

**SOYBEAN SEED COMPONENTS AS AFFECTED BY NODAL POSITION,
ENVIRONMENTAL CONDITIONS, AND IRRIGATION**

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by

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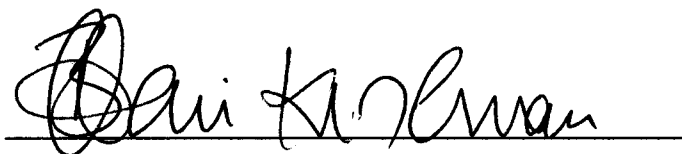
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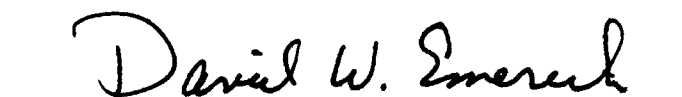
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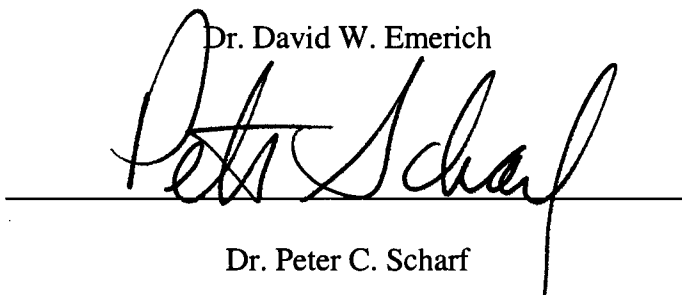
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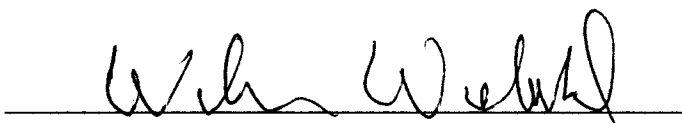
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ABSTRACT

Soybeans (*Glycine max* [L.] Merr.) are a major source of vegetable protein and edible oil. The nutritional quality of these seed components depends upon the relative abundance of specific proteins and fatty acids. Additionally, secondary metabolites such as isoflavones, which are present in soybeans, have been shown to impact human health. Genetics, environmental conditions, and agronomic practices have a bearing on accumulation of each of these seed components. Work presented here reveals that the constituents of the protein and oil components vary with the nodal position of seed development. Sodium dodecyl sulfate polyacrylamide gel electrophoresis provided evidence that proteins rich in the sulfur amino acids accumulate preferentially in seed from the basal nodes while proteins poor in these amino acids are found in the apical nodes. Fatty acid content determined by gas chromatography showed a nodal dependent difference in accumulation of monounsaturated and polyunsaturated fatty acids but no difference in that of the saturated fatty acids. A long-term crop rotation study revealed that environmental factors and putative changes in soil ecology could affect seed protein and oil content. Protein content notably increased with a concomitant decrease in oil over the 11-year span of the study.

Using one- and two- dimensional gel electrophoresis and gas chromatography, we determined that the protein and fatty acid profiles respectively, of soybeans cultivated in an early planting system were comparable to that of a traditionally cultivated crop. Although irrigation did not improve the protein and oil accumulation, it did elicit a dramatic increase in the isoflavone content of the seed. Continued research devoted to the elucidation of soybean genetics, physiology, and biochemistry is crucial for breeding and development of this vital food crop.

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CHAPTER 1

INTRODUCTION AND OVERVIEW OF SOYBEAN PROTEIN, OIL, CARBOHYDRATES, AND SECONDARY METABOLITES

Soybean (*Glycine max* [L.] Merr) has the distinction of providing a preponderance of the world's protein and oil trade and thus has become one of the most valuable of cultivated crops. World production of soybeans in 2003/2004 was 189.8 million metric tons (1). Processed seed from this crop provided 31% of vegetable oil and 69% of the protein meal consumed worldwide (2). Protein and oil from soybeans are major contributors to human nutrition either directly or through use as animal feeds. In addition to the primary seed storage compounds, protein, oil, and carbohydrate, soybeans contain a variety of minerals, vitamins, and secondary metabolites, which contribute to human health. Accumulation of the aforementioned seed components is influenced by genetics and environmental factors (3-6). Soybean seeds are composed of approximately 20% oil, 36% protein, 30% carbohydrate, 9% crude fiber, and 5% ash (7, 8). Current research in the various disciplines of plant science is enhancing our knowledge of the genetic, physiological, and biochemical processes underlying the accumulation of the seed components. The ongoing research will facilitate production of soybean seeds with desired attributes such as increased levels of essential amino acids for monogastric nutrition, favorable distributions of fatty acids for food or industrial use, and enhanced accumulation of vitamins, minerals, and secondary metabolites. Thus, the soybean could be considered as a veritable factory, producing food for animal and human consumption and components that can be utilized in industrial and pharmaceutical applications.

The major storage proteins are the globular 11S glycinins and 7S conglycinins which together account for over 70% of the total seed protein (9). Relative accumulation of glycinins and β -conglycinins affects the nutritional quality of the seed protein and characteristics important in the production of foods (10). Accrual of soybean proteins is influenced by both genetics and the environmental conditions (11-14). Glycinins, encoded by a gene family designated Gly1 to Gly5, are hexamers with an approximate mass of 320 to 375 kDa comprising 60% of the total globulin fraction (15-17). Synthesized as precursors on the rough endoplasmic reticulum, these polypeptides are transported through the Golgi as trimers, and then moved to protein storage vacuoles where they are cleaved into 40 kDa acidic and 20 kDa basic polypeptides (18). The second major seed storage protein, the β -conglycinins, are trimers consisting of α' , α , and β subunits (9, 19). β -conglycinins are glycoproteins and are encoded by a gene family comprised of at least 15 genes located in six regions of the soybean genome (20). Other seed proteins include protease inhibitors, lipoxygenases, and lectins (21-25).

Enhancing nutritional value of seed protein involves increasing total protein, enhancing content of particular subunits, increasing specific amino acids, in particular those that are limiting in monogastric nutrition, and minimizing the proteins that have been shown to have deleterious effects.

The mechanisms involved in the synthesis of soybean oil are complex due to the involvement of a multitude of genes and organelles, which include chloroplasts, mitochondria, and endoplasmic reticulum (26, 27). Twelve forms of glycerolipids are found in the soybean each having at least one fatty acid esterified to the glycerol backbone (28). Polar lipids such as phospholipids are involved in cell membrane

structure and are metabolically active during seed development and subsequent germination (28). The non-polar glycerolipids, triacylglycerols, are storage lipids and the major components of soybean oil derived from mature seeds. Triacylglycerols (TAG) are stored in oil bodies, which are surrounded by a glycerolipid monolayer containing the protein oleosin (29). Soybean oil contains five commercially important fatty acids: palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3). The relative content of fatty acids influences the physical and chemical characteristics of the oil thus the suitability of the oil for a particular use. Soybean lines are currently being developed to express amended fatty acids thus increasing potential uses of the oil (30-35). Oil with low content of polyunsaturated fatty acids has enhanced shelf life and heat stability thus has applications in both food preparation and industry. Chemical hydrogenation of soybean oil reduces the polyunsaturated fatty acid content but the process introduces trans-isomers that have been deemed detrimental to human health (36, 37). Soybean lines with an enhanced content of stearic acid potentially obviate chemical hydrogenation for production of edible lipids that are solid at room temperature (38, 39). Soybean oil containing modified fatty acids along with a high percentage of oleic acid are comparable in quality for use as lubricants to that of the petroleum based oils (31, 40). Other industrial uses of soybean oil include synthesis of plastics, coatings, and adhesives (31, 41, 42). An understanding of the complexity of lipid biosynthesis is paramount for the production of soybean seed containing specific fatty acid profiles, which endues the oil with characteristics suitable for both food and industrial applications.

Structural and non-structural carbohydrates account for approximately one third of the soybean dry matter. At maturity, nonstructural carbohydrates comprise 12% of the

dry seed weight with the remaining carbohydrates involved in formation of cell wall material. Non-structural carbohydrates include sucrose (41-68%), stachyose (12-35%), raffinose (5-16%), and starch (1-3%). Other low molecular weight carbohydrates, such as monosaccharides, D-pinitol, D-onoitol, *myo*-inositol, galactinol and their galactosyl derivatives are present in the mature seed (43). Carbohydrate composition at the beginning of seed fill is primarily monosaccharides with very little sucrose or oligosaccharides (44). As development proceeds the sucrose and starch increase with concomitant decrease in the monosaccharides. Prior to physiological maturity raffinose and stachyose begin to accumulate (45). The accumulation of the raffinose oligosaccharides and the galactosyl cyclitols have been characterized during soybean seed development (46). These carbohydrates aid in desiccation tolerance and provide cold tolerance (47-50). Consumption of these carbohydrates is detrimental to monogastric digestive processes, thus these sugars are considered anti-nutritional components of soybean seed (51). Research directed toward minimizing these compounds to improve the nutritional quality of the soybean while maintaining agronomic viability is ongoing (51).

Cell wall polysaccharides are the second major group of carbohydrates found in soybean seed. Synthesized from monosaccharide precursors, cell wall polysaccharides form a complicated arrangement of associated polymers primary of which are cellulose, hemicellulose and pectin (52). Cell wall composition of soybean seeds has been ascertained by first segregating its polysaccharide polymers on the basis of solubility, followed by a determination of the monomeric subunits comprising the individual polymers (53-55). The composition and properties of the cell wall are ultimately

dependent upon the combination of the monomeric units, which form these polymers. Compared to other aspects of seed composition, a dearth of information exists concerning the cell wall polysaccharides, ostensibly due in part to the complexity of analytical procedures necessary for elucidation of these structures (56). Accumulation of total protein and oil has been found to be inversely proportional to that of cell wall material (57). Since there is genetic variation in cell wall polysaccharides, an opportunity exists to shift carbon utilization from cell wall to the more desirable protein and oil (58).

Research in the past decades has brought to attention a multitude of compounds synthesized by plants termed secondary metabolites. Isoflavones and saponins comprise the majority of secondary metabolites in soybean (59). Isoflavones are derived from the phenylpropanoid pathway while the saponins are synthesized in the terpenoid pathway. Soybean synthesizes three isoflavones, daidzein, genistein, and glycitein, which are stored in the vacuole as glycoside conjugates (60). Accumulation of these compounds is influenced by genetics and environmental factors (61-63). Isoflavones possess antimicrobial activity and are involved in the crucial communication between the soybean plant and the nitrogen fixing rhizobia (64). Exhibiting estrogen-like properties in animal species, isoflavones have garnered considerable interest as having an impact on human health (65-67). Saponins, which are derived from triterpenoids, exhibit a wide range of biological functions (68, 69). The biochemical pathways involved in saponin synthesis have not been as fully characterized as those of the flavones and thus knowledge concerning their assimilation is not as complete (70, 71). Consumption of soy foods containing saponins has been reported to have specific health benefits (72, 73).

Summary of presented work

The difference in protein and oil composition between the apical and basal region of the plant possibly results from a combination of environmental and temporal or spatial signals that affect gene expression in the developing seed. Nutrient availability is a major factor influencing the relative accumulation of glycinins and conglycinins. However, β -conglycinin content was 4 fold higher in the apical nodes when compared to basal nodes even after application of exogenous nitrogen suggesting that nutrient availability alone does not determine relative accumulation of seed proteins.

Environmental conditions and soil ecology appear to influence the composition of the seed. Ostensibly, evolution of soil ecology as influenced by a long term four crop rotation affected protein and oil accumulation in the soybean. However, separating the effects of changing soil ecology and ambient environmental conditions is difficult.

Protein and oil concentrations did not respond to irrigation, however, a dramatic increase in the isoflavone concentration in soybeans was observed. Higher concentration of isoflavone indicates increased flux through the synthetic pathway possibly resulting from the enhanced growing conditions provided by irrigation.

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CHAPTER 2

POSITIONAL EFFECT ON PROTEIN AND OIL CONTENT AND COMPOSITION OF SOYBEANS

ABSTRACT

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 was four-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 the oleic (18:0) and linoleic (18:2) acids showed variability in accumulation at different nodes. Neither the abundance nor distribution of the fatty acids was altered by nitrogen application.

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 seed 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 affect 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 external nitrogen sources to 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 and 11S, and are referred to as β -conglycinin and glycinin respectively. The 11S proteins are considered 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 affect 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 in a multi-year study at the Bradford Research and Extension Center near Columbia, Missouri, in 76-centimeter 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.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (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 dodecylsulfate (w/v), 20% glycerol (v/v) and 0.03 mM bromophenol blue. After centrifugation, supernatants were transferred to clean microfuge tubes and 50 μ L of 2-mercaptoethanol was added. Prior to electrophoretic analysis, the samples were heated in a boiling water bath for 5 minutes 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 hour 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 minutes at room temperature after which the slurry was subjected to centrifugation (14300g x 15 min) and the supernatant collected. Prior to overnight storage at 4° C, 0.98 g/L NaHSO₃ was added to the supernatant and the pH adjusted to 6.4. After centrifugation (7500g x 20 min) the supernatant was decanted into a clean tube and the precipitated glycinin fraction was lyophilized. Decanted supernatant was treated with 0.25M NaCl and the pH adjusted to 5.0. Following centrifugation (14300g x 30 min), the supernatant was collected and diluted two-fold with distilled water. The pH of the solution was adjusted to 4.8 and the β -conglycinin was recovered by centrifugation at (7500g x 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.

Fatty acid methyl ester (FAME) analysis of fatty acids. Approximately 100 mg of sample were extracted overnight in one mL hexane:chloroform:methanol (8:5:2 v/v/v) extraction solution. The following day 150 μ L of the extract were pipetted into a reaction vial and the fatty acids were methylated with 75 μ L 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 \times 0.53 mm \times 0.5 μ m AT-Silar capillary column (Alltech, Deerfield

III.) 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.

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 higher protein content than those from the basal region, seeds were harvested from the top three and bottom three nodes and protein content determined by near NIR spectroscopy. Seeds from the top nodes contained $40 \pm 0.8\%$ protein while those from the bottom nodes were $36 \pm 1.2\%$ protein. Similar differences in seed protein content between top and bottom nodes were observed in plants from the 1999 through 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. A preliminary 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. Although there was variability among intervening nodes, protein content generally decreased in seeds harvested from the top to the bottom of the plant (**Figure 1**). 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 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 3A**). 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**). 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 5**, Panel B) while oleic acid (16:0) content was highest in seeds from the upper nodes (**Figure 5**, Panel A). Soil application of nitrogen did not affect the distribution of the fatty acids (data not shown).

DISCUSSION

Work presented in this paper showed a four-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 upon 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 the 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 affect of this ratio on 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 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 by 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 specific times 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 affect 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 oleoyl and linoleoyl desaturases (35) and higher O₂ 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. Based on 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.

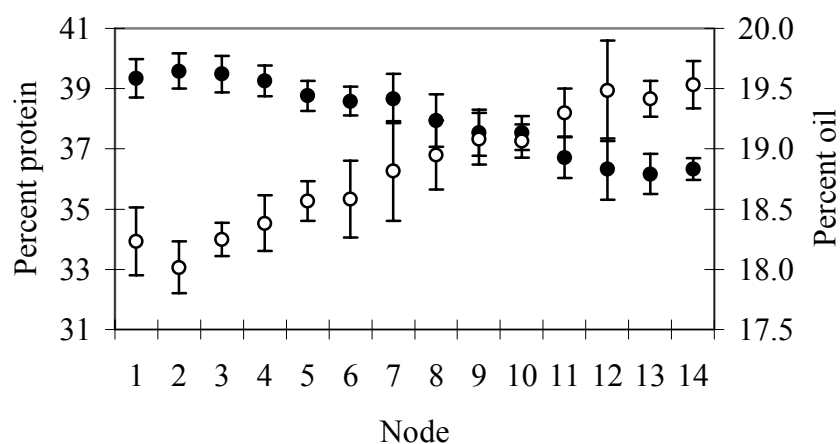


Figure 1. Protein and oil content from seeds harvested from individual nodes of control plants. Near infrared reflectance spectroscopy analysis of seed harvested from each node depicts the general decrease in percent seed protein from the apical to basal nodes while the oil shows an opposite pattern of accumulation. The closed circles represent protein and the open circles represent oil. Standard deviation is indicated by vertical bars in each data set.

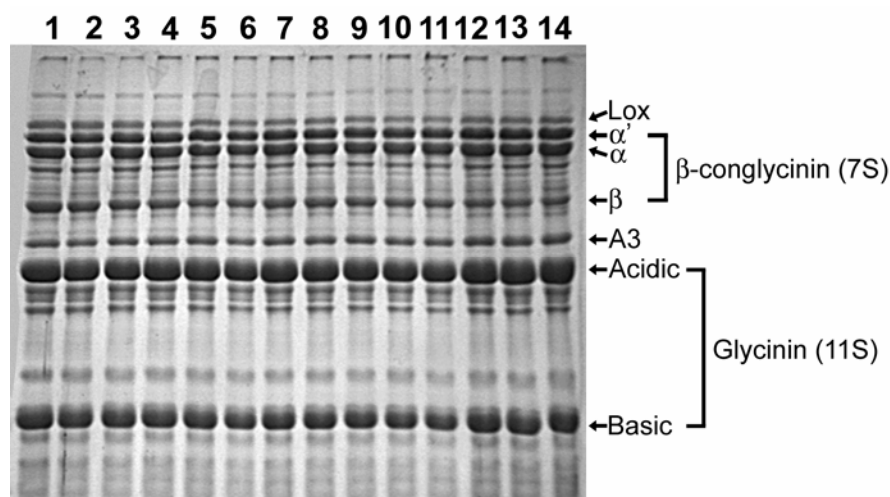


Figure 2. SDS-PAGE gel of total seed proteins from each node of control plants. Soybean seed proteins from the top (lane 1) and bottom (lane 14) nodes were fractionated on 12.5% polyacrylamide gel and visualized by staining with Coomassie Blue R-250. Note that the β -subunit of β -conglycinin accumulated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

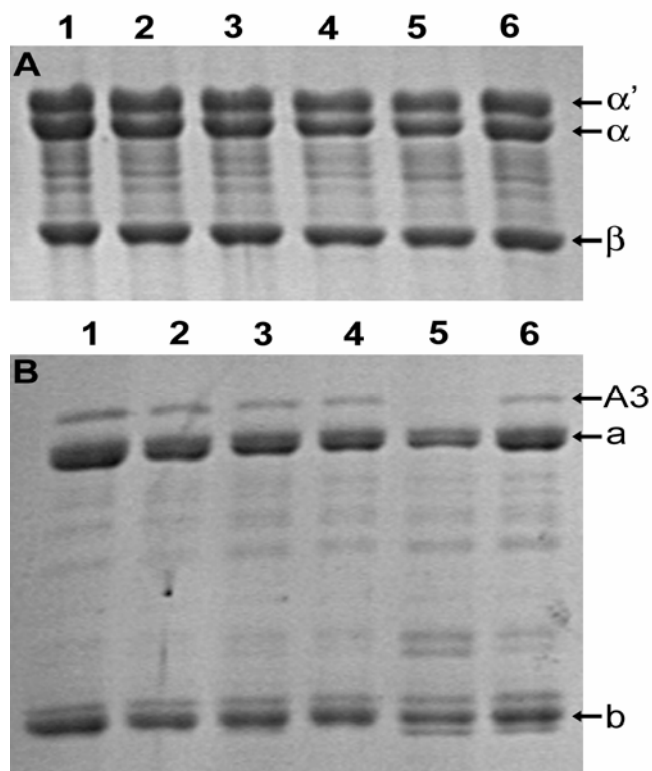


Figure 3. Affect of exogenous nitrogen on accumulation of glycinin polypeptides and β -conglycinin subunits. Purified β -conglycinin (Panel A) and glycinin (Panel B) extracted from seed gathered from the whole plant were fractionated on 12.5% polyacrylamide gel. Proteins were visualized by staining with Coomassie Blue. Lanes 1 through 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.

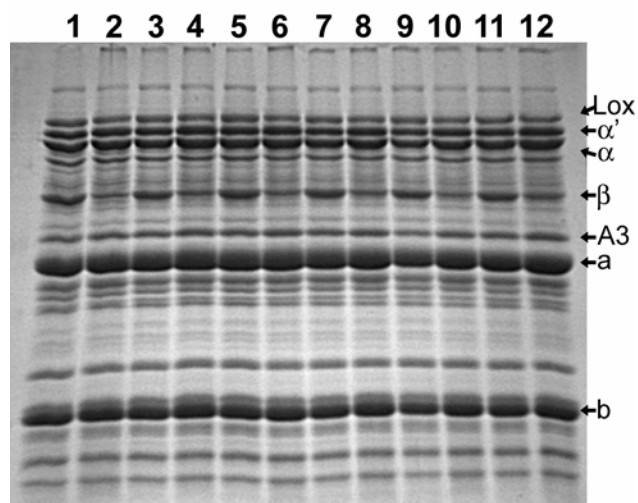


Figure 4. Affect of nitrogen on β -conglycinin accumulation. Total protein from seeds harvested from top and bottom nodes were fractionated by 12.5% SDS-PAGE and visualized by staining with Coomassie Blue. Lanes 1 through 12 depicts proteins from top and bottom nodes of control, planting, V3, R1, R3 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.

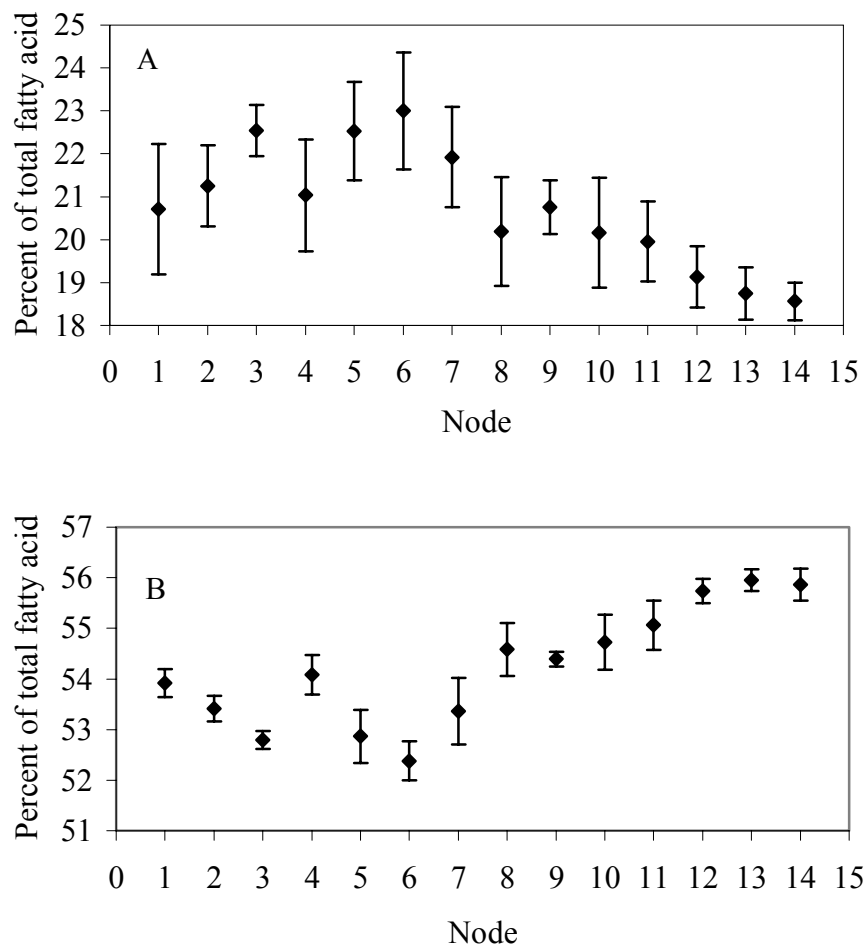


Figure 5. Distribution of oleic and linoleic acid in seed at each node. Esterified fatty acids were separated by gas chromatography and the relative accumulation of oleic acid (Panel A) and linoleic acid (Panel B) was determined. Number 1 on the X-axis represents the apical node and number 14 the basal node. Standard deviation is indicated by vertical bars in each data set.

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CHAPTER 3
ACCUMULATION OF GENISTEIN AND DAIDZEIN, SOYBEAN
ISOFLAVONES IMPLICATED IN PROMOTING HUMAN HEALTH, IS
SIGNIFICANTLY ELEVATED BY IRRIGATION

ABSTRACT

To circumvent drought conditions persisting during seed fill in the Mid-south United States soybean production region, researchers have developed the early soybean (*Glycine max* [L.] Merr.) production system (ESPS) which entails early planting of short-season varieties. Since soybean supplies a preponderance of the world's protein and oil and consumption of soy-based foods has been associated with multiple health benefits, the effects of this agronomic practice on seed quality traits such as protein, oil, and isoflavones should be investigated. Four cultivars of soybean, two from Maturity Group IV and two from Maturity Group V, were planted in April (ESPS) and May (traditional) in a two-year study at Stoneville, Mississippi and grown either under dryland or irrigated conditions. Near-infrared analysis of soybean seed was utilized to determine the percentages of protein and oil. Dependent upon variety, the oil content of the early-planted crop was increased by 3 to 8%, while protein was not significantly changed. Visualization of protein extracts fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and fluorescence 2-D difference gel electrophoresis revealed that early planting did not affect the relative accumulation of the major seed-storage proteins, thus protein composition was equal to that of traditionally cultivated soybeans. Maturity Group IV cultivars contained a higher percentage of oil and lower percentage of protein than did the Maturity Group V cultivars regardless of planting date. Gas

chromatographic separation of fatty acids revealed that the percentages of saturated and unsaturated fatty acids were not significantly altered by planting date. Methanol extracts of seed harvested from different planting dates when analyzed by high-performance liquid chromatography showed striking differences in isoflavone content. Depending upon the variety, total isoflavone content was increased as much as 1.3 fold in early-planted soybeans. Irrigation enhanced the isoflavone content of both early-and late-planted soybeans as much as 2.5-fold. Accumulation of individual isoflavones, daidzein and genistein was also elevated by irrigation. Because this cultural practice improves the quality traits of seeds, ESPS provides an opportunity for enhancing the quality of soybean.

INTRODUCTION

Soybean (*Glycine max* [L.] Merr.) production in the southern regions of the United States is beset by moisture deficit and high temperature during reproductive stages of plant development. A system of early planting, utilizing soybeans selected for production in northern regions and shorter growing seasons was developed to alleviate yield loss because of environmental stresses especially moisture deficit. These earlier-maturing varieties would enter and conclude critical reproductive stages before the onset of non-optimal soil moisture, thus enhancing the possibility of increased production. The protocol, known as the early soybean production system (ESPS), (1-5) has been successful improving yields in irrigated and non-irrigated soybeans in the Mid-south region (6).

Maturity groups, growing regions, and yearly weather variation individually and cumulatively affect characteristics of soybean seed in a manner yet to be elucidated (7). Studies designed to determine if a correlation existed between temperature and protein and oil accumulation indicated that higher temperatures diminished oil content and increase protein. When protein and oil data from soybeans grown at different latitudes were analyzed, temperature was implicated as a factor in the distribution of these seed storage compounds. Seeds from the northernmost regions of the test were lower in protein and higher in oil than those grown farther south (8, 9). A similar study compared protein and oil of seed originating from diverse soybean producing regions. Seeds originating from the northern and western areas of the soybean production locales were higher in oil and lower in protein than those from the southern and eastern regions (10). The average day and night temperature, or mean temperature of each region, was found more significant in relation to protein and oil accumulation than maximum or minimum temperatures during the seed-fill stage of plant development (7, 11).

Northern and Southern Uniform Tests for 1948 to 1998 showed average protein content of soybeans varied significantly between early- and late-maturity groups. Plants adapted for shorter growing seasons in the northern regions produced seed lower in protein than those in the southern regions (12). In contrast, the difference in oil content was not as obvious between the two extremes in maturity groups (12). Although soybeans harvested from maturity zones 1 to 3 (northernmost) were lower in protein than those from zone 7 (southernmost), a definitive and incremental increase in protein from cooler to warmer climate was not seen. A significant difference in oil content was not evident among the seeds harvested from these diverse maturity zones (13, 14). Since field conditions

present a plethora of variables, experiments in controlled environments have been conducted in an attempt to elucidate the factors affecting protein and oil content of soybean. Data from these experiments indicated that temperature at the onset and duration of seed-fill affected protein and oil content (11, 15).

In addition to the major seed-storage compounds, soybeans also contain isoflavones, which serve a variety of biological functions. The principal isoflavones of soybean seeds, daidzein, genistein, and glycitein, are synthesized from the phenylpropanoid pathway and stored as glucosyl- and malonyl-glucosyl- conjugates in vacuoles. Accumulation of these compounds in soybean is cultivar-dependent and influenced by environmental conditions during the seed fill (16-20). Cool temperature during onset and duration of seed-fill has been shown to increase the isoflavone content of the soybean several-fold (21).

Since hectareage of soybeans produced under the ESPS protocol is considerable, the quality traits of the resulting crop should be evaluated. This study was designed to ascertain whether crops grown under the ESPS regimen met or exceeded quality traits expected and to assay the effect of the system on accumulation of isoflavones. Since a portion of the hectareage in the Mid-south is irrigated, we also determined the effect of this practice on protein, oil, and isoflavone accumulation.

MATERIALS AND METHODS

Plant material and growth conditions. Field studies were conducted in 2002 and 2003 at the Delta Research and Extension Center near Stoneville, Mississippi (lat. 33°26' N). The pH at the study site ranged from 6.5 to 7.7. Soil tests indicated phosphorus and

potassium levels were adequate thus needing no supplementation (22, 23). Non-irrigated (NI) and irrigated (IRR) experiments were conducted in the same location each year. A randomized complete block design with four replications was used each year. Cultivars were randomly assigned to plots within each of two planting date blocks on both NI and IRR sites in 2002, and remained in the same location for 2003. Two cultivars representing Maturity Group IV (MG IV), 4403 and 4891, and two from Maturity Group V (MG V), 5701 and 9594, were chosen for this study. Seeds were treated with mefenoxam [(R)-2-{2, 6-(dimethylphenyl)-methoxyacetylamino}-propionic acid methyl ester] fungicide (Syngenta Corp, Wilmington DE) at 0.11 g active ingredient per kg seed prior to seeding each year. Irrigation was applied when soil water potential at the 30-cm depth decreased to approximately -50 kPa. The plots were irrigated through physiological maturity of each cultivar.

Near-infrared reflectance spectroscopy (NIR) of seed protein and oil. An aliquot consisting of approximately 40-50 whole seeds from each replication of each treatment was divided into three portions and assayed for protein and oil content using an Infratech 1255 Food and Feed Analyzer, NIR spectrophotometer (Tecator AB Hoganas, Sweden). The average value of three readings was taken as the protein and oil content of that replication and treatment.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A 15-mg aliquot of finely-ground seed was extracted with 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 removal of cellular debris by centrifugation (5000 g, 15 min) the supernatant containing the total seed protein was transferred to new micro-

centrifuge tubes and combined with 50 μ L 2-mercaptoethanol. Samples were heated in a boiling water bath for 5 min and cooled on ice until used. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (24) was conducted at room temperature on a 13.5% resolving gel (w/v) at 20 mA using a Hoefer SE 260 minigel apparatus (Amersham Bioscience, Piscataway, NJ). Gels were stained overnight with Coomassie Blue R-250. After destaining in 50% methanol/10% glacial acetic acid (v/v), gels were preserved in 10% glacial acid (v/v) prior to visualization. The relative content of seed protein components, separated by SDS-PAGE, was determined by computer-assisted densitometry using the Gene Wizard System (Syngene, Cambridge, UK).

Fluorescence 2-D difference gel electrophoresis (DIGE). Dry soybean seed (1 g) was ground to a fine powder and extracted with 2.5 ml buffer (0.1M Tris-HCl, pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9% sucrose). The suspension was mixed with an equal volume Tris-HCl buffered phenol (pH 8.8), stirred for 30 min at 4 °C and then centrifuged (5000 g, 10 min, 4 °C). The phenolic phase was decanted into a new tube and the aqueous phase re-extracted with 2.5 ml buffered phenol. Phenolic phases were combined and proteins precipitated by mixing 5 volumes 0.1 M ammonium acetate with 100% methanol chilled to -20 °C. After storing overnight at -20 °C, the proteins were precipitated from suspension by centrifugation (20000g, 20 min, 4 °C). The protein pellet was washed twice in 0.1M ammonium acetate in methanol, twice in ice-cold acetone and finally a single wash in cold 70% ethanol. Prior to electrophoresis the pellet was dispersed in 1 ml isoelectric focusing buffer (8 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2% Triton X-100, 50 mM dithiothreitol (DTT), 0.5% ampholytes pH 3-10). A 50 μ g aliquot of the protein was

labeled with 200 pmole of either Cy3 or Cy5 fluorescent dyes and kept on ice for 30 min. The nucleophilic labeling reaction was quenched by adding 10 mM lysine. Fluorescently labeled proteins were combined and applied to immobilized pH gradient (IPG) strips by active re-hydration at 50 V for 12 h, 500 V for 1 h, 1000 V for 2 h, and 8000 V for 1 h. Strips were equilibrated for two dimension electrophoresis in SDS-PAGE buffer (50 mM Tris-HCl pH 6.8, 6 M urea, 30% glycerol, 5% SDS) to which 2% DTT was added. Equilibration was repeated supplementing the buffer with 2.5% iodoacetamide. After rinsing in SDS-PAGE running buffer the strips were placed on an 11-17% acrylamide gradient gel and overlaid with agarose solution (60 mM, Tris-HCl, pH 6.8, 60 mM SDS, 0.5% agarose, 0.01% bromophenol blue). Following electrophoretic separation, the gels were imaged using the FLA-5000 Fluorescent Image Analyzer (Fuji Photo Film Co. Tokyo, Japan).

Fatty Acid Methyl ester (FAME) analysis of fatty acid components of soybean oil. An aliquot of three seeds was selected from each replication of treatment. Seed coats were cracked and seeds placed in a test tube containing 1 ml of hexane/chloroform/methanol (8:5:2 v/v/v). After an overnight extraction, 150 μ l of the solution was pipetted into a vial containing 75 μ l sodium methoxide-methanol/petroleum ether/ethyl ether solution (1:4:2 v/v/v) for fatty acid methylation. The methyl esters of the fatty acids were separated on a 30 m \times 0.5 μ m AT-Silar capillary column (Alltech, Deerfield, IL) used in conjunction with an Agilent 6890 gas chromatograph (Agilent, Palo Alto, CA). Fatty acid esters in the eluate were detected by flame ionization. Standards of each esterified fatty acid analyzed, palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) (Matreya, State College, PA) were used for

calibration. Each compound was reported as the normalized percentage of the total fatty acid content.

Isoflavone extraction and analysis. Approximately 1 g of soybean seed from each cultivar was ground to a fine powder using a General Electric seed grinder (Model 5XBG008, New York City, NY). The powder was extracted with 4 ml of 80% methanol at 80 °C. After centrifugation (5000 g, 15 min) the supernatant was filtered using 0.45 µm polytetrafluoroethylene (PTFE) Acrodisc syringe filters (Gelman Laboratory, Portsmouth, UK). Samples were analyzed by reverse-phase HPLC on a System Gold high performance liquid chromatography (HPLC) system (Beckman Coulter, Fullerton, CA) using a Luna C18 (2), 5µm, 4.6 × 150 mm column (Phenomenox, Torrance, CA). Separation and elution were accomplished employing an 18 min linear gradient initiated with 20% methanol /80% 10 mM ammonium acetate (v/v) (pH 5.6) and completed with 100% methanol at a flow rate of 1 ml/min. Detection of the metabolites was accomplished by photodiode array following published procedures (25, 26). Identification and quantification of each isoflavone component were based on available standards (Indofine Chemical Co., Somerville NJ).

RESULTS

Early planting system and irrigation effects on protein and oil. Protein and oil are the principal storage compounds of soybean. To ascertain if the ESPS affects the content of these macromolecules, seed aliquots were collected from field plots and analyzed by NIR spectroscopy. Protein content of soybeans from ESPS was comparable to that of traditionally planted crop (**Figure 1A**). Irrigation of early- and late-planted soybeans did

not influence the protein content (**Figure 1A**). Oil content of seed from the MG IV cultivars 4403 and 4891 was increased by 8% and 9%, respectively, in the early-planted crop, while maturity Group V cultivar 5701 showed a 7% increase in oil. Irrigation did not significantly alter the oil content of the MG IV cultivars from either planting date, but did affect oil accumulation in the MG V cultivars. Irrigated early- and late-planted 9594 showed a 5 % increase in oil and late-planted 5701 demonstrated a 7% increase in oil (**Figure 1B**). Maturity Group V cultivars contained a higher percentage of protein and lower percentage oil than the MG IV cultivars regardless of treatment (**Figures 1A and 1B**).

Influence of early planting and irrigation on seed protein composition. Because the total protein content was not affected by planting date and irrigation, the question as to whether the relative abundance of seed storage polypeptides had been affected by the ESPS regimen was addressed. Total seed protein was extracted from an aliquot of seed representing each treatment, separated by SDS-PAGE, and stained with Coomassie blue. Visual observation of the gels revealed no obvious differences in the seed protein profiles. The relative content of α' -(72 kDa), α -(70 kDa), and β -(52 kDa) subunits of β -conglycinin (7S) was not affected by irrigation or planting date. Acidic (40 kDa) and basic (20 kDa) subunits of glycinin (11S) were found in equal abundance in all treatments. The accrual of lipoxygenase (94 kDa), which is present in significant quantities in soybean, was similar in all treatments (**Figure 2**). Comparison of gels using computer-assisted densitometry revealed similar accretion of the seed storage proteins (data not shown). Since one-dimensional gels indicated that protein profiles did not vary among treatments, more sensitive, two-dimensional fluorescence difference

electrophoresis (27) was performed to detect subtle variations in protein content (Figure 3). Since one-dimensional gels indicated that protein profiles did not vary among treatments, two-dimensional fluorescence difference electrophoresis (27) was performed to detect subtle variations in protein content (**Figure 3**). Seed proteins from non-irrigated and irrigated plants were labeled with Cy3 and Cy5 fluorescent dyes, respectively. Relative content of individual proteins were determined by spot colors of the superimposed images. If the resulting color was yellow, the particular protein accumulated equally in the two treatments being compared. A red image spot indicated more protein in seed from non-irrigated plants, while a green image spot noted seed protein from irrigated plants was present in greater amount. Mostly, the yellow color was visible in gel image overlays, supporting the premise that planting date and irrigation did not significantly affect protein profile (**Figure 3**). However, a few green fluorescent spots were seen indicating that accumulation of these proteins was enhanced in seeds from irrigated plants (**Figure 3**). Two prominent green spots with an apparent molecular weight of 52 kDa and isoelectric points comparable to that of the β -subunit of β -conglycinin were found, suggesting the content of this polypeptide was elevated in the seed from irrigated soybeans (**Figure 3**, shown by arrows).

Fatty acid partitioning affected by planting date and irrigation. Soybean oil contains five fatty acids, palmitic, stearic, oleic, linoleic, and linolenic, which differ considerably in their physical properties. Altering the relative concentration of these compounds changes the characteristics of the oil, thus making it suitable for specific uses. Oils, which contain more oleic and less linoleic and linolenic acids, have higher oxidative stability making them suitable for both food preparation and industrial processes. High

linolenic acid content imparts predisposition to oxidation rendering this soybean oil useful in applications requiring fast drying oils. The fatty acid components of soybean oils were examined to ascertain whether the ESPS regimen affected the relative content of these compounds. Quantitative analysis of methylated esters using gas chromatography indicated that the accumulated percentage of fatty acid was consistent; however, subtle differences did occur in the profiles (**Figure 4**). Neither planting date nor irrigation significantly affected the fatty acid contents of MG IV or MG V.

Isoflavone content increased by irrigation. Environmental conditions during the reproductive stages of soybean are known to affect the isoflavone content of the seed. Since plants under the ESPS regimen will enter the reproductive stage at a different time, and ostensibly different environmental conditions, than those planted according to traditional protocol, the effect on isoflavone content was examined. After methanol extraction, isoflavones and their respective conjugates were individually quantified by HPLC and the total metabolite content was determined. Results indicated that isoflavone content of the early-planted crop and late-planted crop was comparable. The early planting of MG IV cultivar 4403 contained 123 ± 16 $\mu\text{g/g}$ isoflavone and the late planting 157 ± 11 $\mu\text{g/g}$ of the metabolite. Representing the MG V cultivars, early-planted 5701 seed contained 213 ± 20 $\mu\text{g/g}$ isoflavone and the late-planted seed contained 193 ± 63 $\mu\text{g/g}$ isoflavone (**Figure 5**). In contrast, irrigation consistently and significantly increased the seed isoflavone content. When irrigated, seed from cultivar 4403 contained 355 ± 60 $\mu\text{g/g}$ isoflavone, while that from non-irrigated plants had 123 ± 16 $\mu\text{g/g}$ total isoflavone (**Figure 5**). The MG V 9594 plants, which were irrigated, produced seed containing 370 ± 20 $\mu\text{g/g}$ isoflavone, while seed from non-irrigated plants contained 213

$\pm 20\mu\text{g/g}$ of the metabolite. Irrigated early-planted MG IV and V cultivars showed a 2.8-fold and 1.7-fold increase in isoflavone content, respectively, while the same late-planted cultivars each exhibited a 1.6-fold increase (**Figure 5**). The response of individual compounds, daidzein and genistein to planting date and irrigation was similar to that of total isoflavone accumulation, with the exception of glycitein which showed an increase only in the irrigated early-planted MG IV cultivars (**Figure 6**).

DISCUSSION

Epidemiological studies have shown a reduced risk of cancer, heart disease, and other chronic illnesses in populations that consume soybeans and soy products (28). Studies have indicated the isoflavones may be partially responsible for the health benefits associated with soybean consumption (29, 30). Results from this study asserted that irrigation dramatically increased the isoflavone content of soybean seed. The mechanism by which this increase was facilitated is unknown. In soybean, isoflavones are principally found in roots and seeds, however the metabolite has also been isolated from leaf and stem tissue (31, 32). Ostensibly, an increase in isoflavone content, as the result of irrigation, could arise solely from increased synthesis in the seed components or possibly be translocated from distal production sites. Previous reports suggest flavanoids that accumulate after ultra-violet irradiation and pathogenic induction are produced by the cells directly exposed to the environmental stimuli rather than being transported from cells of other tissues (33, 34). However, recent work suggests while seeds are the principal site of isoflavone synthesis, some accumulation is due to transport from other plant organs including maternal tissues (35). In the seed tissue, isoflavone synthase is

expressed only in embryos and seed coats, and not in the developing cotyledons, suggesting the majority of the isoflavones in the cotyledons are transported from other tissues (36, 37). Translocation of glucosinolate from leaf to seed in *Brassica napus* (L.) (38, 39), and similar transport of plant alkaloids provides a precedent for movement of the isoflavones through the vascular system. Soybean-pod exudates collected from the juncture of the marginal veins has been found to contain isoflavones indicating putative vascular transport. In addition, soybean embryos have been shown *in vitro* to assimilate exogenous isoflavones, thus demonstrating the possibility of movement from source to sink tissue (35). Clearly, the mechanisms underlying the effects of irrigation on isoflavone accumulation require further investigation.

Results presented in this study indicate that protein and oil content of ESPS produced soybeans are comparable or enhanced with respect to a crop grown under the traditional regimen. Although protein content of MG IV and V cultivars did not vary significantly with planting date, oil accumulation was higher in early-planted MG IV cultivars. Field studies have shown that oil content is affected by temperature during seed fill, but protein levels does not correlate with temperature during this period (7). Because the temperature during seed fill of early- and late-planted crops in this two-year study was similar (40), the increased content of oil in MG IV cultivars could be attributed to genetic make-up of these cultivars (41) as both MG IV cultivars had higher oil content than the MG V cultivars regardless of treatment. Irrigation in the Midsouth region is a management practice that can be utilized where water resources are readily available. Previous studies have shown protein and oil accumulation do not respond to irrigation (42, 43). Results from our study are consistent with the previous work with respect to

protein, but irrigation did increase the oil content in late-planted MG IV and early- and late-planted MG V cultivars. Alleviation of possible moisture deficit during the reproductive stages is a possible reason for the increase in oil content. Planting dates and irrigation appeared to have subtle effect on fatty acid composition in the current study. For example, an increase or decrease in oleic acid content was countered by reciprocal change in polyunsaturated fatty acids. This could be related to changes in the activity of the desaturase enzyme, which is influenced by both temperature (44) and light quality (45).

Soybeans planted according to the ESPS have shown a significant yield advantage in the Midsouth growing region (2). In this study, we demonstrate that protein and oil contents of the crop are comparable to those of soybeans grown under traditional cultural practices. When irrigated, both ESPS and traditionally-planted soybeans consistently reveal a minimum 1.5-fold increase in isoflavone content. Since isoflavone consumption has been attributed to reduced incidence of certain types of cancer and other chronic illness, producing soybean cultivars that accumulate higher isoflavone levels under a variety of growing conditions is desirable. Determination of the biochemical means by which irrigation improves isoflavone content could aid in the development of cultivars, which have an enhanced and uniform content of the metabolite.

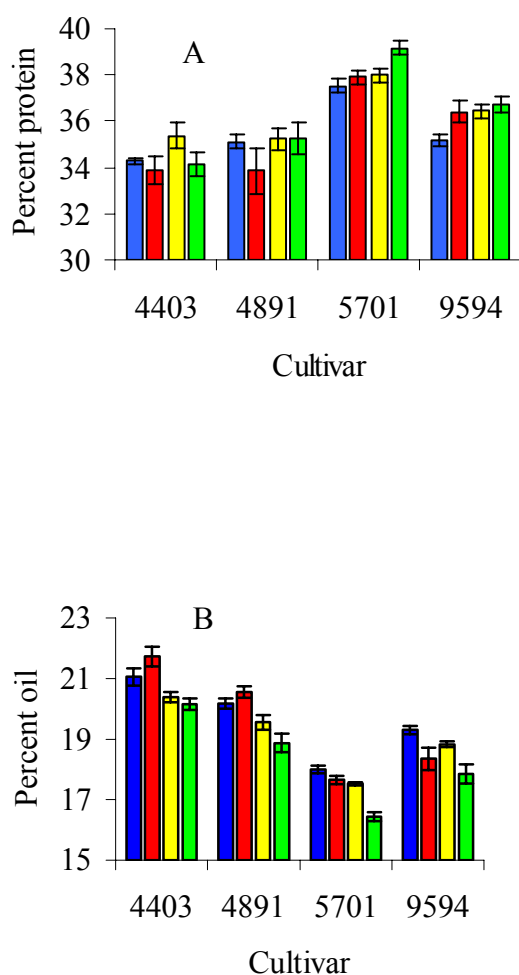


Figure 1. Percent oil and protein as influenced by planting date and irrigation. Analysis of seed by near-infrared spectroscopy was conducted to determine the percentages of seed oil and protein from each cultivar, as a response to planting date and irrigation. The treatments are represented by colored bars: blue designates early-planted, irrigated; red denotes early-planted, non-irrigated; yellow symbolizes late-planted, irrigated and green represents late-planted, non-irrigated. Standard deviation is indicated by vertical bars in each data set.

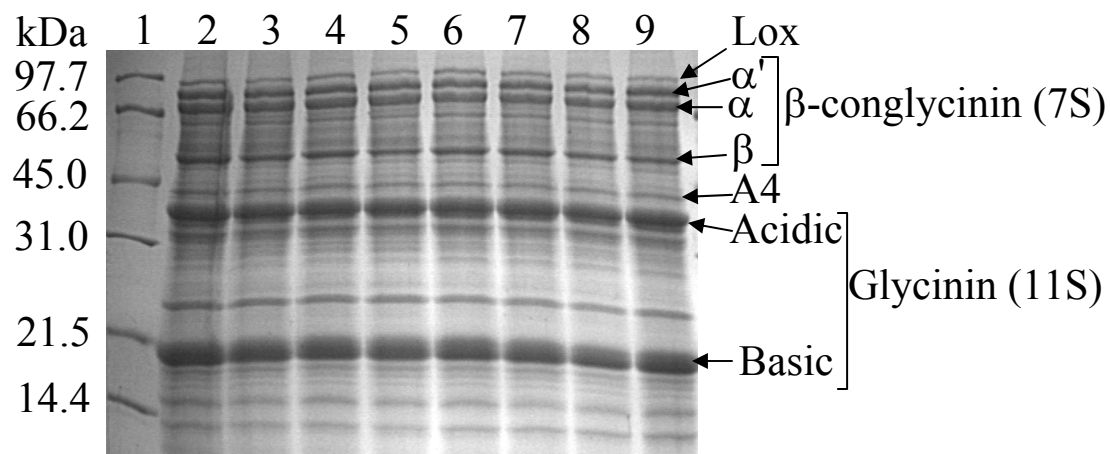


Figure 2. Effect of planting date and irrigation on accumulation of seed proteins. Total seed proteins were fractionated by 13.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized by Coomassie blue. Lane 1 is the molecular weight marker. Lanes 2 through 5 depict seed proteins of cultivar 5701 and lanes 6 through 9 those of cultivar 9594. Lanes 2 and 6 depict early-planted, irrigated; lanes 3 and 7 early-planted, non-irrigated; lanes 4 and 8 late-planted, irrigated and lanes 5 and 9 late-planted, non-irrigated. The sizes of molecular weight of markers in kDa are indicated on the left side of the figure.

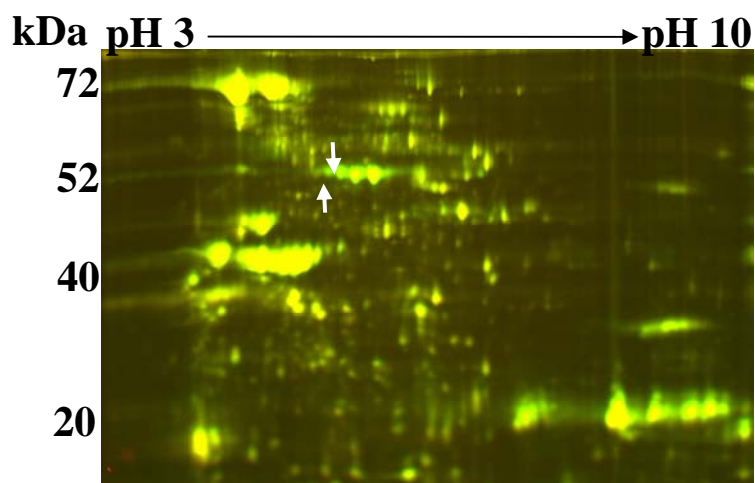


Figure 3. Comparison of seed protein profiles by difference gel electrophoresis (DIGE). Seed protein from irrigated and non-irrigated plants of soybean cultivar 4403 were labeled with Cy3 and Cy5 respectively, mixed, and then subjected to 2-D DIGE. The gel was scanned at emission wavelengths specific for each dye and the resulting images were overlaid and visualized using the FLA-5000 laser analyzer. Arrows point to the β -subunit of β -conglycinin.

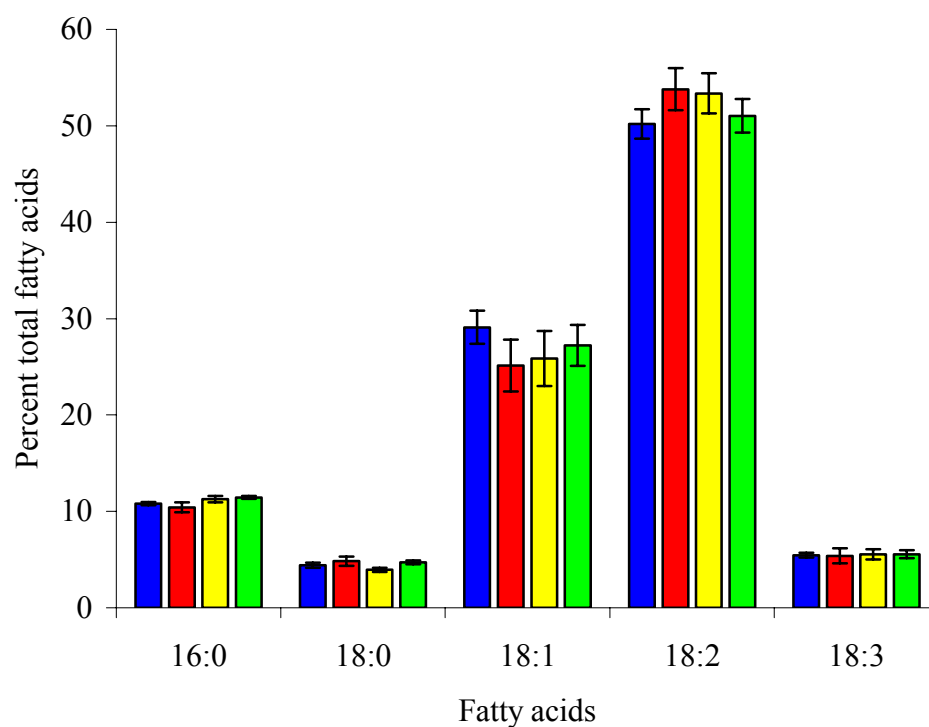


Figure 4. Distribution of fatty acids in seed. Fatty acids were separated and quantitated by gas chromatography and the amount of each reported as a percentage of the total. The treatments are represented by colored bars: blue designates early-planted, irrigated; red denotes early-planted, non-irrigated; yellow symbolizes late-planted, irrigated and green represents late-planted, non-irrigated. Standard deviation is indicated by vertical bars in each data set.

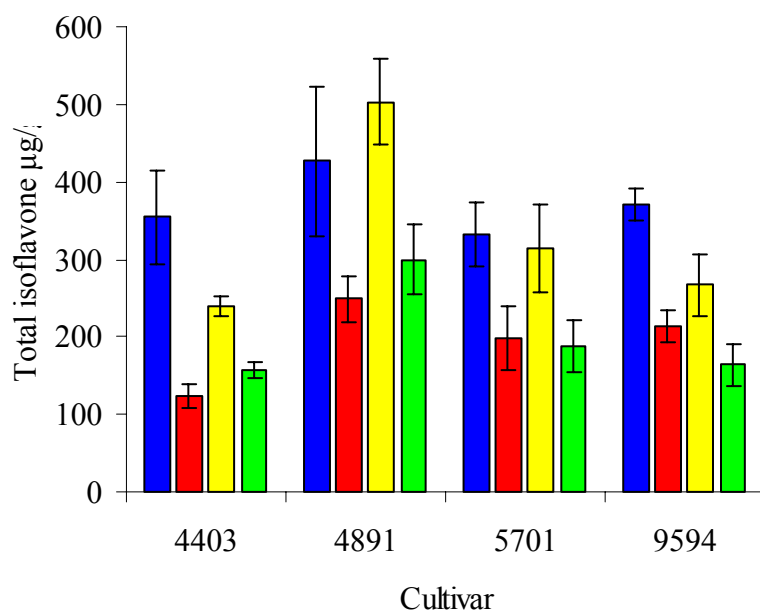


Figure 5. Assimilation of total isoflavone in soybean seed. High performance liquid chromatography was utilized to determine accrual of isoflavones in seed as affected by planting date and irrigation. The treatments are represented by colored bars: blue designates early-planted, irrigated; red denotes early-planted, non-irrigated; yellow symbolizes late-planted, irrigated and green represents late-planted, non-irrigated. Standard deviation is indicated by vertical bars in each data set.

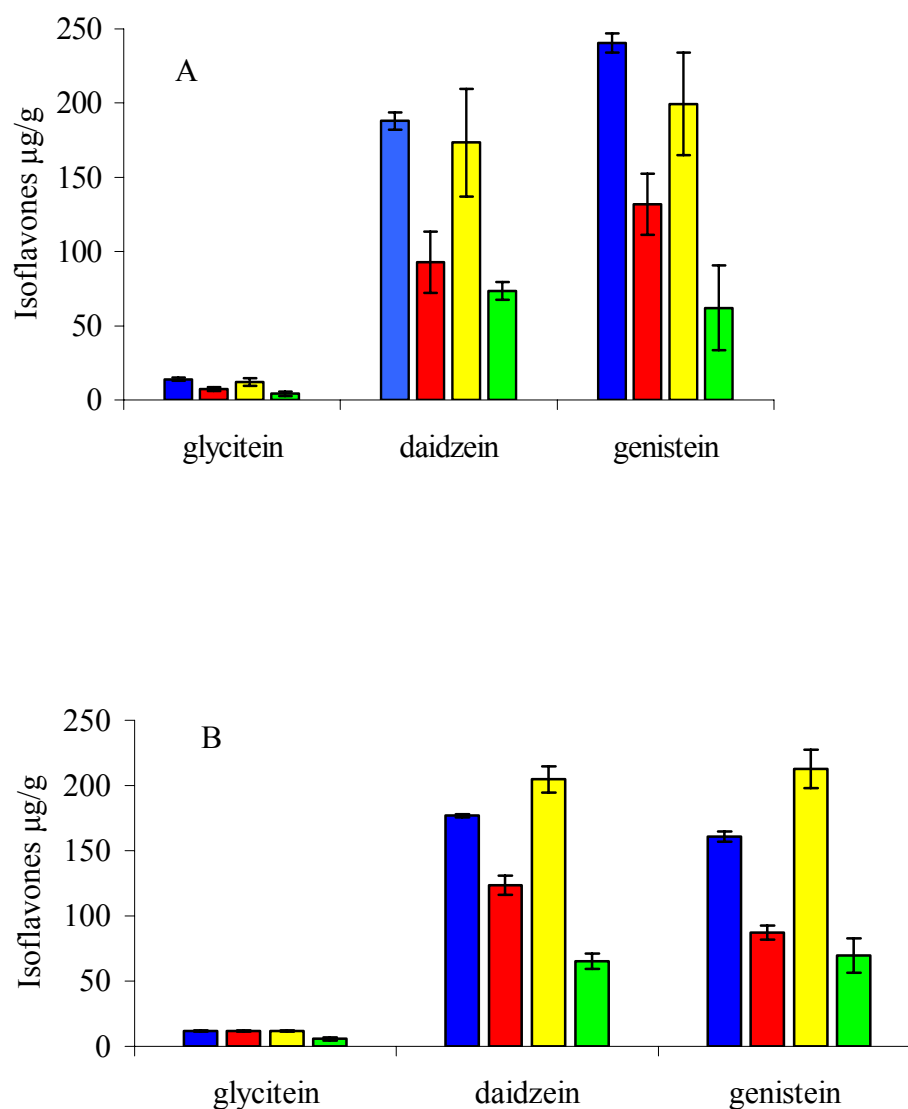


Figure 6. Accumulation of isoflavone components as influenced by planting date and irrigation. Isoflavones glycitein, daidzein, and genistein were separated and quantitated by high performance liquid chromatography using designated standards. Depicted in Figure 6A are the isoflavone contents of MG IV cultivar 4403, and in Figure 6B, those of MG V cultivar 9594. The treatments are represented by colored bars: blue designates early-planted, irrigated; red denotes early-planted, non-irrigated; yellow symbolizes late-planted, irrigated and green represents late-planted, non-irrigated. Standard deviation is indicated by vertical bars in each data set.

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CHAPTER 4

LONG-TERM STUDY OF ENVIRONMENTAL EFFECTS ON SOYBEAN SEED COMPOSITION

ABSTRACT

A long-term study initiated in 1989 at Sanborn Field, Columbia, Missouri, was designed to evaluate the affect of environmental factors, nitrogen application, and crop rotation on soybean (*Glycine max* [L.] Merr.) seed composition. Soybeans were grown as part of a four-year rotation which included corn (*Zea maize* L.), wheat (*Triticum aestivum* L.), and red clover (*Trifolium pratense* L.). Results from soil tests made prior to initiation of the study and subsequently every five years, were used to calculate application rates of nitrogen, phosphorus, and potassium necessary for the target yield of pursuant crops. In the experimental design, nitrogen was applied to one-half of the plot on which the non-leguminous crop, either corn or wheat was to be grown. Analysis of soybean seed by near infrared reflectance spectroscopy collected over an 11-year period revealed a linear increase in protein and decrease in oil content. Application of nitrogen fertilizer to non-leguminous crops did not have an apparent effect on total protein or oil content of subsequent soybean crop. Analysis of soybean seed proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis in conjunction with computer-assisted densitometry revealed subtle changes in the accumulation of seed proteins. Immunoblot analysis using antibodies raised against the β -subunit of β -conglycinin showed a gradual increase in the accumulation of the 7S components during successive years of the experiment. A linear increase in temperature and decrease in rainfall was observed from the onset of data collection. Higher temperatures during the growing season have been

linked to increased protein and diminished oil content of soybean, thus changes observed in this study are possibly related to climatic conditions. However, crop rotation and subsequent changes in soil ecology may contribute to these observed changes in the seed composition.

INTRODUCTION

Production economics are factors in the selection of agronomic practices (1). Although rotation of cereals, such as wheat and corn with legumes is a long-standing cultural method (2, 3), the advent of mechanization has led to a predominance of monoculture systems in today's agricultural production. Research devoted to improving soil and environmental quality while maintaining the cost of production, and enhancing grain yield and quality involves long-term experiments. Increased awareness and knowledge of human and animal nutrition compel plant scientists to develop cultivars whose composition provides optimal nutrition. Selection and breeding, and more recently genetic engineering, are tools being utilized to this end.

Soil ecology is a factor in the mineralization and availability of nutrients to the soybean (4) which in turn influences content of seed storage compounds that are important in human and animal nutrition (5). Soil organic carbon (SOC) is one indicator of soil quality and has an influence on the chemistry and biology of soil (6-9). Since crop rotation influences this parameter (10, 11), the potential exists for a crop rotation system to influence nutrient quality of crops. Higher microbial carbon and nitrogen levels were reported to occur in a four-crop rotation system, which included a forage legume as opposed to a monoculture system (12, 13).

Enzymatic activity necessary for mineralization of plant nutrients has been found correlated to SOC (14). Mineralized sulfur and nitrogen are essential for the production of amino acids, which are components of seed storage proteins. Availability of both sulfur and nitrogen has been associated with relative accumulation of seed protein and thus quality (15-18). Protein and oil content of soybeans are also affected by symbiotic associations with vesicular mycorrhiza and symbiotic bacteria. Protein content was increased in plants that formed symbiotic relationship with arbuscular mycorrhiza (19). Rhizobial nodulation factors have been found to enhance the colonization of soybean roots by mycorrhizobia (20). Tripartite symbiosis involving plant mycorrhiza and bacteria benefits from an increased soil organic carbon (20).

Soybean seed composition is influenced by environmental conditions during the growing season (21- 25). In a general sense, soybeans growing at higher temperatures contain an increased percentage of protein while those from cooler conditions contain higher percentage of oil (23, 26). Soybeans of maturity groups II through IV exhibited the highest oil content while those of maturity group VIII were highest in protein (23). The protein and oil content data were compiled from a multitude of soybean lines submitted for evaluation in uniform tests (23). Recent work has shown that conditions existing at specific times during the growing season can be correlated with the final protein and oil content of soybeans (27). Since variety and growing conditions have been reported to affect seed composition (21-24), utilizing a single variety at one location in a long-term study will provide information as to the specific effects of environment on seed components. In this study, the soybean 'Williams 82' was utilized and the cumulative

effects of nitrogen application, environmental conditions, and crop rotation on protein and oil content were investigated.

MATERIALS AND METHODS

Field plot fertility and design. Williams 82 soybeans were grown beginning in 1989 on Sanborn field in Columbia, MO, in a four-year rotation consisting of wheat, red clover, corn, and soybeans. Data for this experiment were collected from 1991 to 2002, exclusive of 1999, for which samples were not available. A split-plot design was selected to ascertain, in part, the effect of nitrogen carryover on seed storage compounds in subsequent soybean crops and the cumulative effect of crop rotation on content of seed storage compounds. Each 30.8 m \times 9.4 m plot in the study was surrounded by a grass-seeded alley. Soil tests were conducted prior to the onset of the trial and every five years subsequently. Annual fertilizer application was based upon yield goals of 8.15 t ha⁻¹ for corn, 4.7 t ha⁻¹ for wheat, 4.0 t ha⁻¹ for soybeans, and 9.4 t ha⁻¹ for red clover hay (28). Calculated amount of nitrogen was applied to one-half of the plot to be planted in either corn or wheat. Red clover forage was harvested in the fall and re-growth was moldboard-plowed in spring as a green manure crop prior to corn planting.

Near infrared reflectance spectrographic (NIRS) analysis of seed protein and oil. Protein and oil content of seed aliquots was determined by NIRS using the Infratech 1255 Food and Feed analyzer (Tecator AB, Hoganas, Sweden). The reported protein and oil was based upon normalized moisture content of 135 g kg⁻¹ in each sample. Data from NIRS analysis for protein was corroborated by University of Missouri Experimental Station Laboratory using the nitrogen combustion method, 990.03, as outlined in the 17th

edition of Official Methods of Analysis of the International Association of Analytical Communities (AOAC).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

separation of seed protein. An aliquot of 10 seeds was selected from each year's soybean harvest and ground with mortar and pestle. A 15-mg aliquot of the powdered seed was extracted in 1 mL of a solution comprised of 4% sodium dodecyl sulfate (w/v), 20% glycerol (v/v), and 0.03 mM bromophenol blue solubilized in 125 mM Tris-HCl buffer (pH 6.8). Cell debris was sedimented by centrifugation at $14300 \times g$ for 15 min, the supernatant decanted to clean centrifuge tube, and combined with 50 μ L 2-mercaptoethanol. Proteins were fractionated by SDS-PAGE (29) on a 12.5% resolving gel (w/v) at 20 mA for 1 hr using a Hoefer SE260 minigel apparatus (Amersham Biosciences, Piscataway, NJ). The gels were stained overnight with Coomassie blue.

Densitometry. Proteins were separated on a 12.5% polyacrylamide gel as described in the previous section and the relative densities of the bands were determined by computer-assisted densitometry employing the Gene Wizard System (Syngene, Beacon House Nuffield Road, Cambridge, UK). The ratio of the acidic and basic subunits of glycinin with respect to α' , α , and β -subunits of β -conglycinin was determined.

Western blot analysis. Total protein extract was resolved by SDS-PAGE on a 10% gel (w/v) at 20 mA for 1 hr using a Hoefer SE260 minigel apparatus. After 10 min equilibration in transfer buffer (0.02 M Tris, 0.15 M glycine, 200 mL methanol), proteins were electrophoretically transferred to a nitrocellulose membrane, incubated for 1 hr in $1 \times$ Tris buffered saline (TBS) (0.02 M Tris, 0.5 M NaCl) which contained 5% non-fat-dehydrated milk to block non-specific binding. Subsequently, the membrane was

incubated overnight in 1× TBS solution containing 5% non-fat-dehydrated milk (w/v) to which the primary antibody had been added in a final 1 to 5000 dilution. After three 10 min washes in 1× TBS containing 0.05% Tween 20 (TBST) the membrane was exposed to a secondary antibody, goat anti-rabbit-HRP conjugate (Pierce Chemical Co., Rockford, IL), for 1 hr and then washed four times in 1× TBST. Relative amounts of proteins present were determined using the chemiluminescent, Supersignal®, HRP substrate (Pierce Chemical Co., Rockford, IL).

Fatty Acid Methyl Ester (FAME) analysis of oil components. An aliquot of seeds was selected from each year of soybean production. The seed coat was cracked and seed was extracted overnight in 1 mL hexane/chloroform/methanol (8:5:2 v/v/v). Fatty acids from a 150 aliquot of the extract were methylated with 75 µL of sodium methoxide-methanol/methanol/petroleum ether/ethyl ether solution (1:4:2 v/v/v). Methyl ester derivatives of the fatty acids were separated on a 30 mm × 0.53 mm × 0.5 µm AT-Silar capillary column (Alltech, Deerfield, IL) in the Agilent 6890 gas chromatograph (Agilent, Palo Alto, CA). The methylated fatty acids were detected in the effluent stream by flame ionization. A standard containing the methyl ester derivatives of the five fatty acids in soybean, palmitic, stearic, oleic, linoleic, and linolenic acids was used for determining relative amounts of each fatty acid.

Statistical analysis. The effect of years, rainfall, temperature, and nitrogen application on soybean seed protein, protein components, oil, and fatty acids was assessed by analysis of variance (ANOVA). Years, rainfall, and temperature were treated as random effects while nitrogen application was treated as a fixed effect. Relationships between aforementioned seed traits and environmental conditions and between seed traits

and nitrogen application were evaluated using regression analysis. Statistical analyses were determined using the 8.2 version of General Linear Model SAS software (30).

RESULTS

Near infrared reflectance spectroscopy analysis of protein and oil. Accumulation of seed storage components in soybean is influenced by environmental conditions prevailing during the growing season. To monitor the changes in seed composition during the course of this study, we determined the protein and oil content of seeds by NIRS analysis. This analysis revealed that the total protein increased from 35 to 37.5% of seed weight, while that of oil declined from 19.5 to 18.5% over the 11-year period (**Figures 1A and 1B**). We also examined the residual effect of exogenous nitrogen provided to non-leguminous crops in the rotation on soybean seed composition. Application of nitrogen fertilizer did not have an apparent affect on accumulation of these seed storage compounds (**Figures 1A and 1B**). During the course of this experiment, an increase in protein ($P \leq 0.05$) was noted in seed from plants that did not receive supplemental nitrogen. Seeds from nitrogen fertilized plots did not exhibit a significant response (**Figures 1A and 1B**). The year effect putatively reflects a combination of environmental factors and changes soil ecology. Since protein and oil content are inversely correlated, the significant increase in protein should be accompanied by a decrease in oil. Oil content decreased from 19.5% of seed weight to 18.5% over the course of the study. Application of exogenous nitrogen had no significant effect on the seed oil content.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot analysis of seed storage proteins. SDS-PAGE fractionation of proteins supported the observation that an increase in protein content had occurred during the course of the experiment (**Figure 2A**). The α' , α , and β subunits of β -conglycinin and the A4 subunit of glycinin appeared to increase over the course of the trial in both fertilized and non-fertilized plots (**Figure 2A**). Immunoblot analysis was used to assay this putative increase in protein content (**Figure 2B**). The use of polyclonal antibodies, raised against the β -subunit of β -conglycinin, which cross-react with the α' and α subunits, confirmed an increase in accumulation of these proteins. Information provided by the western blot analysis also indicated that application of nitrogen fertilizer did not have a consistent effect on accumulation of β -conglycinin in particular the β -subunit, which has been shown previously to respond to nitrogen application (8, 31). Application of exogenous nitrogen increased the content of this subunit during only three years of the study 1996, 2001, and 2002 (**Figure 2B**). Conversely, plants grown without exogenous nitrogen appeared to accumulate more glycinins than did those from fertilized plots. This was particularly evident in 1992, 1997, 1998, 2001, and 2002 (**Figure 1A**). A densitometry analysis revealed that although total protein increased during the course of the trial, the glycinin /conglycinin ratio did not vary appreciably (data not shown).

Gas chromatographic analysis of fatty acid components of seed oil. Fatty acid composition is integral to soybean oil utilization. Resistance to oxidation is enhanced by increasing the oleic acid content, thus making the oil amendable for cooking and storage. Alternatively, oil containing more of the polyunsaturated linolenic acid is purportedly beneficial to human health. The decrease in oil content of the soybeans observed in this

study was not at the expense of one particular fatty acid. Analysis of variance of the fatty acid components, palmitic, stearic, oleic, linoleic, and linolenic acids, indicated a lack of correlation between year and accumulation of these long-chain compounds (**Figure 3**). Application of nitrogen fertilizer did not alter the total fatty acid content or profile.

Environmental effects on protein and oil. Moisture availability and temperature are crucial parameters in soybean reproduction and ostensibly, are involved in the quality of seed produced. Temperature and rainfall data during the reproductive period of July through September were recorded and the effect on accumulation of seed storage compounds determined. The maximum mean temperature occurring during pod-fill increased $0.25^{\circ}\text{C year}^{-1}$ ($P \leq 0.05$) (**Figure 4**). Additionally, a positive correlation was noted between year and recorded temperature. Although differences in rainfall during pod fill was not significant over the time span of the experiment, a negative correlation did exist between rainfall and years (**Figure. 4**). The effect of rainfall and average maximum temperature during pod fill period was examined by analysis of variance, treating these environmental conditions as random factors. Neither parameter was found to influence the accumulation of seed storage compounds. During the 1993 soybean reproductive period, rainfall exceeded 68 cm, which was well above the 30-year average rainfall of 28 cm for Boone County, Missouri for this time period (<http://agebb.missouri.edu/mass/MO30avg.>). Accumulation of seed storage compounds did not appear to be affected in that year by this inordinate rainfall, but in the subsequent year, protein and oil content showed a dramatic decrease and increase, respectively (**Figure 1**).

DISCUSSION

During the course of this field experiment, the total protein component of soybean seed increased from 35 to 37.5% of total seed weight, while oil diminished from 19.5 to 18.5%, regardless of nitrogen treatment. A warming trend during the reproductive stage of plant development was evident from 1991 to 2002, while rainfall diminished slightly. A positive correlation exists between protein accumulation and years. The ratio of protein gain and oil loss observed during this study is worthy of note. An inverse relationship has been shown in the accumulation of these two storage compounds because they compete for carbon and energy necessary for synthesis (32, 33). Since oil contains twice the Kcal g⁻¹ of energy as protein, it has been suggested that a change in accumulation of one unit of oil would be accompanied by a two-unit change in protein (31, 34). This approximate exchange was observed because oil decreased over 1% and protein increased approximately 2.5% over the 11-year period. Assigning specific causes to the increase in protein and decrease in oil is tenuous because both changing environmental conditions and the incipient crop rotation system could be involved. Higher ambient temperature has been associated with increased soybean protein and diminished oil accumulation in both growth-chamber (24) and field studies (21, 23). In this trial, however, changes in protein and oil content could not be entirely assigned to environmental conditions, which allude to other possible causes for the differences observed in the accumulation of these storage compounds.

Cropping systems such as the rotation protocol selected for this trial are known to affect soil ecology (35) which influences plant nutrient availability (4, 36). Whether the chemical, biological, and physical properties of soil (37-40) have been modified

sufficiently as to influence the assimilation of seed storage protein and oil is not certain. Separation of environmental factors from agronomic effects, using statistical methods capable of segregating the influence of these parameters may facilitate determining the specific and underlying reasons for the changes in seed protein and oil content.

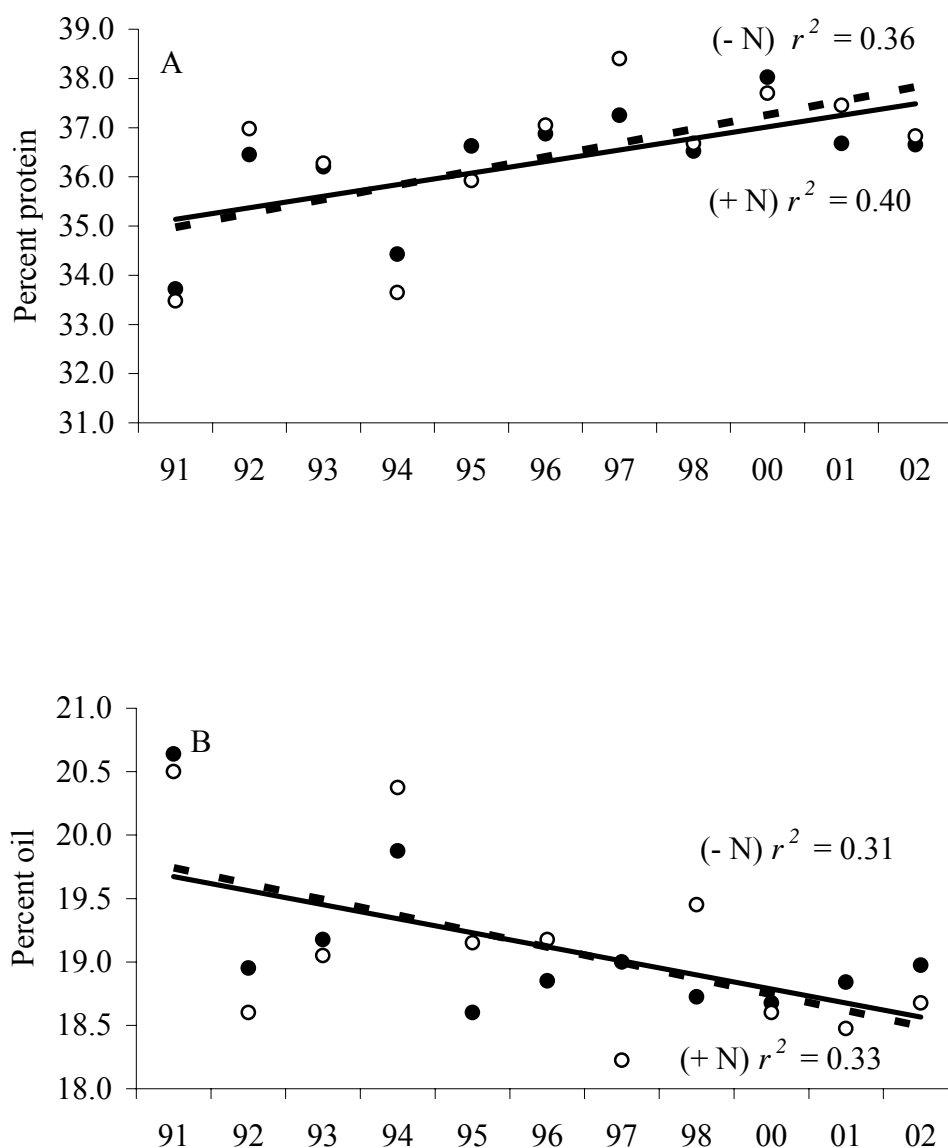


Figure 1. Change in protein and oil content during an 11-year study. Near infrared reflectance spectroscopy analysis revealed changes in protein (1A) and oil (1B) content. Comparison of seed components from nitrogen fertilized (●) and non-fertilized (○) plots shows the effect of years (1A and 1B). Solid regression lines represent data acquired from plots receiving nitrogen, while broken lines correspond to non-fertilized plots.

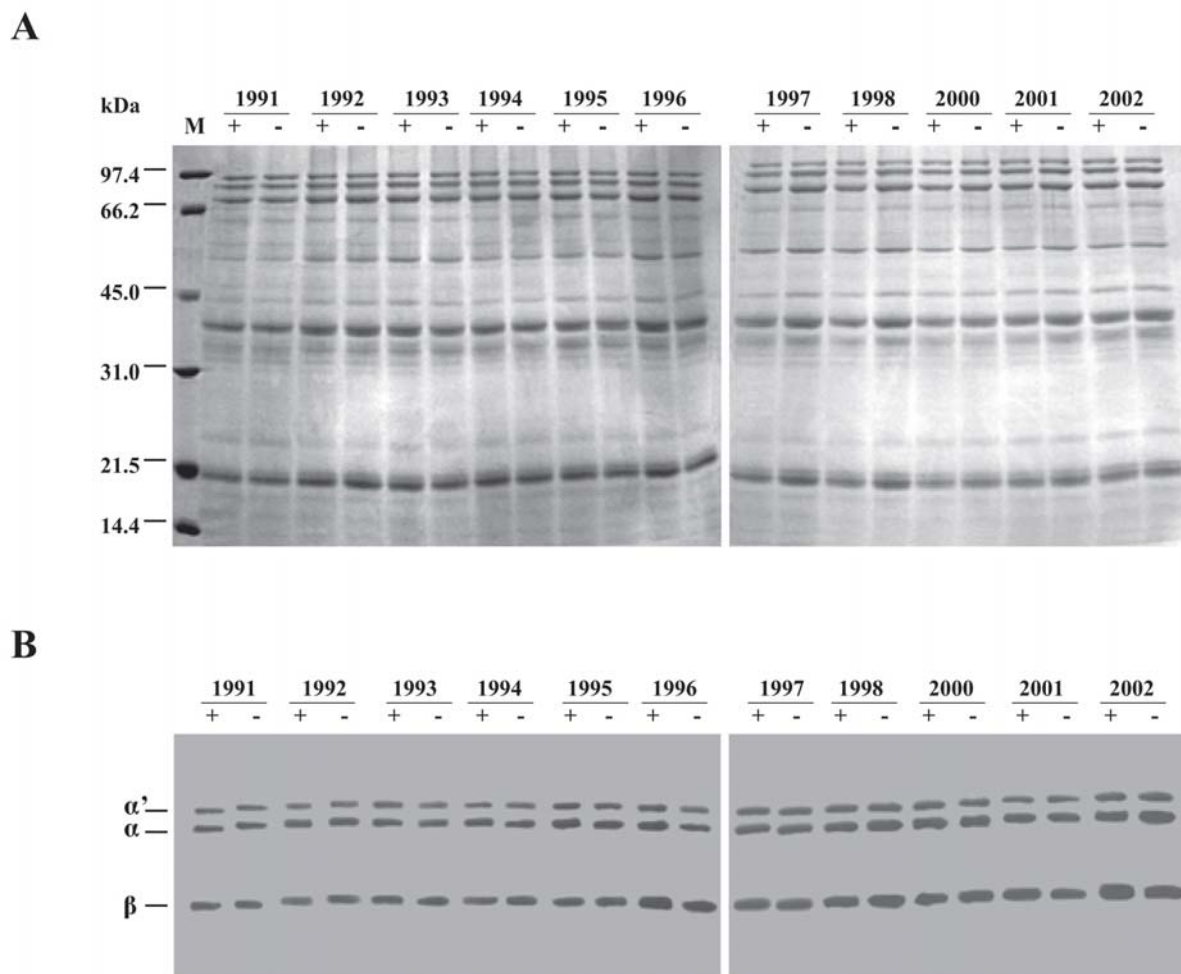


Figure 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of seed storage protein. Seed proteins were fractionated on 12.5% polyacrylamide gel and stained with Commassie blue (1A). Proteins were electrophoretically transferred from a 10% gel to a nitrocellulose membrane for immunoblot analysis (1B). Immunoblot analysis of seed protein shows that the accumulation of the β -subunit of β -conglycinin was notably higher in some years in seeds from nitrogen-fertilized plots. Conversely, application of nitrogen fertilizer was noted to depress the accumulation of the glycinins in several years (1B).

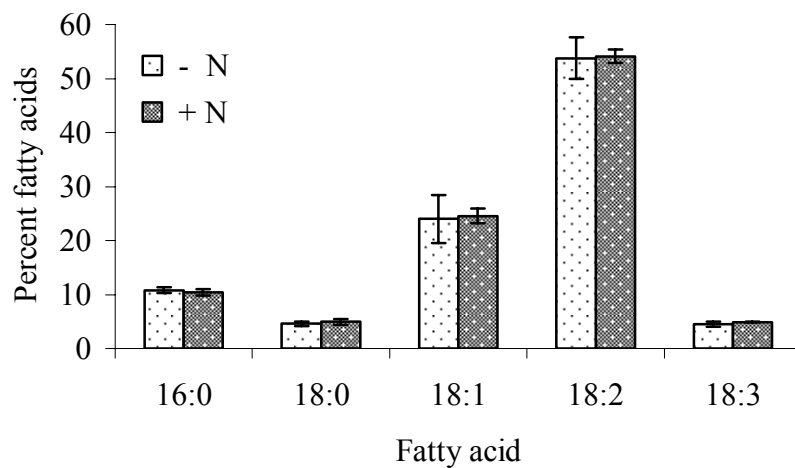


Figure 3. Gas chromatographic determination of fatty acid content. Fatty acids were solvent extracted and methylated in preparation for separation and quantification by gas chromatography. Error bars represent standard deviation of the mean of three replications of analysis.

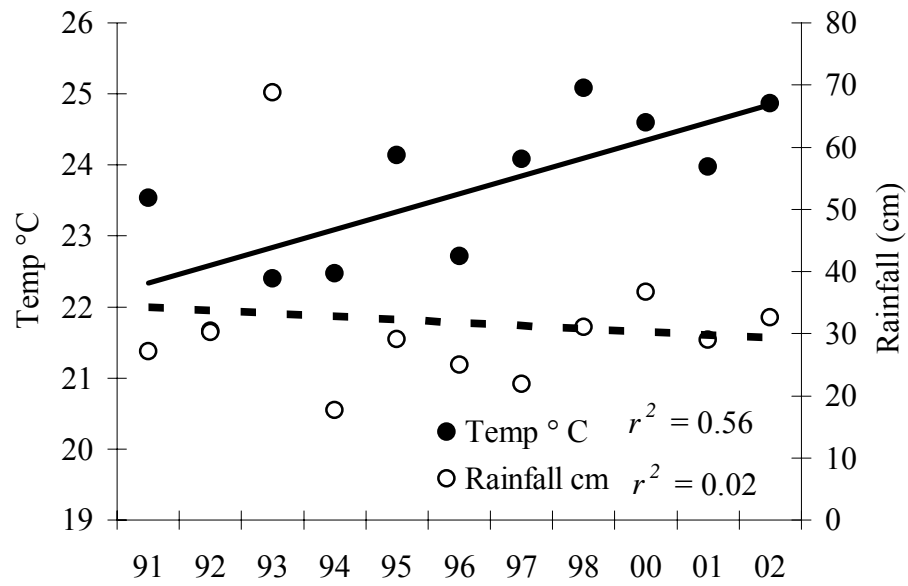


Figure 4. Environmental conditions during an 11-year study. Average daily temperature and rainfall were recorded over the span of the experiment. Temperature (●) during the span of the trial increased an average 0.25 °C per year ($P \leq 0.05$).

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