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Long-Term Study of Weather Effects on Soybean Seed Composition

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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 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 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.

***Keywords*:** crop rotation, seed composition, soybeans

Production economics are factors in the selection of agronomic practices (Katsvairo & Cox, 2000). Although rotation of cereals, such as wheat and corn with legumes is a long-standing cultural method (Campbell *et al.*, 1991; Vanotti & Bundy, 1995), the advent of mechanization has led to 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 (Dodor & Tabatabai, 2003) which in turn influences content of seed storage compounds that are important in human and animal nutrition (Sexton *et al.*, 1998). Soil organic carbon (SOC) is one indicator of soil quality and has an influence on the chemistry and biology of soil (McCallister & Chein, 2000; Kristensen *et al.*, 2003; Sparling *et al.*, 2003; Shukla *et al.*, 2004). Since crop rotation influences this parameter (Reeves, 1997; West & Post, 2002), 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 (Anderson & Domsch, 1989; Anderson & Gray, 1990).

Enzymatic activity necessary for mineralization of plant nutrients has been found correlated to SOC (Jordan *et al.*, 1995). 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 (Kim *et al.*,1999; Krishnan *et al.*, 2000; Nakasathien *et al.*, 2000; Paek *et al.*, 2000). 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 arbusucular mycorrhiza (Bethlenfalvay *et al.*, 1997). Rhizobial nodulation factors have been found to enhance the colonization of soybean roots by mycorrhizobia (Xie *et al.*, 1995). Tripartate symbiosis involving plant mycorrhiza and bacteria benefits from an increased soil organic carbon (Xie *et al.*, 1995).

Soybean seed composition is influenced by environmental

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conditions during the growing season (Piper & Boote, 1999; Grieshop & Fahey, 2001; Yaklich *et al.*, 2002; Fehr *et al.*, 2003; Grieshop *et al.*, 2003). In a general sense, soybeans growing at higher temperatures contain more protein while those from cooler conditions contain higher percentage of oil (Wolf *et al.*,1982; Yaklich *et al.*, 2002) Soybeans of maturity groups II through IV exhibited the highest oil content while those of maturity group VIII were highest in protein (Yaklich *et al.*, 2002). The protein and oil content data were compiled from a multitude of soybean lines submitted for evaluation in uniform tests. 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 (Yaklich & Vinyard, 2004). Since variety and growing conditions have been reported to affect seed composition (Piper & Boote, 1999; Grieshop & Fahey, 2001; Yaklich *et al.*, 2002; Fehr *et al.*, 2003), 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, 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 × 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 (Buchholz, 1983). 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 × *g* for 15 min, the supernatant decanted to clean centrifuge tube, and combined with 50 µL 2-mercaptoethanol. Proteins were fractionated by SDS-PAGE (Laemmli, 1970) 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× 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.

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 (Figs. 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 (Figs. 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 (Figs. 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.

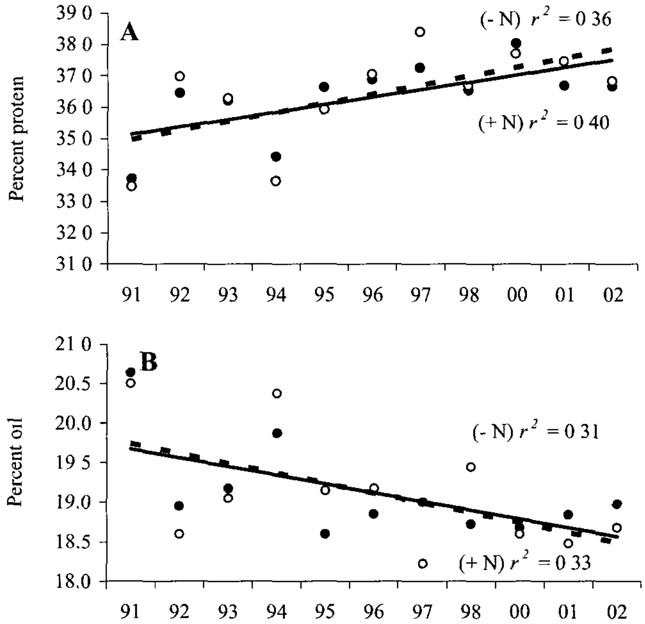

Fig. 1. Change in protein and oil content during an 11-year study Near infrared reflectance spectroscopy analysis revealed changes in protein (1A) and oil (1B) content Comparison of seed components from nitrogen fertilized (●) and non-fertilized (O) plots shows the effect of years (1A and 1B). Solid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

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 dur-

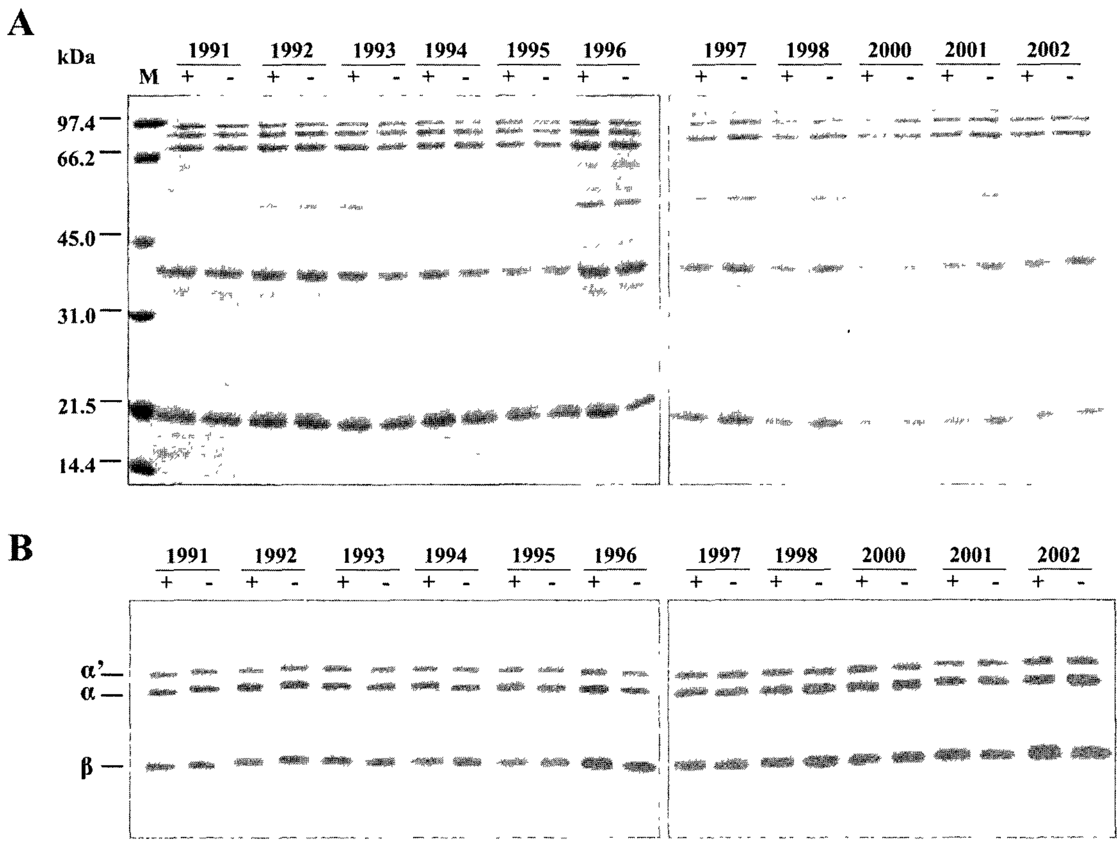

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of seed storage protein Seed proteins were fractionated on 12 5% polyacrylamide gel and stained with Commassie blue (1A) Proteins were electrophoretically transferred from a 10% gel to a nitrocellulose 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 (Fig. 1B)

ing the course of the experiment (Fig. 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 (Fig. 2A). Immunoblot analysis was used to assay this putative increase in protein content (Fig. 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 (Paek *et al.*, 2000; Ohtake *et al.*, 2002). Application of exogenous nitrogen increased the content of this subunit during only three years of the study 1996, 2001, and 2002 (Fig. 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 (Fig. 2B). 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 (Fig. 3). Application of nitrogen fertilizer did not alter the total fatty acid content or profile.

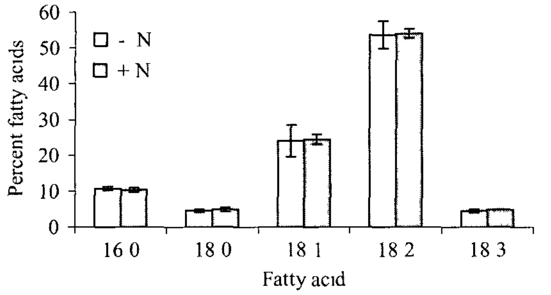

Fig. 3. Gas chromatographic determination of fatty acid content Fatty acids were solvent extracted and methylated in preparation for separation and quantification by gas chromatography. Error bars represent standard deviation of the mean of three replications of analysis

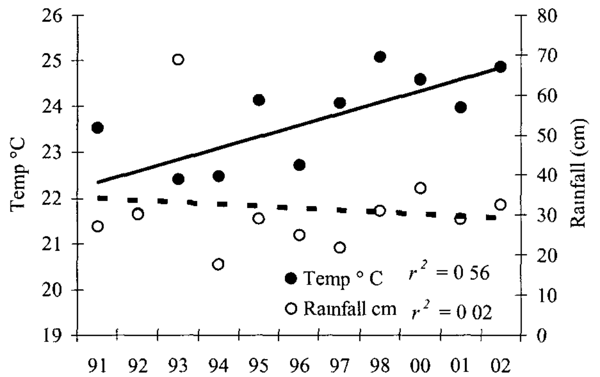

Fig. 4. Environmental conditions during an 11-year study. Average daily temperature and rainfall were recorded over the span of the experiment. Temperature (●) during the span of the trial increased an average 0.25°C per year ($P \leq 0.05$)

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 °C year^{-1} ($P \leq 0.05$) (Fig. 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 (Fig. 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.pdf). 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 (Fig. 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 (Hanson *et al.,* 1961; Simpson & Wilcox, 1983). Since oil contains twice the Kcal g^{-1} 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 (Hanson *et al.*, 1961; Chung *et al.*, 2003). 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 (Wolf *et al.*, 1982) and field studies (Piper & Boote, 1999; Yaklich *et al.*, 2002). 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 (Lavelle, 2000) which influences plant nutrient availability (Moore *et al.*, 2000; Dodor & Tabatabai, 2003). Whether the chemical, biological, and physical properties of soil (Hsieh, 1992; Haynes & Tregurtha, 1999; Hickman, 2002; Eivazi *et al.*, 2003) 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.

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REFERENCES

Anderson, T-H and K. H. Domsch 1989. Ratios of microbial biomass carbon to total organic carbon in arable soils. Soil Biol Biochem. 21 : 471-480.

Anderson, T-H and T. R. Gray. 1990. Soil microbial carbon uptake characteristics in relation to soil management. FEMS Microbiol. Ecol. 74 11-20

Bethlenfalvay, G J., R. P. Schreiner, and K. L. Mihara. 1997. Mycorrhizal fungi effects on nutrient composition and yield of soybean seeds J. Plant Nutr. 20 : 581-591.

Buchholz, D. D. 1983. Soil test interpretations and recommendations handbook Dept of Agronomy. University of Missouri Columbia, MO

Campbell, C. A., M. Schnitzer, G P Lafond, R. P. Zentner, and J E Knipfel 1991 Thirty-year crop rotations and management practices effects on soil and amino nitrogen Soil Sci. Soc. Am. J. 55 · 739-745.

Chung, J., H. L. Babka, G L. Graef, P E. Staswick, D. J. Lee, P B. Cregan, R C. Shoemaker, and J. E. Specht 2003 The seed protein, oil, and yield QTL on soybean linkage group I Crop Sci 43 : 1053-1067

Dodor, D E. and M. A. Tabatabai 2003 Effect of cropping systems on phosphatases in soils. J. Plant Nutr. Soil Sci 166 7-13

Eivazi, F., M R Bayan, and K. Schmidt 2003. Select soil enzyme activities in the historic Sanborn Field as affected by long-term cropping systems Commun Soil Sci. Plant Anal. 34 · 2259-2275.

Fehr, W. R., J. A Hoeck, S. L Johnson, P. A Murphy, J D. Nott, G I Padilla, and G A Welk. 2003 Genotype and environment influence on protein components of soybean. Crop Sci 43 · 511-514.

Grieshop, C M. and G C. Fahey 2001. Comparison of quality characteristics of soybeans from Brazil, China, and the United States J Agric. Food Chem. 49 : 2669-2673.

Grieshop, C. M., C T. Kadzere, G M Clapper, E A Flickinger, L L Bauer, R. L Frazier, and G C Fahey. 2003 Chemical and nutritional characteristics of United States soybeans and soybean meals. J Agric Food Chem 51 · 7684-7691

Hanson, W. D , R C Leffel, and R. W. Howell. 1961 Genetic analysis of energy production in the soybean Crop Sci 1 : 121-126.

Haynes, R J. and R. Tregurtha 1999 Effects of increasing periods under intensive arable vegetable production on biological, chemical and physical indices of soil quality. Biol Fert Soils 28 . 259-266.

Hickman, M. V. 2002. Long-term tillage and crop rotation effects on soil chemical and mineral properties. J. Plant Nutr. 25 1457-1470.

Hsieh, Y-P. 1992. Pool size and mean age of stable soil organic carbon in cropland. Soil Sci. Soc. Am J. 56 460-464.

Jordan, D., R. J. Kremer, W A Berfield, K. Y Kim, and V. N. Cacnio. 1995. Evaluation of micobial methods as potential indicators of soil quality in historical agricultural fields. Biol Fert Soils 19 : 297-302

Katsvairo, T. W and W. J Cox 2000. Economics of cropping systems featuring different rotations, tillage, and management. Ag J 92 : 485-493.

Kim, H., M. Y. Hirai, H Hayashi, M Chino, S. Naito, and T. Fujiwara. 1999. Role of O-acetyl-L-serine in the coordinated regulation of the expression of a soybean seed storage protein gene by sulfur and nitrogen nutrition. Planta 209 . 282-289.

Krishnan, H., G Jiang, A. M. Krishnan, and W J. Wiebold. 2000 Seed storage protein composition of non-nodulating soybean (*Glycine max* (L) Merr.) and its influence on protein quality Plant Sci. 157 : 191-199.

Kristensen, H , L. K. Debosz, and G W McCarty 2003. Short-term effects of tillage on mineralization of nitrogen and carbon in soil. Soil Biol Biochem 35 : 979-986

Laemmli, U. K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature 227 680-685

Lavelle, P 2000 Ecological challenges for soil science. Soil Sci 165 73-86

McCallister, D. L. and W. L. Chein. 2000 Organic carbon quantity and forms as influenced by tillage and cropping sequence Commun. Soil Sci Plant Anal 31 465-479

Moore, J M., S Klose, and M A.Tabatabai. 2000 Soil microbial biomass carbon and nitrogen as affected by cropping systems. Biol Fert Soils. 31 200-210.

Nakasathien, S., D W Israel, R. F. Wilson, and P Kwanyuen 2000. Regulation of seed protein concentration in soybean by supra-optimal nitrogen supply Crop Sci 40 1277-1284

Ohtake, N., T Kawachi, I Okuyama, H. Fujikake, K Sueyoshi, and T.Ohyama 2002 Effect of short-term application of nitrogen on the accumulation of beta-subunit of beta-conglycinin in nitrogen-starved soybean (*Glycine max* L) developing seeds Soil Sci Plant Nutr 48 31-41.

Paek, N C., P. J Sexton, S L Naeve, and R Shibles 2000. Differential accumulation of soybean seed storage protein subunits in response to sulfur and nitrogen nutritional sources. Plant Prod Sci. 3 268-274.

Piper E. L and K. J. Boote. 1999. Temperature and cultivar effects on soybean seed oil and protein concentrations. J. Am. Oil Chem Soc 76 1233-1241.

Reeves, D W 1997 The role of soil organic matter in maintaining soil quality in continuous cropping systems. Soil Tillage Res 43 . 131-167.

SAS Institute 1996. SAS user's guide. SAS Institute, Cary, NC.

Sexton, P. J , S L. Naeve, N. C. Paek, and R. Shibles. 1998. Sulfur availability, cotyledon nitrogen:sulfur ratio, and relative abundance of seed storage proteins of soybean Crop Sci. 38 · 983-986

Shukla, M. K., R Lal, and M. Ebinger. 2004. Soil quality indicators for the North Appalachian Experimental Watersheds in Coshocton Ohio. Soil Sci 169 · 195-205.

Simpson A. M. and J. R.Wilcox. 1983. Genetic and phenotypic associations of agronomic characteristics in 4 high protein soybean *Glycine-Max* populations. Crop Sci. 23 : 1077-1081.

Sparling, G., R. L. Parfitt, A. E Hewitt, and L. A Schipper. 2003. Three approaches to define desired soil organic matter contents. J Environ Qual. 32 : 760-766.

Vanotti, M. B. and L. G Bundy. 1995. Soybean effects on soil nitrogen availability in crop rotations Ag. J 87 . 676-680.

West, T. O. and W M. Post. 2002. Soil organic carbon sequestration rates by tillage and crop rotation· A global data analysis. Soil Sci Soc Am. J. 66 : 1930-1946

Wolf. R B.. J. F. Canvins. R Kleiman. and L. T. Black. 1982. Effect of temperature on soybean seed constituents: oil, protein, moisture, fatty acids, amino acids, and sugars J. Am Oil Chem. Soc. 59 : 230-232

Xie, Z -P, C. Staehelin, H. Bierheilig, A Wiemken, S Jabbouri, W.J. Broughton, R Voegeli-Lange, and T Boller. 1995. Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. Plant Physiol. 108 : 1519-1525

Yaklich, R W., B Vinyard, M. Camp, and S. Douglass 2002. Analysis of seed protein and oil from soybean Northern and Southern Region Uniform Tests. Crop Sci. 42 : 1504-1515

Yaklich, R. W and B T. Vinyard. 2004 Estimating soybean seed protein and oil concentration before harvest J. Am Oil Chem. Soc 81 : 189-194.