ABSTRACT

NAEGLE, ERIN ROCHELLE. Seed nitrogen content of soybean: mobilization of nitrogen reserves and its relationship to seedling growth. (Under the direction of Dr. Thomas Rufty.)

Leguminous crops such as soybean are commonly grown in the relatively infertile soils of the southeastern U.S. The primary source of N for soybean growth and development in these environments is N_2-fixation, which requires a symbiotic relationship that does not develop until 3 to 4 weeks after germination. Prior to N_2-fixation, plants are largely dependent on seed reserves and they often experience a period of N stress. The purpose of this thesis was to investigate mobilization of seed N and its impact on soybean seedling development.

Sixteen soybean lines differing in seed N content were grown hydroponically for 27 days without external N. Higher seed N was associated with increased seedling growth and reduced expression of N deficiency symptoms. Three of the 16 lines were selected for detailed characterization of seed protein degradation and N mobilization, and their relationship with seedling developmental responses during progression into and recovery from N stress. Leaf expansion and initiation were restricted more severely in soybean lines with lower seed N content. Depressed canopy development was the primary factor leading to decreased shoot:root growth ratios in all 3 lines. The soybean line with the lowest seed N content had a higher S/R ratio as the N stress progressed. The shoot and root growth changes were different than those in previous N deficiency studies, where adjustments have been proportional to the severity of N stress. When external N was supplied to plants deprived of N for 15 or 23 days, the induction period of the nitrate uptake process was extended and growth recovery rates were correlated with initial seed

N contents. There was no delay, however, in stimulation of leaf initiation rates, which responded rapidly to the presence of external N. Individual leaf expansion during the recovery from N stress was dependent upon a leaf's developmental stage.

The majority of N was mobilized out of cotyledons within 12 days in three soybean lines with differing seed N contents. Mobilization was complete before differences in seedling growth were measurable. Mobilization rates were lower when external N was present, suggesting the involvement of source/sink relations on the mobilization process. Differences in proteolysis of glycinin and β-conglycnin, the main storage proteins in soybean seeds, between N treatments were not detectable. Storage protein content and proteolysis rates were proportional to differences in seed N content.

SEED NITROGEN CONTENT OF SOYBEAN: MOBILIZATION OF NITROGEN RESERVES AND ITS RELATIONSHIP TO SEEDLING GROWTH.

by

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

DEPARTMENT OF CROP SCIENCE
(PLANT PHYSIOLOGY PROGRAM)

Raleigh

2002

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BIOGRAPHY

Erin Rochelle Naegle was raised at the base of the majestic Book Cliffs in Helper, Utah. The curiosity and independence instilled by her parents during this time were an integral part of her decision to become a scientist. Upon her graduation from Carbon County High School, she began attending Utah State University (USU) in Logan, Utah. After three long years of deliberation and a six-month study abroad in Israel, she chose to concentrate her academic efforts in the plant sciences. Erin graduated magna cum laude with a Bachelor of Science in Crop Science with minors in Chemistry and Plant Biology from USU in 1999. While attending USU, she worked under the direction of Dr. William Campbell investigating the basis for sterility in dwarf wheat grown on the Russian Mir space station. Her work as part of the 'wheat in space' team fostered an interest in plant stress physiology. An interest that she pursued further as a Master's student in the Crop Science Department at North Carolina State University under the supervision of Dr. Thomas Rufty.

ACKNOWLEDGEMENTS

". . . there are so many whose names or even faces I do not know. For one's attitude can be changed by a passing conversation, a passage in a book. Hundreds--no, thousands--of human beings, yes, and animals too, have helped me to reach this point in time and space. How grateful I am to each and every one of them, and I only wish I had the space to list the names of all those who have helped me so much throughout the years."

Jane Goodall, Reason for Hope

I find my heart echoing such sentiments, but I would undoubtedly further include plants to the sculpting of who I am today. Though I cannot list them all, I would like to extend my sincere appreciation to all those who have made the completion of this thesis possible. Thanks to my major advisor, Tom Rufty, for his tireless encouragement to achieve excellence and pursue truth. Thanks to Prachuab Kwanyuen for his patient and candid mentoring in the way of proteins and life. Thanks to Judith Thomas and Jim Holland for serving on my committee, and to Joe Burton for providing seeds and advice for my research. Thanks to my dear family and friends who provided a living well of encouragement, support, and perspective. Thanks to Alexander Graham Bell, whose keen invention made it possible to receive such blessings. An especial mention to my fellow residents of the Vanderbilt Palace: Kathy, Dianne, and Mary Lou, who provided such a palace with all the warmth, love, and security of home. How pleasing it is to have such a symbiotic relationship, your baking and my eating.

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CHAPTER 1. EFFECTS OF SEED NITROGEN CONTENT ON SOYBEAN SEEDLING DEVELOPMENT

INTRODUCTION

Leguminous crops such as soybean are commonly grown in the sandy, relatively infertile soils of the southeastern United States. Often, nitrogen fertilizer is not applied and nitrogen fixation provides adequate N for sustained growth and maximized yields. In the absence of N fertilizer, soybean seedlings are dependent on cotyledonary N during the critical establishment phase. Soybean plants typically experience a transient N stress as the cotyledonary reserves are depleting, which extends until development of the N_2-fixation system 3.5 to 4 weeks after germination (Israel, 1981). Little information is available on the relationship of seed N reserves to the early development of soybean, especially in low N conditions.

Soybean plant breeding programs are producing genotypes with varying seed oil and protein contents, two components that are heritable and inversely related (e.g. Shorter et al, 1976; Burton, 1984; Burton and Wilson, 1994; Burton et al., 1999). Limited seed N reserves in high oil and low protein seed lines could predispose seedlings to differing degrees of N stress prior to N_2-fixation. The results from a number of studies with other crop species have indicated that seed N can have a strong impact on seedling vigor (Ries, 1971; Bulsani and Warner, 1980; Nedel et al., 1996; Hara and Toriyama; 1998).

In this series of experiments, we examined seed N mobilization and early growth of soybean genotypes with different seed N contents in the absence of external N. The focus was on growth and developmental changes as seedlings progressed into N stress and recovered when external N was supplied. Many studies have examined growth

alterations with N stress, and experimental systems typically involved limiting N availability from well-nourished plants or growing plants at sub-optimal N levels. The N stress responses include changes in shoot to root dry weight ratio and the production of fewer and smaller leaves (Morton and Watson, 1948; Brouwer, 1962; Greenwood, 1976; Ingestad, 1979; Marschner, 1998). In this seedling experimental system a precise characterization of those developmental changes and an examination of their relationship with a variable endogenous N supply were performed.

MATERIALS AND METHODS

Soybean (*Glycine max*) seeds were wrapped in germination paper moistened with 0.1 mM $CaSO_4$ and placed in a dark germination chamber at 28 C and 98% RH for three days. Seedlings with roots 8 to 12 cm in length were selected for uniformity and placed into 50-L continuous-flow hydroponics systems. The systems were located in a walk-in growth chamber in the Southeastern Plant Environmental Laboratory (Raleigh, NC, USA) programmed for a day/night temperature of 26/22 C. Plants were exposed to a nine-hour light period with cool white fluorescent and incandescent light, PPFD of 550 $\pm$ 50 μmol $m^{-2}s^{-1}$. A three-hour night interruption with incandescent light of insignificant PPFD of 30.5 $\pm$ 3.4 μmol $m^{-2}s^{-1}$, provided sufficient photomorphogenic irradiance, 11 $\pm$ 1 Wm^{-2}, to repress flowering.

The nutrient solution temperature was maintained at 24 $\pm$ 0.5 C and pH at 6.0 $\pm$ 0.1 with automated monitoring and additions of KOH (0.01 mM) and H_2SO_4 (0.01 mM). The complete nutrient solution composition was: 200 μM KH_2PO_4, 600 μM KNO_3, 300 μM $MgSO_4$, 800 μM $CaSO_4$, 19 μM H_3BO_3, 3.7 μM $MnCl_2H_2O$, 317 nM $ZnSO_4$, 132 nM $CuSO_4$, 50 nM 85% H_2MoO_4, and 35.8 μM Fe as Fe-Sequestrene. When plants were

grown without an external N source, KNO_3 was replaced with 300 μM K_2SO_4. Nutrients were monitored and adjusted so that depletion was minimized to less than 30% of the initial solution concentrations.

Seedlings of 16 lines of soybean (*Glycine max* [L.] Merr. cv. Clifford, Dillon, Haskell, Holladay, Prolina, Ransom, Young, NC-101, NC-104, NC-105, NC-106, NC-110, NC-111, NC-112, N93-1264, and D68-0099) were grown for 27 days without N. At the end of the experiment, eight plants of each line were harvested, dried at 55 C, and weighed. Three of the 16 lines, NC-106, Young, and D68-0099, representing a wide variation in seed N content and growth response, were selected for more detailed experiments. Seed of all the soybean lines were obtained from USDA soybean field plots grown at the Central Crops Research Station in Clayton, N.C. in 1997.

Three types of experiments were conducted. In one, plants from the three lines were grown in the complete nutrient solution with or without a N source for 27 days. Four randomly selected plants from each treatment were harvested every two to three days. In a second experiment, plants were grown in -N solutions for either 14 or 23 days, at which point KNO_3 was added to the nutrient solution to establish a N concentration of 600 μM. Four randomly selected plants of each line were harvested at four or five day intervals over a 15 day recovery period.

For the first two types of experiments shoot, root, and cotyledon tissues were separated at harvest, and leaves were counted. Shoot apical meristems were examined using a dissecting microscope to include primordia emerging from the apical dome; thus, leaf initiation estimates include macroscopic and microscopic leaves. Areas of individual leaves $\geq$ 2.00 cm^2 were measured with a Li-Cor 3100 leaf area meter (Li-Cor

Instruments, Lincoln, NE). Tissues were oven dried at 60 C, weighed, and ground. Tissue nitrogen was measured using a CHN Elemental Analyzer (Model 2400, PerkinElmer Corp., Norwalk, CT, USA).

A third type of experiment was conducted to characterize nitrate uptake by N-stressed plants. Seedlings of NC-106, Young, and D68-0099 were grown in –N nutrient solutions as described previously for 15 days, at which time KNO_3 was added to the solution three hours into the light period to establish a N concentration of 600 μM. At specified times, four plants of each line were removed from the hydroponics units and placed into four liter beakers containing aerated solutions with ^{15}N labeled nitrate. At 0, 4, 12, 24, 48, and 77 hours after addition of nitrate, plants were exposed to 98 atom % ^{15}N- nitrate for three hours. On days 4, 5, 7, 9, 11, and 13 after addition of nitrate, plants were exposed for five hours during the light period to solutions containing 10 atom % ^{15}N- nitrate. The ^{15}N treatments were within the same growth chamber as the hydroponics units. Immediately before and after exposure to ^{15}N, plant roots were dipped 5 times in 1.0 mM $CaSO_4$ to remove apoplastic nitrate. After exposures, roots and shoots were separated, dried, weighed, and ground. Ground tissues were analyzed for total N and ^{15}N enrichment using elemental N analysis and ratio mass spectroscopy.

RESULTS

Growth of seedlings from the 16 soybean lines varied in the nutrient solutions without N. At the end of 27 days, plant dry weights ranged from 300 to 1000 mg and the weights were positively correlated with original seed N contents (Fig. 1A). The correlation between plant growth and seed dry weight was much lower (Fig. 1B).

Although not portrayed in Fig. 1, the ordering of genotypes on the horizontal axis was different in the two graphs, thus seed N content was not predictable solely by seed weight. All of the seedlings exhibited typical N deficiency symptoms including leaf chlorosis and senescence of older leaves. The expression of N-stress symptoms was inversely related with the initial seed N content and growth.

To more precisely characterize the relationship between seed N and early growth, three soybean lines were selected (from the group of 16) that had a range of seed N contents: NC-106, Young, and D68-0099 (Table 1). The N contents ranged from 13.5 to 6.4 mg N seed^{-1}. To determine if inherent growth differences existed, the lines were grown for 27 days with N included in nutrient solutions. During that time, plant dry weight accumulation, leaf area expansion (Fig. 2), and N content (data not shown) were not statistically different among the three lines. Thus, growth differences in the absence of external N could be attributed to differing levels of internal N in the seed. For visual clarity, lines of +N plants were combined for the remainder of the graphs.

Dry Weight Accumulation

When the three lines were grown without external N, differences in growth became apparent on about day 15 (Fig. 2). The three-fold difference in dry weight accumulation at 27 days between the high seed N line, NC-106, and the low seed N line, D68-0099, was a magnification of the two-fold difference in initial seed content (Table 1). In all three lines, shoot growth was limited more than root growth, and growth of both was more severely affected with lower initial seed N content (Fig. 3). After an initial decline, associated with early root growth, shoot to root dry weight ratios of +N plants increased over time (Fig 4A). In contrast, the S/R ratios of –N plants decreased steadily

(Fig. 4B). The shoot:root of the low seed N line, D68-0099, was higher than the other two lines for both N treatments until the end of the experiment.

Nitrogen was rapidly mobilized out of the cotyledons (Fig. 5). Although only the data for –N are plotted, the pattern of exponential decay was similar under the two N regimes. Because cotyledonary N depleted to less than 1 mg N plant^{-1} in all three lines, the N content of developing seedlings mirrored the initial differences in seed N content.

Leaf Growth

The differences in initial seed N content and amount of N mobilized out of the cotyledons led to different degrees of restriction in individual leaf development. Primary leaf expansion of NC-106 was relatively unaffected compared to the +N control, while expansions of Young and D68-0099 were 81 and 66% of controls (Fig 6A). A similar pattern of restriction was present in the first and second trifoliolate leaves, with leaf expansion of the low seed N line, D68-0099, consistently more severely inhibited (Figs. 6B and 6C). Leaf initiation also was affected by the level of internal N (Fig 7, Table 2). A decline in leaf number was apparent in the –N treatment towards the end of the second week, and the total number of leaves initiated by NC- 106, Young, and D68-0099 at 27 days was 11.3, 10, and 8.5, respectively. The combination of decreased leaf number and individual leaf areas in –N plants was responsible for the severely reduced total leaf areas compared to +N plants (cf. Fig 2B).

Recovery from N Stress

Growth of N-stressed plants generally was slowing down by day 15 of the experiment (Fig. 2A). When external N was supplied to N deprived plants after 15 days, growth increased in all three soybean lines, but the recoveries were different as D68-0099

lagged behind (Fig 8A). When external N was supplied to N deprived plants at day 23, differences in growth of NC-106 and Young were present, and growth of D68-0099 was minimal (Fig 8B).

Nitrate uptake rates were measured using ^{15}N when external N was supplied after 15 days. Uptake rates g^{-1} root increased for about four to five days and then stabilized (Fig 9A). Data plots indicated that D68-0099 initially had a somewhat slower uptake rate but tended to have a slightly higher uptake rate when maximum rates were obtained. The stabilized rates were much higher than those of control plants, which had been continually exposed to nitrate in solution. Translocation of ^{15}N to the shoot mirrored the increases in uptake g^{-1} root for about four days (data not shown). Thereafter, about 70% of the absorbed ^{15}N was found in the shoot for the three soybean lines, so there was no indication of a separate seed N effect on the translocation process. With plants supplied with nitrate on day 23, nitrate uptake g^{-1} root was estimated from total N accumulation (Fig. 9B). The calculated N uptake rates for the three soybean lines increased over the 15-day recovery period. Again, genotypic differences were apparent, as slower uptake occurred with lower N status of the initial seed and slower growth.

Even though nitrate uptake rates were not maximized for several days, canopy leaf areas increased rapidly when N was supplied to the N-stressed seedlings (Fig. 10A). Total leaf area expansion of NC-106 and Young were only about 10-15% lower than the +N controls at 27 days (cf. Fig. 2B). Leaf area expansion of D68-0099 was slower than the other genotypes, particularly when nitrate was supplied after 23 days. Leaf initiation rates responded quickly to the N supply, and the rates of initiation were similar to those for controls (Fig. 10B; Table 2). The recovery was immediate, as about two leaves were

initiated in all three lines during the first four days after N was supplied on day 14. The same response occurred when nitrate was supplied on day 23 in the two higher seed N lines, with D68-0099 lagging somewhat.

Though expansion of the entire canopy increased at similar rates between the two recovery periods, individual leaf expansion was notably different as demonstrated in the 1st and 2nd trifoliate of NC-106 (Fig. 11). Supplying N at day 14 increased the expansion of both leaves compared to the –N treatment, the 2nd more so than the 1st. When N was supplied on day 23, the 1st trifoliate remained the same size, equal to that of the –N treatment (Fig. 11B), while that of the 2nd trifoliate increased.

DISCUSSION

This research was conducted to investigate the influence of seed N content on soybean seedling development in low N fertility conditions. Results from the genotype comparisons indicate that there were notable differences in growth as seedlings progressed into and recovered from N stress, with higher seed N leading to more rapid growth in both circumstances. The growth effects included differences in shoot and root growth rates and canopy leaf area expansion.

The nutrient solution without N was used to accentuate physiological responses associated with varying seed N contents. It is rare, of course, that field situations would be encountered where N would be entirely absent. Nonetheless, the sandy soils of the coastal plain in the southeastern US, where soybean commonly are grown, are ultisols (Buol et al., 1973) that contain little inorganic N and the organic matter content is <1.0%. Consequently, N availability to plants is extremely low. Also, in greenhouse experiments

and in the field, soybean typically exhibits decreased growth and symptoms of N stress between the time that seed N reserves deplete and the N_2-fixation system develops (DW Israel, personal communication; Israel 1981). Furthermore, physiological studies have consistently shown that plant responses to different degrees of N stress are similar, primarily differing in magnitude (Rufty et al., 1984; Ingestad and Lund, 1979). It is assumed that the physiological responses observed in the –N treatment as plant vigor declined also would occur during N stress in the field, but to a more moderate degree.

Canopy Development

In general terms, the decrease in leaf canopy expansion with N stress was not surprising. Decreases in leaf size and (Fig. 6) and number (Fig. 7) have long been recognized as important responses in N stressed plants (e.g. Watson, 1947; Greenwood, 1976; Rufty et al., 1984). There have been few detailed descriptions of growth changes in individual leaves within a canopy under N stress. The response pattern with the soybean seedlings seems to indicate that leaf size was determined by the degree of N stress and the stage of leaf development. At all leaf positions, the greater amounts of N released from the cotyledons led to larger final leaf size, i.e. the leaves of NC-106 were affected the least and D68-0099 the most (Fig. 6). That was true for all leaves, even though some were expanding after the seed N reserves were depleted. The importance of leaf developmental stage in the N stress response can be seen by comparing the expansion restrictions among leaf positions. For each genotype, expansion of older leaves was affected more severely.

Leaf expansion is a function of cell division and cell enlargement, and restriction of both can contribute to smaller leaves under N stress. In detailed anatomical

experiments with castor bean, it was shown that N stress early in development inhibited both cell division and enlargement, whereas N stress later in the expansion phase affected only cell enlargement (Roggatz et al., 1999). The mechanistic basis for the restriction of cell division and enlargement remains largely unresolved. It has been proposed that the cause of decreased cell enlargement size may be decreased hydraulic conductance in roots coupled with decreased water flow to the shoot (Radin and Parker, 1979; Radin and Boyer, 1982) or decreased cell wall extensibility (Palmer et al., 1996).

Regardless of the exact mechanism(s) involved in the restriction of leaf expansion, once a certain stage of development was reached, the restriction was irreversible. That effect is seen most clearly in leaf expansion plots for NC-106, detailed in Fig. 11. The 1st trifoliolate leaf reached its expansion plateau in the days just prior to the external N addition on day 23, and no further expansion occurred, i.e. meristematic activity or cell enlargement did not resume. The effect of external N addition on day 14 was different, as expansion increased somewhat and final size was closer to the control. A similar response pattern occurred with the 2nd trifoliolate leaf for both recovery periods.

The irreversibility of meristematic activity that seemed to exist in individual leaves certainly did not apply to the shoot apical meristem. N-stress led to a marked down regulation of leaf initiation that was detected around day 15 (Fig. 7), and the rate of initiation was 10 to 30% of the control rate after that time (Table 2). When external N was supplied, however, initiation recovered immediately in all three genotypes to rates that were similar to +N control plants. That was true whether N was introduced into the system on day 14 or on day 23 when the plants were in advanced stages of stress (Fig. 10,

Table 2). The two genotypes with higher initial seed N contents, NC-106 and Young, initiated about two leaves within the first four days after N supply at both dates, similar to the controls. The leaf initiation rate of the low seed N genotype, D68-0099, was somewhat lower with N supply after 23 days, but leaf initiation still was higher than rates when N was continually withheld. The quick recovery of leaf initiation suggests that the meristem assumed a quiescent state during the N stress progression, and was capable of rapidly up-regulating cell division rates when N became available. Although no photographs are shown here, the apical dome was examined microscopically throughout the experiment during leaf counts and, as in an earlier P stress study (Chiera et al. 2002), there were no indications of structural changes occurring at the growth center.

Shoot:Root Growth Ratio

One aspect of the growth response in this seedling system that was not consistent with past N-stress experiments was the lack of a positive correlation between the degree of N stress among the genotypes and alterations in the shoot to root growth ratio. It has been seen in many studies that the S/R growth ratio is lowered soon after a stress is imposed and the degree of adjustment reflects the degree of stress (Brouwer, 1962; Raper et al., 1977; Ingestad, 1979; Rufty et al., 1984). That was not the case here, as the most severe N stress was in D68-0099, but its S/R ratio remained higher than the other genotypes during and after seed N mobilization occurred (Fig. 4). Leaf growth was reduced to a greater extent in D68-0099, so limited root growth was responsible. The most obvious explanation is that the seed is the primary source of N and carbon driving root growth early on, and the limited amount of nutritional reserves in the seed of D68-0099 prevented more extensive root growth. This notion is supported by the growth

pattern in the +N plants, where root growth during the first two weeks was lower in D68-099 even with the external N supply (data not shown). The initial linkage between seed reserves and root growth could reflect vascular system arrangement and, in the case of the +N treatment, the limited ability to absorb N near the growth center at the root tip (Lazof et al., 1992; Henriksen et al., 1992).

N Transport and Leaf Initiation

One of the most interesting aspects of these experiments was the very rapid up-regulation of meristematic activity when external N was supplied on day 14 or 23, while nitrate uptake rates were initially low and relatively slow. Uptake of nitrate by roots is subject to both inducible and feedback effects (reviewed by Clarkson, 1986; Imsande and Touraine 1994; Crawford and Glass, 1998; Tischner, 2000). In most experiments with crop seedlings including soybean, induction of the nitrate uptake system occurs rapidly and maximal uptake rates obtain within about 4 to 12 hours (Jackson et al., 1973; Touraine et al., 1992; Siddiqi et al., 1989). In the present experiment, when 600 uM ^{15}N-nitrate was supplied at 14 days, uptake rates did not reach a maximum until four to five days later, and when unlabeled nitrate was supplied at 23 days, there were indications that the uptake rate had not reached a maximum even after 12 days (Fig. 9). Likewise, white spruce seedlings starved of N for 3 weeks took 2 to 3 days to reach maximal uptake rates (Kronzucker et al., 1995). The slower induction probably was caused by the low N status of the root and impaired protein synthesis; specifically, the formation of functional membrane proteins. This explanation was given previously to explain results from experiments with N deprived arabidopsis and maize (Doddema and Otten, 1979; Teyker et al., 1988).

After plants were exposed to nitrate, there were no indications of strong feedback effects within the time frame of this experiment, as uptake rates of all three genotypes remained much higher than the +N control plants (Fig. 9A). It would be expected that the rates would eventually decline as the N status of the plants increase, nitrate and amino acids accumulate, and feedback controls become engaged (Siddiqi et al., 1989; King et al., 1993; Imsande and Touraine, 1994; Gojon et al., 1998).

The immediate recovery of leaf initiation indicates that the apical meristem is highly sensitive to N uptake and translocation to the shoot. Translocation of N was determined primarily by uptake rate, so it followed the same transport pattern when external nitrate was supplied on day 15. For initiation of two leaves in the first four days after external N was supplied (Fig.10), meristematic activity must have responded to the low amounts of N transported to the shoot during early induction. The linkage between N transport to the shoot and leaf initiation could involve at least two factors. One is the delivery of N to the meristem, which could serve as a signal (Crawford, 1995) as well as increasing the availability of N for protein and DNA synthesis. The other is hormonal regulation. When N stress is relieved, decreases in absisic acid and increases in cytokinins have been found (Clarkson and Touraine, 1994).

Field Implications

Plant breeding or environmental effects that cause large differences in seed N content, such as that present in the genotypes examined here can have important consequences for seedling growth during the establishment phase in low N fertility conditions. All seedlings experience N stress, but lower seed N content was associated with decreased vigor. It is logical to think that low vigor would be associated with lower

disease and pest tolerance, decreased competitiveness with weeds, and decreased ability to avoid stresses such as drought.

Though root nodule development and N_2-fixation were not part of this study, establishment of the N_2-fixation system is especially important under low N soil conditions, where fixed N_2 is the major N source (Deibert, 1979). Genotypes that have higher seed N evidently will be able to minimize the impact of the N-stress seedlings experience in the period between mobilization of N reserves and inception of N_2-fixation. Increased vigor is associated with more rapid growth and more favorable energy relations in the root system. Past experiments have shown that seedlings that emerge faster and have greater root mass generally develop more nodules per root length (Smith and Ellis, 1980). Thus, it is reasonable to expect that seedlings derived from seed with higher N content will develop N_2-fixation sooner and the capacity for N_2-fixation will be greater. Nonetheless, from the nitrate uptake response and the growth response associated with it, it seems that the low seed N genotypes would retain the ability to develop the N_2-fixation system and recover, albeit at a slower rate.

With current market pressures dictating the move towards value-added soybean with greater oil content, and given the inverse genetic relationship between protein and oil (Shorter et al, 1976; Burton, 1984), it seems inevitable that new soybean varieties will be released that have lower seed N content. Can there be management adjustments to offset lower seedling vigor? The most obvious would be additions of low amounts of N fertilizer, which would help to carry seedlings until development of the N_2-fixation system. The fertilizer N rates must be low enough to avoid inhibition of the N_2-fixation system (Hardarson et al., 1984; Yinbo et al., 1997).

Table 1. Components of seed N content, the product of averaged seed weight and N concentration. Seed weights are a mean of 50 seeds for each line. The 50 seeds were divided into five groups of ten seeds. Nitrogen was analyzed for each of the five groups. Means are followed by standard error of the mean.

Lines	Seed Weight (mg seed^{-1})	Nitrogen Concentration (%N)	Seed Nitrogen Content (mg seed^{-1})
NC 106	181.9 ± 3.0	7.40 ± 0.149	13.5
Young	155.3 ± 2.9	6.25 ± 0.100	9.7
D68-0099	117.1 ± 3.4	5.45 ± 0.500	6.4

Table 2. Number of leaves initiated per day. Slopes of each line and N treatment were averaged from four linear regressions, all $r^2 > 0.95$. Slopes of the +N and –N columns were calculated from Figure 7 data points from day 15 to 27.

Lines	+N	-N	N Recovery -N for 14d	N Recovery -N for 23d
NC-106	0.50 ± 0.0043	0.15 ± 0.017	0.60 ± 0.014	0.43 ± 0.013
Young	0.51 ± 0.0004	0.07 ± 0.019	0.47 ± 0.022	0.52 ± 0.014
D68-0099	0.50 ± 0.0110	0.05 ± 0.070	0.50 ± 0.018	0.27 ± 0.013

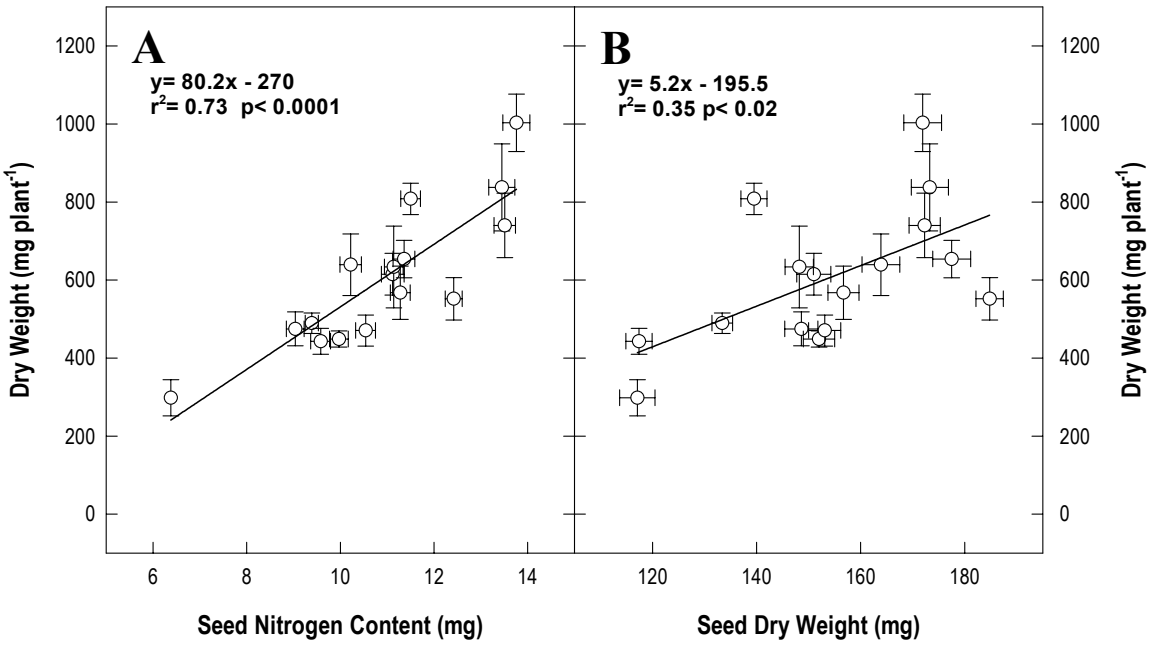

Figure 1. Linear regressions of mean plant dry weight on mean seed N content (A) and mean seed dry weight (B) of 16 soybean lines after 27 days of growth without external N. Circles represent the mean dry weight of eight plants, and the seed N content or dry weight of 50 seeds. As in all proceeding figures, vertical and horizontal bars represent mean standard error.

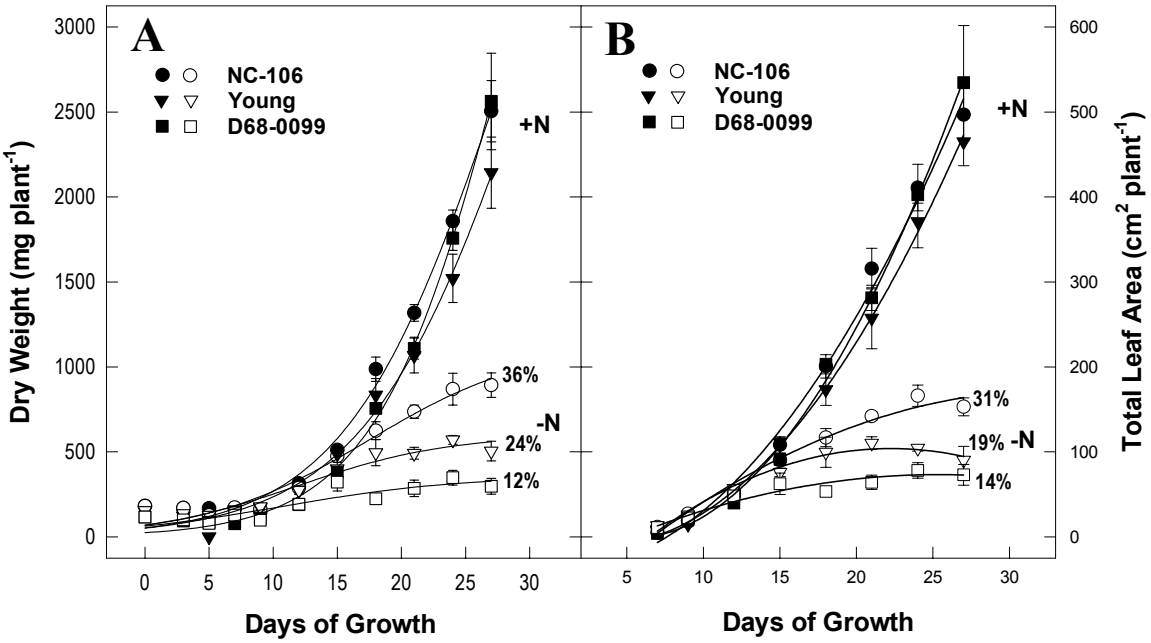

Figure 2. Dry weight (A) and total leaf area (B) of three soybean lines grown in +N (black symbols) or –N (white symbols) solutions for 27 days. Decrease in –N Young leaf area after 21 days of growth was due to senescence. Percent inserts represent the proportion of +N treatment reached by –N plants of respective lines at day 27.

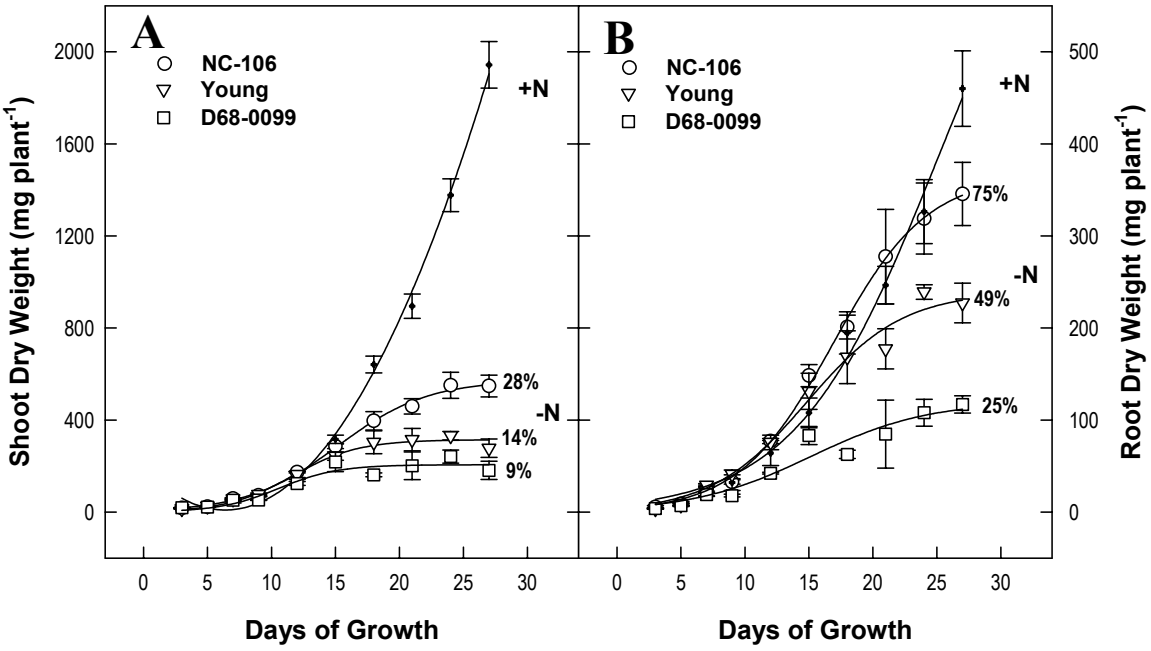

Figure 3. Comparison of shoot (A) and root (B) dry weight accumulation among lines. The primary difference in plant dry weight between N treatments was due to shoot rather than root growth. +N treatments of all three lines were averaged, and plotted as a single line (black symbols). Inserts indicate percent of averaged +N controls at 27 days.

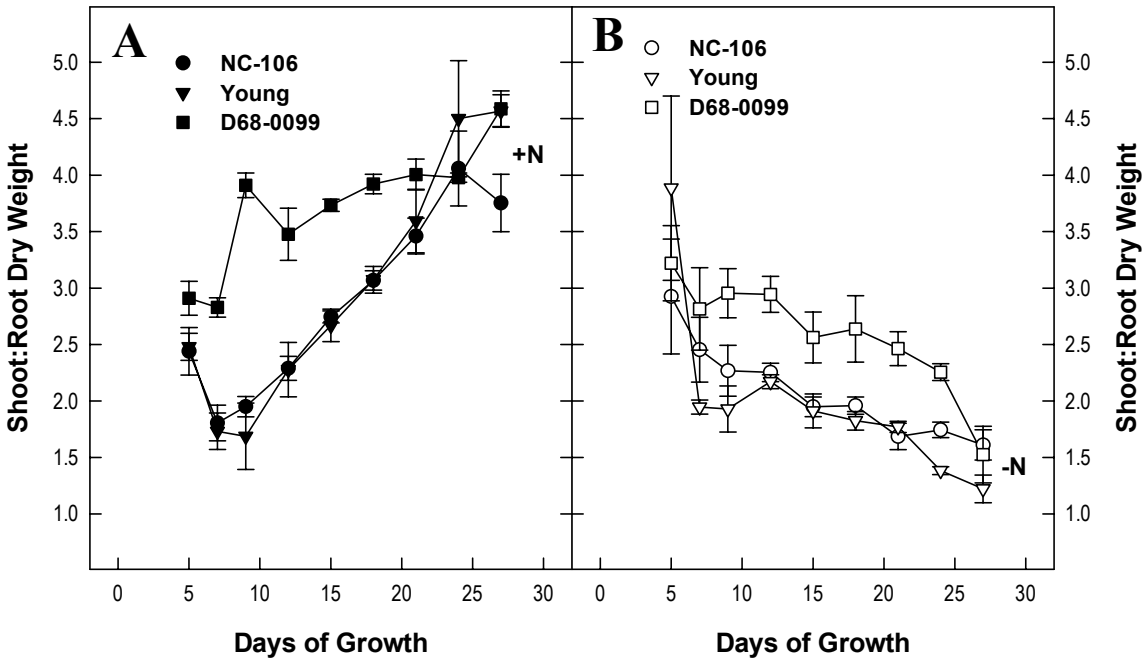

Figure 4. Shoot to root dry weight ratios for plants grown in +N (A) increased over time while those in -N nutrient solutions (B) decreased over time. The low seed N line, D68-0099, had the highest shoot:root growth ratio in both N treatments.

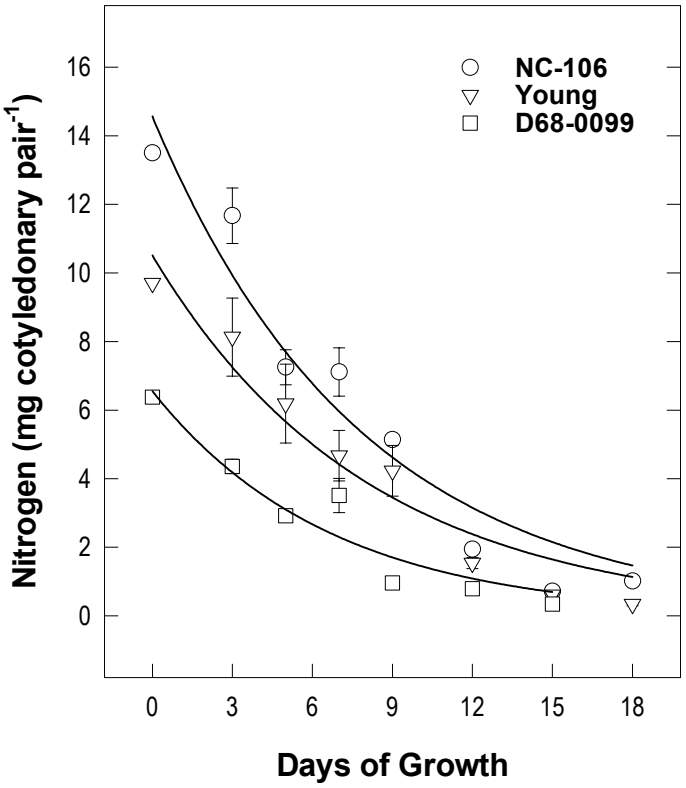

Figure 5. Depletion of storage N out of cotyledons for –N plants.

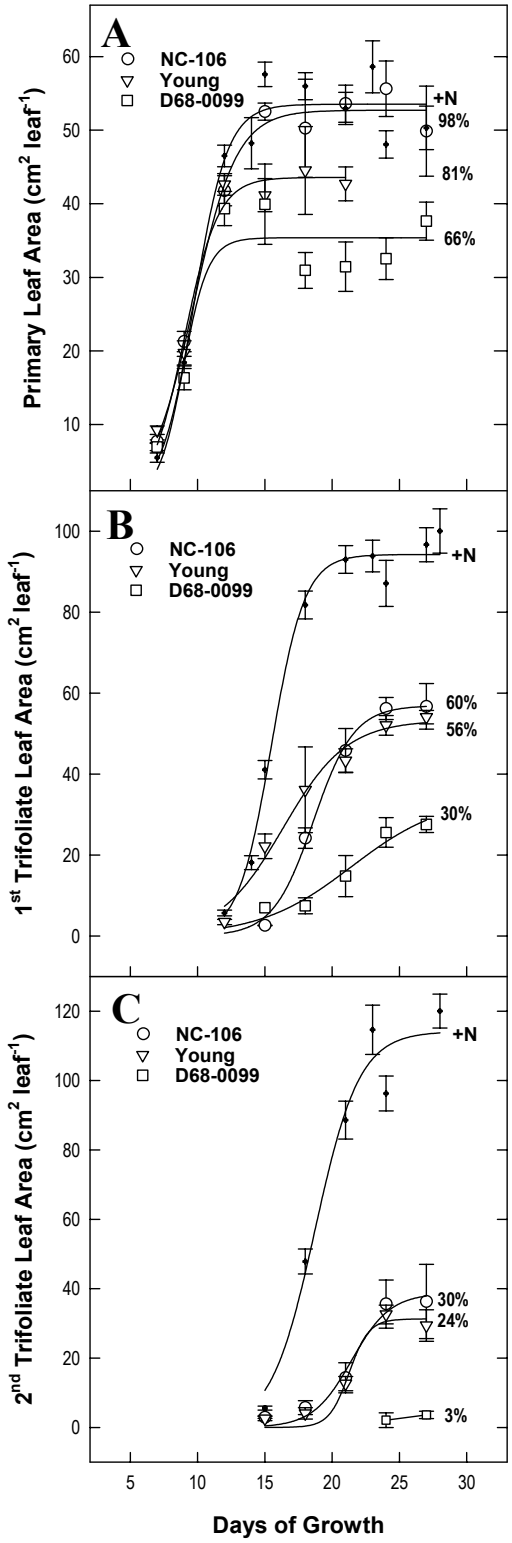

Figure 6. Leaf expansion of the primary (A), 1st trifoliate (B), and 2nd trifoliate (C) leaves over time. Symbols for the primary leaves of Young end due to senescence after day 21. +N treatments of all three lines were averaged, and plotted as a single line. Inserts indicate percent of averaged +N controls at 27 days.

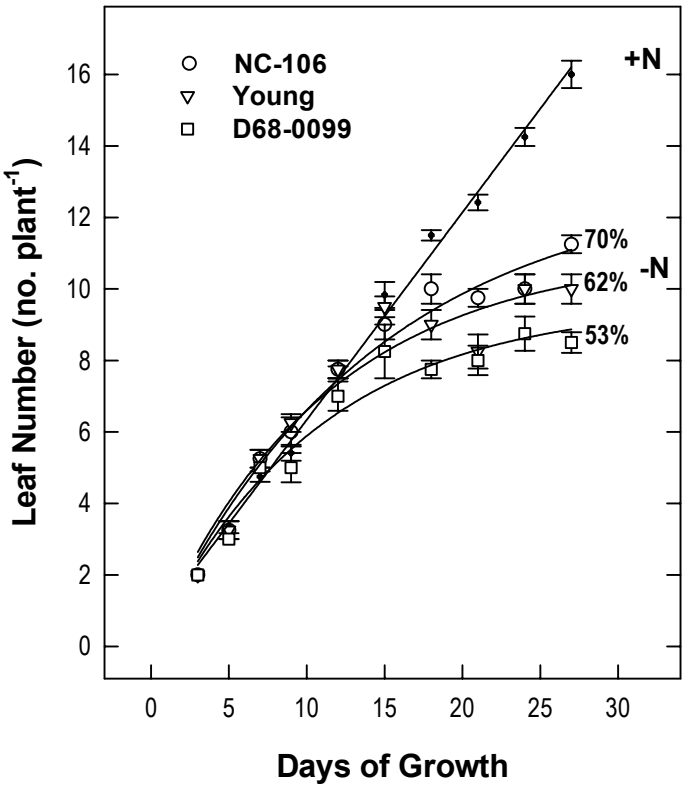

Figure 7. Total leaf number, excluding primary leaves, of plants over time. +N treatments of all three lines were averaged, and plotted as a single line. Inserts indicate percent of averaged +N controls at 27 days.

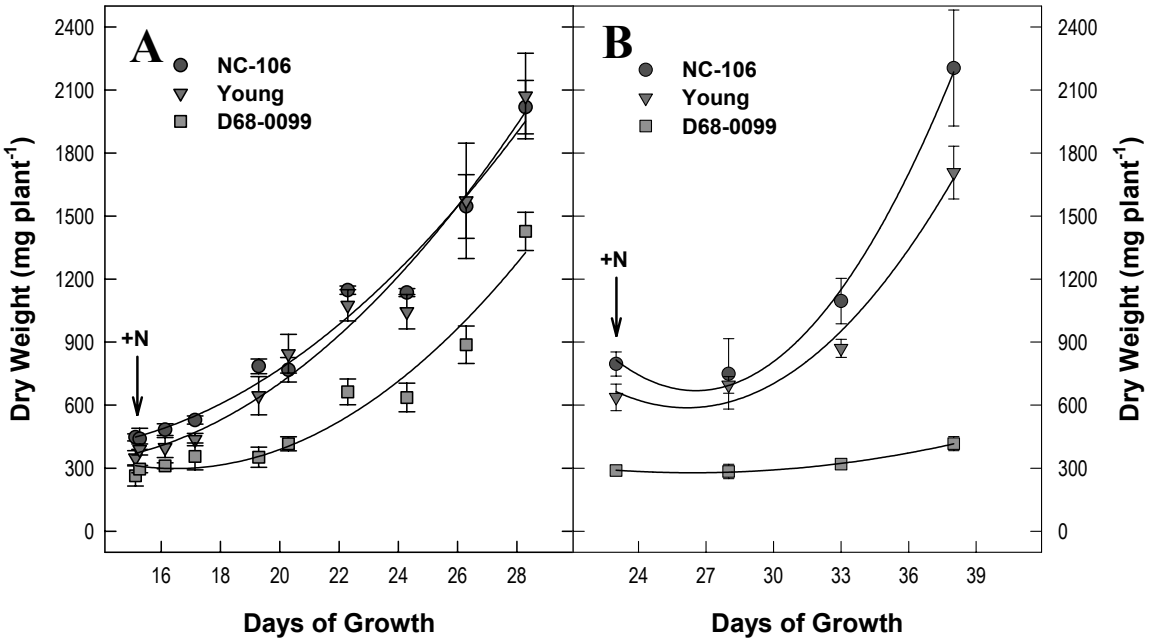

Figure 8. Dry weight accumulation of plants supplied with nitrate after 15 (A) or 23 (B) days of growth in the absence of external N.

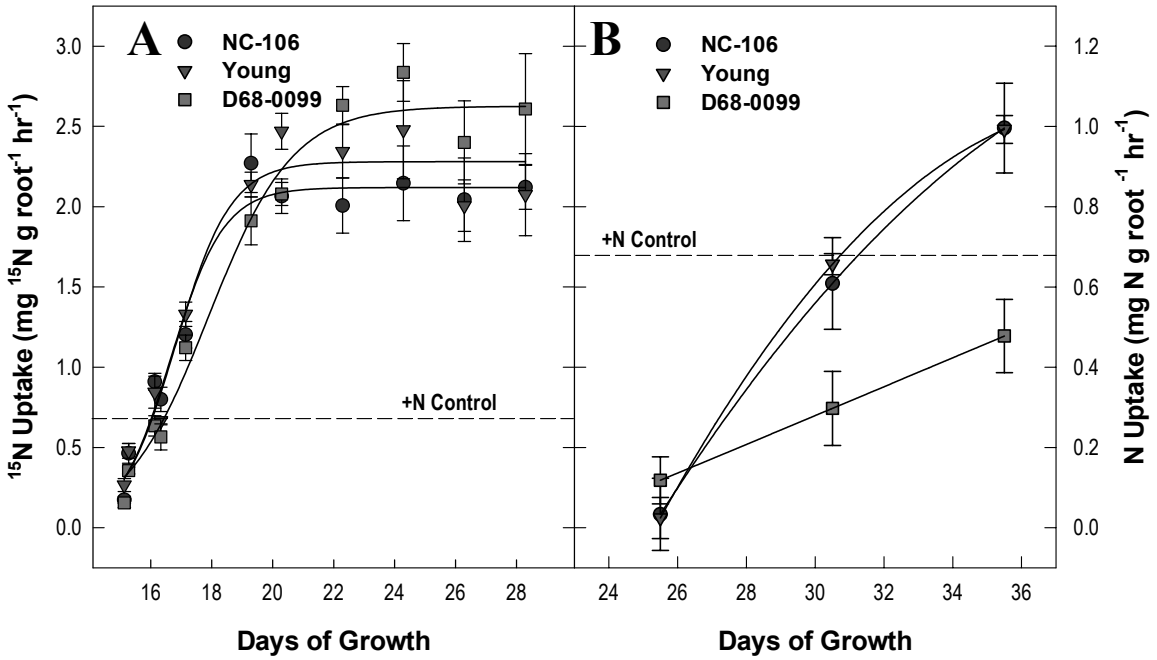

Figure 9. N uptake in N recovery experiments when plants were deprived of N for 15 (A) or 23 (B) days, note differences in vertical scales. Dashed lines represent a calculated N uptake rate of 0.679 $\pm$ 0.0421 for +N plants averaged over time and lines.

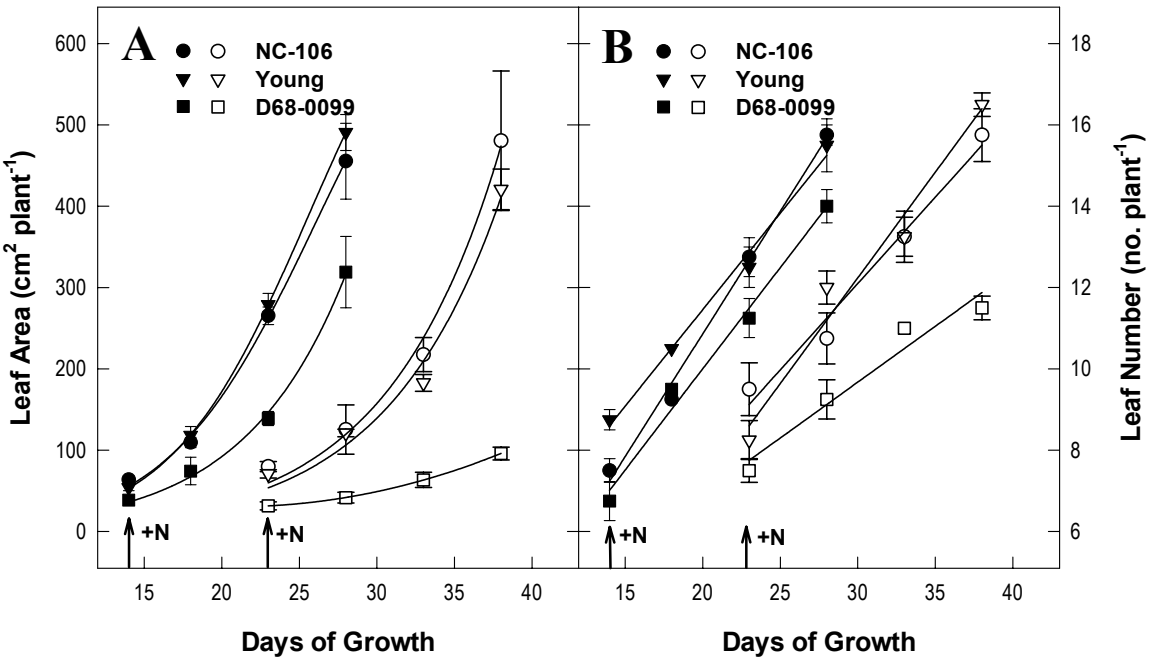

Figure 10. Total leaf area (A) and number (B) of plants grown without N for 14 or 23 days and then exposed to N as indicated by arrows.

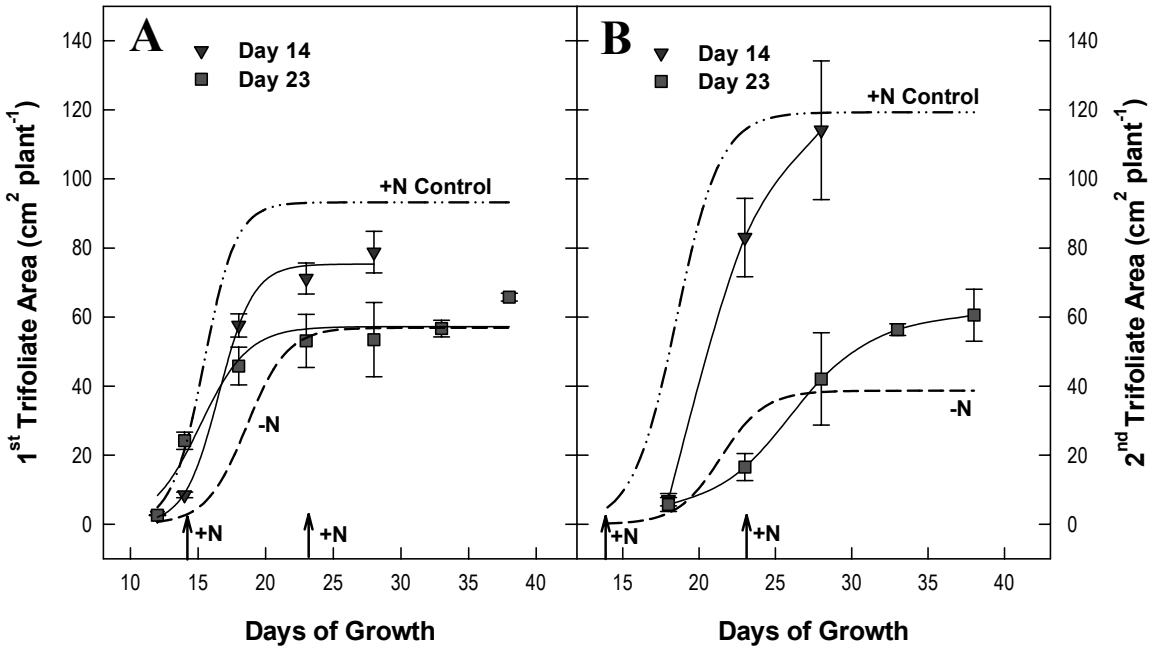

Figure 11. Differences in the extent and timing of the 1st (A) and 2nd (B) trifoliate leaf expansions for line NC-106 when N was added to the nutrient solution on day 14 or 23 as indicated by arrows. Dashed lines in both panels represent respective leaf areas when N was continuously present in or withheld from nutrient solutions as calculated from data in Figure 6.

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CHAPTER 2. SEED NITROGEN MOBILIZATION IN SOYBEAN

INTRODUCTION

Seed protein reserves play an essential role in early seedling development. This is especially true when seeds germinate in poor fertility conditions, which prevail in the highly weathered soils of the southeastern United States. In soybean fields, N fertilizer is typically not added and N_2-fixation provides adequate N for growth and development. The N_2-fixation process becomes active about three to four weeks after germination (Israel, 1981); consequently, seedlings are primarily dependent upon seed reserves for N up to that time.

In a previous study with different soybean lines, it was found that a strong positive correlation existed between seed N content and seedling vigor in low N conditions (Naegle and Rufty, 2002). All soybean lines experienced N stress over a 4 week growth period, but lower seed N content and N mobilization were associated with greater N stress.

Little is known about the relationship between mobilization of seed N reserves and seedling growth with soybean. Much of the N in soybean seeds exists as the storage proteins glycinin (legumin) and β-conglycinin (vicillin), which account for 55% to 75% of the total seed protein (Murphy and Resurreccion, 1984). Glycinin is in greater abundance and is composed of six pairs of basic and acidic subunits that are linked via disulfide bonds (Nielsen, 1985). β-conglycinin consists of three electrostatically associated glycosylated subunits, α, α', and β; six combinations of the subunits have been identified (Thanh and Shibasaki, 1978). The storage proteins are found exclusively

in the seed, have no metabolic activity, and are quickly degraded upon imbibition (Goldberg et al., 1981; Murphy, 1984).

This study was initiated to characterize mobilization of N and proteolysis of storage proteins from cotyledons of soybean lines with varying N contents. The intent was to define their relationship with differential seedling growth in low N fertility conditions.

MATERIALS AND METHODS

Plant Culture

Experiments were conducted with three soybean (*Glycine max* L.) lines, NC-105, NC-112, and D68-0099, which represented a wide variation in seed N content. Seed had been generated in a plant breeding field program at the Central Crops Research Station in Clayton, NC in 1997 and were kept frozen. Seeds were germinated in paper rolls moistened with 0.1 mM $CaSO_4$. The rolls were placed in a dark germination chamber at 28 C and 98% RH for approximately 72 hours. Seedlings with radicals 8 to 12 cm long were placed into 50-L continuous-flow hydroponics systems. The systems contained nutrient solution treatments with or without nitrogen. Treatment solutions were randomly assigned to eight hydroponics units (four per N treatment) and seedlings of each line were randomly distributed among the units. Units were located in a walk-in growth chamber in the Southeastern Plant Environmental Laboratory (Raleigh, NC) programmed for a day/night temperature regime of 26/22 C. Plants were exposed to a nine-hour light period with cool white fluorescent and incandescent light, PPFD of 550 $\pm$ 50 μmol $m^{-2}s^{-1}$. A three-hour night interruption with incandescent light of insignificant PPFD of 30.5 $\pm$

3.4 μmol $m^{-2}s^{-1}$, provided sufficient photomorphogenic irradiance, 11 ± 1 Wm^{-2}, to repress flowering.

The nutrient solution temperature was maintained at 24 ± 0.5 C and pH at 6.0 ± 0.2 with automated monitoring and addition of KOH (0.01 mM) and H_2SO_4 (0.01 mM). The complete nutrient solution contained: 600 μM KNO_3, 200 μM KH_2PO_4, 300 μM $MgSO_4$, 800 μM $CaSO_4$, 19 μM H_3BO_3, 3.7 μM $MnCl_2H_2O$, 317 nM $ZnSO_4$, 132 nM $CuSO_4$, 50 nM 85% H_2MoO_4, and 35.8 μM Fe as FE-Sequestrene. When plants were grown without an external N source, KNO_3 was replaced with 300 μM K_2SO_4. Nutrients were monitored and adjusted so that depletion was less than 30% of the initial solution concentrations. Nutrient solutions were completely changed every ten days.

Seedlings were harvested periodically over a 24-day interval after seed imbibition. See Results section for exact harvest days. At each harvest, eight plants per line and N treatment were sampled, two plants from each of four chambers. Plants were immediately divided into shoots, roots, and cotyledons. Cotyledons were kept on ice, and within 30 minutes frozen at –80 C. Cotyledons were freeze-dried, weighed, and ground to a fine powder with a Wig-L-Bug (Reflex Analytical Corp., Ridgewood, NJ) ball and pestle mill. Shoots and roots were dried in an oven at 55 C, weighed, and ground. Nitrogen content was measured using a flash combustion N analyzer (Model Flash EA 1112, ThermoQuest, Rodano, Milano, Italy).

Soluble Protein

Ground cotyledon tissues from the eight plants at each harvest were combined, and 0.35 g of tissue were placed into a 15 ml test tube. Then 0.2 M Tris-HCl buffer, pH 8.0, containing 0.1 M β-mercaptoethanol was added (1:20 w/v) to the tubes. The mixture

was rigorously stirred for one hour at room temperature and centrifuged at 10,000 g for 10 minutes at 4 C. The supernatant (i.e. the crude protein extract) was placed into a separate tube. An aliquot of the supernatant was combined with an equal volume of solution containing 5% SDS and 0.1 M β-mercaptoethanol and boiled in a water bath for 10 minutes to dissociate soluble proteins. Bromophenol blue and glycerol were added to the dissociated proteins for a final concentration of 0.025% and 10%, respectively. Samples were frozen until used for electrophoresis.

Protein concentrations in crude extracts were determined using the Bradford protein assay (Bradford, 1976). Protein content on a per plant basis or per cotyledonary pair was estimated by multiplying protein concentration by the extraction volume and the ratio of the averaged weight of cotyledons to the extraction weight. Nitrogen content within protein was calculated using a conversion factor of 5.71 (USDA, 2001). Nitrogen content of crude protein extract was measured by flash combustion N analysis on dried extract sample, with the N in the Tris-HCl buffer measured and subtracted out. Soluble protein buffer extraction of the tissue recovered 95.2% $\pm$ 1.65 of the total N.

Dissociated proteins were separated utilizing a Bio-Rad (Richmond, CA) Protean II vertical slab gel apparatus according to Chua (1980), with the proceeding modifications. The gel dimensions were 14x16x0.15 cm with a linear gradient of 10 to 20% polyacrylamide. A mold with 15 wells was used in the stacking gel. Protein was loaded into every other well to avoid cross contamination between lanes and maximize clarity for scanning densitometry. Gels were loaded with 80-100 μg of protein per well, 10-20 μl, based on concentrations obtained from the Bradford assay. Electrophoresis was conducted at room temperature for 14-16 hours at 7.5 mA/gel.

Gels were fixed for thirty minutes in a solution of 40% (v/v) methanol and 10% (v/v) acetic acid, and stained with 0.25% (w/v) Coomassie Brilliant Blue in 40% methanol and 10% acetic acid. Gels were destained with the methanol, acetic acid solution until the background of the gel was nearly clear of blue dye. This consisted of changing the destaining solution every 2 hours at least four times. Fixation, staining, and destaining of gels occurred on an orbit shaker at room temperature. After destaining gels were sandwiched between cellophane sheets, and dried in a Bio-Rad (Richmond, CA) GelAir dryer.

Gels were scanned with a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA) equipped with a HeNe laser light source. Volume integration was performed using ImageQuant software. Background absorbance was subtracted from total absorbance of protein bands. Relative amounts of identified bands were expressed as percent of total protein in a lane. Bands identified as soybean storage protein subunits were α, α', and β for β-conglycinin, and A3, acidic, and basic for glycinin. Concentrations of storage protein subunits were multiplied by the calculated total protein to estimate protein subunit content (mg protein plant^{-1}).

RESULTS

The N content of seeds from the three lines utilized in this study ranged from 4.1 to 14.6 mg seed^{-1}, over a three-fold difference (Table 1). When seedlings were grown in the presence of external N over a 27-day period, plant dry weight accumulation among the three lines was similar. When plants were grown in the absence of N, however, dry

weight accumulation was different among lines, and growth differences were positively related to seed N content (Table 1).

Seed N Mobilization

Total N in the seed and cotyledonary tissues was measured to characterize N mobilization. The + and –N treatments were combined to show differences among the three soybean lines (Fig. 1). The N content decreased exponentially over 21 days, at which time most cotyledons had senesced. Greater amounts of N were mobilized out of cotyledons with higher initial N contents, and most was depleted within 12 days.

The tissue N data were separated into + and –N treatments to determine whether the presence of the external N had an impact on mobilization from the seed. The N mobilization response over time was linearized by transforming the N content of cotyledons with a natural log. Examination of log transformations of the data indicated that mobilization rates were significantly higher ($p < 0.0001$) in –N than +N treatments in all three lines (Fig. 2, Table 2). All r^2 values (>.95) were highly significant. Close inspection of the data points revealed temporal differences in the treatment effects among the lines. Differences between –N and + N treatments became apparent with NC-105 after day 12, but after day 9 with NC-112, and after day 6 with D68-0099 (Fig. 2). Also, cotyledons of –N treatments tended to senesce three days earlier (day 18) than +N treatments.

Protein Degradation

The seed and cotyledonary tissues were analyzed for total soluble protein. Protein degradation rates were different among the soybean lines, with higher seed N associated with faster protein breakdown. No statistical separation was possible between the + N and

–N treatments. The great majority of the protein was degraded by day 8 (Fig. 3). Protein breakdown occurred noticeably faster than N mobilization from the cotyledons, as depicted in the Fig. 4 summary graph.

Degradation of the subunits of the primary storage proteins, glycinin and β-conglycinin, was followed over time using polyacrylamide gel electrophoresis. Higher N content was associated with higher levels of the storage proteins (Fig. 5), which accounted for ~ 47 to 64% of the total soluble protein in the seed. The breakdown rates were greater with higher protein. The majority of the glycinin was degraded within 6 days and β-conglycinin within 4 days in the three lines.

Each soybean line contained approximately equal quantities of acidic and basic subunits of glycinin at seed (Fig. 6). Acidic subunits depleted faster than the basic subunits (Fig 6). Breakdown of the acidic subunits occurred within about 4 days and breakdown of the basic subunits lagged behind by about 2 days, although the low amounts in D68-0099 made degradation time frames somewhat difficult to distinguish. In seed of NC-105 and NC-112, α subunits of β-conglycinin were present in much greater amounts than the β subunits (Fig. 7). The low N line, D68-0099, contained very little of either subunits. The α subunits degraded very rapidly and little was present after 2 days. The slower breakdown of the β subunit extended out for about 5 days. There did not appear to be any differences in proteolysis of any of the subunits between the +N and –N treatments.

DISCUSSION

This study examined mobilization and proteolysis of N reserves in cotyledons with varying N contents. The focus derived from previous experiments, which indicated that differences in seed N content were associated with seedling vigor when seedlings were grown in the absence of external N and solely dependent on endogenous seed N reserves (Naegle and Rufty, 2002). Higher seed N led to seedlings with greater growth rates, leaf canopy development, and ability to recover from N stress. When the soybean lines were grown in the presence of external N, growth and development were similar among the lines. Thus, differences in vigor did not stem from inherent differences in growth potential among the lines. Though only dry weight at the end of 27 days was reported here (Table 1), the same growth characteristics among lines and between treatments were observed.

Total N mobilization was very different among the soybean lines. Cotyledons of the high N line, NC-105, released more than three times as much N as those of the low N line, D68-0099, over approximately the same period of time (Fig. 1). Several factors could contribute to the different rates, as the process of N mobilization from cotyledonary reserves into the developing seedling involves multiple steps. Proteases must first be activated and/or generated to cleave storage proteins into small peptides and then amino acids, which are loaded into the phloem for transport.

Storage Protein Degradation

The protein separations revealed large differences in breakdown rates of the two storage proteins among the soybean lines. Glycinin was in higher amount, and the great majority (>80%) was degraded within six days of imbibition in all three lines (Fig. 5A).

β-conglycinin degraded within four to five days in all three lines (Fig. 5B). The protein data showed, collectively, that temporal degradation patterns were independent of protein quantity. Because the amounts of storage protein in NC-105 > NC-112 > D68-0099, and the periods of degradation were not different, higher protein was associated with greater breakdown rates. The results imply that the substrate specific proteases responsible for initial storage protein degradation (Muntz, 1996) either had higher enzymatic activity or were in greater abundance with increasing protein content.

Past research has shown that protein subunits of the storage proteins are differentially degraded, with acidic and α subunits being degraded faster than the basic and β subunits (Wilson et al., 1986). Our study shows that, indeed, the majority of the acidic subunits of glycinin were degraded before proteolysis of the basic subunits began (Fig. 6). In glycinin, the disulfide bond remains between the acidic and basic subunits during proteolysis (Wilson et al., 1986). The basic subunits compose the hexamer core (Muntz, 1996). Thus basic subunit degradation is inhibited until the acidic subunits have been degraded. A similar degradation pattern occurred in β-conglycinin between the α/α' and β subunits (Fig. 7). The enzyme responsible for initial proteolysis of β-conglycinin, PC1, cleaves α/α', but not β subunits (Tan-Wilson et al., 1996). The β subunit is smaller than the α subunits, lacking the amino acid sequence recognized by PC1 (Qi et al., 1994).

Lines of decreasing seed N content all had proportional decreases of individual subunits, and the β subunit in D68-0099 was only faintly detectable. D68-0099 is a non-nodulating line (Hartwig, 1994). The β subunit is often absent or in very small quantities in non-nodulating soybean lines due to low N nutrition (Ohtake et al., 1997). The

β subunits form one to two weeks after the majority of the subunits have formed in developing seeds (Meinke et al., 1981). It follows that the β subunit would be most affected by poor N nutrition, as the plants would have exhausted N reserves in constructing other storage subunits.

Role of Source/Sink Relationships

When N was absent in the nutrient solution, the mobilization rate of N increased compared to when N was present in the solution (Fig. 2, Table 2). This was true for all three soybean lines. Changes in N mobilization due to N treatments suggest the involvement of source-sink regulatory factors. Cell division and enlargement serve as N sinks in the developing seedling. Seedlings grown in –N were dependent solely on N from the cotyledonary reserves, placing a greater demand on reserves than when an external N supply was present. The mechanistic basis for altered mobilization rates due to changes in source/sink relations is unclear. Nonetheless there are precedents for source/sink relations influencing mobilization of N out of plant tissues. In experiments with wheat, for example, mobilization of reduced N from flag leaves, the primary source of N for grain fill, decreased when ears were removed from plants (Feller, 1979).

It should be pointed out that in a study with two *Lupinus* species, N mobilized slower out of cotyledons of when plants were deprived of N (Hocking, 1980). The conflicting results may be due to methodological differences. Hocking used distilled water depriving plants of all nutrition, whereas the present study provided all nutrients except N. The *Lupinus* plants may have been more severely stressed, and factors other than N stress may have limited growth, causing the sink strength for N to lessen.

Although certain storage protein proteases have been identified (Qi et al., 1992; Wilson et al., 1988), the mechanisms and regulating step(s) of the complex process of N mobilization remain largely unknown. There is substantial evidence, however, that the embryonic axis plays a key role in regulating mobilization of protein reserves in several dicotyledonous species (reviewed by Davies and Slack, 1981; Ilan and Gepstein, 1980). This is further supported in soybean by research showing that proteolytic activity in cotyledons is first initiated in cells closest to the vascular tissue (Diaz et al., 1993). There is contradicting evidence as to whether the axis provides a signal, primarily cytokinins, which alters enzyme activity or if the developing seedling serves as a sink promoting N transport from storage tissues (Nandi et al., 1995; Davies and Chapman 1980; Gepstein and Ilan, 1980; Davies and Chapman, 1979; Yomo and Srinivasan 1973; Wiley and Ashton, 1967). Because N treatments did not begin until day three of the experiments, it cannot be concluded whether N treatments and thereby source/sink relationships altered proteolysis. It is apparent however, that N or source/sink relationships did alter later transport of N from cotyledons. Protein degradation occurred faster than N mobilization (Fig. 4). Coomassie Brilliant Blue requires a minimum peptide molecular weight in order to bind with protein (Compton and Jones, 1985), leaving free amino acids and small peptides derived from proteolysis undetected. The small proportion of protein compared to total N after day eight lends evidence that the N mobilization differences between N treatments were due to transport of amino acids derived from proteolysis.

Table 1. Differences in seed parameters of three soybean lines. Seed N content is the product of weight and N concentration. Numbers in parentheses are standard error of the mean of 8 seeds.

Lines	Avg Weight (mg seed^{-1})	N Concentration (%N)	Avg N Content (mg seed $^{-1}$)	Plant Dry Weights at 27 Days (mg plant^{-1})	
				+N	-N
NC-105	195.1 (5.09)	7.50 (0.0253)	14.63 (0.389)	1920.7 (148.8)	450.5 (61.6)
NC-112	125.8 (2.28)	7.17 (0.224)	9.01 (0.200)	1897.0 (83.4)	233.8 (12.5)
D68-0099	90.1 (3.98)	4.73 (0.456)	4.46 (0.491)	1879.0 (105.4)	135.6 (17.1)

Table 2. Slopes of linear transformations of cotyledonary N mobilization data. All $r^2 > 0.95$, p values results from F-tests conducted on +N vs. –N within a line. Numbers in parentheses represent standard error of the mean.

Lines	+N	-N	+ N vs –N
NC-105	-0.1315 (0.0040)	-0.1867 (0.018)	p < 0.0001
NC-112	-0.1324 (0.0090)	-0.1937 (0.017)	p < 0.0001
D68-0099	-.01239 (0.0072)	-0.1702 (0.0012)	p < 0.0001

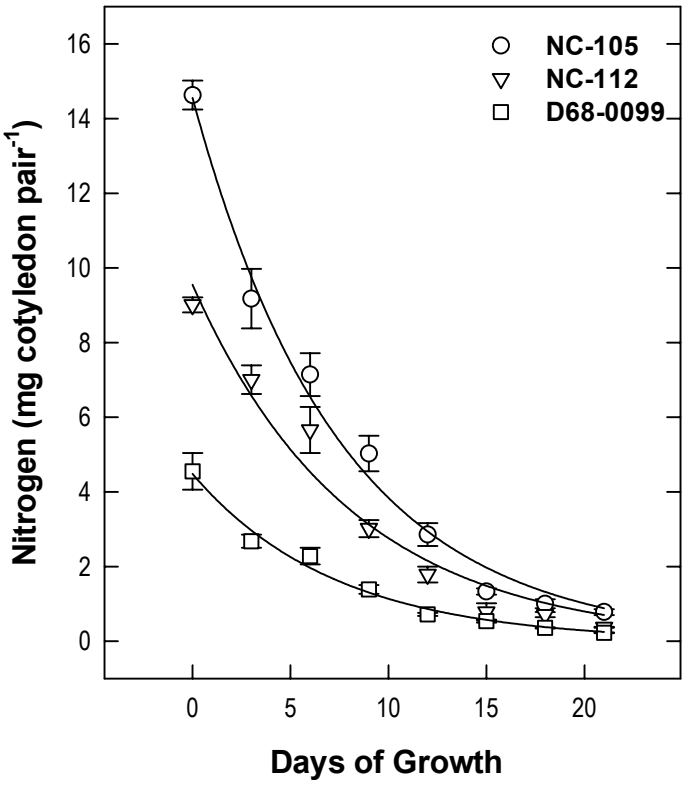

Figure 1. N mobilization out of cotyledons for combined N treatments.

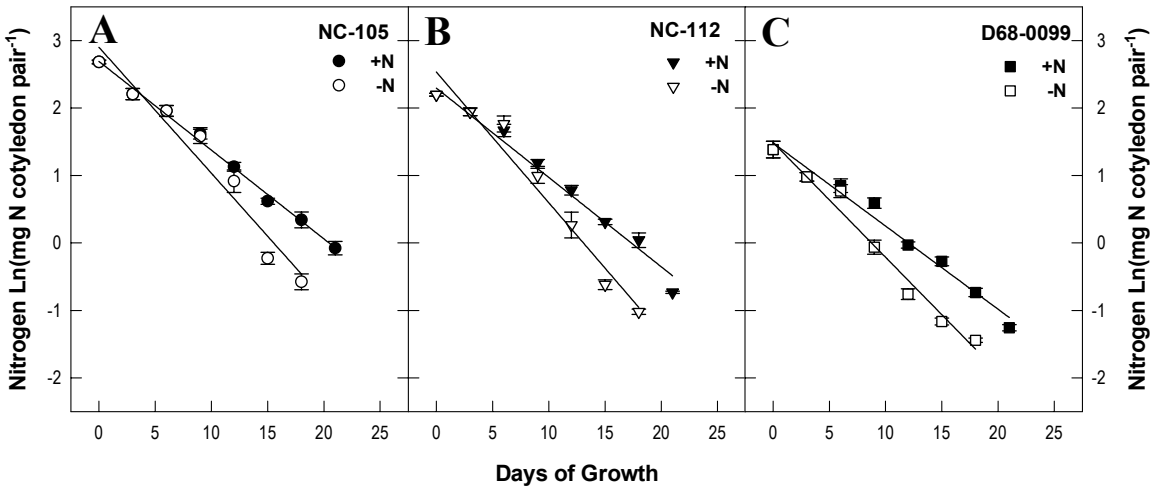

Figure 2. Natural log of cotyledonary N mobilization data. Mobilization was higher within a line in the –N treatment, and slopes between N treatments within a line were all significantly different, $p<0.0001$.

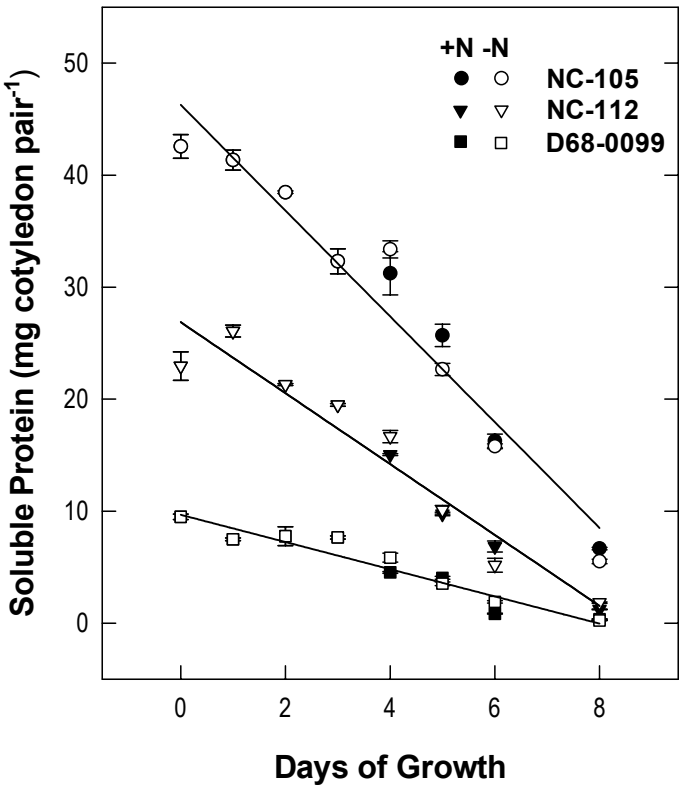

Figure 3. Total soluble protein degradation during the first eight days of growth was significantly different among lines ($p < 0.0001$), but there was no significant difference between N treatments within any of the lines. Mobilization rates (mg N plant^{-1} day^{-1}) of combined N treatments were 1.3 $\pm$ 0.21, 3.2 $\pm$ 0.33, and 4.7 $\pm$ 0.042 for D68-0099, NC-112, and NC-105 respectively. Only –N symbols are visible where symbols overlap.

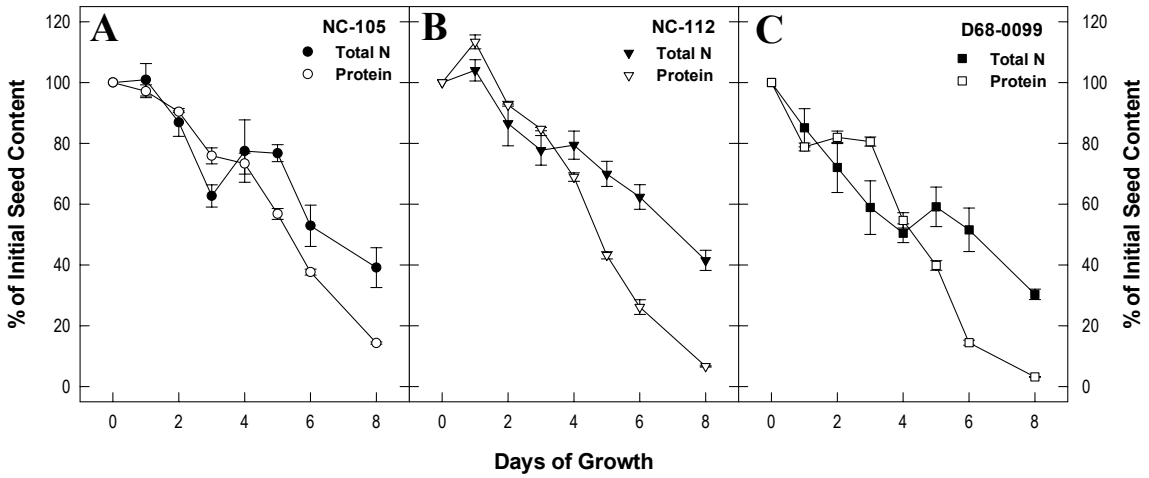

Figure 4. Decline of total N and soluble protein as percent of initial seed content.

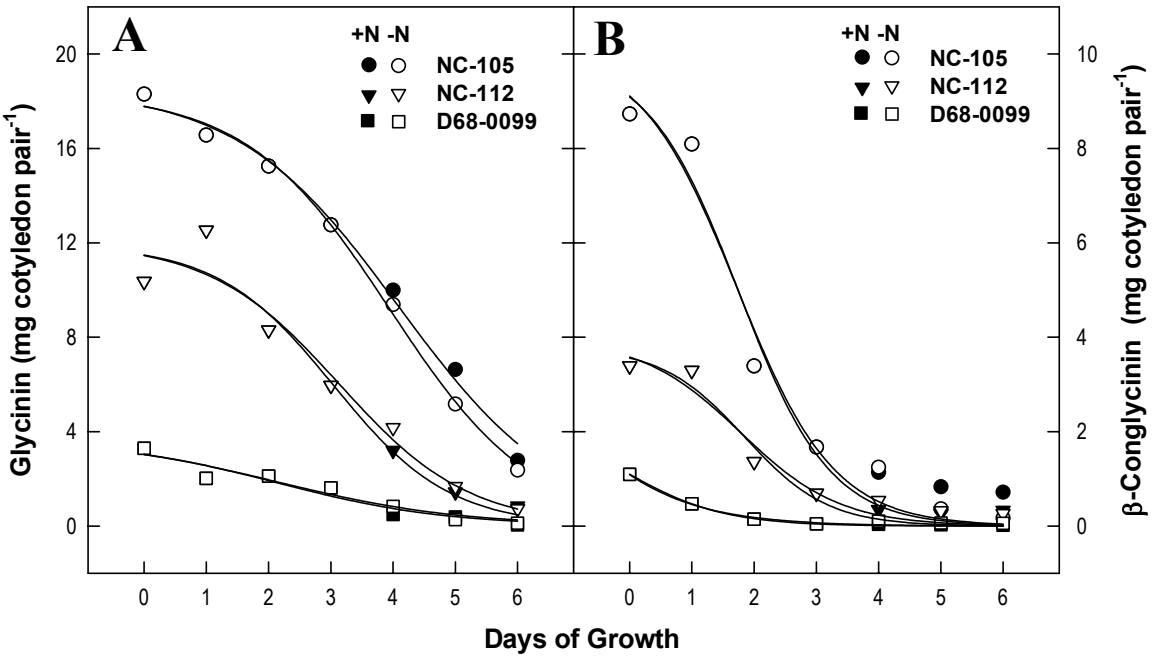

Figure 5. Depletion of storage proteins glycinin (A) and β-conglycinin (B) over the first six days of growth. Note differences in vertical scale between panels. Only –N symbols are visible where symbols overlap.

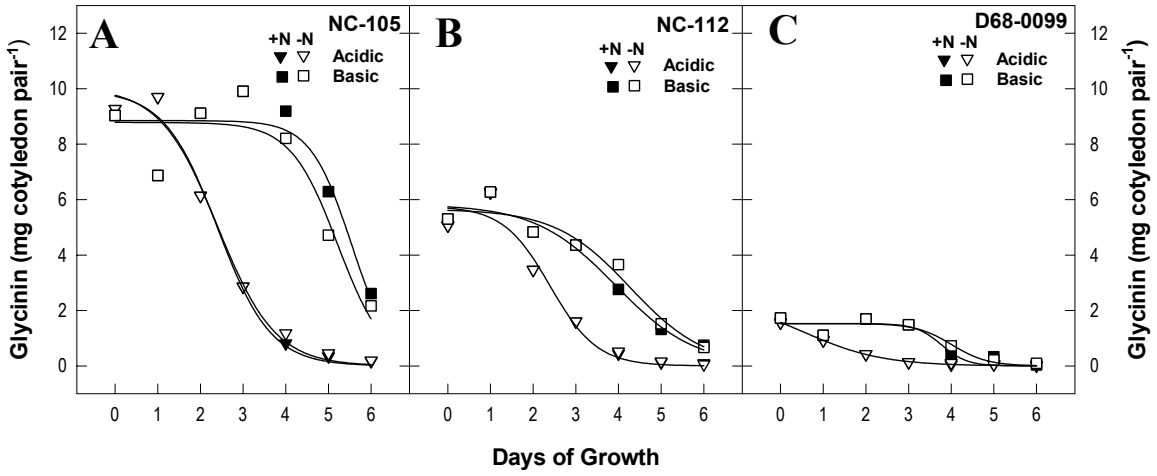

Figure 6. Degradation of the subunits of the glycinin storage protein over time. Individual acidic and basic subunits were combined. Only –N symbols are visible where symbols overlap.

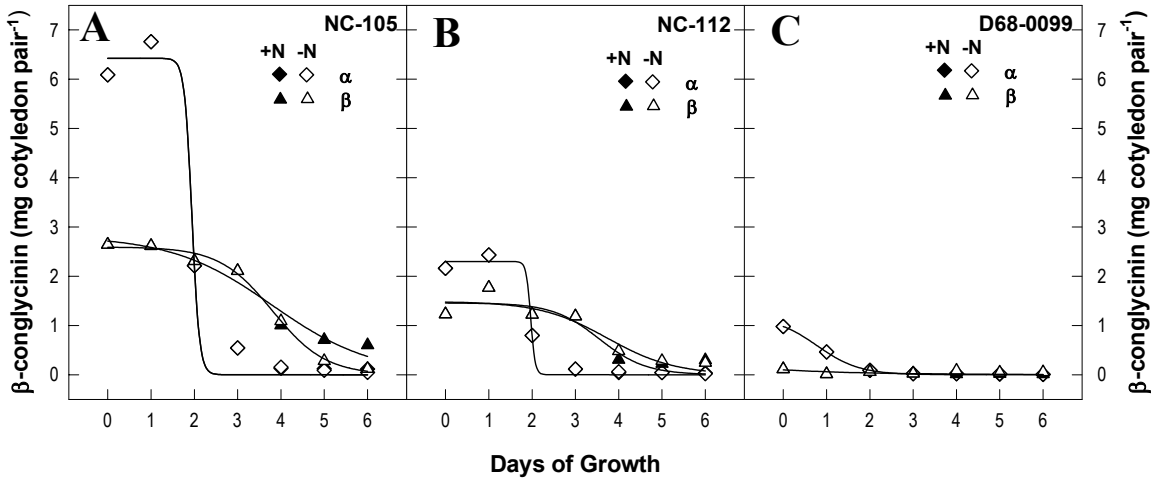

Figure 7. Degradation of the β-conglycinin subunits. The subunits α and α' were plotted as combined α. Only –N symbols are visible where symbols overlap.

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