CHANGES IN NITRATE CONTENT AND NITRATE REDUCTASE ACTIVITY DURING THE GROWTH CYCLE OF WINTER WHEAT

by

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INTRODUCTION

Due to the presence of nitrogen in proteins, nucleic acids and other nitrogenous constituents of the plant cell, nitrogen metabolism is extremely important to plant growth. The principal form of nitrogen absorbed by most higher plants is nitrate. However, nitrate must be reduced to ammonia before it can be utilized by the plant. The biological process by which nitrate is converted to the ammonium or amino acid level for ultimate synthesis of N-containing cell constituents is called nitrate assimilation.

The first step in this reduction process is the conversion of nitrate to nitrite by the enzyme nitrate reductase. Reduced nitrate reductase activity in the presence of available nitrates has been shown to result in accumulation of nitrates and may influence the quality and/or quantity of other nitrogen fractions in the plant. Several factors have been implicated as being partially responsible for reduced nitrate reductase levels. Molybdenum is known to be an essential component of the enzyme nitrate reductase and, therefore, could be a limiting factor to nitrate reductase activity if deficient.

An investigation was conducted using three varieties of winter wheat during the 1964-65 growing season at the University Agronomy Farm at Manhattan, Kansas. The purpose of this study was to compare nitrate reductase activity with nitrate, water-soluble protein and molybdenum contents of various plant parts of winter wheat through the growth cycle.

REVIEW OF LITERATURE

The biological process by which nitrate is converted to the ammonia or amino acid level for ultimate synthesis of protein, nucleic acids, and other

N-containing cell constituents is called nitrate assimilation. The method by which nitrate is reduced to a useable form was not understood for some time. Early work by Anderson (3) and Eckerson (7) showed that nitrite was formed when expressed plant sap was incubated with nitrate and glucose. However, evidence was not conclusive of enzyme activity because of long incubation periods and the possibility of contamination by microorganisms. Burstrom (4) reported that wheat leaves were capable of assimilating nitrates, but only in the presence of light. He determined that when nitrates were present, carbon dioxide was assimilated and some of it was used to form "CN assimilates."

It was not until 1952, when Evans and Nason (8) partially purified and characterized a nitrate reductase from soybean leaves, that proof was obtained of an enzymatic reduction of nitrate. The enzyme isolated from soybeans was sensitive to concentrations of nitrate, being saturated by a concentration of 2×10^{-2} M potassium nitrate. The natural prosthetic group of the enzyme was found to be flavin adenine dinucleotide (FAD) and enzyme activity was not enhanced greatly by flavin mononucleotide (FMN). Spencer (21) isolated a nitrate reductase from wheat in 1958 which was in the soluble cytoplasmic fraction and highly specific for the reduced cofactor nicotinamide adenine dinucleotide (NADH). This was in contrast to the enzyme isolated by Evans and Nason (8) which was active with nicotinamide adenine dinucleotide phosphate (NADPH) and to a lesser extent with NADH. The enzyme isolated from wheat had an optimum activity of pH 7.4 and the activity was increased by the addition of FAD, while FMN gave no response. The presence of a heavy metal was indicated but attempts to isolate it were unsuccessful.

Molybdenum has been identified as the metal which plays a role with

nitrate reductase in soybeans (14) and Neuropora (15). However, only minute amounts of molybdenum were required. Barley, oats, and wheat contained from 0.03 to 0.07 ppm molybdenum and were still healthy (11). Nicholas (14) showed a direct proportionality between molybdenum content and specific activity of the enzyme. He also demonstrated the specific reactivation of the dialyzed enzyme by molybdenum. It was shown that flavin and molybdenum served in the transfer of electrons from NADH to nitrate in the following sequence:

$$\text{NADH} \longrightarrow \text{FAD (or FMN)} \longrightarrow \text{Mo} \longrightarrow NO_3^-$$

It should be noted that molybdenum deficiency during growth caused a decrease in nitrate reductase activity which was quite different than the loss of activity brought about by removal of molybdenum from the purified enzyme by dialysis (12). In the latter instance, the activity was restored to the enzyme by adding the metal back to the protein, whereas, in the case of the nutritional molybdenum deficiency, addition of the metal to the cell-free extract was ineffective.

Nason (13) pointed to several steps which pinpointed molybdenum as the metal involved with nitrate reductase: (1) nitrate accumulated in fungi and higher plants deficient in molybdenum; (2) the molybdenum requirement of Neurospora and of Aspergillus was considerably greater when nitrate was the only nitrogen source as compared with nitrite or ammonia; and (3) molybdenum deficiency specifically decreased the enzyme activity. Candella, Fisher and Hewitt (5) also showed an increase in nitrate reductase activity of molybdenum deficient plants when they were infiltrated with molybdenum. Alfridi and Hewitt (1) showed that nitrate reductase could not be stimulated by nitrate

alone but required both nitrate and molybdenum in cauliflower and mustard plants.

Results presented in the literature on the relationship between nitrate reductase and water soluble proteins (WSP) were quite variable. Hageman and Flesher (9), working with corn, found a significant positive correlation between nitrate reductase activity and WSP. Zieserl and Hageman (27), in later work, found no meaningful correlation between nitrate reductase and WSP. Zieserl, Rivenbark and Hageman (28) later found that although there was no overall correlation, there was a distinct parallelism between WSP and nitrate reductase with a 7-10 day lag period. Candella et al. (5) found that nitrate reductase activity was not closely related to the total soluble protein content in cauliflower plants. Toman and Pauli (22) found that nitrate reductase was not correlated with WSP content in crown tissues of winter wheat.

The relationship between WSP and cold hardiness in winter wheat has been studied by several investigators (26, 16, 18). Although a certain amount of association has been found between WSP and cold hardiness (26, 16), it is apparent that changes in water content as the plants harden may in part account for this relationship (17).

MATERIALS AND METHODS

Three varieties of winter wheat (Triticum aestivum L.) 'Minturki,' 'Pawnee,' and 'Ponca,' were planted at the University Agronomy Farm at Manhattan, Kansas, on October 2, 1964. Seed of all varieties was grown on the farm in previous years.

Samples of crown tissue were taken at intervals during the cold

hardening and dehardening season from November 23, 1964, to April 7, 1965, and brought to the laboratory. The crowns (that portion of tissue above the roots and below the soil surface) were removed, washed with tap water, and rinsed with distilled water. The excess surface water was removed by blotting.

Sampling of the aerial portions of the plants began March 20, 1965, and was continued at weekly intervals until maturity. The tillers were cut 1 inch above the surface of the ground and brought to the laboratory. The aerial portion was separated into different plant parts as they developed. The dates at which these separations were made are as follows:

March 20	Leaf material
April 13	Blade and sheath
May 4	Culm
May 11	Heads
May 18	Upper and lower blades and sheaths
June 1	First and second blades and sheaths

Fresh weights were determined on each sampling date. Dry weights were determined by drying for 24 hours at 70 C and reweighing. Dry matter percentages were used to calculate the dry weight of the samples used for the various analyses.

Hardiness Measurements

The specific conductivity method outlined by Dexter, Tottingham, and Graber (4) was used in determining the relative hardiness of the three varieties. Five grams of fresh crown material were placed in an uncovered Petri dish and frozen at -10 C for four hours. Crowns were transferred

to 50 ml deionized water and allowed to stand overnight at 5 C. Resistance readings were made on the solution of exosmosed solutes using a Wheatstone bridge. The crown tissue and the solution containing the exosmosed solutes were transferred to a blending cup and homogenized with a Servall Omnimixer for 2 minutes at 16,000 rpm. The extract was filtered through a fine mesh screen and centrifuged at 5,000 x G for 5 minutes. Resistance readings were made on the extract to determine total solutes in the cells. Hardiness was expressed as the percentage of solutes exosmosed after freezing at -10 C.

Nitrate Reductase

Extraction and assay of nitrate reductase activity were similar to the method employed by Hageman and Flesher (9). From 1 to 5 g of fresh plant material (depending on plant part) were homogenized with 20 ml of blending medium consisting of equal proportions of 0.1 M tris hydroxymethyl amino methane, 0.01 M cysteine and 0.0003 M ethylene diamine tetraacetic acid adjusted to a pH of 7.2. The samples were homogenized in cold (3-4 C) blending medium using a Servall Omnimixer at 16,000 rpm for 1 minute with the cup immersed in an ice bath. The cup was removed and any unmacerated tissue adhering to the side was pushed down before blending for another minute. The homogenate was filtered through a fine strainer and centrifuged at 20,000 x G for 15 minutes at 2 C. The supernatant, maintained at 2-3 C, was used for nitrate reductase assay.

Assay mixture consisted of 2 ml of 0.2 M phosphate buffer of pH 7.2, 0.2 ml of 0.1 M KNO_3, 0.5 ml of 0.00136 M reduced nicotinamide adenine dinucleotide (NADH), and 0.3 ml of enzyme extract. The mixture was incubated at 27 C for 45 minutes in a water bath. The reaction was stopped by

adding 1 ml of 1% (w/v) sulfanilic acid in 1.5 N hydrochloric acid. The color was developed by adding 1 ml of 0.02% N-(1-naphthyl)-ethylene-diamine dihydrochloride and allowed to develop for 10 minutes. The percentage transmittance was read at 540 millimicrons and referred to a standard curve prepared with sodium nitrite. Enzyme activity was expressed as micromoles of nitrite produced per 45 minutes incubation per gram dry weight.

Nitrate

From 0.2 to 0.5 g of dry plant material, ground to 40-mesh size with a Wiley mill, was extracted with 10 ml of boiling water in a test tube and analyzed for nitrate. The contents were mixed thoroughly and allowed to stand for at least 2 hours to allow the plant material to settle.

Determinations of nitrate were made by a modification of the Nelson, Kurtz, and Bray method as reported by Woolley, Hicks, and Hageman (24). Two ml of aqueous extract were added to 10 ml of 20% acetic acid containing 0.2 ppm of copper sulfate. About 0.4 g of reducing powder, consisting of 100 g of barium sulfate, 75 g of citric acid, 10 g of manganous sulfate dihydrate, 4 g of sulfanilic acid, 2 g of powdered zinc, and 2 g of 1-naphthylamine was added. Duplicate aliquots of the extract were analyzed using the same procedure except omitting the reducing powder, thereby correcting for the color imparted to the sample by the plant material. The test tubes were shaken vigorously for exactly 15 seconds and allowed to stand for 3 minutes. After repeating this procedure three times, the mixture was centrifuged at 6,000 x G for 5 minutes. The supernatant was filtered through a loose plug of glass wool and the absorbance was read on a Bausch and Lomb Spectronic 20 colorimeter at a wavelength of 540 millimicrons. Nitrate standard curves

were made by substituting known concentrations of calcium nitrate for the extract. Samples were referred to the standard curve and nitrate concentrations were expressed as micromoles of nitrate per gram of dry weight.

Water Soluble Protein

Water soluble protein nitrogen was determined by a modification of the method used by Pauli and Mitchell (17). A known weight of fresh tissue was blended in approximately 40 ml of distilled water in a Servall Omnimixer at 16,000 rpm for 1 minute with the blender cup immersed in an ice bath. Any adhering tissue was pushed down into the cup and reblended for another minute. The mixture was filtered through Whatman No. 4 filter paper with suction. The cup and cutting assembly was rinsed several times with distilled water and the rinsings were used to wash the macerated tissue held by the filter paper. The filtrate was made to 100-ml volume and a 20-ml aliquot was taken for analysis. The pH of the aliquot was adjusted to 4.5 with dilute glacial acetic acid and the proteins were precipitated in a boiling water bath. The samples were cooled immediately and stored overnight at 3 C. The precipitate was filtered off with Whatman No. 2 filter paper using suction and washed repeatedly. The filter paper with the precipitate was transferred to micro Kjeldahl flasks and nitrogen determinations were made by the Gunning modification of the Kjeldahl method (2) using boric acid in the receiving flask (19).

Molybdenum

Molybdenum concentrations of dry weight samples were determined using a modification of the method used by Ulrich et al. (23). Samples were dry

ashed in pyrex beakers at 650 C for 16 hours in a muffle furnace. Ash was taken up in 10 ml of deionized water and 10 ml of 6.5 N hydrochloric acid containing 50 ppm of ferric chloride. After gentle warming to dissolve the ash, the contents were filtered through Whatman No. 40 filter paper into 60-ml separatory funnels marked to contain 45 ml of solution. Deionized water was added to bring the volume to 45 ml and the solution was saturated by shaking vigorously with 4 ml of a 1:1 mixture of isoamyl alcohol and carbon tetrachloride. Saturating the solutions was necessary to ensure a quantitative recovery of the color-extracting solvent used later. The saturating solution was quantitatively discarded after allowing 10 to 15 minutes for the phases to separate. One ml of 30% sodium thiocyanate was added and mixed. This was followed by adding 0.5 ml of 20% stannous chloride and mixing until any interfering red color was removed. The color was extracted by adding 1 ml of the isoamyl alcohol-carbon tetrachloride mixture and shaking vigorously for 2 minutes. All traces of water were removed from the funnel tip and stopcock bore with suction. One or 2 drops of the extractant were discarded and the remainder was transferred to test tubes. The absorbance was measured at 470 millimicrons with a Beckman DU Spectrophotometer after disappearance of any apparent turbidity. Sample readings were compared to a standard curve prepared with molybdenum trioxide and concentrations were expressed as ppm on a dry weight basis.

Statistical Analysis

Correlation coefficients between paired observations of hardiness, nitrate reductase activity, nitrate and water-soluble protein nitrogen contents were determined for each plant part according to Snedecor (20).

Significance of the correlation coefficients was determined by a tabular test and expressed as significant (5% level) or highly significant (1% level).

EXPERIMENTAL RESULTS

AUTUMN AND WINTER

Hardiness

Results of the cold hardiness tests are shown in Figure 1. All varieties were relatively unhardy when the first sample was taken during November. Minturki showed the greatest hardiness on this date, followed by Ponca and Pawnee, respectively. The percentage of electrolytes exosmosed dropped rapidly during the next four sampling dates, thus indicating that the plants were attaining a higher degree of hardiness. Maximum hardiness was reached on January 4, 1965, in Minturki and Ponca, and on February 2 in Pawnee. Although Ponca was initially hardier than Pawnee, this relationship was true on only one other sampling date. Pawnee was hardier than Ponca on nine of the 11 sampling dates. Thus, the over-all ranking of hardiness was Minturki, hardiest; Pawnee, intermediate; and Ponca, least hardy.

All varieties dehardened rapidly from February 2 until March 6. On the next sampling date, March 20, conductance values dropped greatly, indicating an increase in hardiness. After March 20 all varieties dehardened rapidly and by April 7 had reached a stage of dehardiness equal to or greater than at the initial sampling date, November 23.

Nitrate and Nitrate Reductase

Results of nitrate content and nitrate reductase (NR) activity in the

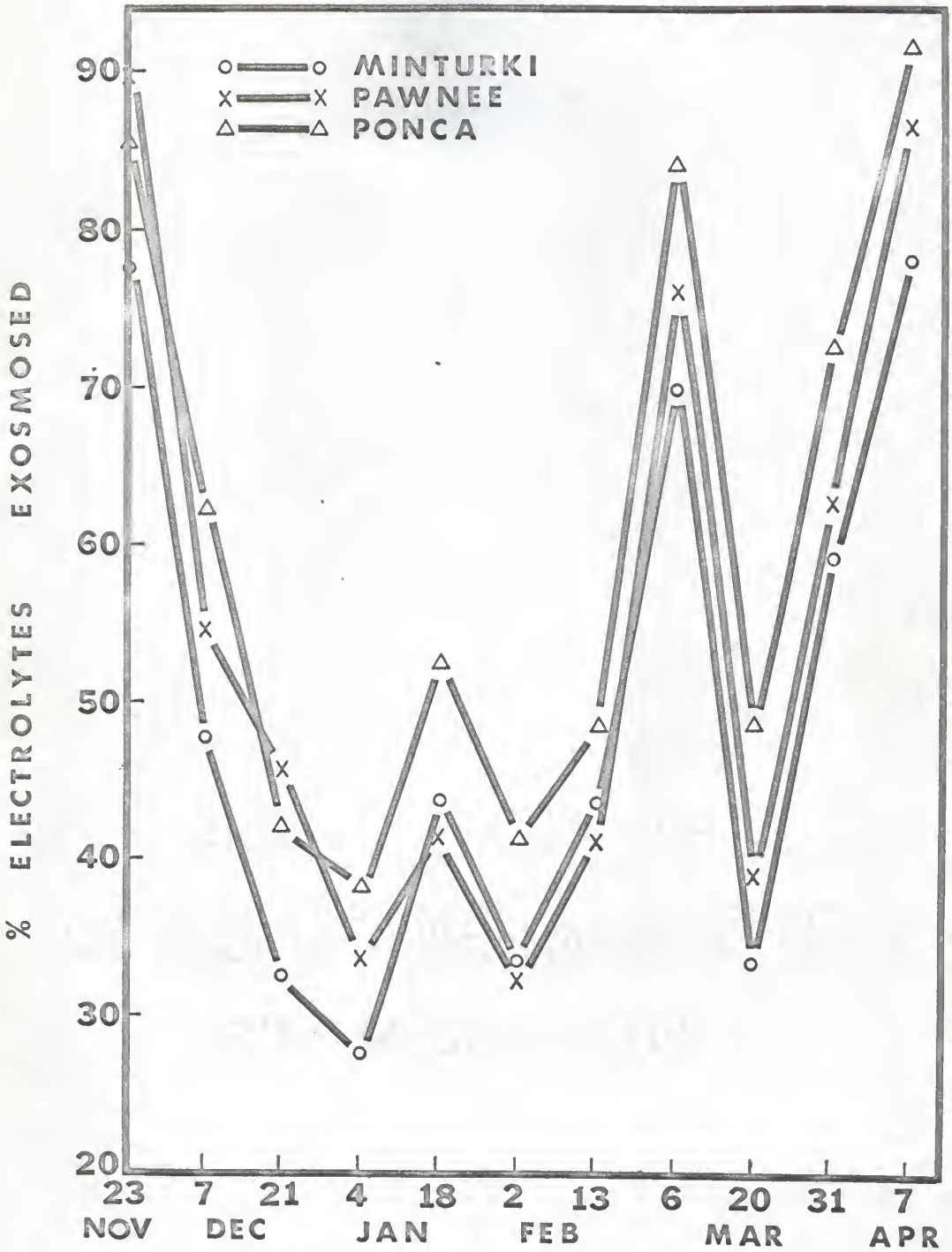

Fig. 1. Cold hardiness as per cent of electrolytes exosmosed from wheat crown tissue after freezing at -10 C.

crown tissue are shown in Figure 2. Concentrations of nitrate were generally high in all three varieties on the first sampling date. The nitrate content then decreased in all varieties as the plants hardened. During dehardening in the spring, nitrate concentrations increased and reached a higher level than was found on the initial sampling date. Differences in the nitrate content of the three varieties were found throughout the sampling period. On nine of the 11 sampling dates, Minturki was markedly lower in nitrate content than either Pawnee or Ponca and Pawnee was lower than Ponca. The general ranking was Ponca, highest; Pawnee, intermediate; and Minturki, lowest.

NR activity was initially at a low level in both Pawnee and Ponca and increased slightly during the period of maximum hardiness. Minturki, on the other hand, had a higher level of nitrate reductase activity initially and activity decreased with the next sampling date. After this date, Minturki closely followed Pawnee and Ponca in NR activity. Enzyme activity in all varieties increased slightly as the plants entered the stage of maximum hardiness. All varieties showed a slight decrease in enzyme activity during the early stages of dehardening but increased rapidly with later stages of dehardening. All varieties attained a considerably higher level of enzyme activity by April 7, than was found during the initial sampling dates in the autumn.

In contrast to the low nitrate content found in Minturki, NR activity was predominately higher in Minturki than in either Pawnee or Ponca. Results showed Ponca as generally having a slightly higher level of nitrate reductase activity than did Pawnee, although these two varieties closely paralleled each other in enzyme activity.

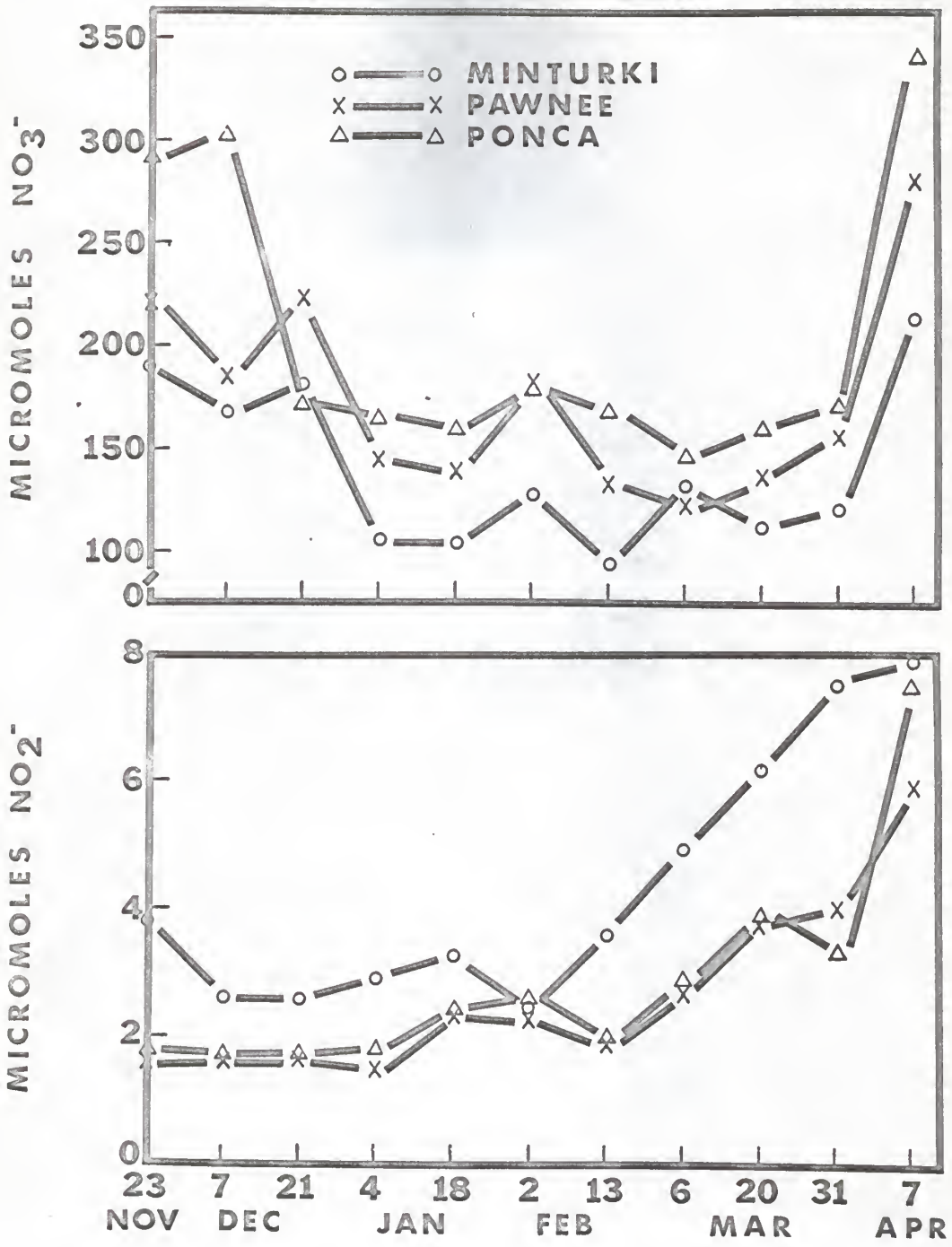

Fig. 2. Nitrate (upper) and nitrate reductase activity (lower) per gram dry weight of wheat crown tissue.

Water-Soluble Protein

Results of analysis for the water-soluble protein-nitrogen (WSP-N) content of crown tissue are found in Figure 3. WSP-N increased in all varieties from November 23 to December 7. After the latter date, a slight decrease in WSP-N content was found in all varieties as they began to harden. Following the period of maximum hardiness, the WSP-N content generally increased through the dehardening stage. Although there was considerable fluctuation during this period, the WSP-N content on the last sampling date was considerably higher than the level found in the autumn during the initial sampling date. Although differences in WSP-N concentrations among the three varieties were not always in the same direction, Pawnee generally contained lower levels than either Minturki or Ponca. The latter two varieties alternated frequently in levels of WSP-N. Minturki, however, contained considerably higher levels of WSP-N on the last two sampling dates than did either of the other varieties.

SPRING

Nitrate and Nitrate Reductase

Results of nitrate contents and NR activity of the leaf and blade tissue are shown in Figures 4 and 5. Samples taken during the first three sampling dates, starting March 20, consisted of leaf tissue since the plants were tillering and no separation was made between blades and sheaths. Beginning April 13, the sheath and blades were analyzed separately, for nitrate content and NR activity.

The nitrate content of the leaf tissue was initially low in all

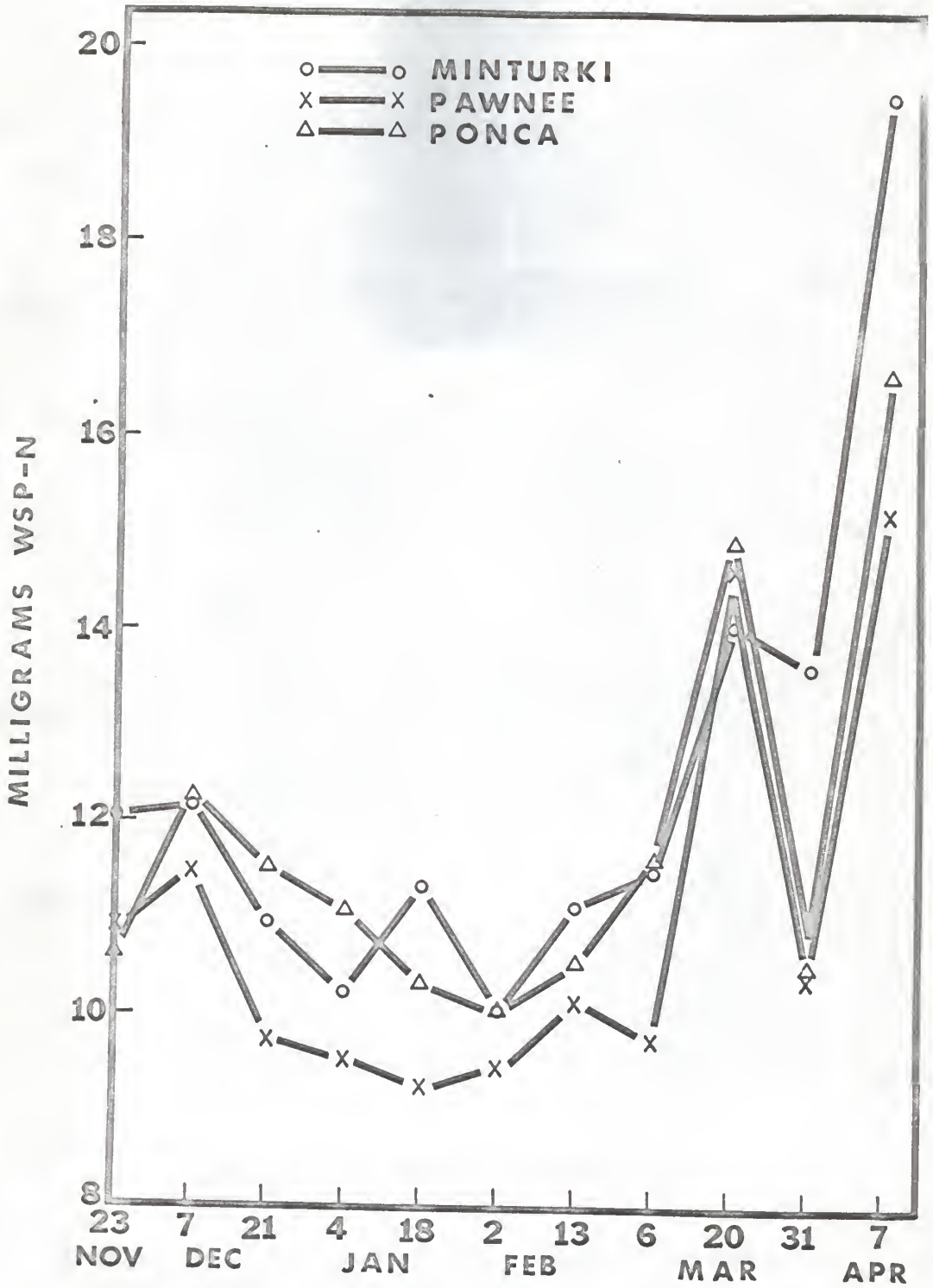

Fig. 3. Water-soluble protein nitrogen per gram dry weight of wheat crown tissue.

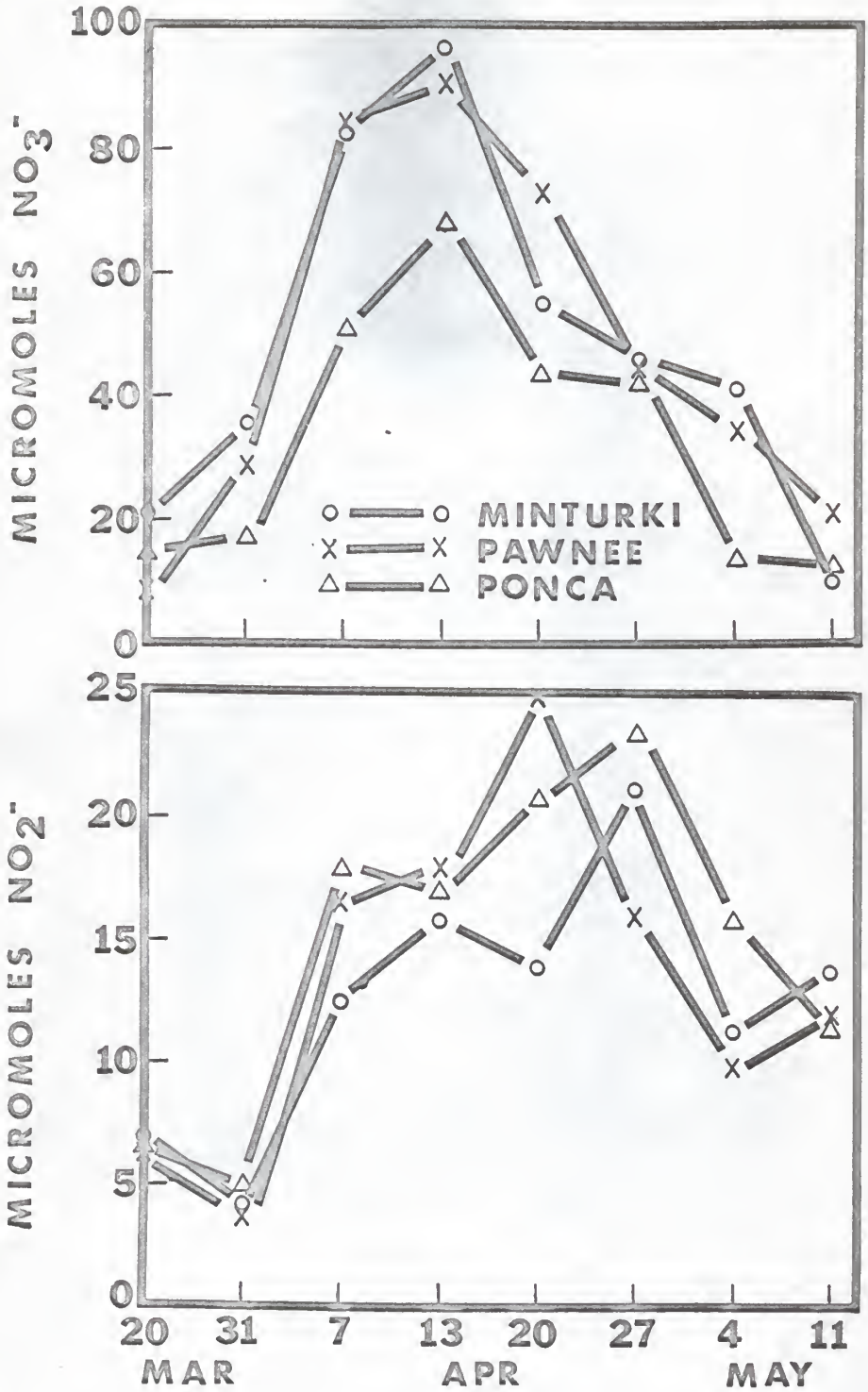

Fig. 4. Nitrate (upper) and nitrate reductase activity (lower) per gram dry weight of total leaf tissue of wheat.

varieties and increased rapidly during the initial sampling dates. After separation of the blade tissue from the sheath tissue, nitrate content of the blade tissue increased until April 13, at which time all varieties reached a maximum. The nitrate content then decreased in all varieties through the remaining sampling dates. Minturki and Pawnee alternated frequently in concentrations of nitrate, and Ponca contained lower levels of nitrate than the other varieties during most of the sampling periods. The differences between nitrate contents of Minturki and Pawnee were not always in the same direction. Pawnee generally had greater concentrations during the earlier stages of spring growth and Minturki had higher concentrations during the latter sampling dates.

The NR activity of all three varieties generally increased in samples of leaf tissue taken from March 20 through April 7. NR activity in the blade tissue increased and reached peaks on April 20 in Pawnee and on April 27 in Minturki and Ponca. During the following sampling dates until May 11, NR activity generally decreased in all varieties. NR activity of the leaf and blade tissue was generally higher in Ponca than in either Pawnee or Minturki.

Figure 5 shows results of analysis for nitrate and NR activity of the separate portions of blade tissue. Blade tissue was separated initially into upper and lower portions. The upper portion consisted of the blades of the upper two leaves. The lower portion was comprised of the blades of the remaining leaves.

Initially, all varieties showed greater concentrations of nitrate in the lower blades than in the upper blades. This relationship was generally true for the remainder of the sampling dates until maturity, although the difference decreased considerably as the plants neared maturity. After an initial

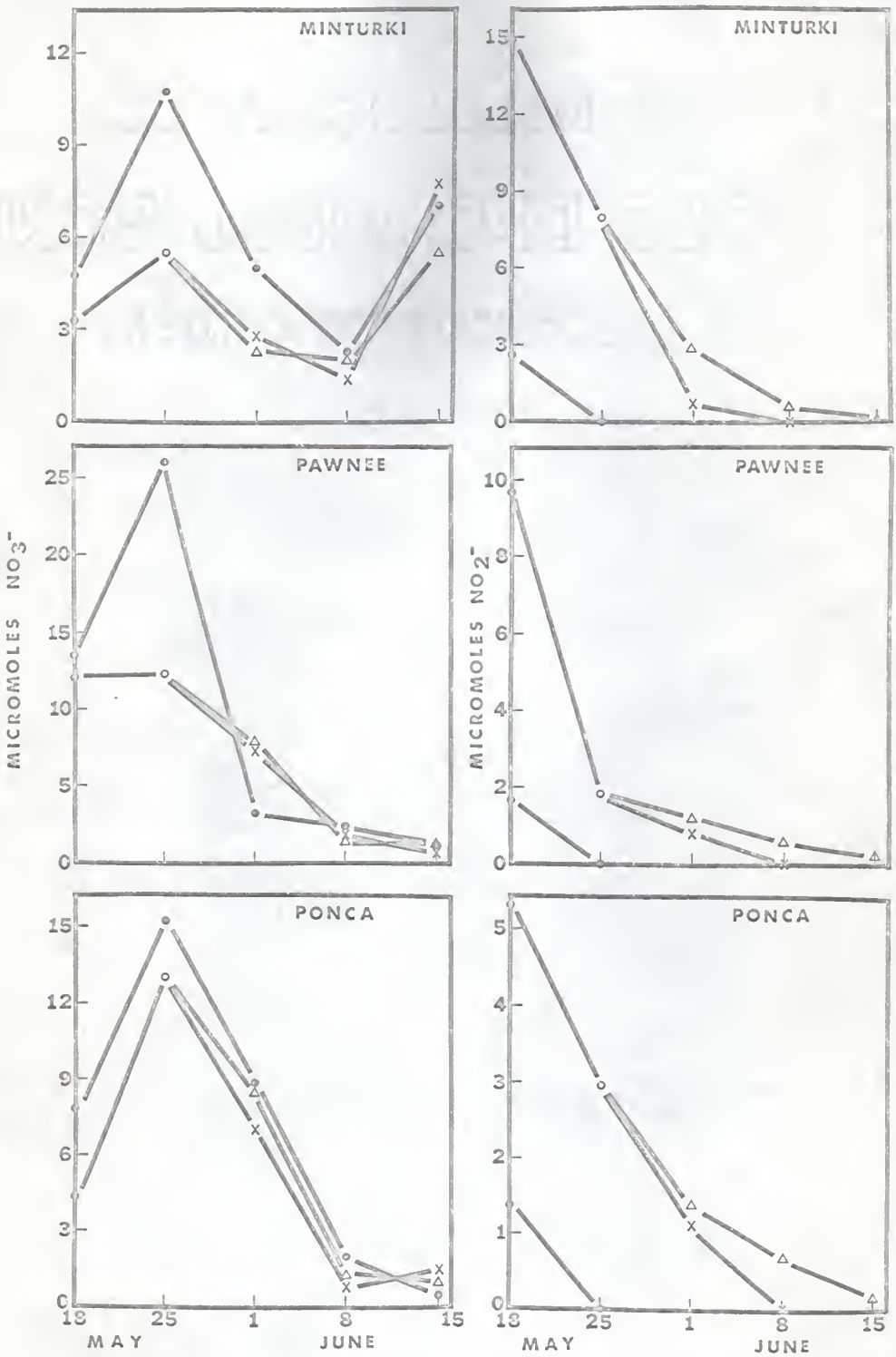

Fig. 5. Nitrate (left) and nitrate reductase activity (right) per gram dry weight in upper (o---o), first (Δ---Δ), second (X---X), and lower (•---•) blade tissue of wheat.

increase in the nitrate content of the lower blades, there was a general decrease with samples taken later in the season.

The nitrate content of the upper blades followed the trend seen in lower blades in all varieties with an initial increase from May 18 to May 25. Upon separation of the upper blades into first and second blades, on June 1, nitrate analysis failed to show any consistent difference between these plant parts. The differences found were slight and not always in the same direction. As was seen with lower blades, the nitrate content of the upper blades generally decreased with maturity.

Nitrate reductase activity was markedly higher in the upper blades than in the lower blades. The lower blades of all varieties declined rapidly in activity and, by May 18, NR activity was no longer detectable. The level of enzyme activity also dropped rapidly in the upper blades from May 18 to May 25 in all varieties. Upon separation of the upper blades into first and second blades, results showed the first blade of all varieties to have higher levels of enzyme activity on all sampling dates. As the plants approached maturity, enzyme activity levels decreased in all varieties in both the first and second blades. The first blade retained enzyme activity the longest. It lost all detectable activity by June 15, while the second blade lost enzyme activity on June 8. All varieties lost enzyme activities in a given blade part on the same dates. The levels of NR activity were generally higher in Minturki, intermediate in Pawnee, and lowest in Ponca, although the range decreased with maturity.

The nitrate contents and NR activities of sheath tissue are found in Figures 6 and 7. The sheath tissue was initially treated as a composite sample considered as total sheath (Fig. 6). On May 18, the sheath tissue

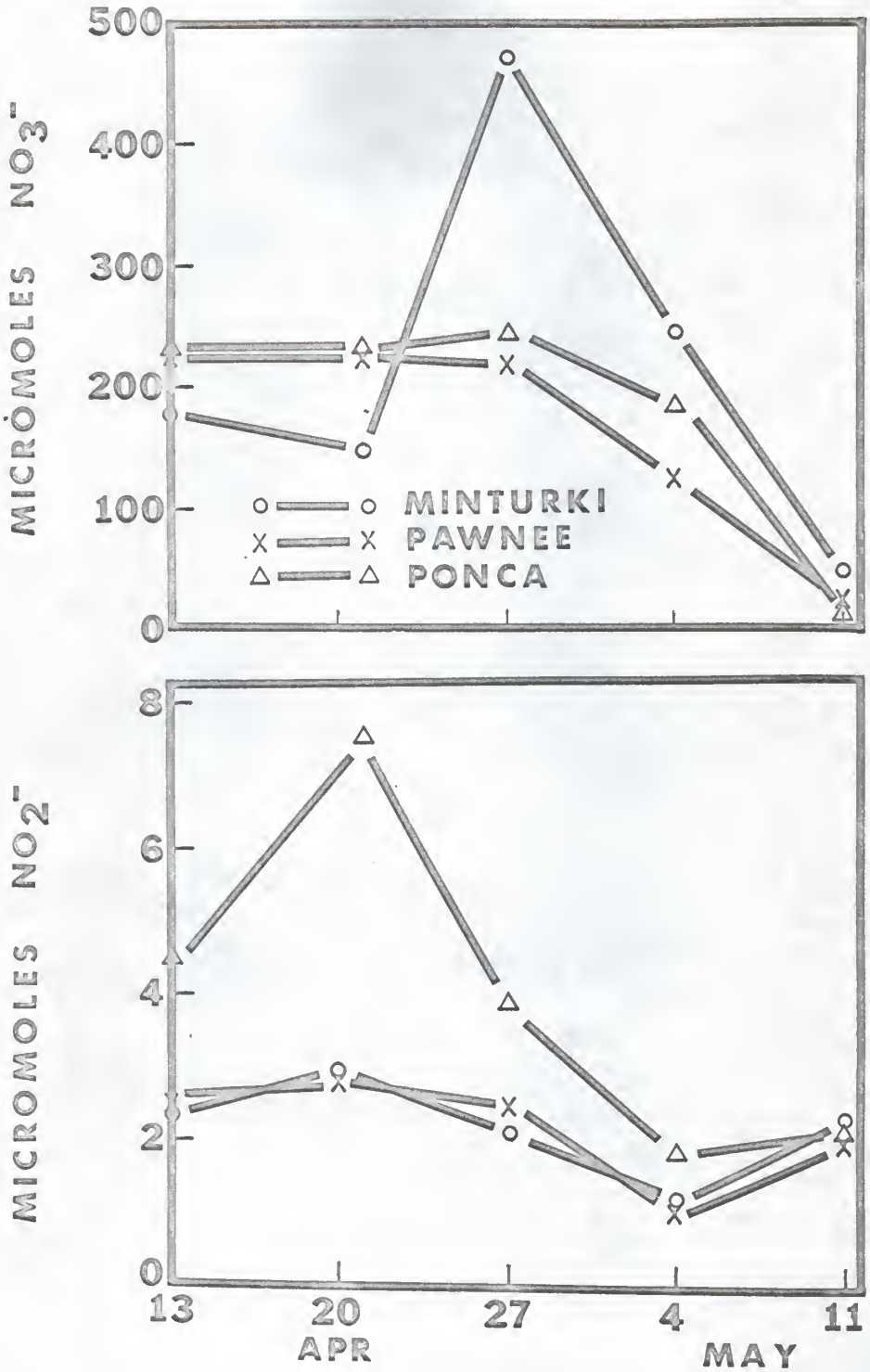

Fig. 6. Nitrate (upper) and nitrate reductase activity (lower) per gram dry weight of total sheath tissue of wheat.

was separated into upper and lower portions. Upper sheaths were associated with the two upper most leaves and the lower sheaths with the remaining lower leaves (Fig. 7). The upper portion was further separated into first and second sheaths and analyzed separately on June 1 (Fig. 7).

The nitrate content of the total sheath tissue generally decreased in all varieties from April 13 through May 11. Minturki fluctuated considerably within these sampling dates. Ponca and Pawnee closely paralleled each other in nitrate content, but Ponca had a slightly higher level than Pawnee. Minturki contained a lower concentration of nitrate on the first two sampling dates, then rose sharply and remained at a higher level than either Pawnee or Ponca through May 11.

As was true for nitrate, there was a general decrease in NR activity in the total sheath tissue with samples taken later in the season. Analysis showed the total sheath tissue of Ponca to be considerably higher in NR activity than either Pawnee or Minturki. The latter two varieties varied in levels of enzyme activity, but closely paralleled each other.

The lower sheath tissue was higher in nitrate content than the upper sheath tissue on all sampling dates until maturity (Fig. 7). There was a general trend of decreasing nitrate concentration in both the upper and lower sheaths as the plants approached maturity. On June 1, separation of the upper sheath portion into first and second sheaths showed all varieties to have a higher level of nitrate in the second sheath than in the first sheath. On the following date, June 8, the differences decreased markedly and in two varieties, Minturki and Pawnee, the trend was reversed with the first sheath having a higher nitrate content than the second sheath. However, the difference in nitrate content between these two plant parts was slight. Again

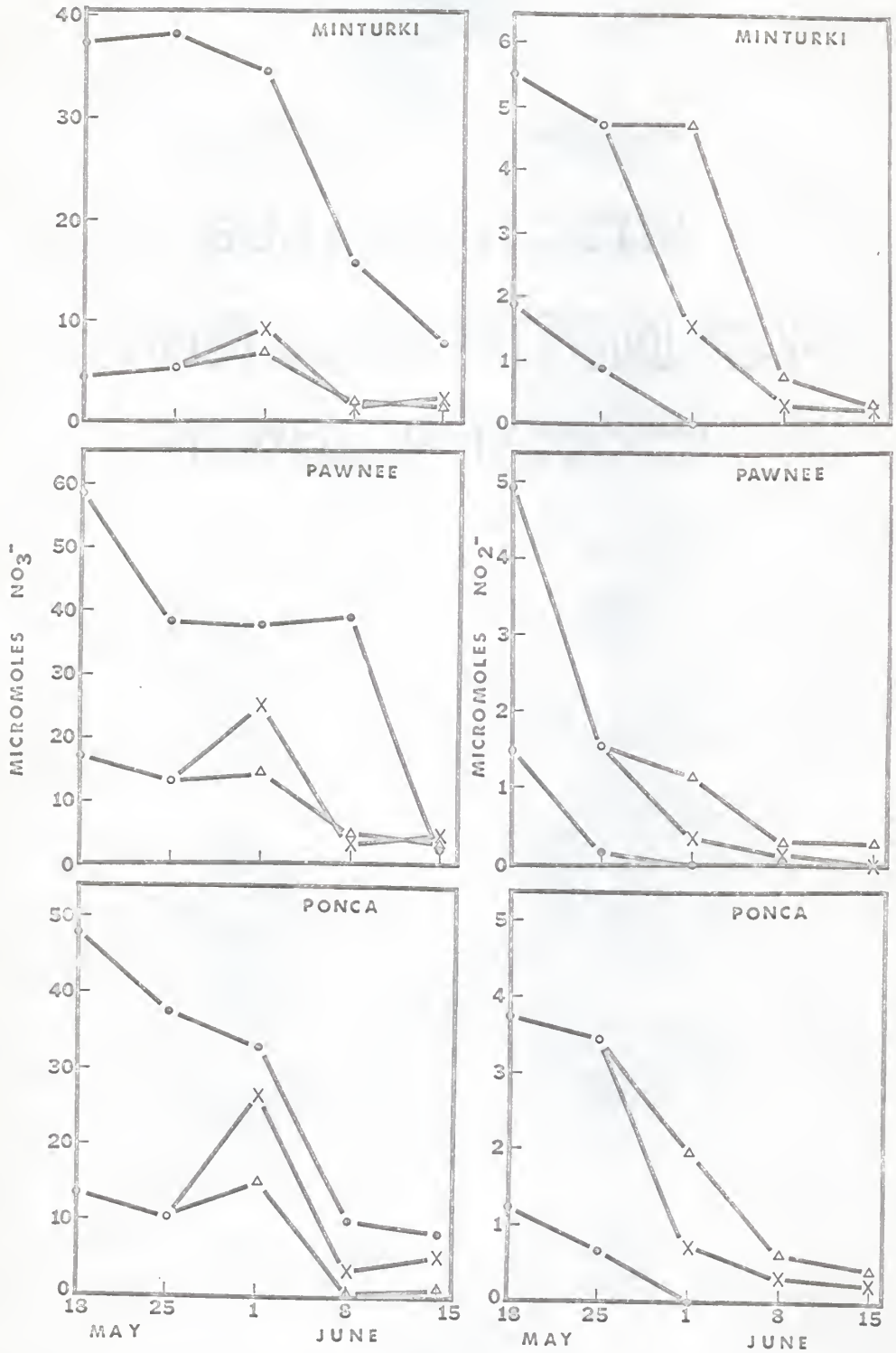

Fig. 7. Nitrate (left) and nitrate reductase activity (right) per gram dry weight in upper (o---o), first (Δ---Δ), second (X---X), and lower (•---•) sheath tissue of wheat.

on June 15, all varieties showed a slightly higher nitrate content in the second sheath than in the first sheath.

In contrast to the high nitrate content in the lower sheath, NR activity was considerably less in the lower sheaths compared with the level found in upper sheaths in all varieties. It was noted that although the lower sheaths decreased in enzyme activity similar to lower leaves, a small amount of activity was still found on May 25 in the lower sheaths of all varieties. The lower blade tissue had lost all activity on this date. NR activity decreased in the upper sheaths in all varieties from May 18 to May 25. Upon separation of the upper sheaths into first and second sheaths, it was noted that the first sheath had higher enzyme activity in all varieties and on all sampling dates. Again, enzyme activity decreased in both first and second sheaths with the onset of maturity. As was seen in the lower sheath, the NR activity was retained for a longer period of time in both the first and second sheaths than in the first and second blades, respectively.

Results of nitrate content and NR activity assays of culm tissue are given in Figure 8. Sampling of the culm tissue began May 4 and was continued until maturity. Culm tissue was extremely high in nitrate content compared with the other plant parts. On the initial sampling date, Ponca contained considerably more nitrate than did either Pawnee or Minturki. Nitrate content dropped rapidly during the next two sampling dates in all varieties. A general decrease in nitrate was noted in all varieties throughout the remainder of the sampling dates although this decrease was very gradual. Minturki generally contained lower levels of nitrate than did Pawnee or Ponca. The latter two varieties alternated frequently in levels of nitrate concentration. However, Pawnee and Ponca were nearly similar in nitrate content

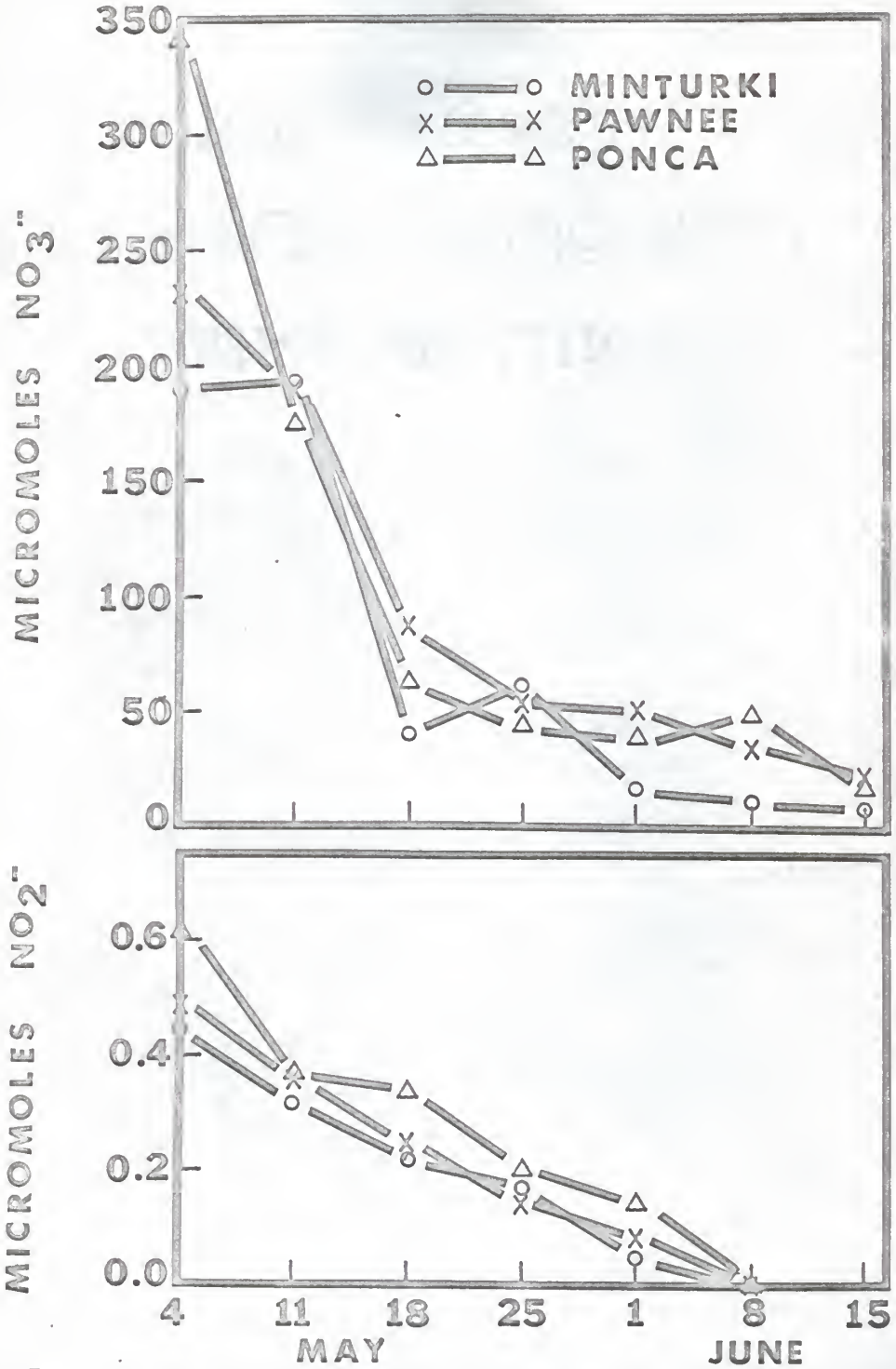

Fig. 8. Nitrate (upper) and nitrate reductase activity (lower) per gram dry weight of culm tissue of wheat.

throughout the sampling periods.

In contrast to the high level of nitrate in the culm tissue, NR activity was extremely low. All varieties decreased linearly in NR activity from May 4 until June 8, at which time the level of enzyme activity had dropped to a point where it could no longer be detected in any variety. Ponca showed a slightly higher level of activity on all sampling dates than did either Pawnee or Minturki. Minturki contained the lowest level of enzyme activity on all except one sampling date, while Pawnee was generally intermediate in enzyme activity. It was noted that although the culm tissue was markedly low in NR activity, this tissue retained detectable activity for a longer period than either the lower leaves or lower sheaths.

Nitrate contents and NR activity of head tissues are shown in Figure 9. Differentiation of tissue to form recognizable heads occurred on May 11 in Pawnee and Ponca and on May 18 in Minturki. The nitrate content of the head tissue fluctuated considerably from the initial sampling date until maturity and was extremely low compared with other aerial plant parts. There was a general trend of reduced levels of nitrate through the first five sampling dates in all varieties. However, there was a trend of increased nitrate content from June 8 until maturity on June 22. The level of nitrate found on June 22 was higher in all varieties than the level found on the first sampling date. No clear-cut varietal differences were noted with respect to nitrate content of head tissue.

NR activity was initially low in the head tissue and generally increased to a maximum level on June 1. Following this peak, NR activity decreased in samples taken progressively later in the season. By the time maturity was reached on June 22, the level of NR found was extremely low. Ponca and

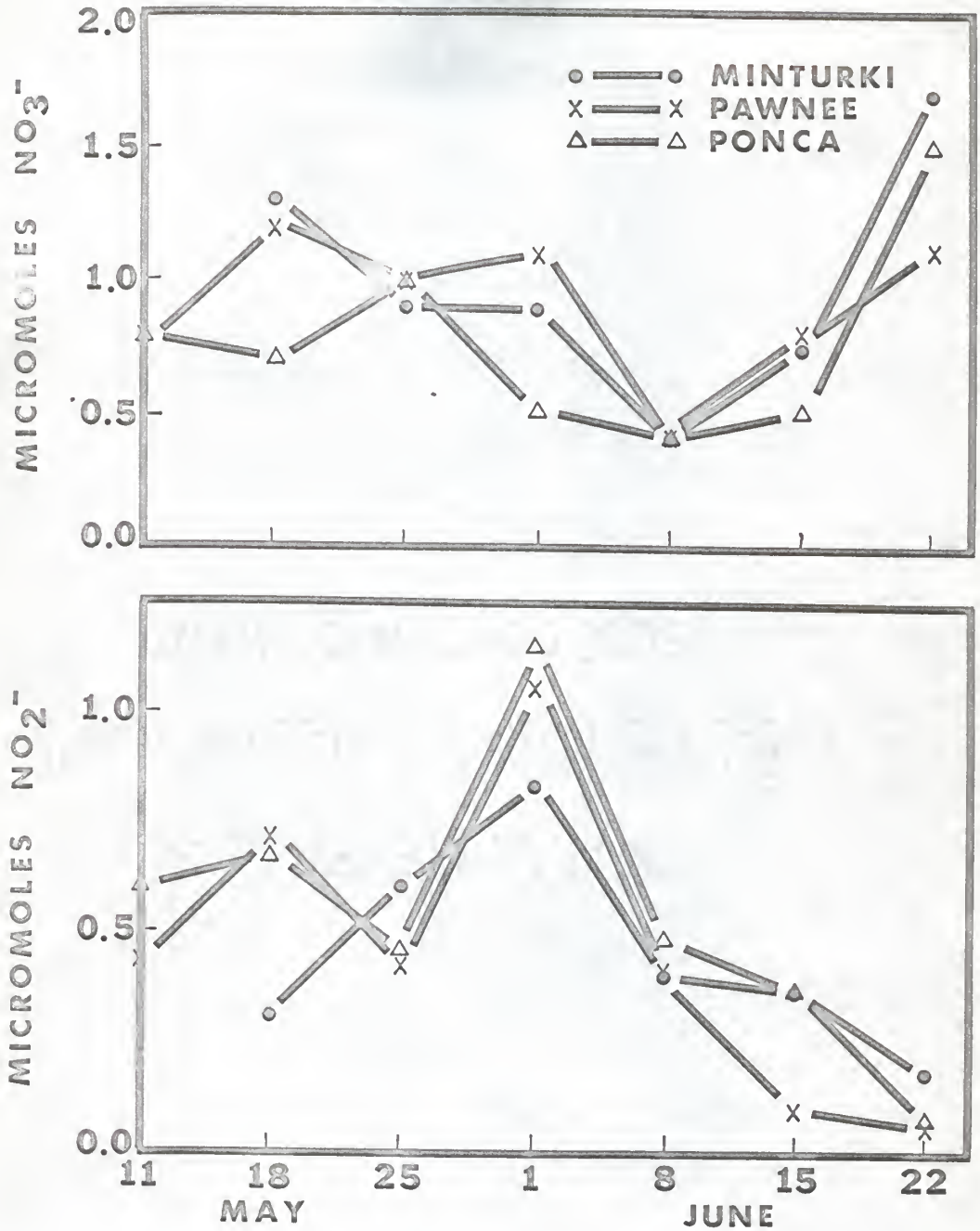

Fig. 9. Nitrate (upper) and nitrate reductase activity (lower) per gram dry weight of head tissue of wheat.

Pawnee generally paralleled each other in NR activity. Minturki was later in maturing and did not follow similar levels of NR activity found in the other varieties. Ponca was generally slightly higher in enzyme activity than Pawnee, while Minturki ranged from having the highest to the lowest activity on different sampling dates.

Water-Soluble Protein

Results of WSP-N analysis for leaf and blade tissue are shown in Figure 10. On the first sampling date, April 7, the sample consisted of both blade and sheath tissues. The WSP-N content on this date was low in all varieties compared with later sampling dates. On April 13, the sheath and blade tissues were analyzed separately and the level of WSP-N in the blade tissue was higher in all varieties than on the previous date when a composite sample was analyzed. WSP-N generally decreased in the total blade tissue with samples taken progressively later in the season. After separation of the blade tissue, results showed a higher level of WSP-N in the upper blades than in the lower blades of all varieties. The lower blade portion decreased in WSP-N content and reached a relatively low value by June 1 in all varieties. This low level of WSP-N was maintained through the remaining sampling dates. Analysis of the first and second blades on June 1 showed a higher WSP-N content in the first blade than in the second blade. This relationship held in all varieties through the remainder of the sampling dates until maturity. All varieties dropped to an extremely low level of WSP-N by the last sampling date, June 15. Minturki showed a lower level of WSP-N during the period in which analysis were run on total blades. However, after separation of the upper blades into first and second blades on later dates, analysis of the

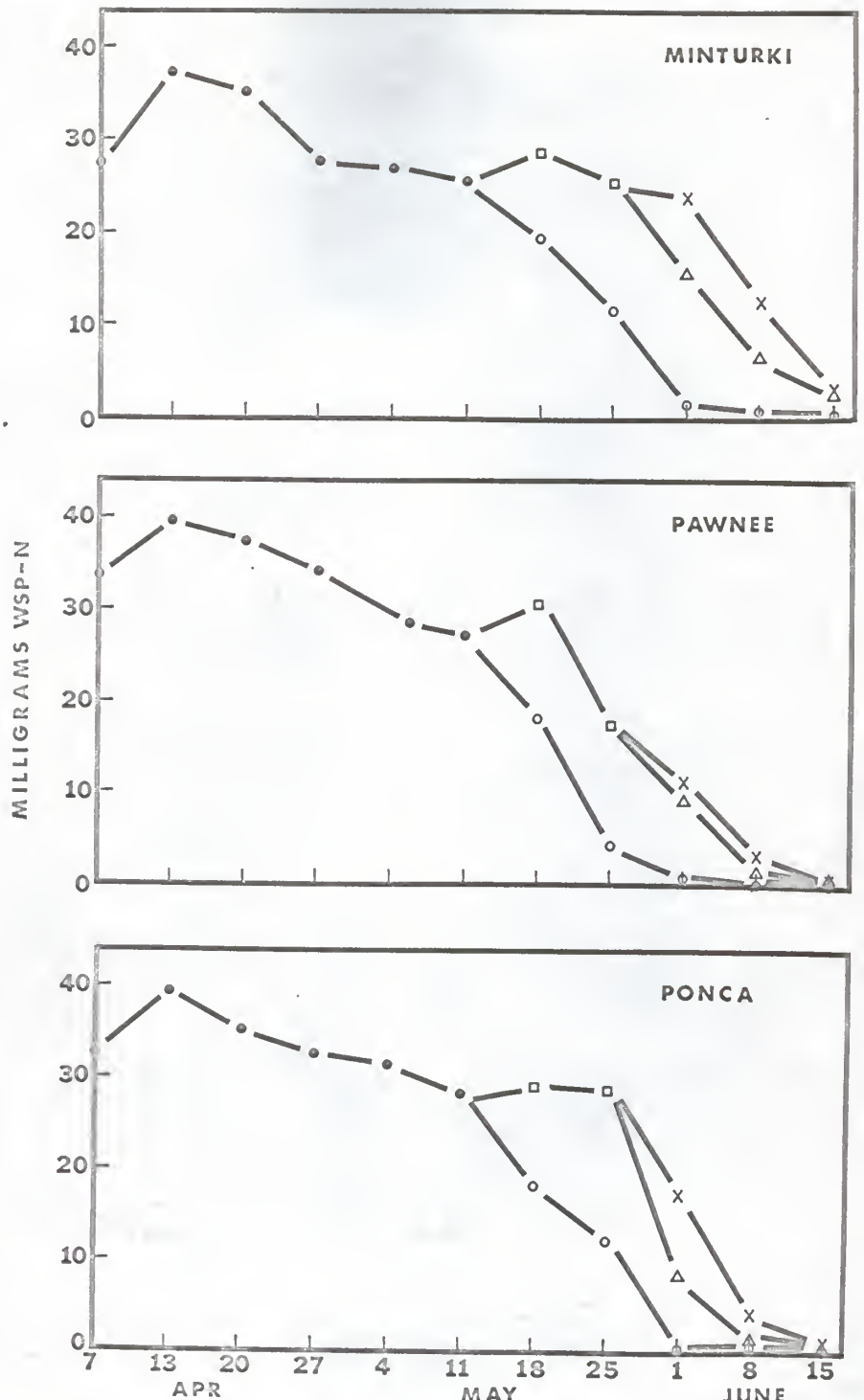

Fig. 10. Water-soluble protein nitrogen per gram dry weight in total (●---●), upper (□---□), first (X---X), second (Δ---Δ) and lower (o---o) blade tissue of wheat.

first and second blades of Minturki showed a generally higher level of WSP-N than the other varieties. Pawnee and Ponca, on the other hand, alternated frequently in WSP-N content and no consistent differences were noted.

The WSP-N content of the sheath tissue is shown in Figure 11. During the first four sampling dates, all sheath material was combined in a composite sample. Analysis of this plant part showed a general decrease of WSP-N in all varieties. On May 18, the upper and lower sheath tissues were analyzed separately. The upper sheaths had a predominantly higher WSP-N content in all varieties than did the lower sheaths. The level of WSP-N in the upper sheaths of Pawnee decreased steadily from the initial separation date until maturity. Minturki and Ponca increased in WSP-N content from May 18 to May 25, followed by a general decrease until maturity. As was true for blade tissue, the WSP-N content was highest in the first sheath compared with the second sheath on all sampling dates at which this separation was made. Pawnee, on most sampling dates, showed a lower level of WSP-N than was found in either Ponca or Minturki. Minturki and Ponca, on the other hand, showed no consistent differences. Although these two varieties frequently changed in ranking they paralleled each other closely in WSP-N content of the sheath material. Although the WSP-N content of the sheaths decreased with maturity, the rate at which this decrease occurred was not as rapid as in blade tissue. For instance, on the final sampling date, June 15, the sheath material of all varieties exhibited greater quantities of WSP-N than were found in blade material.

Results of WSP-N determinations of the culm tissue are found in Figure 12. The WSP-N content of the culm tissue was relatively low compared with levels found in sheath and blade tissue. There was a decrease in WSP-N content from May 4 to May 11 in all three varieties. During the following two

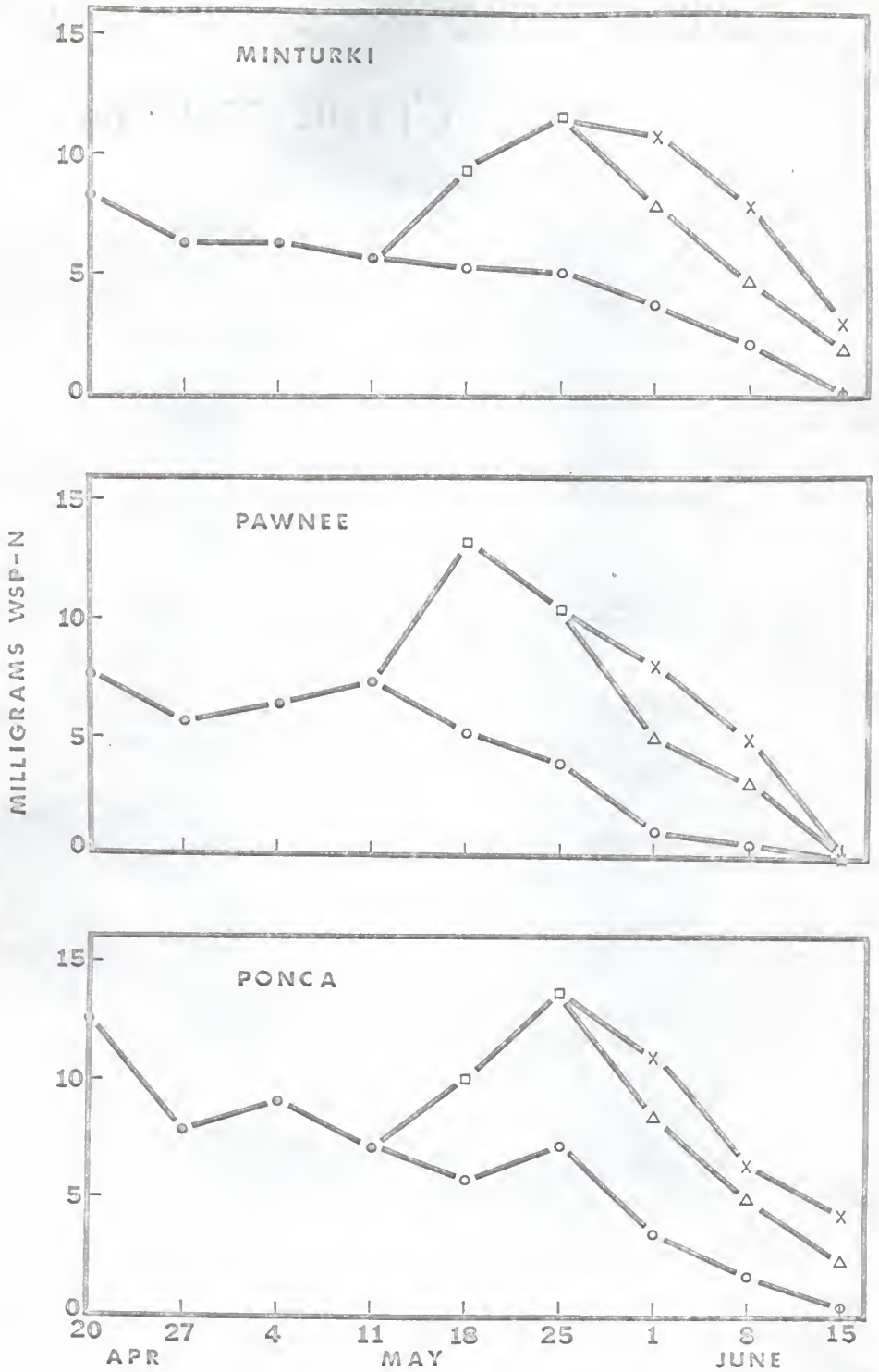

Fig. 11. Water soluble protein nitrogen per gram dry weight in total (o---o), upper (□---□), first X---X), second (Δ---Δ) and lower (o---o) sheath tissue of wheat.

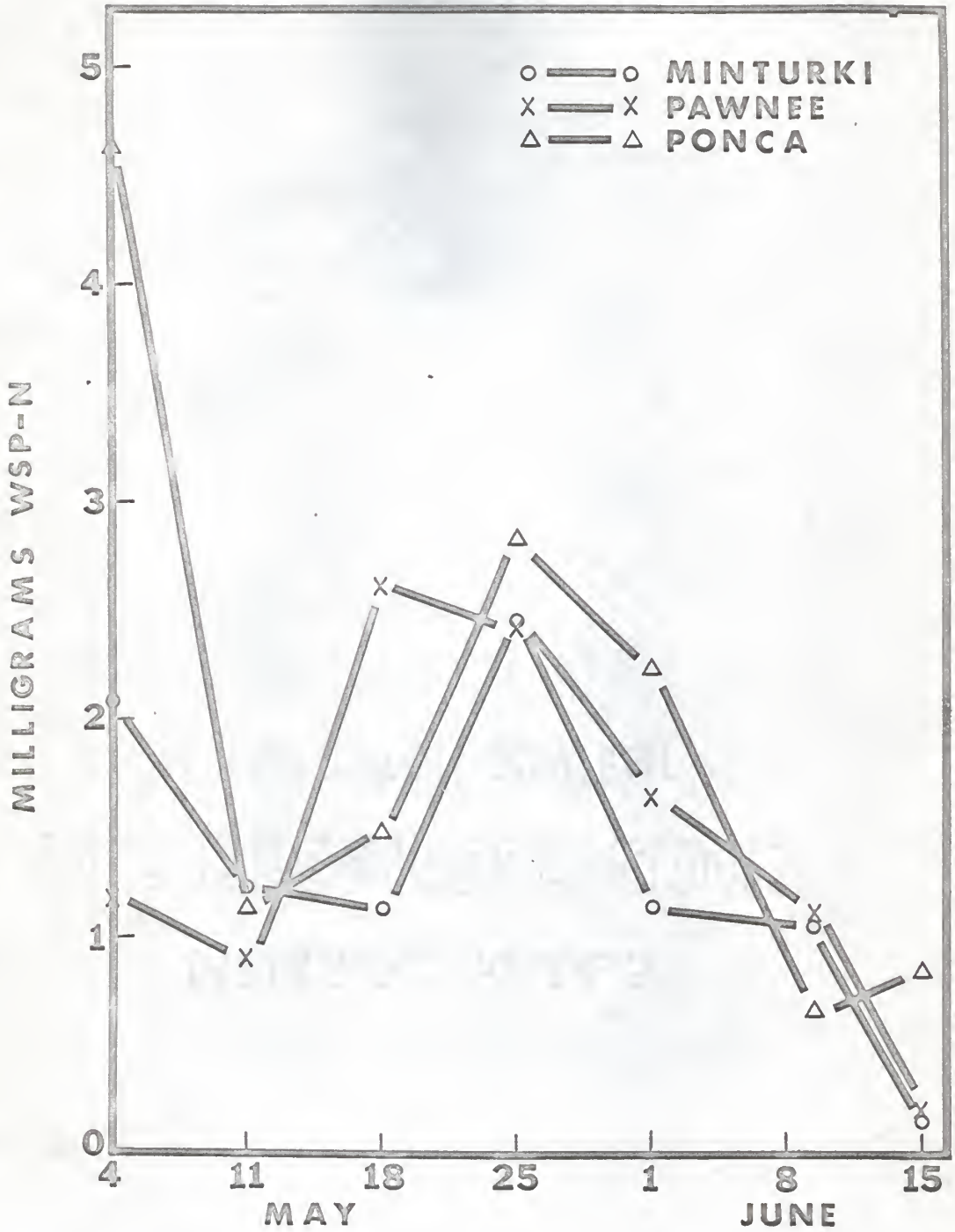

Fig. 12. Water-soluble protein nitrogen per gram dry weight of culm tissue of wheat.

sampling dates, WSP-N content generally increased. WSP-N then dropped off rapidly in all varieties as the plants approached maturity on June 15. No consistent difference was found in varietal contents of WSP-N of the culm. The three varieties frequently varied in content of this component.

Results of the WSP-N determinations of the head tissue are found in Figure 13. Differentiation of the head tissue occurred in Pawnee and Ponca by May 11, but not until May 25 in Minturki. All varieties followed a general downward trend in WSP-N content as the heads approached maturity. Pawnee generally contained a smaller quantity of WSP-N than did either Ponca or Minturki, and Minturki generally contained greater amounts of WSP-N than did Ponca.

Molybdenum

Analysis for molybdenum (Appendix; Tables 1, 2, and 3) showed all varieties to contain relatively low concentrations during the initial sampling dates when the composite leaf sample, consisting of both blades and sheaths, was analyzed. On April 13, when the leaf tissue was separated into blades and sheaths, levels of molybdenum in the blades were considerably higher in all varieties compared with the previous composite samples. A general trend of decreasing molybdenum content was seen in the total blade tissue during later sampling dates. This trend held true for all varieties. No consistent difference in molybdenum content in any variety was detected upon separation of the blade tissue into upper and lower portions. As was true for total blade tissue, maturity generally decreased the molybdenum concentration of the total sheath tissue. Upon separation of the upper and lower sheaths, results showed in most instances a higher concentration of molybdenum in

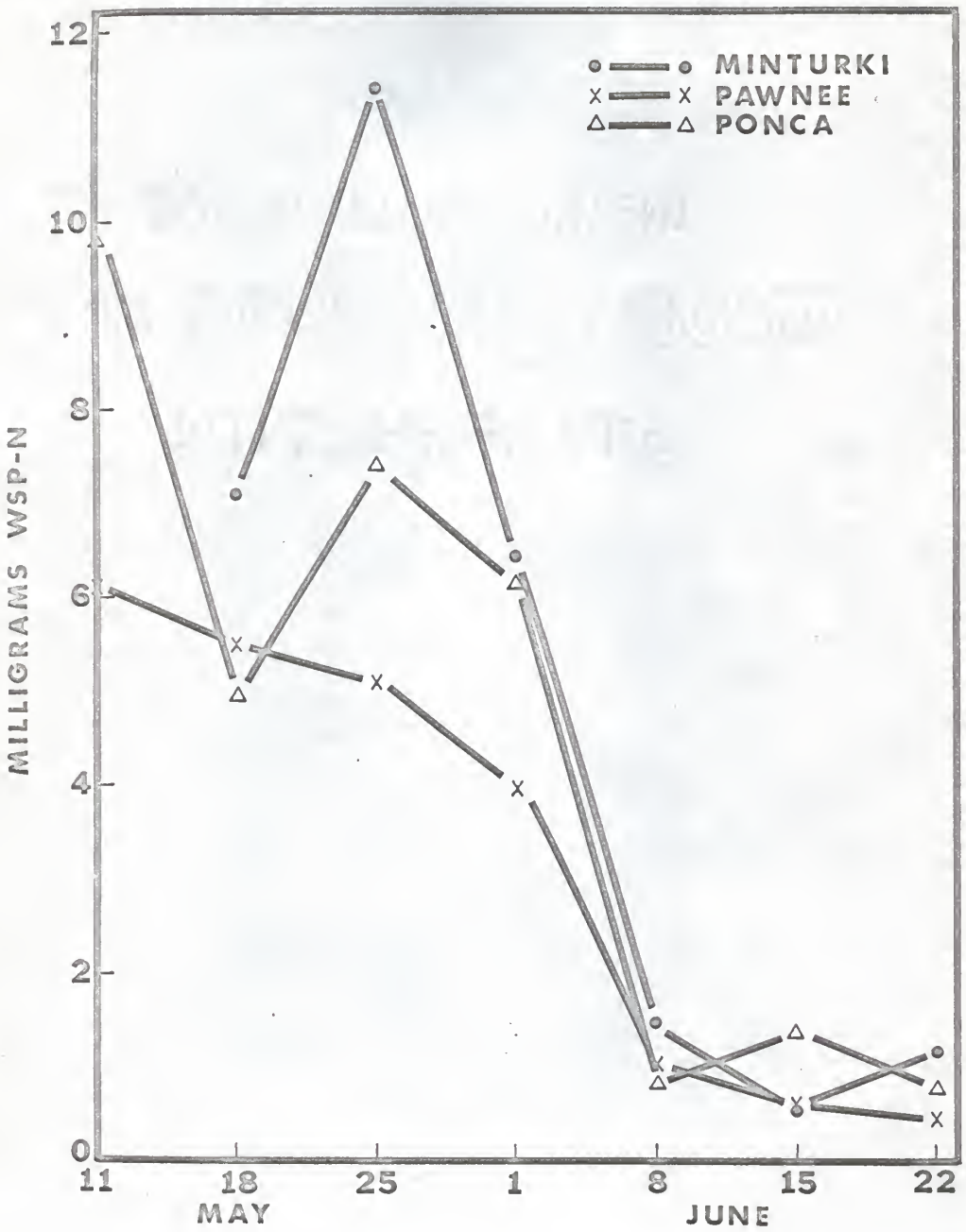

Fig. 13. Water-soluble protein nitrogen per gram dry weight of head tissue of wheat.

upper sheaths of all varieties than in lower sheaths. Again, analysis of the first and second sheaths failed to show any specific trends. A general decrease in molybdenum concentration in the culm tissue was noted in all varieties with samples taken progressively later in the season. It was noted that no plant part was markedly higher in molybdenum content than any other. However, analysis of head tissue generally showed lower levels of molybdenum content than the levels found in other plant parts. Although considerable fluctuation in molybdenum content in the head tissue existed, Ponca and Minturki showed a trend of increased molybdenum contents with maturity.

DISCUSSION

The hardiness patterns generally agreed with previous work with these winter wheat varieties (18, 21). The ranking of cold hardiness, Minturki, hardiest; Pawnee, intermediate; and Ponca, least hardy, as measured by electrical conductivity, corresponded to the observations of winter injury and spring survival.

The high level of nitrate in the crown tissue found during both the autumn and spring was possibly associated with a rapid uptake of nitrate to meet the requirements of the new growth for nitrogen. Although there was a high level of nitrates in the crown tissue initially, the low level of NR activity indicated that the nitrates were not being assimilated to any extent in this tissue portion. It would appear that the nitrates found were merely being translocated through the crowns to the actively growing leaf tissue for ultimate assimilation to meet the metabolic nitrogen requirements of the plant. The slight increase in enzyme activity associated with hardening was also found by Toman and Pauli (22) in earlier work. A possible conditioning of

the enzyme protein to cold temperature similar to that of other proteins was postulated as an explanation of this trend. The marked rise in NR activity with dehardening may have been due, in part, to the increasing levels of nitrate also found during this period.

The trends noted in WSP-N contents of the crown tissue failed to correspond with previous investigations (18, 26). Although no correlation was found between WSP-N and hardiness, there was a highly significant correlation between WSP-N and NR activity in the crown tissue of Minturki and Ponca. This indicated that WSP-N was probably more closely associated with NR activity than with hardiness.

Analysis of the aerial plant parts in the spring revealed that the lower plant parts accumulated greater quantities of nitrate than did upper parts. The low level of nitrate in upper plant parts indicated that rapid reduction of nitrate was occurring. Considering the individual plant parts, culm tissue contained higher levels than any other part. Head tissue, on the other hand, was low in nitrate compared with blades and sheaths. It appeared that the culms were mainly concerned with translocation of nitrates, and that the blades and, to a lesser extent, the sheaths were involved in reduction and assimilation of nitrate. The role of the head was mainly in the storage of the assimilated nitrates as protein. The concentration of nitrate in the aerial plant parts decreased as the plants matured. This relationship was found in all varieties. It is possible that this decrease was due in part to a decreased concentration of nitrates available from the soil and in part to a dilution in the concentrations within the plant with increased dry matter accumulation.

In contrast to the levels of nitrate found in the upper and lower plant

parts, NR activity analysis showed a reverse order. Highest levels of NR activity were associated with upper plant parts and lowest levels of activity with lower plant parts. These findings further supported the theory that the role of the leaves was in the reduction of nitrate, while the culm was mainly concerned with translocation. Higher enzyme activity in upper leaves could be associated with the higher light intensity incident at the surface of upper plant parts. Likewise, comparing the expanded blades with the rolled sheaths, it was evident that more light was incident on the blades than on the sheaths and could partially account for higher NR activity in the blades.

The overall level of NR activity was not consistently higher in any one variety. It was noted, however, that the variety which had the highest NR activity in a specific plant part usually contained a lower level of nitrate in that same plant part. That is, on most sampling dates, an inverse relationship existed between nitrate and NR activity when comparing plant parts within varieties. The decrease in NR activity in all plant parts of all varieties with maturity could be associated with the decreased nitrate content during this time. A highly significant correlation was found between NR activity and nitrate content of the blades of Pawnee and Ponca, while Minturki showed significance at the 5% level. Pawnee and Ponca also had highly significant correlation coefficients between NR activity and nitrate content of the sheath tissue over all sampling dates. This indicated that factors which limited nitrate uptake may also have been involved with decreased NR activity as the plant matured. Higher temperatures have been found to decrease NR activity in corn under controlled environmental conditions (25). Therefore, it was possible that the increase in mean temperature from early spring into June could partially explain the decrease in NR

activity with maturity found in the present experiment.

The low level of WSP-N in the leaf tissue on April 7 was probably due to the fact that this was a composite sample consisting of both blade and sheath tissues. Later analysis of the separate blade and sheath portions supported this contention, since the sheath tissue contained considerably lower levels of WSP-N than did the blade tissue. Considering the levels of WSP-N in individual plant parts among the varieties, there appeared to be a direct association between WSP-N and NR activity. A high level of WSP-N was generally associated with a high level of NR activity. This suggested that NR activity was partially regulating nitrogen metabolism rates within the plant.

As the plants matured, WSP-N contents of the plant parts followed closely the trends seen in NR activity. Thus, it appeared that the decrease in WSP-N contents with maturity was associated directly with decreased NR activity. This was supported by highly significant correlation coefficients between WSP-N and NR activity in the blades of all varieties. Minturki and Pawnee had highly significant correlation coefficients between WSP-N and NR activity in the sheath tissue, while Ponca showed significance at the 5% level.

The data presented in the Appendix (Tables 1, 2, and 3), suggested that molybdenum was not a limiting factor in NR activity. It has been shown that grasses, including wheat, appear normal as judged from foliar symptoms and nitrate concentrations, with molybdenum levels as low as 0.03 ppm (11). In view of this observation, it appeared that adequate molybdenum was present to fulfill the molybdenum requirements of nitrate reduction. Although molybdenum concentrations and NR activity were higher in total blade tissue

than in other plant parts, there was no basis for concluding that higher molybdenum levels were causing increased enzyme activity.

SUMMARY

Changes in nitrate reductase (NR) activity, nitrate, water-soluble protein nitrogen (WSP-N) and molybdenum contents of three varieties of winter wheat 'Minturki,' 'Pawnee' and 'Ponca' were compared through the growth cycle.

Hardiness determinations by the electrical conductivity method showed Minturki, hardiest; Pawnee, intermediate; and Ponca, least hardy. These results verified previous hardiness rankings made by other investigators working with these varieties.

Nitrate and WSP-N contents of the crown tissue decreased as the plants hardened. With dehardening, both of these fractions increased. NR activity was initially at a low level, increased slightly with hardening and increased rapidly with dehardening. NR activity was significantly correlated with WSP-N content of the crown tissue (1% level in Minturki and Ponca; 5% level in Pawnee). No correlation was found between WSP-N and hardiness of the crown tissue.

During the spring, the aerial portion of the plant was separated into plant parts. Concentrations of nitrate were highest in culm tissue, followed by lesser amounts in sheath, blade and head tissue, respectively. The lower sheath and blade tissue contained greater quantities of nitrate than did the upper portions. Nitrate content generally decreased in all plant parts of all varieties with samples taken progressively later in the season.

Blade tissue showed the highest level of NR activity followed by lower

levels in sheath, head, and culm tissue, respectively. The upper blades and sheaths contained greater enzyme activity than corresponding lower fractions. Likewise, the first blades and sheaths, starting with the top blade and sheath, showed higher NR activity than the second blades and sheaths, respectively. NR activity generally decreased with maturity in all plant parts of all varieties. Correlation coefficients between NR activity and nitrate content were highly significant in the blade and sheath tissue of Pawnee and Ponca, while Minturki showed significance at the 5% level in the blade tissue.

The WSP-N content of the aerial plant parts closely followed the trends seen with NR activity. The blades contained the highest levels of WSP-N followed by sheaths, heads, and culms in decreasing order. As was seen with NR activity, higher levels of WSP-N were found in upper plant parts than lower. Likewise, the first blades and sheaths contained higher levels of WSP-N than did the corresponding second blades and sheaths. As the plants matured, the WSP-N content generally decreased. A highly significant correlation was found between WSP-N and NR activity of the blade tissue of all varieties as the plants matured. A significant correlation was also found between WSP-N and NR activity of the sheath tissue (1% level in Minturki and Pawnee; 5% level in Ponca).

Molybdenum analysis indicated that sufficient concentrations were present to support the requirements of NR activity. There was no apparent association between molybdenum concentrations and NR activity.

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APPENDIX

TABLE 1. MOLYBDENUM CONTENT IN PPM (DRY WT. BASIS) OF AERIAL PLANT PARTS OF MINTURKI WHEAT

Plant part	Date												
	March		April				May				June		
	20	31	7	13	20	27	4	11	18	25	1	8	15
Total leaves	.226	.112	.310	----	----	----	----	----	----	----	----	----	----
Total blades	----	----	----	.826	.766	.724	.208	.190	----	----	----	----	----
Upper blades	----	----	----	----	----	----	----	----	.389	.304	----	----	----
1st blades	----	----	----	----	----	----	----	----	----	----	.208	.232	.262
2nd blades	----	----	----	----	----	----	----	----	----	----	.413	.689	.124
Lower blades	----	----	----	----	----	----	----	----	.340	.328	.286	.323	.287
Total sheaths	----	----	----	----	.425	.298	.438	.298	----	----	----	----	----
Upper sheaths	----	----	----	----	----	----	----	----	.450	.358	----	----	----
1st sheaths	----	----	----	----	----	----	----	----	----	----	.383	.352	.280
2nd sheaths	----	----	----	----	----	----	----	----	----	----	.069	.244	.350
Lower sheaths	----	----	----	----	----	----	----	----	.286	.328	.511	.226	.238
Culm	----	----	----	----	----	----	.583	.423	.231	.178	.125	.243	.200
Heads	----	----	----	----	----	----	----	----	.027	.086	.050	.181	.179

TABLE 2. MOLYBDENUM CONTENT IN PPM (DRY WT. BASIS) OF AERIAL PLANT PARTS OF PAWNEE WHEAT

Plant part	Date												
	March		April				May				June		
	20	31	7	13	20	27	4	11	18	25	1	8	15
Total leaves	.383	.244	.280	----	----	----	----	----	----	----	----	----	----
Total blades	----	----	----	.706	.894	.474	.148	.358	----	----	----	----	----
Upper blades	----	----	----	----	----	----	----	----	.130	.226	----	----	----
1st blades	----	----	----	----	----	----	----	----	----	----	.178	.492	.148
2nd blades	----	----	----	----	----	----	----	----	----	----	.364	.431	.160
Lower blades	----	----	----	----	----	----	----	----	.370	.250	.444	.184	.271
Total sheaths	----	----	----	----	.274	.268	.125	.226	----	----	----	----	----
Upper sheaths	----	----	----	----	----	----	----	----	.364	.310	----	----	----
1st sheaths	----	----	----	----	----	----	----	----	----	----	.172	.334	.425
2nd sheaths	----	----	----	----	----	----	----	----	----	----	.172	.069	.444
Lower sheaths	----	----	----	----	----	----	----	----	.280	.358	.148	.190	.214
Culm	----	----	----	----	----	----	.380	.450	.207	.158	.140	.111	.174
Heads	----	----	----	----	----	----	----	----	.077	.028	.085	.088	.075

TABLE 3. MOLYBDENUM CONTENT IN PPM (DRY WT. BASIS) OF AERIAL PLANT PARTS OF PONCA WHEAT

Plant part	Date												
	March		April				May				June		
	20	31	7	13	20	27	4	11	18	25	1	8	15
Total leaves	.286	.377	.383	----	----	----	----	----	----	----	----	----	----
Total blades	----	----	----	.639	.626	.560	.136	.389	----	----	----	----	----
Upper blades	----	----	----	----	----	----	----	----	.268	.364	----	----	----
1st blades	----	----	----	----	----	----	----	----	----	----	.566	.456	.250
2nd blades	----	----	----	----	----	----	----	----	----	----	.383	.425	.081
Lower blades	----	----	----	----	----	----	----	----	.358	.238	.419	.220	.225
Total sheaths	----	----	----	----	.316	.274	.195	.286	----	----	----	----	----
Upper sheaths	----	----	----	----	----	----	----	----	.480	.419	----	----	----
1st sheaths	----	----	----	----	----	----	----	----	----	----	.168	.298	.164
2nd sheaths	----	----	----	----	----	----	----	----	----	----	.142	.136	.274
Culm	----	----	----	----	----	----	.520	.386	.348	.246	.150	.155	.183
Heads	----	----	----	----	----	----	----	----	.041	.089	.072	.107	.176

TABLE 4. CORRELATION COEFFICIENTS

Correlations	Degrees of freedom	Minturki	Pawnee	Ponca
WSP vs. Hardiness	9	.575	.360	.349
WSP vs. NR activity				
Crowns	9	.836**	.709*	.819**
Blades	10	.800**	.753**	.782**
Sheaths	12	.838**	.719**	.627*
Culms	2	.000	-.794	.604
WSP vs. Nitrate				
Crowns	9	.584	.944**	.369
Blades	20	.493*	.856**	.760**
Sheaths	18	.116	-.005	.399
Culms	5	.352	-.110	.703
Nitrate vs. NR activity				
Crowns	9	.153	.789**	.448
Blades	10	.687*	.735**	.734**
Sheaths	12	-.029	.699**	.667**
Culms	2	.914	.949	.945

**Significant at the 1 per cent level

*Significant at the 5 per cent level

CHANGES IN NITRATE CONTENT AND NITRATE REDUCTASE ACTIVITY DURING THE GROWTH CYCLE OF WINTER WHEAT

by

JAMES E. HARPER

B. S., Kansas State University, 1962

AN ABSTRACT OF A MASTER'S THESIS

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Manhattan, Kansas

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Changes in nitrate reductase (NR), nitrate, water-soluble protein nitrogen (WSP-N) and molybdenum contents of three varieties of winter wheat, 'Minturki,' 'Pawnee' and 'Ponca,' were compared through the growth cycle.

Crown tissue was analyzed during the cold hardening and dehardening periods. Hardiness measurements by the electrical conductivity method showed Minturki, hardiest; Pawnee, intermediate; and Ponca, least hardy. NR activity was initially low, increased slightly during maximum hardiness and increased rapidly with dehardening. WSP-N and nitrate contents generally decreased with hardening and increased with dehardening. WSP-N was not significantly correlated with hardiness but was positively correlated with NR activity.

During the spring, the aerial plant parts were analyzed separately. NR activity and WSP-N contents were highest in the blade tissue, followed by decreasing levels in the sheath, head and culm tissues, respectively. The upper blade and sheath tissues contained greater NR activity and WSP-N contents than did the lower blade and sheath tissues, respectively. Levels of NR activity and WSP-N decreased with maturity in all plant parts. A significant positive correlation was found between NR activity and WSP-N as the plants matured.

Concentrations of nitrate were highest in culm tissue, followed by lesser amounts in sheath, blade and head tissue, respectively. In contrast to NR and WSP-N, nitrate concentrations were generally higher in lower blades and sheaths than in corresponding upper plant parts. Nitrate concentrations generally decreased with maturity in all plant parts. A significant positive correlation was found between nitrate content and NR activity as the plants matured.

Molybdenum analysis indicated that sufficient concentrations were present to support the requirements of NR activity. There was no apparent association between molybdenum concentrations and NR activity.