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Positional Effect on Protein and Oil Content and Composition of Soybeans

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Soybean (*Glycine max* [L.] Merr.) protein and oil qualities, with respect to monogastric nutrition, have been linked to the relative abundance of specific protein subunits and fatty acids, respectively. An analysis of field-grown soybean seeds by near-infrared spectroscopy revealed significant differences in their protein and oil contents as a function of nodal position. Seed proteins from the plant apex were high in protein and low in oil content, while those from the basal region exhibited an opposite pattern of accumulation. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis of total seed proteins revealed that the β-subunit of β-conglycinin content was 4-fold higher in seeds from the apical nodes than in seeds from basal nodes. The glycinin A3 polypeptide content gradually increased in successively lower nodes from the top of the plant. Its accumulation was drastically reduced when nitrogen was applied at specific growth stages. Exogenous nitrogen did not alter the pattern of β-subunit accumulation, but accrual of the acidic and basic polypeptides of glycinin was diminished. The remaining seed storage protein components were not influenced by nodal position or nitrogen application. Gas chromatographic analysis of fatty acids indicated that only oleic (18:0) and linoleic (18:2) acids showed variability in accumulation at different nodes. Neither the abundance nor the distribution of the fatty acids was altered by nitrogen application.

KEYWORDS: Fatty acids; nodal position; protein composition; soybean

INTRODUCTION

Both oil and protein content in soybean (*Glycine max* [L.] Merr.) seed have been shown to be subject to a positional effect (*1*). Seeds that develop in the upper one-fourth of the plant contain a higher concentration of protein and lower concentration of oil than seeds from the lower one-fourth of the plant. When the oil content of the soybeans was determined for each node, it was found that both determinate and indeterminate varieties contained more oil in the seeds that had developed on lower nodes (*1*). The authors noted variability existed among the nodes in oil content, but plants of the same variety exhibited a similar pattern of oil accumulation. In successive experiments, Escalante and Wilcox (*2*, *3*) analyzed the seed from each node of normal and high-protein genotypes and seeds from each node of determinate and indeterminate near-isolines. Seeds from both normal and high-protein breeding lines exhibited an increase in protein from bottom to top nodes (*2*). The authors noted that analyzing seed from each node, rather than by regions of the plant, showed that variability in protein content existed among the nodes. In the second experiment, they found variability in protein content among the nodes of determinate and indeterminate plants (*3*). The protein content was lowest in the basal node seeds and increased toward the apical nodes in both types of plants (*3*). The biochemistry underlying this variation in seed protein and oil content among the nodes has not been elucidated. Whether the accumulation of each seed protein or only specific subunits or polypeptides of those proteins varies as a function of nodal position has not been investigated.

Genetic and environmental factors determine yield, protein, and oil concentration of soybeans (*4*, *5*). Field, greenhouse, and environmental-chamber experiments have been conducted to determine the effect of nitrogen fertilization on protein and oil concentration of soybeans. In field experiments, application of nitrogen at various growth stages has not proven effective in improving the protein or oil concentration of soybeans (*6*−*8*).

Hydroponics experiments have shown that external nitrogen sources increase soybean protein concentration. Soybean plants dependent upon nitrogen fixation yielded seeds with a protein concentration of 35%, while those supplemented with 6 mM KNO_3 produced seeds containing 41% protein (*9*). A 30 mM exogenous nitrogen supply increased the protein by 28% in a cultivar that exhibited normal seed protein concentration (*10*). These experiments indicate the potential for increasing protein quantity by increasing nitrogen availability to the plant. Major seed storage proteins of soybean are of two classifications, 7S

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and 11S, and are referred to as glycinin and β-conglycinin, respectively. The 11S proteins are considered to be more nutritious because they contain a higher percentage of sulfur-containing amino acids than the 7S proteins. Nitrogen application has been shown to promote the accumulation of the β-subunit of β-conglycinin, thus lowering the 11S to 7S ratio and protein quality. Nitrogen fertilization also reduces the accumulation of the 11S glycinin, further exacerbating the decline in protein quality (*10*). Since the qualities of soybean protein and oil have been linked to the relative abundance of specific protein subunits and fatty acids, respectively, our objective was to examine the distribution of these components of seed storage proteins and oils in seeds harvested from each node. Nitrogen fertilizer was applied at different plant growth stages to determine its effect on the accumulation of the components of seed protein and oil.

MATERIALS AND METHODS

Plots of soybeans (Round Up Ready Pioneer brand 94B01) were grown at the Bradford Research and Extension Center near Columbia, Missouri, in 76-cm rows in a randomized complete block design. Nitrogen was applied at rate of 45 kg/hectare at planting, vegetative stage 3, and reproductive stages 1, 3, and 5 (*11*). Ten uniform plants were selected from each plot, and the seeds were harvested and separated according to the node on which they developed.

Near-Infrared Reflectance (NIR) Spectroscopy Analysis of Seed Protein and Oil. A representative sample of each treatment was assayed for protein and oil content using NIR spectroscopy (Infratech 1255 Food and Feed analyzer, Tecator AB, Hoganas, Sweden). Moisture content of the sample was adjusted to 13.5% by the spectrometer. A six-seed aliquot of each sample was ground to a fine powder with mortar and pestle for subsequent protein and fatty acid analysis.

SDS−PAGE Fractionation of Seed Protein. Total seed proteins were extracted from a 15 mg aliquot of the ground soybeans in 1.0 mL of a solution containing 125 mM Tris-HCL buffer, pH 6.8, 4% sodium dodecyl sulfate (w/v), 20% glycerol (v/v), and 0.03 mM bromophenol blue. After centrifugation, supernatants were transferred to clean microfuge tubes and 50 μL of 2-mercaptoethanol was added. Prior to electrophoretic analysis, the samples were heated in a boiling water bath for 5 min and then cooled on ice. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (*12*) was carried out on a 12.5% resolving gel (w/v) at 20 mA for 1 h using the Hoefer SE 260 minigel apparatus (Amersham Biosciences, Piscataway, NJ). Protein bands were visualized with Coomassie Blue R-250.

Purification of 11S and 7S Globulins. Glycinin (11S) and β-conglycinin (7S) were isolated from soybean seed powder by the method of Nagano (*13*). The seed powder was extracted with 15 volumes of distilled water for 60 min at room temperature after which the slurry was subjected to centrifugation (14300g × 15 min) and the supernatant collected. Prior to overnight storage at 4 °C, 0.98 g/L $NaHSO_3$ was added to the supernatant and the pH was adjusted to 6.4. After centrifugation (7500g × 20 min) the supernatant was decanted into a clean tube and the precipitated glycinin fraction was lyophilized. Decanted supernatant was treated with 0.25 M NaCl and the pH adjusted to 5.0. Following centrifugation (14300g × 30 min), the supernatant was collected and diluted 2-fold with distilled water. The pH of the solution was adjusted to 4.8 and the β-conglycinin was recovered by centrifugation at (7500g × 20 min) and lyophilized.

Densitometry. Quantitative assessment of relative protein content was made by computer-assisted densitometry. The SDS−PAGE gels were scanned using the Gene Wizard System (Syngene, Beacon House, Nuffield Road, Cambridge, UK) and protein was reported in relative amounts per gel.

FAME Analysis of Fatty Acids. Approximately 100 mg of sample were extracted overnight in 1-mL of hexane/chloroform/methanol (8:5:2 v/v/v) extraction solution. The following day 150 μL of the extract was pipetted into a reaction vial and the fatty acids were methylated with 75 μL of sodium methoxide-methanol/petroleum ether/ethyl ether solution (1:4:2 v/v/v). The fatty acid methyl esters were segregated on a 30 m × 0.53 mm × 0.5 μm AT-Silar capillary column (Alltech, Deerfield, IL) installed in a Agilent 6890 gas chromatograph (Agilent, Palo Alto, CA) equipped with a flame ionization detector. The system was calibrated using standards of palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids (Matreya, State College, PA). Each fatty acid was reported as a normalized percent of the five preceding fatty acids in soybean seed.

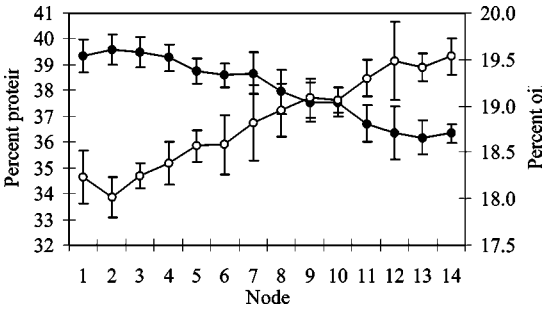

Figure 1. Protein and oil content from seeds harvested from individual nodes. Near-infrared reflectance spectroscopy analysis of seed harvested from each node depicts the general decrease in percent seed protein from the apical to basal nodes while the oil shows an opposite pattern of accumulation. The closed circles represent protein, and the open circles represent oil.

RESULTS

Seeds at the Apical Nodes Accumulate Greater Amounts of the β-Subunit of β-Conglycinin. To verify that the soybean seeds developing at the apex of the plant had a higher protein content than those from the basal region, seeds were harvested from the top three and bottom three nodes and protein content was determined by near NIR spectroscopy. Seeds from the top nodes contained 40 ± 0.8% protein, while those from the bottom nodes were 36 ±1.2% protein. Similar differences in seed protein content between top and bottom nodes were observed in plants from the 1999−2002 growing seasons. To determine if protein composition varied between seeds harvested from top and bottom nodes, the total seed storage protein was isolated and fractionated by SDS−PAGE. Precursory examination of the Coomassie stained gel revealed that the β-subunit (52 kDa) of β-conglycinin accumulated in higher amounts in seeds harvested from the apical nodes than it did in seeds from the basal nodes (data not shown).

Seeds Harvested from Individual Nodes Differ in Protein Content and Composition. Since seeds harvested from apical and basal nodes exhibited significant differences in both protein concentration and composition, seeds from intermediate nodes were analyzed to determine if a gradient in these compounds existed between the top and bottom nodes of the plant. Field-grown plants were selected on basis of uniformity and total number of nodes. Each plant had 14 main stem nodes and a similar branching pattern. The uppermost fruit-bearing node was designated as number one. Seeds from numerically equivalent nodes of several plants were pooled and protein and oil content was determined by NIR. Protein content of seeds from the top node was 4% greater than in those harvested from the bottom node (**Figure 1**). Although there was variability among intervening nodes, protein content generally decreased in seeds harvested from the top to the bottom of the plant. Total proteins were isolated and fractionated by SDS−PAGE from an aliquot representing seeds at each node (**Figure 2**). Even though the seed protein profiles between top and bottom nodes were similar, two differences were noted. The β-subunit of β-conglycinin accumulated to a greater extent in the seeds of the topmost nodes

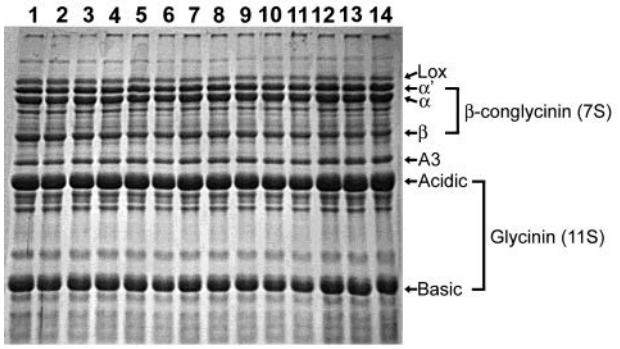

Figure 2. SDS–PAGE gel of total seed proteins from each node. Soybean seed proteins from the top (lane 1) and bottom (lane 14) nodes were fractionated on a 12.5% polyacrylamide gel and visualized by staining with Coomassie Blue R-250. Note that the β-subunit of β-conglycinin accumulated to a greater extent in the top node as compared to accrual in the bottom node as seen in lanes 1–14, respectively. The A3 polypeptide (46 kDa) of glycinin increased gradually in the lower nodes as seen in lanes 1–14. Lox = lipoxygenase. Lanes 1–14 represent total seed proteins from apex to basal nodes, respectively.

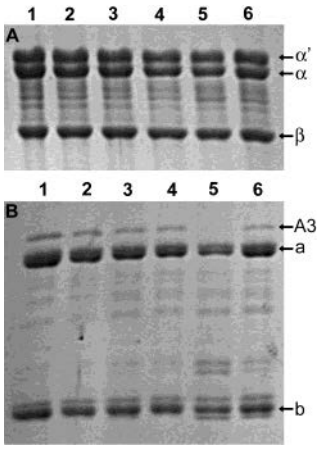

Figure 3. Effect of exogenous nitrogen on accumulation of glycinin polypeptides and β-conglycinin subunits. Purified β-conglycinin (panel A) and glycinin (panel B) were fractionated on 12.5% polyacrylamide gel. Proteins were visualized by staining with Coomassie Blue. Lanes 1–6 of each gel depict proteins from control, planting, V3, R1, R3, and R5 time of nitrogen application, respectively. The Greek letters α', α, and β refer to the three β-conglycinin polypeptides (panel A). The letters A3, a, and b (panel B) refer to the 46-kDa A3 glycinin polypeptide and the acidic and basic subunits of glycinin, respectively.

and declined in aliquots taken from lower nodes. Conversely, a gradual increase in the accumulation of a 46 kDa A3 glycinin polypeptide occurred in seeds analyzed from the same aliquots (**Figure 2**).

Nitrogen Application Does Not Promote the Accumulation of the β-Subunit of β-Conglycinin at the Bottom Nodes. When purified glycinin and β-conglycinin proteins were fractionated by SDS−PAGE, a pattern of varying concentration among the subunits was apparent (**Figure 3**A). Nitrogen application at planting, V3, R1, and R3 lowered the accumulation of the acidic (40 kDa) and basic (20 kDa) polypeptides of glycinin, while application at R5 affected these polypeptides only marginally. Exogenous nitrogen applied at the R3 stage of plant development drastically reduced the accumulation of A3 polypeptide (46 kDa) of glycinin (**Figure 3B**). However, nitrogen application did not generate any detectable changes in the accumulation of the β-conglycinin subunits (**Figure 3A**).

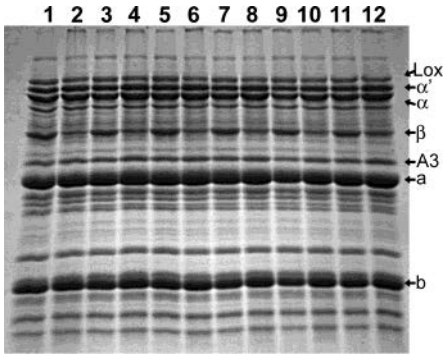

Figure 4. Effect of nitrogen on β-conglycinin accumulation. Total protein from seeds harvested from top and bottom nodes were fractionated by 12.5% SDS–PAGE and visualized by staining with Coomassie Blue. Lanes 1–12 depicts proteins from top and bottom nodes of control, planting, V3, R1, R3, and R5 time of nitrogen application, respectively. Odd number lanes depict protein from top nodes and the even number lanes depict protein from the bottom nodes. The most abundant soybean seed proteins are identified.

Since seeds developing in the basal nodes appeared to contain less of the β-subunit of β-conglycinin, experiments were designed to determine whether soil applied nitrogen would increase the β-subunit accrual in the lower nodes. The accumulation in the lower nodes of β-conglycinin, and in particular the β-subunit, was not affected by the application of external nitrogen (**Figure 4**). Consistent with previous observations, it was noted that the accumulation of the β-subunit of β-conglycinin was significantly higher in the upper nodes (**Figure 4**).

Seeds at Different Nodes Accumulate Different Amounts of Oleic (18:1) and Linoleic (18:2) Acid. In contrast to protein, the oil content of soybean seeds was higher in the bottom nodes compared to that from the upper nodes (**Figure 1).** Palmitic (16:0), stearic (18:0), and linolenic (18:3) acids comprised 11.5, 4.5, and 9.5%, respectively, of the total fatty acid content of the seed, regardless of the nodal position. Linoleic acid (18:2) was most abundant in seed from the lower nodes (**Figure 5B**), while the oleic acid (16:0) content was highest in seeds from the upper nodes (**Figure 5A**). Soil application of nitrogen did not affect the distribution of the fatty acids (data not shown).

DISCUSSION

Work presented in this paper shows that a 4-fold difference in the accumulation the β-subunit of β-conglycinin exists between seeds harvested from the apical and basal nodes. Relative accumulation of the major seed storage proteins glycinin and β-conglycinin ultimately depends on nitrogen and sulfur nutrition of the maternal plant (*9*, *10*, *14*, *15*). If the nitrogen-to-sulfur ratio varied between the apical and basal nodes, differential accrual of the seed storage proteins in these opposite regions of the plant would be expected. During seed development, leaf tissue contributes a significant portion of nitrogenous substrate (*16*, *17*), while a preponderance of sulfur is derived from the growth medium (*18*, *19*). The difference in physical distances from source to sink and relative mobility of each nutrient could generate high nitrogen-to-sulfur ratio in the apical region of the plant. Sulfur deficiency is known to enhance the accumulation of the β-subunit of β-conglycinin (*15*, *19*, *20*), while repressing the accumulation of glycinin (*14*). Conversely, nitrogen availability increases the accumulation of the β-subunit of β-conglycinin (*9*, *10*, *21*−*23*). The possibility of enhanced nitrogen-to-sulfur ratio and resulting effect of this ratio on

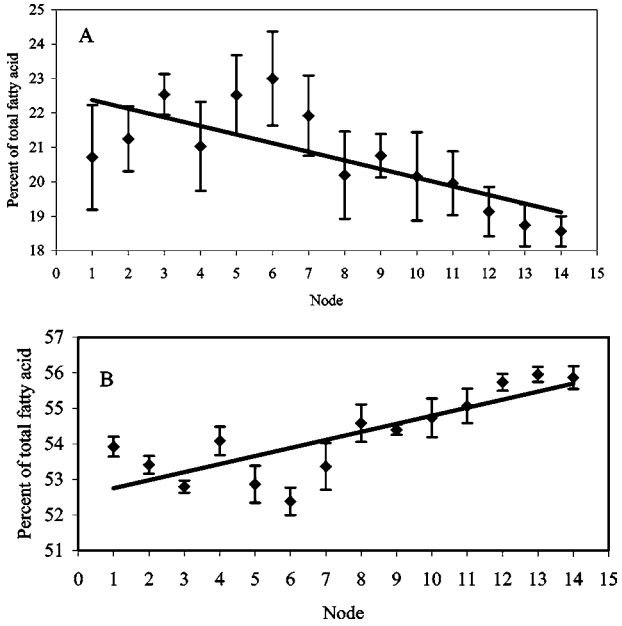

Figure 5. Distribution of oleic and linoleic acid in seed at each node. Esterified fatty acids were separated by gas chromatography and the relative accumulation of oleic acid (panel A) and linoleic acid (panel B) was determined. Number 1 on the *x*-axis represents the apical node and number 14 represents the basal node.

relative expression of protein subunits could generate the increased accumulation of the β-subunit in the apical region of the plant.

Application of nitrogen fertilizer at different growth stages of the plant did not increase the β-subunit accumulation in the lower nodes. If nutrient availability were entirely responsible for the differential accumulation, seeds on the lower nodes, being proximal to the exogenous nitrogen source, should have exhibited an increased amount of the β-subunit. Since additional nitrogen did not increase the accumulation of β-subunit in the lower nodes, the possibility exists that genes coding for this protein are under the influence of a localized environmental factor, such as light quality or other control mechanisms, in addition to nutrient ratios. Experimental evidence suggests that specific metabolites are also are involved in regulating this accumulation of seed storage proteins (*24*−*27*). The concentration of *O*-acetylserine (OAS), an intermediate in cysteine synthesis, plays an important role in the 7S and 11S storage protein accumulation (*27*). Concentration of OAS increases in response to sulfur deficiency, and when applied to cotyledons in culture, stimulates accumulation of the β-subunit, and reduces the amount of glycinin (*27*). Ostensibly, OAS coordinates the signal originating from photosynthate availability and the nitrogen-to-sulfur ratio. Since the possibility exists that the nitrogen-to-sulfur ratio is greater in the upper nodes, OAS accumulation would be enhanced in the upper nodes thus increasing the accumulation of the β-subunit of β-conglycinin.

Although the nitrogen/sulfur status of the maternal plant is the crux of seed storage protein profile, a temporal facet exists in the expression of the genes for these proteins (*28*−*30*). Nitrogen application at flowering has been shown to favor the accumulation of seed oil at the expense of protein (*31*). Seed protein subunits begin to accumulate within general time periods after flowering. The α'- and α-subunits of β-conglycinin appear 20 days after flowering (DAF), followed by the acidic (40 kDa) and basic (20 kDa) subunits of glycinin 25 DAF. Finally, the β-subunit of β-conglycinin begins to accumulate 30 DAF (*29*). Application of nitrogen fertilizer prior to the appearance of the β-subunit did not increase its accumulation, whereas nitrogen application after the subunit appeared resulted in enhanced accrual of this protein subunit (*32*). When nitrogen was applied at the successive growth stages, accumulation of the acidic (40 kDa) polypeptide of glycinin decreased with maximum reduction occurring after application at growth stage R3. Nitrogen application ostensibly would have increased the nitrogen-to-sulfur ratio and thus facilitated accumulation of OAS. *O*-Acetylserine accumulation has been linked to reduced production of the glycinins (*27*). Possibly, there is a window of time when the genes encoding the storage proteins are receptive to environmental signals.

We observed a positional effect involving oleic and linoleic acids. The content of linoleic acid (18:2) was highest in the lower nodes and was found to diminish in seeds from successively higher nodes. Antithetically, oleic acid (18:1) was more concentrated in the seeds from the upper nodes and diminished in linear fashion toward the base of the plant. It is likely that environmental conditions contribute to this differential accumulation of linoleic and oleic acids. Even though the saturated fatty acids do not vary appreciably under different climatic conditions (*4*, *32*, *33*), soybeans grown in cooler climates have higher concentrations of the polyunsaturated linoleic and linolenic acids, while monounsaturated oleic acid prevails in warmer climates (*4*, *33*, *34*). Increased activity of oleolyl and linoleolyl desaturases (*35*) and higher O_2 solubility in the cytoplasm (*4*) have been suggested as possible causes. In addition, light quality has also been shown to have a role in the fatty acid synthesis (*36*). The activity of the cytosolic enzyme omega-6-desaturase, which catalyzes the conversion of oleic acid to linoleic acid, was enhanced in developing seeds under reduced blue light (*37*). The quality of light and temperature variation occurring at different nodes within soybean plants may be one of the contributing factors for the differential accumulation of oleic and linoleic acids observed in our study. On the basis of our study, it appears if seed from upper and lower regions of plant could be segregated at harvest both protein and oil quality would be improved. Seeds harvested from the lower nodes would contain a higher proportion of the sulfur containing amino acids in the protein and oil from seeds in the upper region will have a higher percentage of oleic acid, thus improving its oxidative stability.

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