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Title: Enhancement of 'Scio' Barley Seed Protein by Late Foliar Applications of Urea-Ammonium Nitrate

Abstract approved: Te May Ching

Foliar applications of urea-ammonium nitrate (UAN) around anthesis increased seed protein content by about 40% in 1983 and 1984 in 'Scio' barley (Hordeum vulgare L.). In exploring the mode and the mechanism of this increase, agronomic and physiological studies were conducted.

The late N applications did not affect yield or harvest index, but elevated the seed amino acid content and reduced the sugar content. The 40% increase of total seed protein was unevenly distributed to globulin-albumin, hordein, and glutelin fractions by 27, 67, and 40%, respectively. No new protein subunits were observed after the treatment as revealed by SDS-polyacrylamide gel electrophoresis of the protein fractions. The approximate molecular weights of the hordein subunits were 33, 35.5, 38, 40.5, 42, 44, 50, 52, 54, 57, 61, 72, and 79 kD.

The physiological responses to repeated foliar-UAN sprays were monitored in young and mature leaves. The absorbed urea and ammonia were metabolized in 2 days and stimulated the endogenous activities of nitrate reductase (NR) and urease from 2- to 77-fold within hours with

the higher activity of NR sustained for several days. The activities of glutamine synthetase and glutamate synthase were also increased but much less in extent. These enhanced activities and added N substrates resulted in an increase of leaf contents of amino acids, chlorophyll, soluble protein, and a small decrease of sugars.

Eight hours after the UAN spray, when amino acid content was highest in sprayed-seedling leaves, the absolute weights of glutamine, asparagine, alanine, glutamate, serine, glycine, valine, and histidine were 2535, 511, 460, 373, 361, 240, 129, and 59 ng g^{-1} fresh weight over the control, respectively. The highest increase in weight percent of the extract was found in glycine, which is different from the results of ammonia or nitrate treated leaves, indicating that the absorbed urea may enhance the photorespiratory synthesis of glycine or reduce the degradation of glycine due to the high concentration of ammonia produced by urease hydrolysis of the influxed urea.

ENHANCEMENT OF 'SCIO' BARLEY SEED PROTEIN BY LATE FOLIAR APPLICATIONS OF UREA-AMMONIUM NITRATE

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INTRODUCTION

Scientists for many years have attempted to improve cereal protein content and quality to provide better nutrition to humans and animals. Various approaches have been taken among which are: hybridization and selection of protein-rich lines for the creation of new varieties, use of mutagenic techniques to broaden genetic variability from which high protein lines may be obtained, provision or improvement of symbionts for efficient nitrogen fixation and high productivity, application of fertilizers to increase seed protein by efficient management procedures, e.g., timing, quantity, kind, or method of application. Newer approaches in genetic engineering offer the hope of improving the quality of proteins by inserting more essential amino acid sequences into the genes coding for seed storage proteins and/or increasing the protein quantity by adding more copies of protein genes in the genome, or improving the transcription efficiency of those genes.

If any one of the above approaches were elected to improve seed protein quantity in a given crop variety, several basic aspects should be studied in order to understand the mechanism(s) of the improvement procedure:

(a) the primary physiological processes affected by the manipulation,

(b) timing of those key processes during the plant life cycle,

(c) anatomical location of those key processes, and

(d) means by which those key physiological processes lead to the desired outcome.

Newly developed 'Scio' (CI 11896) barley (Hordeum vulgare L.) was used as a model system to explore the above aspects as they pertain to protein accumulation.

Barley Seed Proteins

Protein Fractions. Barley seed protein is composed of three general fractions, based on solubility in various solvents: albumins and globulins which are soluble in salt solution, hordeins which are soluble in alcohol solution, and glutelins, which are soluble in dilute acid or alkaline solutins (13). In the mature barley seed, the embryo contains over 30% protein by weight, but the embryo mass is small compared to that of the endosperm which contains 8 to 13% protein. In the endosperm, the albumins and globulins comprise approximately 13 to 25% of the total protein, the hordein, 35 to 45%, and the glutelins, 35 to 45% (4,12). Thus, the hordeins and glutelins are the principal proteins for N storage. They are deposited in protein bodies in the later stages of seed development (3,12). The accumulation of hordein does not begin until the seed has reached 40% of its final dry weight (15). With increased N-fertilization, excess N is preferentially stored as hordein, then as glutelin (4,11,12). Because hordein is low in lysine, an essential amino acid for monogastrics, an increase in total seed protein by N fertilization means a relative decrease of lysine stored in the

endosperm compared with the other amino acids. Therefore, the biological quality of the additional protein resulting from N fertilization is lowered (4,12).

Hordein. Because hordein is a major storage protein and is increased the most by supplmental N, it is the most studied of the barley proteins. The hordeins are composed of three groups, according to the apparent molecular weights of the hordein polypeptides as determined by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS): A (below 35,000), B (35,000 to 46,000), and C (55,000 to 75,000) (12). Groups B and C hordeins are also known as hordein-2 and hordein-1, respectively, corresponding to the genes controlling their synthesis: Hor 2 and Hor 1 (3). Groups B and C together account for 95% of the hordein fraction, group B being the larger of the two by about 5 fold (16).

Under conditions of increased N nutrition, the proportion of hordein, among the protein fractions, increases, as evidenced in work by Kirkman et al. (11): hordein increased from 36 to 49% of the total seed N. However, the B-hordein to C-hordein ratio decreased from 6.7 to 4.4. The B-hordein comprises the major portion of the hordeins, but the synthesis of C-hordein increases faster with increased N availability. This is especially true at later times of seed development. Giese et al. (7) observed that synthesis of C-hordein during the later stages of seed development can actually exceed that of B-hordein at the highest levels of N nutrition.

Components of Seed Protein Synthesis

By extraction and estimation of barley hordeins at various developmental times of the seed, it has been shown that hordein is synthesized between 15 to 35 days after anthesis (9). During this period of storage protein synthesis, there is an extensive development of the endoplasmic reticulum to which are attached numerous polyribosomes or polysomes. Polysomes are messenger ribonucleic acids (mRNAs) to which are attached two or more 80S ribosomes, and they are present in greater numbers in cells actively synthesizing protein (1). It is possible that protein synthesis rate can be limited by the total amount of polysomes or number of mRNA's for storage protein. Components of protein synthesis that pose potential limitations are: a) building blocks - amounts and kinds of amino acids, tRNAs and activity of aminoacyl-tRNA synthetases; b) "workbench" - amounts of mRNA and ribosomes, initiation factors, adenosine triphosphate (ATP), guanine triphosphate (GTP), elongation factors, and termination factors; c) post-translational modification capability and rates, transport of protein in endoplasmic reticulum vesicles, and storage in protein bodies (1). All components of the protein synthesis system must be present in adequate amounts and active state for high rates of protein synthesis and accumulation. The supply of N through late foliar applications of nitrogen is an attempt to correct possible deficiencies of substrates, factors, co-enzymnes, RNA's, and enzymes needed for the synthesis of seed protein.

Seed Protein Increase by Late Applied N

Timing and amount of late nitrogen application. Early applications of N (7 to 8 weeks before anthesis) increased wheat yield and sometimes produced a small increase in grain protein content; later applications of N (2 to 5 weeks before anthesis) caused greater increases of seed protein content (8). When N application occurs between heading to several weeks after anthesis, grain protein content is increased but no increase in grain yield occurs (2,6,10,17). Therefore, late nitrogen fertilization (LNF), or the application of N after heading, is a specific crop management procedure for increasing the protein content of small grains.

Finney et al. (6) utilized foliar urea sprays on Pawnee wheat to investigate the effects of nitrogen fertilizer rates, timing of applications, and number of sprays on seed protein content. Seed protein content was increased most at highest N-rates (56 kg ha^{-1}), when applications were made at flowering or 2 days thereafter. The longer before or after flowering the applications occurred, the less effect they had on seed protein content. Although 3 to 15 urea sprays increased seed protein content over 1 spray, the effect of the additional sprays was not additive. Urea sprays applied during the first half of fruiting were more effective than those applied during the last half of fruiting.

As mentioned previously, early applications of N primarily improve grain yields, whereas LNF improves seed protein content. Eilrich and Hageman (5) offered an explanation for this phenomenon. Early N application stimulates vegetative growth, as evidenced by

increased straw yields, which in turn promotes higher carbohydrate production per unit land area; LNF does not increase vegetative growth, so the carbohydrate production is unaffected, but the potential for assimilating more N is increased. When the small grain plant shifts from vegetative to reproductive growth, the only major sink left for the accumulation of supplemental N is the seed.

Foliar versus dry fertilizers for LNF. Foliar N applications are more effective than soil treatments when roots are unable to take up nutrients. This is true for crops entering the reproductive stage when there is a lack of soil moisture, diminishing soil N, or the depression in nutrient uptake by the roots at flowering (18). Pate (14) suggested that fruiting-induced senescence of leaves can lead to reduced supplies of photosynthates for the roots, which causes a reduced uptake of minerals. Thus, LNF is important to study because of its potential for supplying N to developing seeds when the root system is often less effective in N uptake, either for genetic or environmental reasons.

Purposes of Reseach Project

Scio barley was selected as a model system to test the effectiveness of late foliar applications of nitrogen (LFAN) for seed protein enhancement. It has been reported to be responsive to soil applications of N in yield improvement (Ann Corey, personal communication, Department of Crop Science, Oregon State University). Urea-ammonium nitrate was chosen as the liquid fertilizer to apply because it is commonly used in grass and grain crops and has

several forms of nitrogen, namely, urea (non-dissociable), ammonium (cationic), and nitrate (anionic). The following studies were conducted in this thesis to discern the physiological processes that are stimulated by the LFAN and contributed to the increase of protein in Scio barley seed:

(1) Effect on grain yield, straw yield, and protein percent of seed and straw to locate the plant parts that are specifically altered.

(2) Effect on various seed protein fractions by quantitative analysis of polypeptide subunits of each fraction using polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) to identify the molecular species that are particularly changed.

(3) Effect on rates of hordein synthesis using radioactive tracer method to reveal the efficiency of protein synthesis machinery or function in endosperm of sprayed and control plants.

(4) Effect on levels of amino acids and sugars in seeds during development to estimate the difference in substrate levels that are caused by the spray.

(5) Effect on chemical composition and N-assimilating enzymes activity of leaves to determine the extent and timing of primary physiological processes that are perturbed by the spray.

(6) Effect on the amino acid composition of seedling leaves after application to discern the N-assimilating pathways of sprayed leaves.

The first four studies appear in Chapter 1 - Storage protein accumulation in 'Scio' barley as affected by late foliar applications

of nitrogen. The fifth study appears as Chapter 2 - Physiological responses of barley leaves to foliar applied urea-ammonium nitrate. The last study appears as Chapter 3 - The effect of foliar applications of urea-ammonium nitrate on the amino acid composition of juvenile 'Scio' barley leaves.

Summary of Experimental Results

(1) LFAN increased seed protein content 30 to 45% but did not increase grain yield or harvest index indicating late foliar application of N is specifically for seed protein. The protein percents of albumins/globulins, hordeins, and glutelins were increased by 27, 67, and 40%, respectively.

(2) LFAN caused an increase in all polypeptide sub-units of albumins/globulins, hordeins, and glutelins. Within the hordeins, the C-hordeins were increased more than B-hordeins by LFAN.

(3) LFAN caused an increase in hordein synthesis during seed development as revealed by a radiotracer experiment.

(4) LFAN enhanced the amino acid contents of developing seeds, whereas sugar contents were slightly depressed.

(5) Foliar applications of UAN on seedling and mature leaves caused temporary increases in ammonia, urea, amino acids, soluble protein, and activities of NR, urease, and glutamine synthetase to metabolize the influxed UAN. In seedling leaves, chlorophyll content, leaf area, and plant fresh weights were increased by repeated foliar applications of UAN. Sugar contents of treated leaves were slightly depressed from levels in control leaves because

of the use of sugars as carbon backbones in the synthesis of amino acids and as respiratory energy for increased synthesis of amino acids, soluble protein including enzymes, and chlorophyll.

(6) Foliar applications of UAN on seedling leaves elevated the absolute weights of glutamine, asparagine, alanine, glutamate, serine, glycine, valine, and histidine; eight hours after spray the increases were 2535, 511, 460, 373, 361, 240, 129, and 59 ng g^{-1}FW, respectively. Glutamine is therefore the major amino acid for the assimilation of UAN-N in barley leaves and the glutamine synthetase/ glutamate synthase pathway appears to be the major route for UAN incorporation. The increases by weight percent for glycine, alanine, glutamine-asparagine-serine, valine, glutamate, and histidine were to 3420, 1120, 412, 282, 205, and 159%, respectively. The increase in weight of glycine by 3420% in treated leaves may indicate that the spray either stimulated glycine synthesis or inhibited its photorespiratory degradation because of the high influx ofammonia. Based on these experimental results, the mechanism of seed protein enhancement by the foliar UAN spray may be proposed as follows: The UAN spray primarily stimulated the activities of urease, glutamine synthetase, and nitrate reductase in leaves. Some substrate induction might be involved with regard to urease and NR. The activated urease and NR metabolized the absorbed urea and nitrate to ammonia which further stimulated glutamine synthetase and asparagine synthase, resulting in elevated glutamine and asparagine. These amides provided substrates for glutamate (aspartate) synthase and enhanced enzymes for the synthesis of other amino acids. An accumulation of glutamine, asparagine, alanine, glutamate, serine,

glycine, and valine was thus observed in leaves hours after the spray. A concommitant reduction of leaf sugars possibly for the synthesis of amino acid and the energy needs of anabolic activities was observed. Within two to three days of the sprays, the absorbed ammonia and urea were metabolized but the leaf content of amino acids was increased by the UAN spray. These amino acids were evidentally translocated to the developing seeds of sprayed plants, and resulted in elevated protein content with a preferential enhancement of the hordein fraction. The synthesis of hordein appeared to be regulated by a higher temporal level of mRNA in seeds of treated plants.

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CHAPTER ONE

STORAGE PROTEIN ACCUMULATION IN 'SCIO' BARLEY AS AFFECTED BY LATE FOLIAR APPLICATIONS OF NITROGEN

ABSTRACT

Late foliar applied nitrogen (LFAN) can improve seed protein content but the mechanisms involved are not well known. This study was conducted to determine how LFAN is accumulated in small grains. 'Scio' barley (Hordeum vulgare L.) was treated with foliar applications of urea-ammonium nitrate at 9 days before anthesis and 18 days after anthesis at 72 and 87 kg N ha^{-1}, respectively, in 1983. In 1984, a soil application of 28 kg N ha^{-1} of urea was applied 8 weeks before anthesis in addition to foliar applications of urea-ammonium nitrate at 4 and 18 days after anthesis at 50 kg N ha^{-1}. No yield increase was observed either year because of applied N, but seed protein percentage increased from 8.9 to 11.9 in 1983 and from 7.8 to 11.3 in 1984. Harvest index was unaffected. In 1984, rates of hordein synthesis (ug $seed^{-1}$ h^{-1}) in seed from N treated plots were 93, 38, and 40% higher than rates in control seeds at 16, 22, and 35 days after anthesis, respectively. Amino acid contents of seed from N treated plots were 13 and 37% higher than those of seed from control plots at 16 and 22 days after anthesis, respectively. Sugar contents of treated seed were 74 and 69% of those in control seed at 16 and 22 days after anthesis, respectively. Late N application after vegetative growth was completed did not affect yield, but rather, enhanced the content of seed amino acids and reduced the content of sugars. The elevated levels of amino acids apparently favored the synthesis of seed protein which increased 27, 67, and 40% in the globulin and albumin, hordein, and glutelin fractions, respectively. Polyacrylamide gel electrophoresis of hordeins in the

presence of sodium dodecyl sulfate revealed that all hordein polypeptide subunits were increased by late N application. Their approximate molecular weights were 33, 35.5, 38, 40.5, 42, 44, 50, 52, 54, 57, 61, 72, and 79 kD.

INTRODUCTION

Foliar applications of nitrogen to cereal crops near anthesis have increased grain protein content without increasing grain yield (3,11,14,20). Improved grain protein content is primarily a result of an increase in the prolamin fraction, which in barley is known as hordein (9,15,17). Within the hordein fraction are two major groups of polypeptides, called B-hordein and C-hordein, whose apparent range of molecular weights are 35,000 to 46,000 and 55,000 to 75,000, respectively. The B- and C-hordein make up 95% of the hordeins by weight, with the B-hordein accounting for about 80% of the total (19). With increasing N nutrition, greater relative amounts of N are deposited in the C-hordein fraction (12,15,19). The reasons for enhanced seed storage protein by late foliar applied N (LFAN) and for the differential accumulation of various hordein fractions are unknown.

The newly developed barley cultivar 'Scio' (CI 11896) is known to respond to soil-applied N by increased yield (personal communication, Ann Corey, Dep. of Crop Sci., Oregon State University). Scio barley's ability to respond to LFAN by increased protein content was investigated in this study.

MATERIALS AND METHODS

Experimental material. In 1982, Scio barley (Illinois 6219 X Washington 213868) was planted in 1.5 by 4.9 m plots on the Crop Science Field Laboratory, Corvallis, OR. At planting time, the plots were fertilized at a rate of 56 kg N ha^{-1}. The plots received 67 kg N ha^{-1} in early March of 1983 and 28 kg N ha^{-1} in late March. In April, the plots were randomly divided into two groups. The control plots received no additional N, while the treated plots received two foliar sprays of urea-ammonium nitrate (UAN-32, manufactured by Phillips' Petroleum) which was diluted with water to approximately 15% N with the aid of a surfactant. The first spray was 9 days before anthesis at 72 kg N ha^{-1}. The second spray was 18 days after anthesis at 87 kg N ha^{-1}. Material was applied with a backpack hand-operated sprayer (Hardi, model RY2). At the end of the growing season, four control plots and four treated plots were randomly selected. When the grain reached maturity, two 0.46-m^2 samples were taken from each plot for determination of grain yield, straw yield, harvest index, and protein content of grain, straw, and chaff.

In 1984, 16 plots were divided randomly into two groups of 8 plots each. The 1984 crop received the same fall and spring fertilizer applications as those given to the 1983 crop, except that the control plots did not receive the fertilizer application in late March. The treatment plots received two sprays of UAN, diluted to 8% N, each spray delivering approximately 50 kg N ha^{-1}, at 4 and 18 days after anthesis. A Cooper, Pegler and Co., Ltd., model CP-3 back pack sprayer was used. At the end of the growing season, a 0.46-m^2

sample was taken from each plot. The samples were collected as described above.

Yield, harvest index, seed protein content, amino acids, and sugars. Harvested heads were processed with a wheat head thresher (Precision Machine Co.). Harvest index was determined by dividing the weight of grain by the total above ground plant weight . Protein contents of the grain, straw, and chaff were determined by the micro-Kjeldahl method (1). Air-dried grain was ground in a Wiley laboratory mill (intermediate model, #40 sieve). The straw and chaff were each ground in a Wiley Model 4 grinder (1mm screen). Amino acids and sugar contents of seed harvested at different stages were determined from alcohol extracts by the ninhydrin and anthrone method, respectively (4).

Extraction of protein fractions. Globulins-albumins and glutelins were extracted by a modified method of Koie and Nielson (16). Hordeins were extracted by the procedure of Doll and Andersen (8). Samples, consisting of 120 mg of flour, were placed in 15-ml polyethylene tubes. To each was added 4 ml salt solution containing 2.92% NaCl and 0.002% Na-EDTA. Samples were kept at room temperature for 30 minutes, and stirred every 10 minutes. The samples were centrifuged at 12,000 X g for 10 min at 10 C, and the supernatant poured to another tube. This extraction was repeated once. The hordeins were next extracted twice as above, except with an alcohol solution containing 50% isopropanol, 41 mM TRIS, and 40 mM boric acid, with or without 0.6% 2-mercaptoethanol. Last, the glutelins were similarly extracted with alkaline solution containing 0.48% boric acid, 0.17% NaOH, and 0.5% sodium dodecyl sulfate (SDS). The

protein content of each extract was determined by the method of Bradford (2).

SDS-PAGE of protein fractions. Globulins-albumins and glutelins of 1983 material were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using glass tubes (0.5 by 6 cm) by the method of Davis (6), except that SDS was added to the buffer at a final concentration of 0.1%. Standard SDS-PAGE and protein staining for the hordeins were conducted (8) on 12 cm by 10 cm by 0.75 mm slab gels using a BIO-RAD Model 220 or Protean double slab electrophoresis cell unit. Standard proteins from BIO-RAD of molecular weights 14,400, 21,000, 31,000, 45,000, 66,000, and 92,500 Daltons were run on the gels to estimate the molecular weights of the hordein polypeptides. The standard protein solution was diluted 1:50 in sample buffer (pH 8.6), which contained 10% sucrose, 41 mM TRIS, 40 mM boric acid, 1% SDS, and 0.01% bromophenol blue, and heated in a 100 C water bath for 5 minutes. The hordein extracts were further prepared before electrophoresis by reduction, alkylation and dialysis (8). The precipitated hordeins of each sample were collected by centrifugation at 12,000 X g and dissolved in 325 uL of sample buffer. This resulted in a solution containing approximately 0.5 to 1 ug protein uL^{-1} sample buffer. Fifteen uL of protein extract were loaded onto each lane of the slab gel. After electophoresis, the slab gels were fixed and stained, as described by Payne (18), with 0.05% Coomasie Blue-R in a water/ methanol/TCA solution (5:5:1 v/v/w), for at least 2 h. Gels were destained with several changes of water/methanol/TCA (38:3:2 v/v/v) until the background cleared. Some slab gels were scanned with a BIO-RAD Model 1650

transmittance/reflectance scanning densitometer to determine the relative quantities of the hordein polypeptide subunits.

Radiotracer methodology. The rates of hordein synthesis in endosperms of seed from control and treated plots were estimated at 16, 22, 28, and 35 days after anthesis by determining the fraction of radioactivity in the amino acid pool that was incorporated into hordein, after providing the seeds with radioactive glutamate for a 5 h period (5). Spikes were transferred from the field to the lab with their stalks inserted in a solution of 1% sucrose and 0.1% glycine. Two replications of four spikelets (three seeds per spikelet) from each treatment were placed in culture solution containing 1% sucrose, 0.1% glutamate and $U^{14}C$-glutamate (New England Nuclear, sp. act. 7.4 X 10^4 Bq ug^{-1}), and 20 ug ml^{-1} each of penicillin and streptomycin. The specific activity of $U^{14}C$-glutamate in the culture solution was 4.59 X 10^5 Bq mg^{-1} glutamate. Only the rachillas of spikelets were submerged in culture solution, and the spikelets were provided with fluorescent light at a photosynthetic photon flux density of 220 umol m^{-2} s^{-1} (measured with a Li-Cor, Inc. LI 1776 solar monitor). After a 5 h pulse, endosperms were removed from seed, and ground on ice in a mortar with pestle in a total of 10 ml buffer containing 100 mM K_2PO_4, and 5 mM dithiothreitol (DTT) (pH 8) at 4 C. Two 0.1 ml aliquots of the slurry were each counted in 2.5 ml scintillation fluor (Insta-gel by United Technologies) in a Beckman 7000 liquid scintillation counter to determine total uptake of tracer (X) into endosperms. An internal standard was placed in selected samples and the samples recounted to determine the counting efficiency so that disintegrations per minute (dpm) could be calculated. Nine ml of

slurry was centrifuged at 30,000 X g for 10 min at 4 C. The pellet was washed with an additional 10 ml of Pi buffer and centrifuged again. The washed pellet was then suspended in 3.4 ml of solvent containing 50% isopropanol and 4 mM DTT for 1 h with mixing every 20 min, after which it was centrifuged as before. Two 0.1 ml aliquots of the supernatant were placed in fluor and counted for the total incorporation of tracer into hordein (Y). The rates of hordein synthesis were estimated by multiplying the fraction (dpm found in hordein / total dpm taken up into endosperms) by the amount of amino acids in the endosperm and then subtracting 12% of that result to account for the loss of water during peptide formation:

Hordein synthesized in 5 h = (Y/X)(amino acid content)(.88)

The above estimates of hordein synthesis rates were calculated using two assumptions: first, that 50% of the amino acids in the whole seed were found in the endosperm (percentages were 50, 51, and 55 for 16, 22, and 29 days after anthesis), and second, that there was no preferential incorporation of $U^{14}C$-glutamate by the seed into different fractions. The uptake of glutamate from the culture solution into the amino acid pool of the endosperm was taken into account when calculating the amount of amino acids available for hordein synthesis.

RESULTS AND DISCUSSION

Agronomic effects. Applying UAN to Scio barley close to anthesis increased seed protein content 34 and 45% in 1983 and 1984, respectively (Table 1-1). As a result of the improved grain protein

content in treated plants, protein yields were 33 and 47% higher than the control in 1983 and 1984, respectively. Grain, straw and chaff yields were not increased by LFAN. Consequently, harvest index was unaffected (Table 1-1). In 1983, the protein percentage of straw was 3.35 and 4.26 for control and treated plants, respectively. The protein percentage for chaff was 3.99 and 4.83 for control and treated plants, respectively. The increase of N by treatment in straw and chaff combined was only 12% of the increase in total plant N. Increased grain N, due to treatment, accounted for 88%. Based on these data, LFAN does not increase vegetative growth, although protein content was somewhat elevated. Because the crop had already entered the reproductive stage, the principal sink for the utilization of the late-applied N was the developing seed. Scio barley apparently was not different from wheat in this respect (10).

Protein fractions in harvested grain. In 1984, the distribution of N in control seed was 32, 30, and 26% in albumins-globulins, hordeins, and glutelins, respectively (Fig. 1-1A). We assumed that 12% N was non-protein N. This distribution is quite different from other barley cultivars, in which N is distributed 18% to albumins-globulins, 42% to hordeins, 23% to glutelins, and 12% as non-protein N (9). The high content of albumins-globulins in Scio barley may be a good trait for feeding purposes, because of the higher lysine content of this fraction compared to hordeins (9). The hordein fraction was increased most by the supplemental nitrogen, as reported by other researchers (9,15,17).

The polypeptide sub-unit pattern in SDS-PAGE of albumins and globulins did not differ qualitatively as a result of LFAN in 1983

(Fig 1-1B). Similarly, LFAN did not cause qualitative differences in polypeptide subunit pattern in the glutelin fraction except that two bands (indicated by arrows) were much more distinct in treated seed (Fig 1-1B). SDS-PAGE pattern of hordeins from 1984 grain (Fig 1-1C) revealed minor qualitative differences, in that two minor bands of 33 and 72 kD appeared in treated seed. Scanning of the gels showed 2.5-fold more hordein, by weight, in seed from treated plants, which was much greater than that estimated by the Bradford method. Further verification of these results apparently will be needed. The C-hordein peptides of 50 to 79 kD and the B-hordeins of 33 to 44 kD were 5.0-fold and 2.1-fold higher, respectively, than those of the control seed. The B-hordeins, by weight, were the predominant group, being 4.5- and 1.8-fold higher than the C-hordeins in the grain of control and treatment groups, respectively. Supplemental N increased the relative amount of C-hordein, as observed by others (12,15). By running a lane of standard proteins with the hordeins, the major bands of the hordeins were estimated to have molecular weights of 33, 35.5, 38, 40.5, 42, 44, 50, 52, 54, 57, 61, 72, and 79 kD. Scio barley is different in its hordein subunit banding pattern from the cultivars observed by Doll and Andersen (8), but similar to others reported by Doll (7).

Mechanisms of N accumulation in sprayed plants. The synthesis of seed protein is regulated by developmental changes in mRNA levels for the quantity of protein accumlation, by genetic control of qualitative composition, and by environmental influences of temperature and nutrition on both quantity and quality (13). The increased seed protein of sprayed barley plants may, therefore, be

attributed to improved nutrition, which probably elevated substrate and machinery for protein synthesis. To test the levels of substrates, the amino acid contents of the seeds were determined at 3 (one day prior to treatment), 16, 22, 28, and 35 days after anthesis. Amino acid contents of treated seed at 16 and 22 days after anthesis were 13% and 37% higher than those of control seeds (Fig 1-2B). Additionally, sugar content of the treated seed was lower at 16 and 22 days after anthesis, being 74% and 69% of the control, respectively (Fig. 1-2C). This reduction could be the effect of UAN sprays on leaf photosynthesis, but this has not yet been investigated. The above results suggested that foliar applied urea-ammonium nitrate was converted to amino acids in the leaves and green seed parts at the expense of sugars which were incorporated into carbon backbones of amino acids and catabolized as respiratory energy for the incorporation. The amino acids were then transported to the developing endosperm where the amino acids were used in the production of additional protein. The increased seed protein content by foliar N spray without a concomitant increase in grain yield (usually due to increased starch accumulation) may result from the competition for photosynthates by the two pathways.

Hordein synthesis. Since hordein was preferentially increased by the foliar N fertilizer, a radiotracer incorporation experiment was conducted in 1984 to discern the mode of action. Less radioactive glutamate was incorporated into hordein in endosperms from the treated group over a 5 h period (Table 1-2). Only about 66% of the amount of tracer absorbed by the control endosperms was absorbed into the endosperms of treated seeds, indicating that the control seeds

were deficient in amino acid supply in vivo. In addition, more isotopic dilution of the radioactive glutamate probably occurred in treated seed because higher amounts of amino acids were found in treated material (Fig 1-2B). This resulted in lower incorporation rates or the tracer (by 33%) in treated seeds. When the amino acid pool size, and the amount of radioactive glutamate taken up and incorporated into hordein, were inserted in the formula for calculating hordein synthesis rates (method section), these rates in treated seeds were 95, 38, and 40% higher than those in the control at 16, 22, and 35 days after anthesis, respectively (Fig. 1-2A). Using these rates to estimate hordein accumulation per seed over a 25 day period resulted in 0.593 mg hordein in treated seed versus 0.413 mg hordein in control seed, an increase of 43%, which approximates the difference found in mature seeds (Fig 1-1A).

The changes in hordein incorporation rates with developmental stages (Fig 1-2A) indicate that the level of mRNA for hordein synthesis probably was higher at the stage of peak accumulation (16 DAA, Fig 1-2A) in the endosperm of treated seeds. The amino acid contents were higher in both control and treated seeds at 16 DAA than those at 22 DAA; but the rate of hordein synthesis was constant in the control while the treated material declined rapidly. The rate was 95% higher in treated than the control at 16 DAA and only 38% higher at 22 DAA, while the difference in amino acid contents was two-fold higher at 22 DAA. Therefore, in addition to substrate difference, the mRNA level for hordein may also be higher in treated material. Whether the higher mRNA for hordein is attributed to more transcription or stability is difficult to discern.

Conclusions. Late applications of supplemental N, in the form of UAN, caused an increase in seed protein content without affecting grain yield. The principal seed protein sink for LFAN was the hordein fraction, followed by the glutelin fraction. LFAN did not cause appearance of additional major sub-units in any protein fraction. Within the hordein sub-units, the B-hordeins (peptides of 33 to 44 kD) were the dominant sink for both control and N treated seeds, although the relative content of C-hordeins (peptides of 50 to 79 kD) increased in N-treated seed. Seed amino acid content and endosperm mRNA level for seed protein were increased by LFAN, while the sugar content was decreased during the latter portion of seed development. The increased availability of amino acids in the seed and possibly elevated levels of mRNA for storage protein may be responsible for the increased protein content in seed from plants receiving supplemental N sprays. Grain yield was not improved by LFAN because some of the photosynthates or sugars were utilized in the production of amino acids and protein for their carbon backbones and respiratory energy, rather than for starch production. Therefore, the chief agronomic use of LFAN in Scio barley is the improvement of grain protein content, as reported for other small grain crops (3,10,13,19).

Fig. 1-1. A) The effect of late foliar applications of urea-ammonium nitrate on seed content of albumins-globulins, hordeins, glutelins, and total protein. Proteins were extracted from mature Scio barley seed harvested in 1984. Non-protein N was assumed to be 12% of the total seed N for control and treatment. B) SDS-PAGE pattern of albumins-globulins, and glutelins extracted from 1983 seed. Lanes 1-2 are albumins and globulins from 2 replications of control seed; lanes 3-4 are albumins and globulins from 2 replications of treatment seed. Lanes 5-6 are glutelins from 2 replications of control seed; lanes 7-8 are glutelins from 2 replications of treatment seed. C) SDS-PAGE pattern of hordeins, extracted from 1984 seed. Lanes 1-2 contain hordeins from seed of 2 different control plots. Lanes 3-5 contain proteins from seed of 3 different treatment plots. Each lane was loaded with 5% of the hordein extracted from 120 mg of seed meal. Lane 6 contains standard proteins, whose molecular weights are indicated to their right.

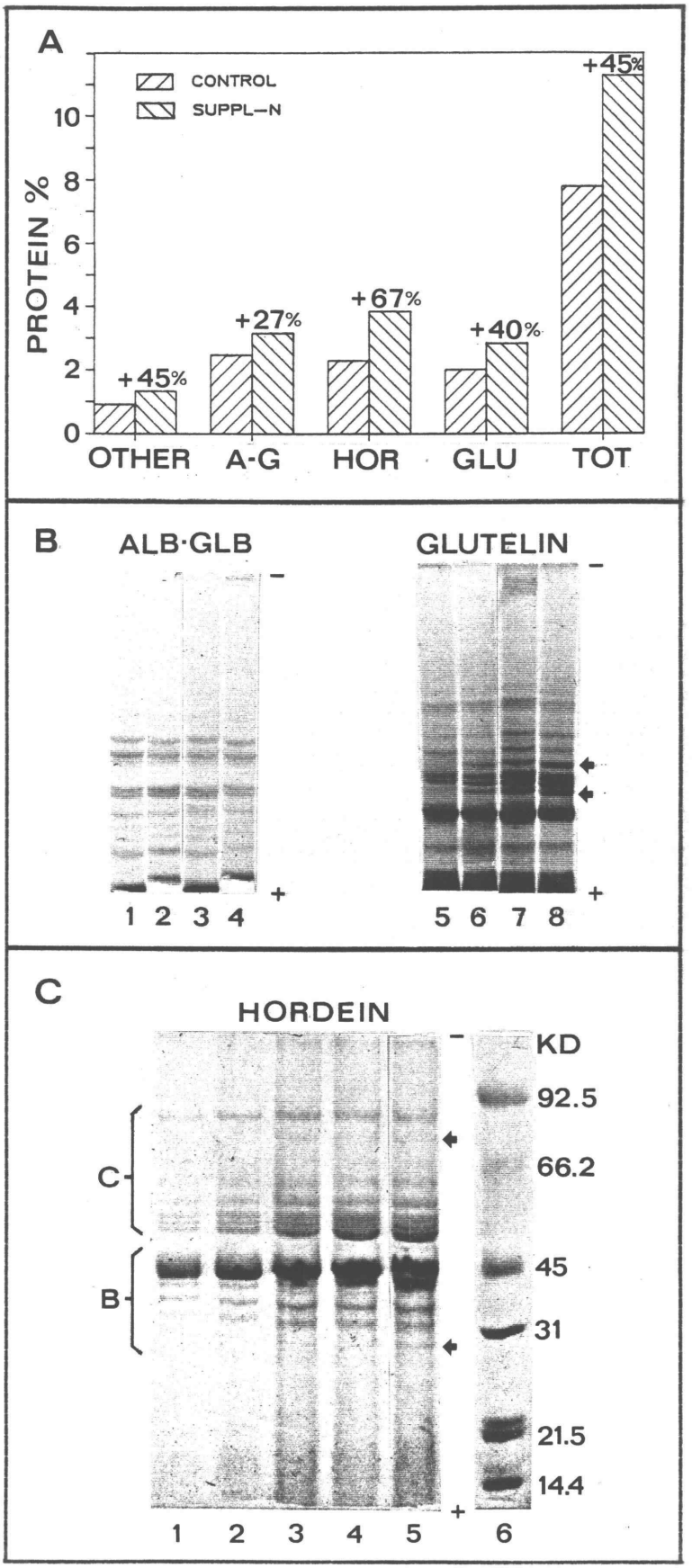
A
CONTROL
SUPPL-N
PROTEIN %
0
2
4
6
8
10
+45%
+27%
+67%
+40%
+45%
OTHER
A-G
HOR
GLU
TOT
B
ALB·GLB
GLUTELIN
1 2 3 4
5 6 7 8
C
HORDEIN
KD
92.5
66.2
45
31
21.5
14.4
C
B
1 2 3 4 5 6

Figure 1-1

Fig. 1-2. The effect of late foliar applications of urea-ammonium nitrate on A) rates of hordein synthesis, B) amino acid content, and C) sugar content of Scio barley seeds during development. Arrows indicate times of fertilizer application. Vertical bars represent the standard errors of the means.

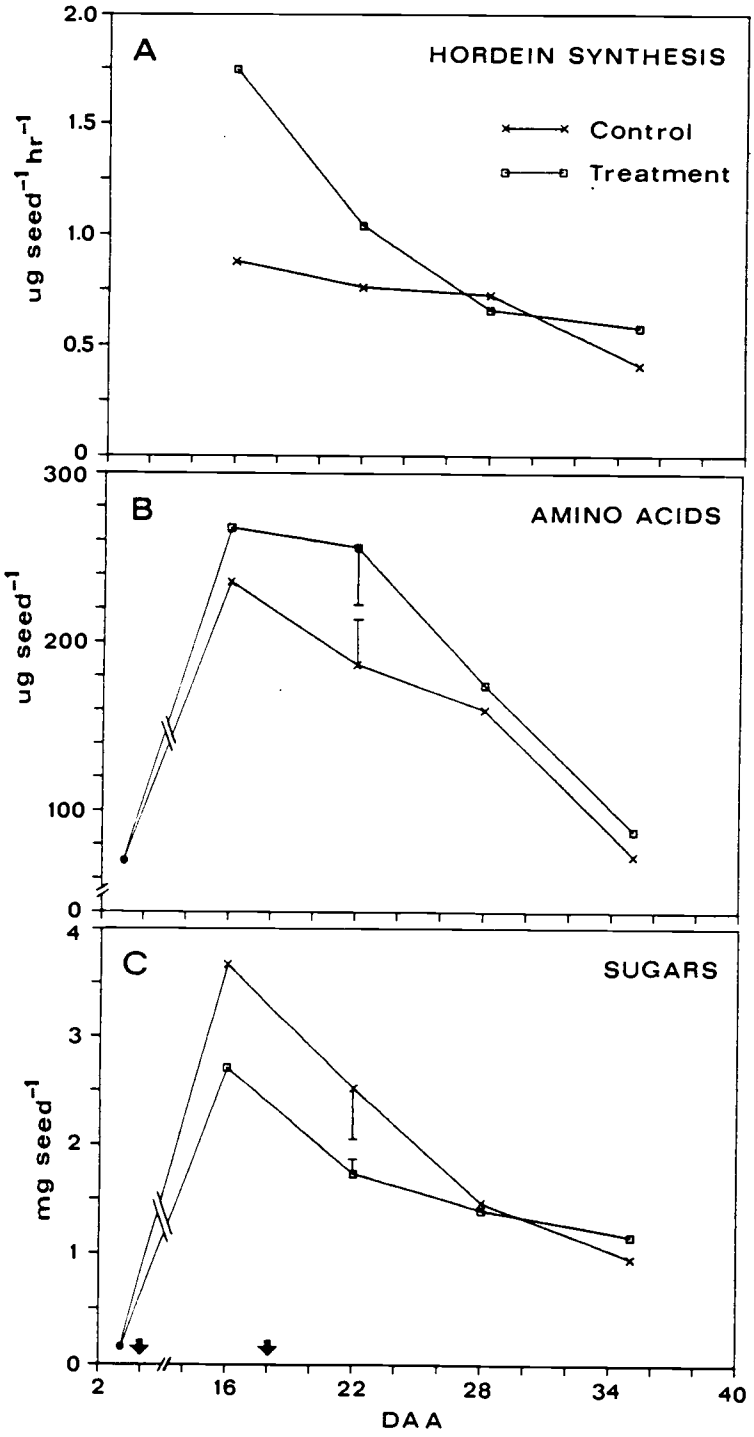
A
HORDEIN SYNTHESIS
Control
Treatment
ug seed^{-1} hr^{-1}
2.0
1.5
1.0
0.5
0
B
AMINO ACIDS
ug seed^{-1}
300
200
100
0
C
SUGARS
mg seed^{-1}
4
3
2
1
0
2
16
22
28
34
40
DAA

Figure 1-2

Table 1-1. The effect of late foliar applications of urea-ammonium nitrate on grain yield, grain protein content, grain protein yield, straw yield, chaff yield, and harvest index of Scio barley.

		Control	Treatment
1983	Grain yield (kg/ha)	2960 ± 1260[†]	2920 ± 1360
	Protein percentage	8.9 ± 1.1	11.9 ± 0.9 **
	Grain protein (kg/ha)	264 ± 85	347 ± 138
	Straw yield (kg/ha)	3440 ± 910	3030 ± 870
	Chaff yield (kg/ha)	853 ± 199	648 ± 169
	Harvest index	0.41 ± 0.05	0.44 ± 0.05
1984	Grain yield (kg/ha)	4240 ± 810	4290 ± 650
	Protein percentage	7.8 ± 0.5	11.3 ± 1.5 *
	Grain protein (kg/ha)	330 ± 72	484 ± 91
	Straw yield (kg/ha)	4910 ± 910	4720 ± 700
	Chaff yield (kg/ha)	1010 ± 150	1010 ± 130
	Harvest index	0.42 ± 0.02	0.43 ± 0.02

*,** Significant at the 5% and 1% level.
[†]Standard error of the mean

Table 1-2. The effect of late foliar applications of urea-ammonium nitrate on total uptake of radioactive glutamate and its incorporation into hordein by spikelets of Scio barley over a 5-h labeling period.

	16 DAA		22 DAA		28 DAA		35 DAA	
	C†	T	C	T	C	T	C	T
	dpm (thousands)							
Total uptake	307.6	190.0	831.8	573.0	464.2	282.9	110.7	82.2
Hordein	13.1	13.8	38.1	26.4	23.8	12.1	7.0	6.1

†C - Control, T - Treatment

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CHAPTER TWO

PHYSIOLOGICAL RESPONSES OF BARLEY LEAVES TO FOLIAR APPLIED UREA-AMMONIUM NITRATE

ABSTRACT

The magnitude and duration of physiological responses in barley (Hordeum vulgare L., var. 'Scio') leaves to foliar applied urea-ammonium nitrate (UAN) were depicted by the changes observed in leaf content of urea, ammonium, sugars, amino acids, soluble protein, and chlorophyll, as well as the activities of nitrate reductase (NR), urease, glutamine synthetase (GS), and glutamate synthase (GOGAT). The earlist response expressed by seedling leaves was observed at 4 hours after spray of 30 mg N g^{-1} fresh weight (FW), when leaf urea and ammonia content were 9.1 and 7.6 umoles g^{-1} FW, respectively, for treatment compared to 2.2 umoles g^{-1} FW each for the control. After sprays of 30 and 50 mg N g^{-1} FW, levels of urea and ammonia in treated leaves declined to those of the control 2 and 4 days later, respectively. Leaf amino acid content increased after each UAN spray, whereas the sugar content was slightly depressed. Significant increases in chlorophyll and soluble protein occurred 2 to 4 days after the sprays. In vivo activities of NR and urease were increased significantly in response to the foliar spray, as were the in vitro activities of GS and GOGAT. Barley seedlings, therefore, were able to use foliar applied nitrogen rapidly and efficiently to produce amino acids, chlorophyll, and soluble protein, including enzymes. Furthermore, the UAN applications on seedlings resulted in greater total plant weight, leaf area, and leaf chlorophyll content. Physiological responses observed in leaves of plants 2 weeks prior to heading after application of 25 mg N g^{-1} FW were similar to those of seedling leaves except that 2 h after spray they already had 8-fold

ammonia, 36-fold urea, 1.8-fold amino acids, 0.8-fold greater urease activity and 1.1-fold greater NR activity over control leaves. An additional spray of 25 mg N g^{-1} FW was applied 1 week after anthesis. Seeds collected from treated plants contained 10% more N than the control.

INTRODUCTION

Foliar applications of nitrogen to cereal crops near anthesis have increased grain protein content (10, 11, 21, 24, 25); and, when availability of N from soil, stem or roots is limited, foliar applied N has been utilized effectively by developing inflorescences and fruits (14, 26). The supplemental N can be applied in conjunction with pesticide sprays to save time and labor, and to reduce soil compaction by ground equipment (16, 18). Thus, foliar fertilization is an important alternative procedure for crop managers.

The mechanisms of enhancing plant growth and seed productivity by soil applied N have been studied extensively (11), but much less attention has been given to foliar applied N. This study was conduct-ed to discern some of the mechanisms by which the foliar application of N could change the composition and metabolism of plant leaf tissue, increase seedling growth, and enhance seed protein yields.

MATERIALS AND METHODS

Seeds of 'Scio' barley were planted in a greenhouse at 26/20 C day/night temperature on 31 August 1984. The seedlings were transplanted, two plants to a pot, on 10 September 1984. On 1 October, the plants were placed under supplementary fluorescent light which provided a photosynthetic photon flux density of 210 umol m^{-2} s^{-1} (measured with a Li-Cor, Inc. LI-1776 solar monitor) at top of canopy with a photoperiod from 0500 to 1900 h. Plants were divided randomly into two groups, one of which received three sprays of diluted urea-ammonium nitrate (UAN-32, manufactured by Phillips Petrolium) on 1 October with 13.3% N in 0.1% Triton X-100 at approximately 50 mg N g^{-1} leaf FW, 10 October with 6.65% N in 0.05% Triton X-100 at approximately 10 mg N g^{-1} leaf FW and 15 October with 6.65% N in 0.05% Triton X-100 at approximately 30 mg N g^{-1} FW. The other group was sprayed with the respective Triton-X solution, but without N. The treatments were applied at 0800 h on each date. For the first two applications, the N solutions were applied with a hand atomizer. On the 3rd spray, a pressurized atomizer was used. The solution was applied to both sides of the leaves. Because of wilting and some leaf-burn after the first spraying, plants were sprayed with water on the next day to reduce the concentration of fertilizer on the leaf surface. On 6 October, control and treatment plants received approximately 10 mg N per pot applied to the soil in a solution identical to that used in the first spray.

Samples of seedling leaf tissue were taken October 1 (before first fertilizer application), 2, 4, 6, 8, 10 (prior to the second

fertilizer spray) 11, 13, 15 (at 4 and 8 h after the third spray) 16 (at 24 and 29 h after the third spray) 17, 18, and 31. Before sampling, the leaves were gently washed three times under running water to remove any fertilizer remaining on the leaf surfaces. Five to 8 g of leaf tissue were collected on ice from four plants (usually two leaves per tiller) of the control and treatment groups at each sampling time, except that samples taken after the third spraying were from two plants. The second and third leaves were used after the first and second sprays, and younger leaves were included in samples collected after the third spray. After transport to the laboratory, the leaves were cut into 1-cm sections, which were mixed thoroughly, and subsamples were used for all the biochemical and enzymatic assays described below.

Enzyme extraction and in vitro assays. All procedures were conducted at 0 to 5 C except as noted. Four samples of 0.5 g leaf sections plus 500 mg buffer-equilibrated insoluble polyvinyl polypyrrolidone and XAD-4 (Amberlite, nonionic polymeric adsorbant to remove phenolics) were ground in a precooled mortar with pestle in 10 ml of grinding buffer (pH 7.8) containing 0.1 M HEPES, 4 mM MgSO4, and 10 mM mercaptoethanol. The slurry was centrifuged at 30,000 X g for 10 min, and the supernatant was used as the enzyme preparation to assay glutamine synthetase (GS) (17) and glutamate synthase (GOGAT) (20). For GS, either 0.1 or 0.2 ml extract was used, with an incubation time of 10 or 15 min at 30 C. GOGAT was assayed by tracing the decrease of NADPH at 340 nm for 3 min at 22 C, using a Cary 219 Spectrophotometer. Soluble protein was estimated in an aliquot of the enzyme preparation by the method of Bradford (4).

In vivo nitrate reductase (NR) activity was assayed by a modification of the method of Brunetti and Hageman (5). Four samples of 0.2 g of leaf sections were placed in 5 ml of 0.1% Triton X-100, 0.005 M K_2HPO_4, pH 7.5 with or without 0.1M KNO_3, and vacuum-infiltrated twice at 2 min each. The vacuum-infiltrated samples in beakers were placed in a rack, covered with aluminum foil to keep out light, and incubated at 30 C for 35 min in a water bath with shaking, after which 2 X 0.5 ml of the incubation mixture were taken to assay the nitrite produced. The samples incubated in buffer without KNO_3 were used to estimate NR activity due to endogenous and absorbed applied substrate (UAN). Samples in buffer with nitrate were used to estimate maximum NR activity under assay conditions.

In vivo urease activity was assayed using four samples of 0.2 g leaf sections in 5 ml buffer without urea and another four with urea. The buffer consisted of 0.1 M K_2HPO_4, 5% n-propanol (v/v), pH 7.5, with or without 0.21 M urea. Incubation time was 45 min, at 30 C, in a water bath with rapid shaking, after which 0.2 ml incubation buffer was assayed for ammonia content (12).

Analysis of leaf chemicals. Three ml of the above enzyme extract were made to 10% TCA solution by adding 3 ml 20% TCA, placed on ice for 10 min, then centrifuged at 10,000 X g for 10 min. The resulting supernatant was titrated to pH 7.0 with NaOH and the volume was made to 6.5 ml. This neutralized solution was used for the determination of sugars, total amino acids, urea, and ammonia. Sugars and amino acids were estimated using the anthrone and ninhydrin methods, respectively (6). Urea and ammonia content were estimated by the indolphenol method (13). Ammonia was analysed in

0.5 ml extract and urea plus ammonia was estimated by incubating 0.4 ml extract with 0.5 unit of urease (Sigma) in 0.1 ml 0.1% EDTA, pH 6.5, at 37 C for 15 min. One ml of 0.1 M phenol, 0.20 mM $NaFe(CN)_5NO-2H_2O$, 1 ml of 0.5% NaOH and 0.04% hypochlorite were added, and the mixture was incubated for 20 min at 37 C to develop the color, which was measured at 560 nm against a neutralized TCA-GS buffer blank. Chlorophyll a + b content was extracted and estimated by the method of Arnon (2).

Seedling growth analysis. The effect of three weekly sprays of 0.05% Triton X-100 with or without 5% UAN-N, at a rate of approximately 25 mg N g^{-1} leaf FW, on growth of 3-month-old seedlings was determined by measuring leaf area, fresh weight of above ground parts, leaf chlorophyll content, and specific leaf weight one week after the last treatment. Three pots of 2 plants each were used for each treatment.

Mature leaves. Young seedlings were vernalized from 18 September to 30 October, 1984 at 8 C with 8 h light/16 h darkness, after which they were transplanted, two per pot, in a greenhouse, with a day/night temperature of 26/20 C, under natural light. The temperature was changed to 22/17 C on 15 November. Plants were placed under supplemental fluorescent lighting on 21 January 1985 which provided a photosynthetic photon flux density of 210 umol m^{-2} s^{-1} with photoperiod from 0500 to 1900 h. On 23 January, two weeks prior to heading, the flag leaves (at least one-half expanded) and the next two lower leaves were sprayed with 0.05% Triton X-100 solution, with or without 5% UAN-N (50 pots per treatment). Both sides of leaves were sprayed and received approximately 25 mg N g^{-1}

FW. Samples of treated leaves were taken at 0, 2, 5, 9, 24, 29, and 48 h after treatment. Assays for biochemical constituents were conducted as stated above for seedling leaves except that 0.7 or 0.75 g leaf pieces were homogenized in 8 ml GS grinding buffer in a polytron (Brinkmann Instruments) for 3 X 10 s. A rinse of 7 ml buffer was combined with the homogenate. In vivo urease and NR assays were conducted as stated above, except that 0.2 M KNO_3 in buffer was used as substrate and an aliquot was taken after 10 min of incubation as the starting point.

Seed amino acids and protein. The transfer of applied N as amino acids from leaves and outer seed parts to the seed was tested by spraying the top two leaves and spike with 0.05% Triton X-100 with or without 6.65% UAN-N. The N-treated tissues received approximately 40 mg N g^{-1} FW. Sampling times after sprays were 0, 7, 26, and 50 h. Amino acid content was determined as stated above. Seed protein content was determined using the microKjeldahl method (1).

RESULTS AND DISCUSSION

Seedlings

Changes in leaf content of ammonia, urea, amino acids, sugars, soluble protein and chlorophyll after sprays of 50 and 30 mg N g^{-1} leaf FW, are illustrated in Fig. 2-1.

Ammonia and Urea. After the first spray, a rapid absorption of ammonia and urea was shown, followed by their assimilation within 2 to 4 days, and a return to the levels of the control (Fig. 2-1 A &

B). A parallel increase and decline in amino acids was also observed (Fig 2-1 C). With more frequent sampling times after the third spray, a dynamic picture of the speed and the duration of urea and ammonia absorption and utilization was shown. Ammonia and urea were about 9-fold higher in treated leaves at 4 to 8 hours after treatment, then declined to control levels by 48 h after the treatment. The rapid reduction of urea indicates that urease (pre-existing in leaves or induced) can effectively and rapidly hydrolyze urea into ammonia and CO_2, within 2 (the third spray) to 4 days (the first spray) following N application. Furthermore, the applied N was assimilated within hours as evidenced by the simultaneous increase of amino acids.

Amino Acids and Sugars. Leaf amino acid contents increased 2- to 4-fold after all foliar UAN sprays, while sugar contents usually decreased (Fig. 2-1 C and D). The changes in amino acid content of treated leaves were similar to those of ammonia and urea, except 2 days after the third spray, when amino acid content began a gradual increase. This last observation may indicate a long-term benefit of repeated foliar N sprays, namely, increased leaf content of amino acids which can be transported to various sinks of the growing plant. Because the applied N was converted to ammonia and utilized for the production of amino acids which requires carbon skeletons, sugar was reduced in treated leaves. Additionally, the respiratory energy demand for increased amino acid synthesis, and subsequent protein synthesis, could also have diminished the levels of sugars in UAN-sprayed leaves. A similar pattern was observed by Wu, et al. (27) in triazine sprayed pea leaves, which utilized the triazine-N to

synthesize amino acids and proteins. A decrease in leaf sugar and starch content resulted, as the carbon was incorporated into amino acids and respired to CO_2. This phenomonon was also observed in barley roots provided with ammonia, where glutamine was readily synthesized with the concommitant reduction in carbohydrates and increased evolution of CO_2 (28). Thus, the decrease in sugar content observed in UAN sprayed leaves was a likely consequence of the assimilation of ammonia and urea into amino acids.

Soluble Protein and Chlorophyll. Soluble protein was 50% higher in the treated material on the 3rd day after the first spray, and continued to be significantly higher than that of the control to day 6 (Fig. 2-1 E). The soil application of 10 mg N on day 6 increased the soluble protein for both control and treated seedling leaves on day 8, whereas the leaf spray of 10 mg N g^{-1} FW on day 10 only elevated amino acid content of the treated materials. One day after the third N spray, soluble protein was significantly higher in the fertilized leaves. This difference continued until the end of the experiment. It appears, then, that the nitrogen substrates used for amino acid synthesis, begin to be incorporated into soluble protein within one day. A significant increase of chlorophyll a + b (Fig. 2-1 F) was observed in the treated leaves on the 4th day after the first spray and 2nd day after the third spray. The two small N applications did not cause a quantitative difference in chlorophyll content.

Enzymes. Urease hydrolyzes urea to CO_2 and NH_4^+ (22); NR reduces NO_3^- to NO_2^-, which is further reduced to ammonium by nitrite reductase (3). Ammonium is then incorporated into glutamine by GS

(15). Urease, NR, and GS, therefore, are key enzymes in the leaves for the utilization and assimilation of foliar applied urea and/or ammonium nitrate. Assays of these leaf enzymes after UAN sprays revealed increased activities because of the treatment (Fig. 2-2 and Table 2-1). The assays with exogenous substrate at excess levels were used in estimating maximum enzyme activities in treated leaves including original and induced enzyme and for preexisting enzyme only in control leaves. The assays without exogenous substrate were used to estimate the activities of urease and NR in leaf sections based on available endogenous substrate; thus, the measured activities estimate the rates in situ. When in vivo assays were conducted with exogenous substrates, urease activity at 8 h after the third spray increased from the control to 4-fold and NR to 2-fold in UAN sprayed leaves (Fig. 2-2). In assays without exogenous substrate supplied, urease activity increased from the control to 24-fold at 8 h after third spray, and NR to 29-fold at 4 h after third spray in treated leaves (Table 2-1). These differences are apparently attributed to the in situ substrate concentration. This is in agreement with Croy and Hageman (7) who observed that the major factor in controlling leaf NR activity was the nitrate content of leaves, and that NR activity was only casually related to leaf protein content. Guerrero (9) suggested that NR probably preexists in the leaves at constituitive levels and that additional copies of the enzyme would be synthesized in response to substrate induction. In this experiment it appears that urease and NR did exist at constituitive levels and immediately utilized foliar applied N substrates for the production of ammonia for amino acid synthesis. Additional enzyme copies were

synthesized in response to the increased amino acids as evidenced by the increase of NR specific activities as follows: 1.7-fold 5 days after the first spray and 1.2 to 1.4-fold at 48 h after the third spray. Induction of urease was evidenced by its specific activity increase to 3.1 and 1.2-fold at 1 and 3 days, respectively, after the first spray, and to 1.9 to 2.9-fold at 29 h after the third spray in treated leaves. These increases in specific activites could be a result of enhanced transcription of the enzymes (substrate induction) or the protection of the enzyme from inactivation by the presence of added substrate, as mentioned in the case of NR by Guerrero (9), or a combination of the two. In this experiment, enzyme induction could not be differentiated from protection against enzyme degradation, however.

Repression of NR induction by the presence of NH_4^+ occurs in lower plants, but not commonly in higher plants (3). In some cases, NO_2^- and amino acids can be effective inhibitors of NR (19). In this study, increased levels of ammonia and amino acids did not appear to repress the inferred induction of NR. Although little work has been done with leaf urease as affected by applied N, it appears that urease induction, like that of NR, is not repressed by the presence of NO_3^-, or by presence of ammonia or amino acids.

The large increase in leaf NR activity in control and treatment plants 2 days after application of 10 mg N to the soil, without a similarly large increase in urease activity, can be accounted for by the lack of urea being transported to the leaves. Although we did not study the constituents of the xylem stream, the lack of urea is supported by the findings of others: a) nitrate is the primary form

of soil N since hydrolysis of urea to ammonium carbonate and the nitrification of ammonia is rapid in most soils (3, 11, 23), and b) any NH_4^+ taken up by roots is utilized *in situ*, as evidenced by the lack of NH_4^+ in the xylem stream leaving the roots (3). Thus, it is reasonable that leaf NR was induced by the soil application, but leaf urease was not.

The *in vitro* activity of leaf GS was enhanced by fertilizer treatment (Fig 2-2 C). It was about 50% higher 2 and 4 days after the first spray, and declined to 30% 6 days after. After the third spray, a small increase of 30% in GS activity over the control was observed in treated leaves 2 and 3 days after. GOGAT activity was measured only after the third spray and was significantly higher in treated leaves 1 and 2 days after spraying (6.0 and 5.8 umole NADPH utilized min^{-1} g^{-1}FW, respectively, in treated leaves and 4.3 umole NADPH min^{-1} g^{-1} FW in the control leaves). The increases observed in GS and GOGAT activities resulted primarily from an increase in all constituitive enzymes in the tissue in conjunction with the increase in soluble protein by the fertilizer treatment. Specific activities of GS and GOGAT were never substantially higher in fertilized leaves, except on day 4 when GS specific activity was 1.3-fold of control.

UAN Sprays and Yield Potential. Application of 3 weekly sprays of UAN to 3-month-old seedlings increased leaf area, plant weight, and chlorophyll content (Table 2-2). This increase of vegetative growth by foliar N applications when soil N is deficient can contribute to improved canopy development. Rapid canopy development is essential for increased utilization of yearly and seasonal incident sunlight for the production of photosynthates. Currently, the

rapidly established canopy is considered as a more effective means for increasing crop yields than manipulations of photosynthetic capacity at the chloroplast level (8).

Mature Leaves, Spikes, and Seed Protein

Ammonia, urea and amino acids were significantly higher in sprayed leaves during the first 24 h after the UAN-spray (Table 2-3). Decreases in sugars by the N application were evident only during the first 24 h after spray. Soluble protein was signficantly higher in treated leaves at 28 h after spray. In vivo urease activity was increased by UAN-spray about 2-fold 2 to 9 h after the spray, while in vivo nitrate reductase was about 2-fold higher than the control 2 and 5 h after spray and 4 to 5-fold higher up to 48 h after the spray (Table 2-4). Based on their specific activities, urease was induced about 2-fold 2 and 5 h after UAN-spray, while NR was induced about 4 fold 9 to 48 h after the spray. NR induction appeared to be greater in mature leaves than in seedling leaves. Glutamine synthetase activity was increased by 6 to 20% 2 to 28 h after UAN-spray and by 30% at 48 h (Table 2-4). Endogenous in vivo activities of urease and nitrate reductase were increased 2 to 29-fold and 17 to 76-fold, respectively, after N application (Table 2-5). Thus, changes in chemical consituents and enzyme activities by UAN sprays were quite similar to those observed in seedling leaves. A second fertilizer treatment on upper leaves and spikes 1 week after anthesis yielded seed which was significantly higher by 10% in N than that of the control, which agrees with unpublished results of a 1983 greenhouse

experiment. This increase of N content was much smaller than the 40% increase in seed protein observed in the field (25).

Leaves and spikes (10 days after anthesis) were capable of utilizing UAN to produce additional amino acids, some of which were tranported to the developing seed. After an application of 40 mg UAN-N g^{-1} leaf FW to top two leaves and head, amino acid contents of treated whole seeds were 44 % and 34 % higher than control levels, at 7 and 26 h, respectively. In the field, seed from N-sprayed plants were significantly higher in amino acids (% of dry weight) at 16 and 22 days after anthesis. The increased availability of N substrate in the seed as amino acids may account for the increase of seed protein from 7.8 to 11.0% (25).

Conclusion

Supplementary N provided by foliar sprays was rapidly absorbed and assimilated by leaves resulting in increased growth of seedlings and higher seed protein.

Fig. 2-1. Changes in contents of A) ammonia, B) urea, C) amino acids, D) sugars, E) soluble protein, and F) chlorophyll a + b in Scio barley seedling leaves after three foliar and one soil application of Triton X-100 with or without urea-ammonium nitrate. Vertical bars represent the standard errors of the means.

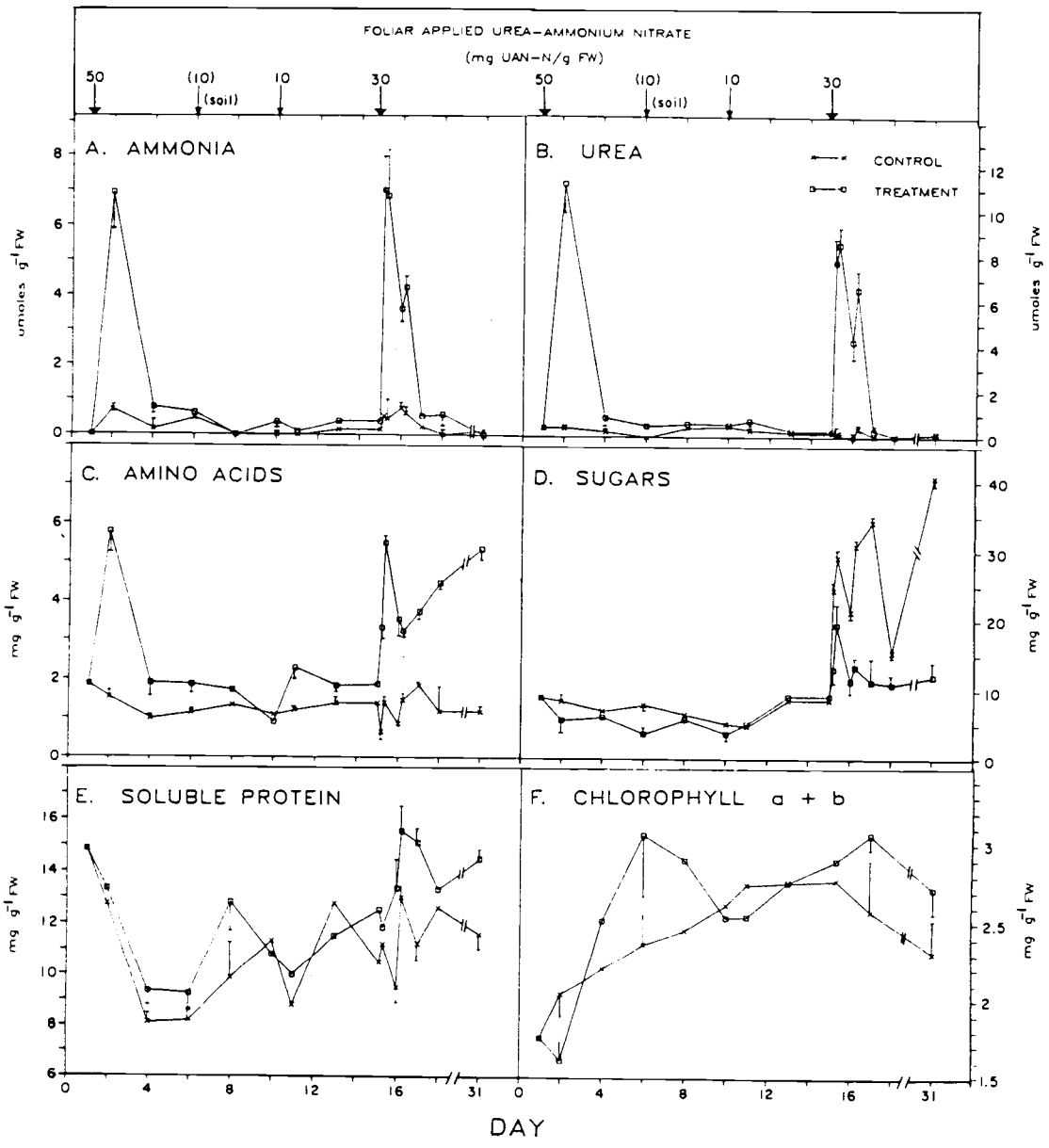
FOLIAR APPLIED UREA–AMMONIUM NITRATE
(mg UAN–N/g FW)
50
(10)
(soil)
10
30
50
(10)
(soil)
10
30
A. AMMONIA
B. UREA
CONTROL
TREATMENT
umoles g⁻¹ FW
C. AMINO ACIDS
D. SUGARS
mg g⁻¹ FW
E. SOLUBLE PROTEIN
F. CHLOROPHYLL a + b
0
4
8
12
16
31
DAY

Figure 2-1

Fig. 2-2. Changes in activities of A) *in vivo* urease, B) *in vivo* nitrate reductase, and C) *in vitro* glutamine synthetase in Scio barley seedlings leaves after three foliar and one soil application of Triton X-100 solution with or without urea-ammonium nitrate. Vertical bars represent the standard errors of the means. The arrows indicate times of fertilizer applications.

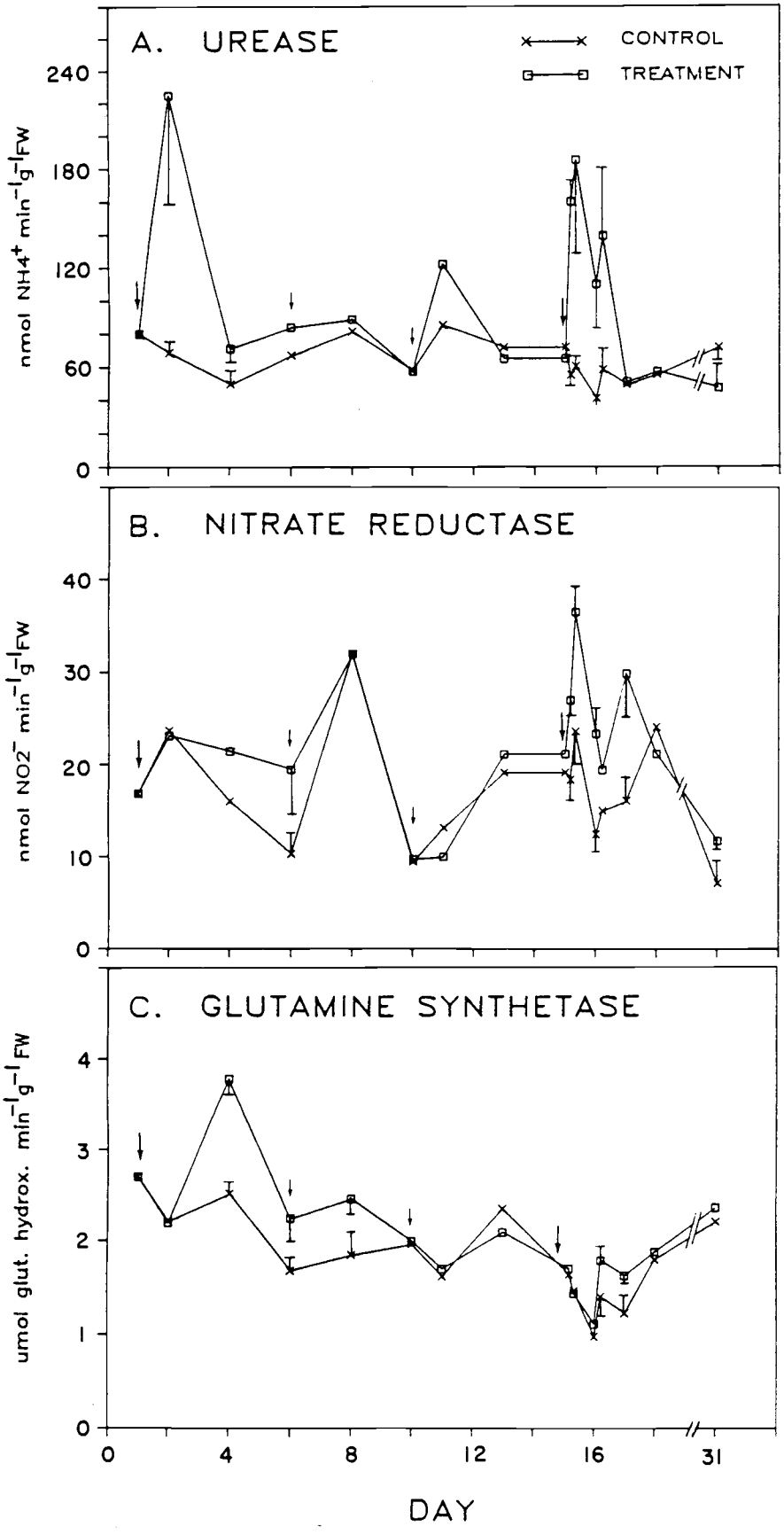
A. UREASE
CONTROL
TREATMENT
nmol NH4+ min-1g-1FW
240
180
120
60
0
B. NITRATE REDUCTASE
nmol NO2- min-1g-1FW
40
30
20
10
0
C. GLUTAMINE SYNTHETASE
umol glut. hydrox. min-1g-1FW
4
3
2
1
0
0
4
8
12
16
31
DAY

Figure 2-2

Table 2-1. The effect of foliar applications of UAN-N on endogenous *in vivo* activities of urease and nitrate reductase in Scio barley seedling leaves.

UAN-N spray	Days after spray	Hours after spray	Urease			Nitrate reductase		
			C†	T	T	C	T	T
mg g^{-1} FW			nmol NH_4^+ min^{-1} g^{-1}FW		% of C	nmol NO_2^- min^{-1} g^{-1}FW		% of C
50	0		28.6		---	8.43		---
	1		32.3	163	505	9.21	14.2	154
	2		11.4	29.7	261	3.36	9.12	281
	4		7.87	25.9	329	0.904	9.06	1000
10 (soil)								
	2		17.6	17.1	97	5.38	11.1	206
	4		8.72	9.78	112	3.16	4.50	142
10								
	1		12.2	48.1	396	1.85	6.24	338
	3		9.45	20.0	211	4.06	7.81	192
30								
		4	9.22	163	1770	0.656	18.9	2870
		8	9.06	213	2360	1.65	25.8	1570
		24	16.0	120	1180	1.70	14.9	878
		29	7.14	84.4	1180	3.48	11.5	329
	2		5.92	16.7	282	0.764	17.1	2240
	3		7.60	23.3	306	4.02	14.5	360
	16		9.56	20.0	---	0.743	7.01	9.43

†C - Control; T - Treated.

Table 2-2. Effect of 3 weekly sprays of 25 mg N g^{-1} FW leaf tissue on leaf area, fresh weight, chlorophyll content, and specific leaf weight of 3-month-old Scio barley seedlings.

	Control	Treatment
Leaf area (cm^2) per pot	396 ± 40†	669 ± 90
Fresh weight (g) per pot	15.6 ± 1.1	23.9 ± 2.3
Chlorophyll (mg g^{-1} FW)	2.99 ± 0.43	3.74 ± 0.15
Specific leaf wt. (mg cm^{-2})	17.3 ± 0.8	17.7 ± 1.1

† Standard error of the mean

Table 2-3. The effect of a foliar spray of 25 mg UAN-N g^{-1} FW on mature leaf contents of ammonia, urea, amino acids, sugars, and soluble protein of Scio barley.

Hours after spray	Ammonia		Urea		Amino acids		Sugars		Sol protein	
	C†	T	C	T	C	T	C	T	C	T
	nmol g^{-1} FW				mg g^{-1} FW					
0	1.04		1.15		2.54		6.71			
2	1.28	11.60	0.33	12.30	1.94	5.58	8.19	7.85	7.89	7.53
5	1.16	13.40	0.10	12.10	2.33	6.28	14.60	8.86	6.30	5.94
9	1.23	3.27	1.06	5.64	2.36	5.91	13.20	10.40	5.43	6.31
24	1.22	2.07	0.49	1.50	3.22	3.98	14.40	8.03	9.80	9.60
28	0.60	0.96	0.66	0.97	2.01	5.38	11.90	11.90	9.60	10.50
48	0.26	0.62	0.72	0.79	3.01	4.74	10.50	11.40	9.70	9.68

†C - Control; T - Treated.

Table 2-4. The effect of a foliar spray of 25 mg UAN-N g^{-1} FW on *in vivo* activities of urease, and nitrate reductase, and on *in vitro* activity of glutamine synthetase in mature leaves of Scio barley.

Hours after spray	Urease		Nitrate reductase		Glutamine synthetase	
	C†	T	C	T	C	T
	nmol product min^{-1} g^{-1} FW					
0	124		13.2		2810	
2	221	391	21.7	46.8	3000	3580
5	119	287	12.7	20.0	3220	3340
9	186	272	13.1	63.7	3820	3890
24	217	238	---	---	4330	5160
28	186	277	21.3	110.0	5750	6130
48	193	174	20.7	93.1	3310	4250

†C - Control; T - Treated.

Table 2-5. The effect of a foliar spray of 25 mg UAN-N g^{-1} FW on endogenous *in vivo* activities of urease and nitrate reductase in mature leaves of Scio barley.

Hours after spray	Urease			Nitrate reductase		
	C†	T	T	C	T	T
	nmol NH_4^+ min^{-1} g^{-1} FW		% of C	nmol NO_2^- min^{-1} g^{-1} FW		% of C
0	9.44			0.36		
2	16.0	375	2340	0.48	23.8	4947
5	24.0	265	1100	0.12	9.22	7680
9	4.72	136	2880	1.32	26.5	2010
24	16.0	35.2	220	----	----	----
28	9.07	62.1	685	1.68	29.1	1730
48	4.72	22.1	468	0.36	12.3	3430

† C - Control; T - Treated.

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CHAPTER THREE

THE EFFECT OF FOLIAR APPLICATIONS OF UREA-AMMONIUM NITRATE ON THE AMINO ACID COMPOSITION OF JUVENILE 'SCIO' BARLEY LEAVES

ABSTRACT

Amino acid composition was analyzed in barley leaves after a spray of urea-ammonium nitrate (UAN) to discern the metabolic pathways of N-assimilation perturbed by the special management procedure. Eight h after a foliar application of 30 mg N g^{-1} fresh weight to leaves of greenhouse-grown 'Scio' barley seedlings, amino acid content of treated leaves was highest. There was an increase in weight of glycine, alanine, glutamine-asparagine-serine, valine, glutamate, and histidine in the extract of treated leaves over that in control leaves of 3420, 1120, 412, 282, 205, and 159%, respectively. Upon hydrolysis of the treatment extract, the glutamine-asparagine-serine peak was resolved; the absolute weights of glutamine, asparagine, alanine, glutamate, serine, glycine, valine, and histidine were increased in treated leaves by 2135, 511, 460, 373, 361, 240, 129, and 59 ng g^{-1} fresh weight, respectively. Thus, glutamine is the predominant metabolite for assimilating, transporting, and perhaps temporarily storing applied N in seedling leaves of barley as in other plants. Data indicated that the glutamine synthetase-glutamate synthase system is the predominant pathway for UAN assimilation as observed in other plants after ammonium nitrate application. The weight increase of glycine in treated leaves by 3420%, however, was different and may indicate that either the photorespiratory synthesis of glycine could be enhanced or the degradation of glycine could be inhibited by the high influx of ammonia which is produced specifically by urease hydrolysis of the applied urea.

INTRODUCTION

The ability of a plant to absorb inorganic nitrogen, convert it to reduced form, and assimilate it into amino acids, enzymic proteins, structural proteins, nucleic acids, and other N-compounds is essential for growth and development. When nitrate and/or ammonia (AN) are applied to plants, nitrate reductase and nitrite reductase are induced to metabolize nitrate to ammonia. The ammonia is combined with glutamate by the enzyme glutamine synthetase to form glutamine, which serves as an efficient carrier or transport form of reduced N in plants (2,6,7,9). Asparagine, another amide, is formed from ammonia and aspartate by the enzyme asparagine synthetase (1,2,6). Glutamate, formed from glutamine by the enzyme glutamate synthase (or glutamine oxoglutarate amino transferase - GOGAT), can provide its amino group for the synthesis of a number of other amino acids by the process of transamination (2,6).

Turley and Ching (10) observed that foliar applications of urea-ammonium nitrate (UAN) to seedlings of 'Scio' barley increased the leaf area, plant weight, and leaf chlorophyll content of the seedlings 4 weeks after foliar spray. Transitory responses of leaves to the UAN applications were increased levels of amino acids, soluble protein, ammonia, and urea; and increased activities of nitrate reductase, urease, and glutamine synthetase. Leaves of older plants, within 2 weeks of anthesis, responded similarly in leaf chemical contents and enzymatic activities, and resulted in enhanced levels of seed protein.

Although urea is a common fertilizer, its influence on amino

acid metabolism has not been explored in detail. An analysis of the amino acid composition of the seedlings may enlighten the subject. This paper reports the quantitative changes in individual amino acids of seedling barley leaves between 4 and 72 h after UAN spray. The results indicate that the major pathways of N assimilation are similar in plants treated with AN or UAN except that glycine is specifically accumulated because of the high influx of ammonia from urease hydrolysis of applied urea.

METHODS AND MATERIALS

Experimental material. Leaves of seven-week-old 'Scio' barley plants grown in the greenhouse were sprayed with a solution of 0.05% Triton X-100 with 50, 10, and 30 mg N (urea-ammonium nitrate) g^{-1} fresh weight (FW) on day 1, 9, and 14, respectively. Control seedlings were sprayed with Triton X-100 solution without N. Leaves were collected on ice at 48 h before and at 4, 8, 24, 29, 48, and 72 h after the third foliar application.

Protein-free leaf extracts. Leaves of control and treated plants were washed, chopped, weighed and ground in cold buffer with mortar and pestle as previously described (10). Supernatants of centrifuged slurries were made to 10% TCA, placed on ice for 10 minutes, and centrifuged for 10 minutes at 10,000 X g at 4 C. Supernatants were neutralized with 5N NaOH and used for the analysis of individual amino acids.

Amino acid analysis. The total amino acid contents of protein-free leaf extracts were estimated by the ninhydrin method (3).

Extracts of the 48 h before treatment sample and all the control material were concentrated by heat and vacuum for 4-6 h in a Speed Vac Concentrator (Savant Instruments, Inc.). The amino acid analysis of these samples was then conducted at the laboratory of Dr. R.R. Becker (Dept. of Biochemistry and Biophysics, Oregon State University) using a custom built amino acid analyzer and the post-column orthophthalaldehyde fluorescence detection method (5). The amino acid analyzer was equiped with a Glenco 3.0 mm glass column packed with 25 cm of Dionex DC-4A and employed a four step sodium elution gradient with Dionex Hi-Phi sodium citrate buffers. A solution of borate buffered o-phthalaldehyde at pH 10.5 (4) was mixed with the eluents to generate fluorescence, which was detected by a Gilson Spectra/Glo Fluorescence Detector using excitation and emission frequencies of 340 and 455 nm, respectively. Integration of peaks was performed by a SpectraPhysics Autolab IVB Integrator, using external standards. A 22 h hydrolysate (constant boiling in 6N HCL in vacuo at 110 C) of an aliquot of the extract from N-sprayed leaves at 8 h after treatment also was analyzed to quantify glutamine, asparagine, and serine. These three amino acids had comparable retention times and eluted as one peak in the physiological sample (Fig 3-1 A). After hydrolysis, they were clearly separated as indicated by the relative increases in peak areas of aspartate, glutamate, and ammonia, and the decrease in the peak area of serine (Fig. 3-1 B). The above detection method of amino acid is not sensitive to proline, hydroxyproline, or cysteine (8).

RESULTS AND DISCUSSION

Amino acid composition in the physiological extract. At 8 h after application of 30 mg N g^{-1} FW, amino acid content of treated leaves was highest and represented a 291% increase over the control (Table 3-1). On a weight basis, the percent increases for the amino acid fractions of the physiological extract at 8 h after spray were 3420, 1120, 412, 282, 205, and 159% for glycine, alanine, glutamine-asparagine-serine, valine, glutamate, and histidine, respectively. This order of percentage weight increase was relatively consistent throughout the experiment, except for alanine and glutamate which exchanged positions 48 h after spray. Although glycine and alanine showed large percentage increases over the levels in control leaves, glutamine and asparagine were the major metabolites produced because of the UAN spray.

Amino acid composition in the hydrolysate. Based on the distribution of glutamate, aspartate, and serine in the hydrolysate and the others in the original extract, the increases in glutamine, asparagine, alanine, glutamate, serine, glycine, valine, and histidine at 8 h after spray were 2135, 511, 460, 373, 361, 240, 129, and 59 ng g^{-1} FW, respectively. Thus, glutamine is the principal sink for assimilation of ammonia from urea, ammonium, and nitrate, and may function as a storage form of N as well as an efficient transporter of N.

The high percentage increases in weight for alanine and glycine may be explained by the pathways of their synthesis and metabolism. In general, when amino acid synthesis is increased, respiratory

processes are stimulated by the depletion of organic acids (2). Pyruvate, the product of glycolysis, would be enhanced similarly and is the immediate precursor of alanine, which is rapidly synthesized in the chloroplast by transamination of glutamate (2,6). Although valine also is synthesized from pyruvate, the pathway is longer and tightly regulated (6). The rapid synthesis of alanine in leaves also was observed in pea seedlings after ^{15}N-NO_3 feeding to roots. Ammonia, glutamine, and glutamate were labeled first, then followed by alanine (1). When ^{15}N-NO_3 was supplied to leaves, most rapid incorporation of N was into ammonia, then glutamine, followed by glutamate, asparagine, alanine, aspartate, and others. Thus, alanine may accumulate in barley leaves of this study because its turnover rate is not as rapid as glutamine and glutamate. Glycine synthesis or accumulation involves the photorespiration process in leaves; its substrates are glyoxylic acid and glutamate (7). Glycine is converted to serine and ammonia during photorespiration to supply ammonia (7). In times of plentiful ammonia supply such as after the urea spray, the photorespiratory pathway perhaps is enhanced and the degradation of glycine is inhibited. An accumulation of glycine results. Glycine is an efficent carrier of N (1N:2C) and its increased synthesis when N is readily available may be a subtle mechanism for more efficient N incorporation, as carbon supply could be a limiting factor in amino acid production. Additional study in this area would be meaningful.

Effect of urea spray. Most studies have investigated the effects of nitrate or ammonia on N assimilation in plants, but this study included urea as a rapid and economical source of ammonia. Urea

comprised half of the N supplied in the foliar spray used in this study. Its hydrolysis in the leaf is less expensive in terms of respiratory energy than the reduction of nitrate. At a reasonable concentration (i.e., 30 mg N g^{-1} FW), urea may be a more efficient source of ammonia than nitrate. The assimilation of applied UAN as amino acids and amides was approximately 5.5 umoles ammonia g^{-1} FW h^{-1} during the first 8 h, based on the difference between amino acid content of the control and treatment at 4 and 8 h. This estimate may be somewhat conservative because the amino acids would be utilized continually for the synthesis of proteins, nucleotides, and nucleic acids in leaves for growth and for transport to sinks, e.g., seeds. However, it is of the same order of magnitude as the estimate of maximum N assimilation of 9 umoles N g^{-1} FW h^{-1} in leaves, based on rates of nitrate and nitrite reduction (7). The amount of ammonia in leaves was 6.86 umole g^{-1} FW at 4 and 8 h after spray while urea was found to be at 8.5 umoles g^{-1} FW during the same time period (10). Evidentally, the absorption rate for the applied fertilizer was close to that for maximum ammonia assimilation, although a total of 2140 umoles of N g^{-1}FW was applied to the leaves. The difference may be attributed to incomplete absorption and/or slow utilization.

Unknown amino acids. Three unknown amino acids were found in the extracts and were labeled X1, X2, and X3 in order of their elution from the column. The unknown amino acid X1 appeared in all extracts of control leaves and in treated leaves 48 h before the UAN spray. After the UAN spray, X2 appeared instead of X1. These amino acids were in the acidic portion of the analysis. An alternate explanation is that the concentration of the samples by heat under

vacuum caused a change in the amino acid, X1, so that it then appeared as X2. The unknown amino acid X3 appeared only in control samples except at 48 and 72 h, and in the treatment sample 48 h prior to N application. These unknowns are small in quantity and are not central to the effects of UAN.

Fig. 3-1. Profiles of free amino acids in Scio barley leaves 8 h after a foliar application of 30 mg N g^{-1}FW: A) scan of the extract representing physiological levels of different amino acids, B) scan of the hydrolysate of above extract, which was separated to glutamate, aspartate, and serine, and provided the quantitative data for estimation of the amides and serine.

Figure 3-1

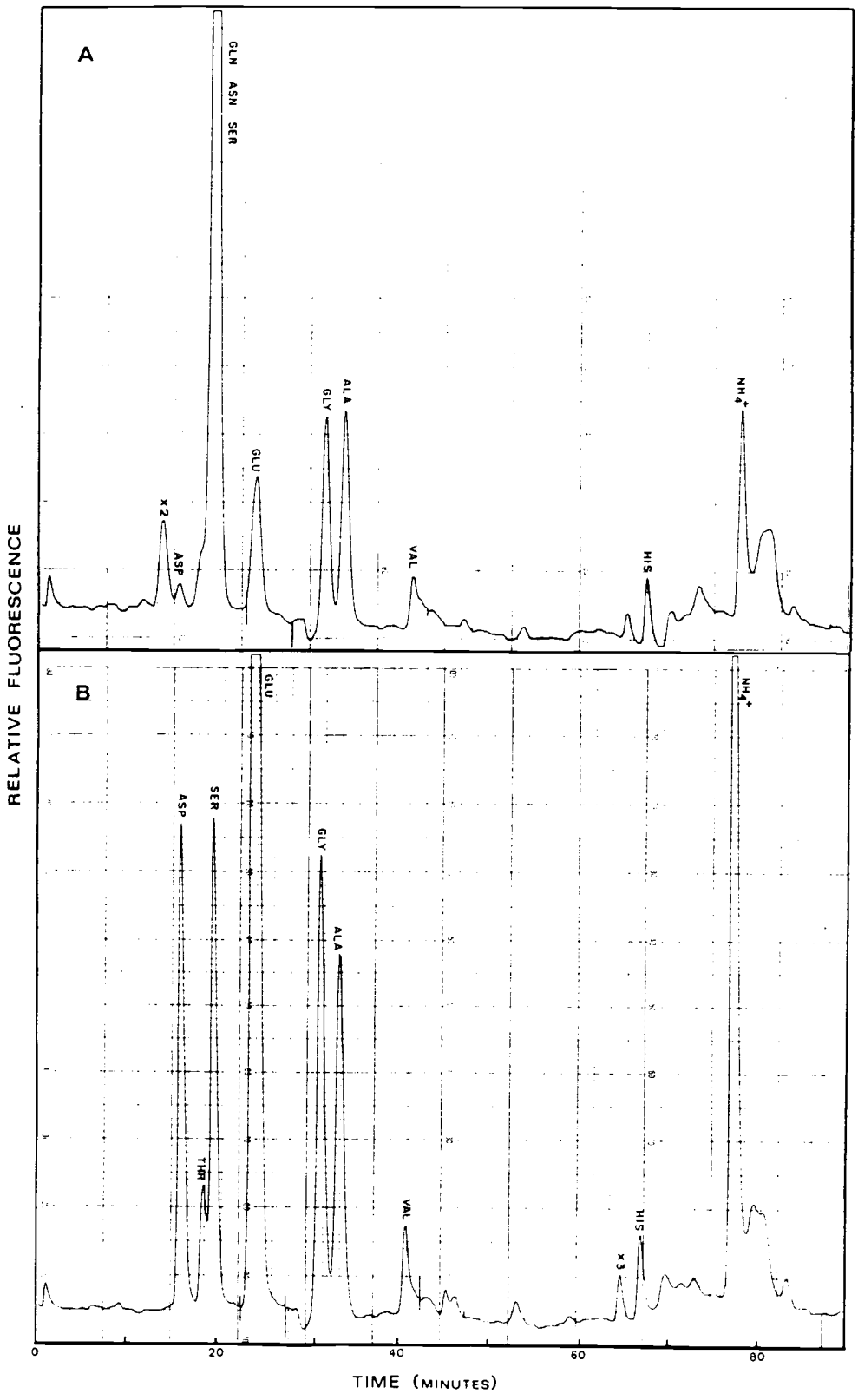
A
GLN ASN SER
GLU
GLY
ALA
VAL
HIS
NH4+
x2
ASP
B
GLU
ASP
SER
GLY
ALA
THR
VAL
HIS
x3
NH4+
RELATIVE FLUORESCENCE
0
20
40
60
80
TIME (MINUTES)

Table 3-1. The effect of foliar applications of urea-ammonium nitrate on the amounts of free amino acids in 'Scio' barley seedling leaves.

	Hours after spray						
	-48	4	8	24	29	48	72
	ng g^{-1} FW						
CONTROL							
X1†	106	125	205	142	156	298	120
ASP	-‡	-	62	-		107	
THR						-	
GLN/ASN/S	439	334	729	403	1062	830	602
GLU	567	35	181	112	89	182	194
GLY	16	3	7	10	5	20	10
ALA	105	17	41	97	15	219	142
VAL	59	34	45	44	39	57	44
ILEU					-		-
LEU	-	-	-	-	-	-	-
TYR				-	-	-	-
PHE				-	25	-	
X3	61	72	53	32	49	70	36
HIS	44	36	38	29	31	68	35
LYS	-	-	47	-	-	-	-
ARG	-	-	-	-	-		-
TOTAL	1397	656	1407	867	1471	1851	1183
TREATMENT							
X1	182		-		-		
X2		126	198	122	93	151	178
ASP		-	-	-	-	-	124
THR		-	-		-	-	-
GLN/ASN/S	1241	2014	3736 §	2676	2203	1916	2778
GLU	204	710	554	358	462	1176	889
GLY	77	113	247	71	83	89	158
ALA	93	274	501	239	287	231	263
VAL	33		170	75	65	99	90
ILEU	-			-	-		
LEU	-		-	-	-	-	-
TYR							
PHE	-		-		-	-	
X3	25	-	-	-	-	-	-
HIS	30	87	97	-	47	76	-
LYS			-	-	-	-	-
ARG	-	-	-	-	-		-
TOTAL	1884	3326	5503	3541	3240	3738	4480

† X = unknown

‡ "-" = trace

§ This amount comprised of 2653 ng glutamine, 635 ng asparagine, and 448 ng serine (Fig. 3-1 B).

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