enzyme was extracted from below the soil level, indications are that light is not a requirement for all nitrate reducing systems.