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Ecophysiological Effects of Nitrogen on Soybean [*Glycine max* (L.) Merr.]

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Abstract

Soybean [*Glycine max* (L.) Merr.] is a leguminous plant with high nutritional and medicinal value. The goal of this research was to determine the optimal concentration of nitrogen, using Hoagland nutrient solution, which will enhance the productivity of soybeans. The specific objective of the study was to assess the effect of variation of nitrogen concentration on soybean growth and leaf chlorophyll concentrations. Soybeans were grown under three soil nitrogen amendments: low, medium, and high concentration of Hoagland nutrient solution and a control group. Soybeans were grown under controlled environmental conditions in the Biotronette® environmental chamber. Temperature of the environmental chamber was regulated at 27°C and the photoperiod was set to 10 L: 14D. Soybeans grown in the low treatment group had the highest growth rate (1.03 ± 0.03 cm/day) compared to the control, medium, and high treatment groups. During the first chlorophyll analyses, the control group had the highest total chlorophyll concentration (216.25 ± 4.09 µg/mL/g). During the second chlorophyll analyses, the low treatment group had the highest total chlorophyll concentration (102.81 ± 14.54 µg/mL/g). Although no finding was statistically significant between groups, the low nitrogen treatment conditions had a trend towards producing more favorable physiological outcomes on soybeans.

Keywords

Soybeans, Nitrogen, Growth, Chlorophyll

1. Introduction

The soybean [*Glycine max* (L.) Merr.] plant is a member of the Leguminoceae family which includes other legumes such as peas, beans, lentils, peanuts, and other podded plants [1] [2]. Soybean has a history as a domesti-

cated plant with earliest accounts of its use dating as far back as the eleventh century BC in China [1]. Today, soybean is a major crop of global importance due to its nutritional and medicinal value [3]. As of 2007, annual global production of soybeans reached 206.4 million tons compared to 27 million tons produced annually across the globe during the 1960s [1].

Soybeans, as with all legumes, are well recognized as excellent sources of dietary protein [2]. Soybean protein is considered a complete protein because it contains ample amounts of the essential amino acids that are found in animal protein [4]. Isolated protein from soybean has a protein digestibility-corrected amino acid (PDCAA) score of 1.0, which is similar to casein and egg protein [4] [5]. This high quality protein content from soybean has contributed to its dietary popularity and has led to the proliferation of soy-based food products such as soy flour, soy tofu, soy protein concentrate, soy milk, soy-based medical nutrition products, and soy-meat products such as hotdogs and sausages [6] [7].

Soybeans are a unique source of the isoflavones, genistein and diadzein [5]. These isoflavones are naturally occurring phytoestrogens similar to mammalian estrogens and selectively bind to and activate estrogen receptor beta more than estrogen receptor alpha, and thus may have similar action as selective estrogen receptor modulators (SERMs) with beneficial effects on bone and heart without detrimental effects on breast and tissues [8]. Soybean and soy foods have potentially multifaceted health promoting effects including cholesterol reduction, improved vascular health, preserved bone mineral density and reduction of menopausal symptoms [5]. A study by Ho *et al.* indicated that soy protein and soy isoflavones independently had a modest raise in hip bone mineral density as well as total bone mineral concentration in women who have had menopause for 4 or more years [9]. Soybean oil has also gained clinical attention and was found to show no different effect than olive oil on infectious and noninfectious complications, glycemic control, inflammatory and oxidative stress markers, and immune function in critically ill patients [10]. There is enduring scientific interest on the health benefits of soybeans in both *in vitro* and *in vivo* studies.

Nitrogen is an essential mineral macronutrient required in the greatest amounts by plant [11]. Nitrogen is a major limiting factor for plant development, growth and overall crop yield [11]-[13]. Soybeans, as well as most leguminous plants, possess the ability to acquire nitrogen for growth through nitrogen fixation and inorganic nitrogen uptake from the soil [14] [15]. Soybeans perform nitrogen fixation through its symbiotic association with *Bradyrhizobioum japonicum* bacteria [16] [17]. *Bradyrhizobioum japonicum* form nodules in soybean roots and facilitate the process of nitrogen fixation [16]. Nitrogen fixation is a biological phenomenon characterized by conversion of atmospheric molecular dinitrogen (N_2) to ammonium ion (NH_4^+) which is further assimilated by cytosolic plant enzymes [17]. In addition, soybeans can also absorb and assimilate inorganic nitrogen, in the form of nitrates, from the soil [18]. The combined process of nitrogen fixation from atmospheric nitrogen and nitrogen assimilation from the soil ensure that soybean meet its large nitrogen demand for the production of protein rich seeds [18].

Previous studies comparing sources of nitrogen for soybeans suggest that nitrogen fixation, facilitated by *Bradyrhizobioum japonicum*, is ideal for soybean growth and productivity [14] [19]. Two studies by de Veau *et al.* and Kaschuk *et al.* have reported that *Bradyrhizobium* nodulated soybeans, in the absence of soil nitrogen supplementation, have higher photosynthetic rates compared to soybeans subjected to soil nitrogen supplementation [14] [19]. Other studies have also shown that soil nitrogen supplementation, albeit at low nitrogen concentration, may be beneficial for the growth and productivity of soybeans. More specifically, a study by Sabaratnam *et al.* noted an improved relative growth rate and higher net photosynthetic rate in soybeans supplemented with low concentration of nitrogen solution [20] [21]. Invariably, the study observed a decline in both relative growth rate and net photosynthetic rate in soybeans supplemented with higher concentration of nitrogen solution [20] [21].

The goal of this research was to determine the optimal concentration of nitrogen, using Hoagland nutrient solution, which will enhance the productivity of soybeans. The specific objective of the study was to assess the effect of variation of nitrogen concentration on soybean growth and leaf chlorophyll concentrations. The findings from this study will contribute vital information on the ideal concentration of nitrogen required for optimum soybean productivity.

2. Materials and Methods

2.1. Experimental Design

This is an experimental study with three treatment groups (low, medium, and high) and a control group. The

three treatment groups' designation was based on variation of nitrogen concentrations using Hoagland nutrient solution. Four replicates, containing two plants per replicate, were assigned to each group: control group, and low, medium, and high treatment groups to maintain the statistical validity of the data.

2.2. Soil Preparation

Soil was prepared by using the ratio of 5:2:8:1. Five pots of potting soil, two pots of garden soil, eight pots of top soil, and one pot of river sand were mixed thoroughly for homogeneity and to give the soil a loamy quality. The soil mixture was then placed into 16 plant pots (~2 lbs capacity), with four pot replicates for each of the three treatment groups and the control group.

2.3. Sowing of Soybean Seeds

Soybean seeds were washed with five percent Clorox® bleach for approximately five minutes to eliminate contaminants. Five seeds were sown in each pot and 100 mL of distilled water was added to each pot and left under room conditions (26˚C). As seedlings started sprouting, the plants were thinned to two plants per pot and were ready for the experiment.

2.4. Hoagland Nutrient Solution Preparation

The Hoagland nutrient solution [22] was used to make the desired concentration of nitrogen for the three experimental treatment groups. The compounds and concentration used for the preparation are presented on **Table 1**. The final volume of each of the three treatment group solution and the control group was 500 mL.

The concentration of the compounds for the low treatment group is the standard concentration for the Hoagland Nutrient Solution [22]. The concentrations of the compounds used for the medium and high treatment group are the modified concentrations of the Hoagland Nutrient Solution. It is worth noting that the concentration of Ca $(NO_3)_2$ and KNO_3 were increased exponentially in the treatment group pots because of their contribution in modifying the nitrogen concentration of the Hoagland nutrient solution. The concentration of the non-nitrogen contributing compounds was not altered as depicted on **Table 1**.

2.5. Application of Modified Hoagland Nutrient Solution to the Soil

The nutrient solution was applied to the soil on two occasions separated by a 7-day interval. The first nutrient application occurred 7 days after seeds were sown. In the first nutrient application, 50 mL of distilled water was added to the control and 50 mL of the low Hoagland nutrient solution, 50 mL of the medium Hoagland nutrient

Table 1. Concentration of compounds used for treatment groups based on the Hoagland Nutrient Solution.

Compounds	Control (ppm)	Low (ppm) (Standard) [22]	Medium (ppm) (Modified)	High (ppm) (Modified)
$Ca(NO_3)_2$	0	1653	3310	6611
KNO_3	0	506	1011	2022
KH_2PO_4	0	326	326	326
$MgSO_4$	0	493	493	493
Trace Elements Constituents: H_3BO_3—2.8 gm/L $MnCl_2{\cdot}4H_2O$—1.8 gm/L $ZnSO_4{\cdot}7H_2O$—0.2 gm/L $CuSO_4{\cdot}5H_2O$—0.1 gm/L $NaMoO_4$—0.025 gm/L	0	4.93	4.93	4.93
FeEDTA Constituents: EDTA·2Na—10.4 gm/L $FeSO_4{\cdot}7H_2O$—7.8 gm/L KOH—56.1 gm/L	0	74.3	74.3	74.3

solution, and 50 mL of the high concentrated Hoagland nutrient solution was added to each pot in the low, medium, and high treatment groups, respectively. A week later, a second 50 mL of the nutrient doses were applied to each pot similar to the first nutrient application. On the same day of the second application, 50 mL of distilled water was added to each of the 16 pots to ensure the soil remain moist.

2.6. Environmental Regulation for Plant Growth

Following the first application of the nutrient solutions to the soil, plant pots were transferred into the Biotronette® Environmental chamber for photoperiod and temperature regulation. Temperature was regulated at about 27˚C and photoperiod was regulated at 10 light (L): 14 dark (D) hours because soybeans are short day plants [23].

2.7. Measurement of Plant Growth

Three plant growth measurements were taken on days 7, 17, and 31 after sowing of the soybean seeds. Plant growth rate per day was calculated by dividing the average plant growth per treatment group by the number of days until the last plant growth measurement (31 days after seeds were sown).

2.8. Leaf Chlorophyll Analysis

Soybean leaves were collected twice for the chlorophyll analysis using the method outlined by Einhellig and Rasmussen [24]. The chlorophyll analysis was conducted in two sets (17^{th} and 31^{st} days of growth). After leaf collection, leaves were weighed using an analytical balance and transferred into a 50 mL test tube with 10 mL of 95% ethanol. The test tubes were labeled according to their respective experimental unit and wrapped in aluminum foil to avoid light. The test tubes were kept in the dark at room temperature. After 48 hours in ethanol solution, chlorophyll extract was decanted into a cuvette for absorbance (A) measurement at both 649 and 665 nanometer (nm) wavelengths using the Spectronic 20D+ spectrophotometer. Chlorophyll a and chlorophyll b concentrations were calculated using the following equations:

[µg Chlorophyll a/mL solution = (13.70) (Absorbance [A] at 665nm) – (5.76) (Absorbance [A] at 649nm)

[µg Chlorophyll b/mL solution = (25.80) (Absorbance [A] at 649nm) – (7.60) (Absorbance [A] at 665nm)

Due to variation in the weight of the soybean leaves used for chlorophyll analysis, chlorophyll concentration of the soybean leaves was calculated per gram using the formula:

Chlorophyll a concentration = [µg Chlorophyll a/mL ÷ weight (g)]

Chlorophyll b concentration = [µg Chlorophyll b/mL ÷ weight (g)]

2.9. Statistics

Data analysis was performed using IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corporation. Analysis of variance (ANOVA) was used to compare the differences between the means in the three treatment groups and the control group. Data are expressed as mean values ± standard deviation (SD).

3. Results and Discussion

3.1. Results

3.1.1. Growth Measurement

Soybean growth was measured on three occasions as described in the experimental section. The data on soybean growth rate and 31-day growth are presented on **Table 2**. Soybeans grown under the low treatment group had the highest growth rate (1.03 ± 0.03 cm/day) compared to the control (0.935 ± 0.05 cm/day), medium (0.959 ± 0.07 cm/day), and high (0.928 ± 0.11 cm/day) treatment groups. The 31-day growth findings were similar to the growth rate results as shown in **Table 2**. There was no statistically significant difference between the groups when evaluating soybean growth rate and 31-day growth ($P = 0.147$ for both). There was, however, a trend towards better growth in the low treatment group.

3.1.2. Chlorophyll Analysis

Chlorophyll a, chlorophyll b, and total chlorophyll were assessed twice as described in the experimental section.

There was no statistically significant difference between chlorophyll a, chlorophyll b, and total chlorophyll in either the first or second set of chlorophyll analysis as depicted in **Tables 3** and **4**. There was a general decline in the leaf chlorophyll concentration in the second chlorophyll analysis when compared to the first chlorophyll analysis as depicted in **Tables 3** and **4** and **Figures 1** and **2**.

Table 2. Growth measurement result.

	Control	Low	Medium	High	P-Value
Growth Rate (cm/day)	0.935 ± 0.05	1.038 ± 0.03	0.959 ± 0.07	0.928 ± 0.11	0.147
31-Day Growth (cm)	28.975 ± 1.40	32.175 ± 0.96	29.725 ± 2.07	28.775 ± 3.31	0.147

Table 3. First chlorophyll analysis result.

	Control	Low	Medium	High	P-Value
Chlorophyll a Concentration [µg/mL/weight (g)]	82.62 ± 2.93	81.36 ± 2.38	83.71 ± 8.12	77.75 ± 12.63	0.723
Chlorophyll b Concentration [µg/mL/weight (g)]	133.63 ± 2.27	131.51 ± 7.97	116.65 ± 33.02	125.531 ± 20.25	0.633
Total Chlorophyll [µg/mL/weight (g)]	216.25 ± 4.09	212.87 ± 8.67	200.36 ± 38.23	203.28 ± 32.85	0.791

Table 4. Second chlorophyll analysis result.

	Control	Low	Medium	High	P-Value
Chlorophyll a Concentration [µg/mL/weight (g)]	34.80 ± 2.59	38.36 ± 5.53	36.09 ± 3.86	36.40 ± 8.75	0.847
Chlorophyll b Concentration [µg/mL/weight (g)]	55.28 ± 4.55	64.45 ± 9.04	61.56 ± 7.39	65.14 ± 12.43	0.411
Total Chlorophyll [µg/mL/weight (g)]	90.08 ± 7.03	102.81 ± 14.54	97.65 ± 11.23	101.54 ± 21.14	0.610

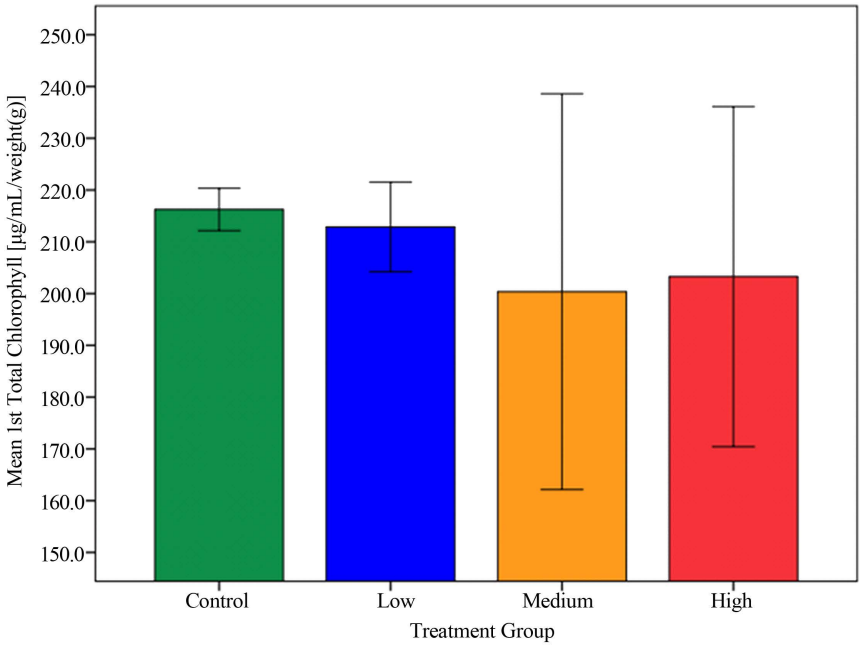

Figure 1. Mean total chlorophyll concentration [µg/mL/g] from the first chlorophyll analysis. All values are mean ± SD. Vertical bars indicate the SD within each treatment group.

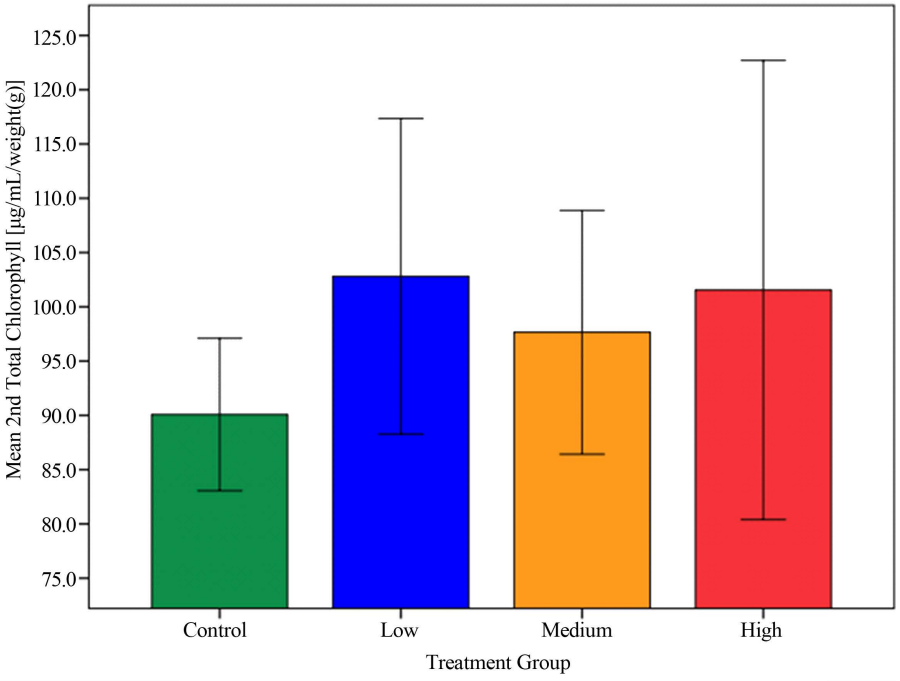

Figure 2. Mean total Chlorophyll concentration [µg/mL/g] from the second chlorophyll analysis. All values are mean ± SD. Vertical bars indicate the SD within each treatment group.

The results of chlorophyll a, chlorophyll b, and total chlorophyll from the first set of chlorophyll analysis are presented in **Table 3** with **Figure 1** showing the total chlorophyll concentration from the first chlorophyll analysis. The soybeans grown under the control group had the highest total chlorophyll concentration (216.25 ± 4.09 µg/mL/g) compared to soybeans grown under the low (212.87 ± 8.67 µg/mL/g), medium (200.36 ± 38.23 µg/mL/g) and high (203.28 ± 32.85 µg/mL/g) treatment groups. The soybeans treated in the low treatment group had the second highest total chlorophyll concentration. Although there was no statistically significant difference between the groups, there was a trend suggesting higher total chlorophyll concentration in the control and low treatment groups compared to the medium and high treatment groups. The soybean in the medium treatment group had the lowest total chlorophyll concentration in the first chlorophyll analysis.

The results of chlorophyll a, chlorophyll b, and total chlorophyll from the second set of chlorophyll analysis are presented in **Table 4** with **Figure 2** showing the total chlorophyll concentration from the second chlorophyll analysis. In general, there was a decline in the leaf chlorophyll concentration in the second chlorophyll analysis compared to the first chlorophyll analysis. In the second chlorophyll analysis, the soybeans in the low treatment group had the highest total chlorophyll concentration (102.81 ± 14.54 µg/mL/g) compared to the control (90.08 ± 7.03 µg/mL/g), medium (97.65 ± 11.23 µg/mL/g), and high (101.54 ± 21.14 µg/mL/g) treatment groups. Although there was no statistically significant difference between the groups, there was a higher total chlorophyll concentration in the low treatment groups compared to the control, medium and high treatment groups. The soybeans in the control group had the lowest total chlorophyll concentration in the second chlorophyll analysis.

3.2. Discussion

In our study, there was no statistically significant difference observed between the treatment groups when evaluating growth rate and 31-day growth. There was however, a trend to higher growth rate in the low treatment group. A study by Sabaratnam and colleagues evaluated growth characteristics of soybean subjected to different concentration of nitrogen as relative growth rate (RGR) [21]. The study revealed a statistically significant reduction in RGR by 47% in soybean treated with higher concentration of NO_2 (0.5 ppm) compared to soybean treated with lower concentrations of NO_2 (0.1, 0.2, and 0.3 ppm) and the control group [21]. The finding from this study supports our finding because the soybeans grown under the high concentration of nitrogen in our study had the lowest growth rate and 31-day growth compared to the soybeans under the control, low, and me-

dium treatment groups. Our finding provides supporting evidence that supplementation of soybean with low concentration of nitrogen may improve the growth of soybeans.

Another study by de Veau *et al.* assessed leaf area of soybean grown under three different nitrogen regimens [14]. The three regimens include: a) Nod+/+ group which was inoculated with *Bradyrhizobium japonicum* and received a nutrient solution containing 6 millimolar NH_4NO_3; b) Nod+/– group which was inoculated with *Bradyrhizobium japonicum* and did not receive a nutrient solution containing nitrogen; c) Nod– group was not inoculated with *Bradyrhizobium japonicum* but received a nutrient solution containing 6 millimolar NH_4NO_3 [14]. This study found that the Nod+/– group which did not receive a nutrient solution containing nitrogen had a statistically significant smaller leaf area which is an indication that the growth of the Nod+/– group was nitrogen limited [14]. The finding from this study suggests that supplementation of soybeans with lower concentration of nitrogen may enhance the growth characteristic of soybeans.

In our study, we observed no statistically significant differences between the treatment groups in the first and second chlorophyll analyses. In the first chlorophyll analysis, the control and low treatment groups had the highest total chlorophyll concentration compared to the medium and high treatment groups although this finding was not significantly different between the groups. In the second chlorophyll analysis, the low treatment group had the highest total chlorophyll concentration compared to the control, low, and medium treatment groups. Overall, when assessing the cumulative concentration of leaf chlorophyll from the two chlorophyll analyses, there was a trend to increase in leaf chlorophyll concentration in the low treatment groups compared to the control, medium and high treatment groups.

Sabaratnam *et al.* in their study observed a significant reduction in chlorophyll a and total chlorophyll content in soybeans treated with higher concentration of NO_2 (0.5 ppm) compared to soybeans treated with lower concentrations of NO_2 (0.1, 0.2, and 0.3 ppm) and in the control group [20] [21]. The reduction in chlorophyll a and total chlorophyll were 45% and 47%, respectively [20] [21]. This finding is similar with our finding and suggests that with higher concentrations of nitrogen there is a decline in leaf chlorophyll concentration. Sabaratnam *et al.* also reported decline in net photosynthetic rate in soybean treated with 0.5 ppm NO_2 compared to soybean treated with lower concentrations of NO_2 (0.1, 0.2, and 0.3 ppm) and controls [20] [21]. This finding supports prior studies reporting photosynthetic rate decline with lower chlorophyll content [25] [26].

deVeau *et al.* in their study assessed the leaf chlorophyll content of the soybean grown under three different nitrogen regimens [14]. The three regimens have been described earlier and include: a) Nod+/+, b) Nod+/– and c) Nod– [14]. The study found that the Nod+/– group which did not receive a nutrient solution containing nitrogen had a statistically significant lower leaf chlorophyll content compared to soybean grown with nutrient solution, which is an indication that the leaf chlorophyll content of the Nod+/– group was nitrogen limited relative to Nod+/+ and Nod– plants [14]. This study, however, did find a statistically significant higher photosynthetic rate in the Nod+/– soybeans suggesting that these plants may be more efficient in utilizing their chlorophyll for photosynthetic CO_2 uptake [14].

Certain limitations are applicable to our study. First, we did not evaluate the effect of variation of nitrogen on soybean photosynthetic rate. Second, this study is susceptible to a type II error and may not have adequate power to detect a statistically significant difference between the three treatment groups and the control group. Our future research will focus on the photosynthetic rates of soybean under different concentrations of nitrogen and its impact on overall yield.

4. Conclusion

This study evaluated the effects of variation of nitrogen concentration, using Hoagland nutrient solution, on soybean growth and leaf chlorophyll concentrations. There was no statistically significant difference between groups when assessing growth rate, 31-day growth, and leaf chlorophyll concentration. This study, however, provides some positive evidence to support that supplementation of soybean with low concentration of nitrogen (based on Hoagland nutrient solution) may produce relatively ideal physiological outcomes for soybeans.

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