Agriculture and Food

AIMS Agriculture and Food, 9(4): 1004–1026.
DOI: 10.3934/agrfood.2024054
Received: 01 July 2024
Revised:16 August 2024
Accepted: 19 September 2024
Published: 14 October 2024

http://www.aimspress.com/journal/agriculture

Research article

Effects of nitrogen topdressing fertilization on yield and quality in soybeans

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Abstract: Soybean [*Glycine max* (L.)] has higher nitrogen requirements than other crops. We investigated the effects on soybean yield and quality of topdressing with nitrogen fertilizer. Nitrogen fertilizer was applied as a topdressing to soybeans at 0, 20, 30, and 40 kg ha^{-1} (N0, N20, N30, and N40 treatments, respectively); half of the total topdressing treatment was applied at the pre-flowering (R 1) stage and the other half at the post-flowering (R 2) stage. Yield was highest in the N20 treatment and decreased with larger quantities of topdressing. The protein and total amino acid content were highest in the N20 treatment but tended to decrease with a greater quantity of topdressing. contents of most individual amino acids peaked in the N20 or N30 treatments and decreased as topdressing quantity increased, although proline and arginine contents increased with quantity of topdressing. Isoflavone content tended to be highest in either the N30 or N20 treatment. The agronomic efficiency of nitrogen (AE_N) was highest in the N20 treatment. There was a positive correlation between AE_N and yield, protein, isoflavone, and amino acid content. Topdressing with 20 kg ha^{-1} N produced the highest yield, protein, and amino acid content. Topdressing with greater quantities of nitrogen fertilizer decreased the yield and quality of soybeans.

Keywords: amino acid; isoflavone; protein; nitrogen fertilizer; topdressing; yield

1. Introduction

Soybean [*Glycine max* (L.)] is one of the most important crops grown worldwide as a source of vegetable oil and protein. Soybeans contain 40% protein, 30% carbohydrates, and 20% fat, as well as a variety of other functional substances such as isoflavones, phytic acid, and lutein. Fertilizers have an important impact on the growth and development of soybean, which are known to have higher nitrogen requirements than other crops [1]. Soybean plants demand a notably high quantity of nitrogen, requiring 500 kg of nitrogen to produce a yield of 5 Mg ha^{-1} [2]. Soybeans mainly absorb nitrogen in two ways: nitrogen fixation by rhizobia and nitrogen uptake from the soil [3]. Although much controversy surrounds the subject, it is widely accepted that a relationship exists between biological nitrogen fixation (BNF) and uptake from the pool of minerals in the soil, such that N fixation is inhibited when the N content in the soil is high, and N fixation is high when the N content in the soil is low [4,5]. BNF activity alone is insufficient to improve soybean yield and quality. Many researchers have found that it is necessary to apply a topdressing of the appropriate amount of nitrogen fertilizer (starter N) to increase BNF activity [6,7]. As soybeans do not form root hairs until 9 days after emergence and begin nitrogen fixation 14 days after sowing, early nitrogen application as a base fertilizer is required to ensure growth [8,9]. It is also necessary to apply nitrogen (N) topdressing fertilizer as the nitrogen requirements of soybeans are highest during the flowering and seed-filling stages [10,11]. Some reports suggest that nitrogen topdressing is ineffective and that BNF microorganisms, alone or in combination with other nitrogen-fixing microorganisms, can supply a sufficient quantity of nitrogen [12–15]. It is generally accepted, however, that nitrogen fertilizer has a significant impact on soybean yield and that soybean has a high nitrogen requirement during its later growth stages when BNF activity alone cannot supply the plant's requirements [16]. A single application of NPK fertilizer as a base fertilizer within the entire soybean growth period resulted in very low yields but applying an N or NPK topdressing fertilizer increased yield [6,17]. Khan et al. [18] also reported that applying organic matter and NPK as a topdressing fertilizer increased soybean oil content by 31% and yield by 73%, compared with a single treatment with base fertilizer. Gan et al. [19] reported that applying N as a topdressing fertilizer increased yield by 21–26%, compared with a treatment without N topdressing.

Insufficient moisture and nutrients during the reproductive growth stages, particularly during the flowering period and seed-filling stages, have a significant impact on soybean yield [20,21]. In areas of continuous soybean cultivation, BNF activity supplies, on average, 50–60%, although it can be up to 90% of the required nitrogen [22]. BNF activity, however, has little effect when soybeans are grown in an area for the first time [23]. It is, therefore, necessary to determine how much nitrogen supplementation is required during the flowering period (R1 and R2 stages), when soybeans have a high absorption capacity, of soybean plants that are being cultivated at a site for the first time. Many researchers have addressed the effects of variety and biotic treatments on protein content, functional component content, and amino acid content of soybeans, but far fewer researchers have focused on the component changes associated with nitrogen topdressing. This study was therefore conducted to investigate the effect of applying N fertilizer as a topdressing on the quality and yield of soybeans grown in soil that had not previously been used for their cultivation.

2. Materials and methods

2.1. Experimental site

Experiments were conducted in 2022 (first year) and 2023 year (second year) under the rain shelter of the plastic house at Gyeongsang National University located in Gajwa, Jinju, Gyeongsangnam-do, Korea. The experimental soil was sandy loam soil that had not been used previously for growing soybean; to determine the effects of nitrogen topdressing, it was not inoculated with nitrogen-fixing bacteria. The soil used in all experiments was analyzed and its chemical and physical properties are shown in Tables 1 and 2. Weather data were downloaded from the Korea Meteorological Administration Weather Data Open Portal [24].

2.2. Analysis of chemical and physical properties of experimental soil

Table 1. Chemical properties of the experimental soil during the 2022 and 2023 growing seasons.

pH (1:5)	EC (dS m^{-1})	P_2O_5 (mg kg^{-1})	T-N (%)	O.M. (g kg^{-1})	K^+	Ca^{2+}	Mg^{2+}	Na^+
					Ex. cation (cmol kg^{-1})			
7.15	0.08	265.0	0.09	13.3	0.22	7.25	0.58	0.44

Table 2. Physical properties of the experimental soil during the 2022 and 2023 growing seasons.

Three phases of soil (%)			Soil hardness (mm)	Soil characteristic (%)				
Solid	Liquid	Gas		Clay	Sand	Silt	Bulk density (g cm^{-3})	Soil texture
56.5	14.0	29.5	7.2	10.2	56.7	32.2	1.5	Sandy loam

The soil samples were air-dried at room temperature and sieved prior to chemical analyses. Soil pH and electrical conductivity (EC) were determined in a soil: water (1:5) suspension using a pH/conductivity meter. Available phosphorus was analyzed by the Lancaster method using a continuous flow spectrometer. The total nitrogen and organic matter content were analyzed by the Kjeldahl method and the Tyurin method, respectively [25]. Exchangeable cations (K^+, Ca^{2+}, Mg^{2+}, and Na^+) were extracted by 1 N NH_4OAc (adjusted pH 7.0) and quantified using inductively coupled plasma spectroscopy. The physical properties, three phases, and bulk density of the topsoil samples were analyzed according to the core method [26]. Soil hardness was measured at a depth of 20 cm in the experiment field using a Yamanaka soil hardness tester. Soil texture, including its clay, sand, and silt content, was investigated using the micropipette method, according to the USDA soil taxonomy method [27]. All physical and chemical analyses of soil were conducted at the National Institute of Crop Science, Rural Development Administration (RDA), according to the soil standard analysis methodology of the National Institute of Agricultural Science and Technology in Korea [28].

2.3. Experimental design and treatments

The cultivar Daewon was used in all experiments, which is a mid-late ripening variety of soybean widely cultivated in Korea. The standard amount of fertilizer (N-P_2O_5-K_2O: 32-32-36 kg ha^{-1}; Chobi, Seoul, Korea) and 15 Mg ha^{-1} (N ratio: 0.41%) of composted livestock manure (Seung Green Tec Co., LTD, Miryang-si, Korea) were applied prior to sowing. Nitrogen fertilizer was topdressed with solid urea (N content 46%). Different quantities of nitrogen (N) topdressing were applied in four treatments: N0 (0 kg ha^{-1}; control or nontreatment: Application of base fertilizer only), N20 (20 kg ha^{-1}), N30 (30 kg ha^{-1}), and N40 (40 kg ha^{-1}). Each topdressing treatment was divided into halves; one half was applied at the pre-flowering (R1) and the other at the post-flowering (R2) stage (Table 3). Soybean sowing was performed using a 15-cm plant spacing and an 80-cm row spacing. Two seeds were sown into each hole and were treated as one plant. The sowing density was 16.6 seeds m^{-2}. The emergence rate of soybeans was 95%. In 2022, seeds were sown on June 20, topdressing was applied on July 25 and August 5, and soybeans were harvested on October 21. In 2023, sowing occurred on June 25, topdressing was applied on July 29 and August 9, and harvesting of soybeans took place on October 25. Wang et al. [29] suggested that the optimal soil moisture content for soybeans should be greater than 15.5% (v/v). The soil moisture level of all treatments during the growing season was therefore maintained at 16% (v/v) at a 20 cm soil depth *via* automatic irrigation by a soil moisture sensor (WT-700, Mirae Sensor, Seoul, Korea). The experimental plot (total area 50 × 12 m) was laid out in a randomized block design containing three replicates with 10 plants per replicate. Agronomic efficiency of nitrogen (AE_N) was calculated for each quantity of nitrogen topdressing fertilizer applied using the Dobermann method, as follows [30]: $AE_N = (Y - Y°)/F$, where F represents the quantity of N topdressing fertilizer applied (kg ha^{-1}), Y represents yield according to the quantity of N topdressing fertilizer applied, and Y° represents the untreated yield (*i.e.*, without application of N topdressing fertilizer).

Table 3. The amount of nitrogen fertilizer applied during the growing season.

Treatment	Base fertilizer (NPK) (kg ha^{-1})	N topdressing (kg ha^{-1})			Total (N) (kg ha^{-1})
		R1 stage	R2 stage	total	
N0	32-32-36	0	0	0	32
N20	32-32-36	10	10	20	52
N30	32-32-36	15	15	30	62
N40	32-32-36	20	20	40	72

Note: The application amount of nitrogen fertilizer refers to the ingredient content.

2.4. Evaluation of soybean growth and yield

The growth parameters and yield components of soybeans in the different topdressing treatment groups were determined at harvest every year. The growth parameters measured were stem height (SH; measured from the base of the stem to the top of the canopy), stem diameter (SD; diameter at the base of the stem), number of branches (NB), and number of nodes (NN); and the yield components were number of pods (NP), number of seeds per pod (NSP), hundred seed weight (HSW), and yield (Y). NP was calculated per plant. NSP was the mean number of seeds in each pod, and HSW was the mean weight of 100 seeds. Yield (Y) was calculated by examining the yield per plant and converting this to hectares. The yield calculation method based on the soybean yield components of the RDA in Korea was as follows: Y = (number of plants ha^{-1} × NP × NSP × HSW)/100. The number of plants investigated was expressed as the mean value of three replicates with 10 plants (20 individual plants) in each replicate.

2.5. Protein analysis

Protein analysis was performed using the method of Kim et al [31]. Soybeans were ground to a fine powder using a mill. A sample of the powder (0.5 g) was mixed with 10 mL 85% n-hexane at room temperature and agitated for 10 min. After agitating, the sample was left to settle before the supernatant was discarded. This process was repeated five times. Residual n-hexane was then volatilized using nitrogen gas, and 0.1 g of extracted sample was added to 1.0 mL distilled water. Protein was extracted by stirring for 10 min and then centrifuging the sample at 15,000 rpm for 15 min at 4 °C. The supernatant was collected and placed in a water bath at 100 °C for 3 min before analysis. For protein quantitative analysis, 30 µL of the sample was added to 1.5 mL Bradford solution in a 96-well plate, allowed to react for 20 min, and then absorbance at 595 nm was measured using a spectrophotometer. A standard curve was established using bovine serum albumen at concentrations of 0.125, 0.25, 0.50, 0.75, and 1.0 mg mL^{-1}.

2.6. Isoflavone analysis

A 1.0 g sample of ground soybean powder was mixed with 40 mL 80% methanol (MeOH) and extracted for 2 h in a sonic water bath at 20 °C [32]. Subsequently, 4 mL 2M sodium hydroxide (NaOH) was added to saponify the extract, together with 1.0 mL acetic acid, and 80% MeOH was added to make a final volume of 50 mL. The mixture was left to react for 30 min, then 5.0 mL of the supernatant was collected and mixed with 4 mL distilled water; 80% MeOH was added to make a total volume of 10 mL. Each sample was filtered using a 0.45 µm membrane filter and analyzed using high-performance liquid chromatography with diode array detection (HPLC-DAD) on a ZORBAX Eclipse XDB-C18 column. The mobile phase consisted of 65% water and 35% methanol at a flow rate of 1.0 mL/min. The measurements were made under ultraviolet light (260 nm) with an injection volume was 5.0 µL and a column temperature of 40 °C. A standard curve was constructed using daidzin, genistin, and glycitin standards diluted to concentrations of 10, 20, and 30 ppm, respectively.

2.7. Amino acid analysis

A 0.5 g sample of ground soybean was added to 10 mL 6 N hydrochloric acid (HCl) and purged with nitrogen gas for 1 min. The test tube was sealed and placed in a dry oven at 110 °C for 22 h to hydrolyze [33]. The samples were allowed to cool at room temperature and concentrated at 50 °C using a rotary evaporator. The concentrated sample was diluted five-fold by adding 8.0 mL 0.02 N HCl to dissolve the sample. The diluted sample was filtered using a 0.22 µm membrane filter and analyzed by HPLC-DAD on a ZORBAX Eclipse AAA column; the column temperature was 40 °C, and the injection volume was 20 µL. The mobile phase consisted of 40 mM Na_2HPO_4 as solvent A and ACN: MeOH: water (45: 45: 10) as solvent B at a flow rate of 1.0 mL/min. In the mobile phase, solvent B was 0% for

0–1.9 min, 57% for 1.9–18.1 min, 100% for 18.1–22.3 min, and 0% for 22.3–30.0 min. The levels of 19 primary amino acids were determined at 338 nm with a bandwidth of 10 nm; the levels of two secondary amino acids were determined at 262 nm with a bandwidth of 16 nm.

2.8. Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) in SPSS version 21 (SPSS Inc., Chicago, IL, USA). All data were tested ($P > 0.05$) for normality (Shapiro-Wilks' method) and homogeneity (Levene's method). Statistical differences between mean values were determined using Duncan's multiple range test (DMRT) at the $P < 0.05$, $P < 0.01$, and $P < 0.001$ levels. R (R studio server pro 4.2.3) was used to calculate Pearson's correlation coefficient ($P < 0.05$, 0.01, 0.001) and perform principal component analysis (PCA) to identify the effects of amino acid content in each quantity of N topdressing. In the PCA analysis, analysis of components by section (N0 and N20, N0 and N30, N0, and N40) was determined to use the value of component loading of PC1, which has high explanatory power of the amino acid content on the level of topdressing and is represented as a mean value over 2 years.

3. Results

3.1. Meteorological conditions during the experimental period

The temperature was summarized as the monthly mean climatic data and compared with the mean long-term data for the city of Jinju in Korea (Table 4). The mean temperature in June, the sowing season for soybeans, was higher than the long-term season mean (LT) in both the first (`22) and second years (`23). In July, the flowering stage (R1) of soybean, the mean temperature was 1.3 and 0.4 °C higher than LT in both years, and the minimum temperature was also 0.6 and 0.7 °C higher than LT in both years, the maximum temperature was 1.8 °C and 0.1 °C higher than LT in the first and second years, respectively. In August, when soybean is in the R2 stage, the mean temperature was 0.4 °C and 1.4 °C higher than LT, the minimum temperature was 0.7 °C and 1.3 °C higher than LT, and the maximum temperature was 0.4 °C and 1.5 °C lower and higher than LT, respectively. In September and October (R5–R8 stages), the ripening and harvesting seasons, the mean and maximum temperatures were higher than LT in both years, but the minimum temperature was similar to LT in both years. Overall, the mean, minimum, and maximum temperatures all tended to be higher than LT during the experimental period, and the maximum and minimum temperatures of the second year were significantly higher than those of the first year.

3.2. Effects of nitrogen topdressing on growth and yield of soybean

We investigated the effects of different N topdressing treatments on the growth of the soybean cultivar Daewon. Although there were no statistically significant differences between treatments in SH for the first year, and in SD for the second year, the growth parameters SH and SD differed overall ($P < 0.05$) between treatments in both the first and second years, as their values tended to increase following N topdressing in comparison with the N0 treatment (Table 5). SH tended to be greatest in the N20 topdressing treatment, and SD tended to be thickest in the N30 treatment. The growth parameters NB and NN differed between years as, although there were no significant differences between treatments, NB tended to be slightly higher in the N topdressing treatments than in the N0 treatment. Similarly, although NN was lower in the N0 treatment than in the topdressing treatments, there were no statistically significant differences between treatments.

Table 4. Historical monthly and growing season climatic data for the experimental area.

Months	Mean temperatures (°C)								
	Minimum			Maximum			Average		
	`22	`23	LT	`22	`23	LT	`22	`23	LT
Jun.	18.4	17.8	17.1	27.8	28.4	27.4	22.7	22.7	21.8
Jul.	22.5	22.6	21.9	31.4	29.7	29.6	26.5	25.6	25.2
Aug.	22.9	23.5	22.2	30.3	32.2	30.7	26.3	27.3	25.9
Sep.	17.0	19.9	16.7	27.3	28.3	27.0	21.8	23.5	21.4
Oct.	8.8	9.0	8.9	22.2	22.7	22.2	15.2	15.1	15.0
Average									
Jun. ~ Oct.	17.9	18.5	17.4	27.7	28.2	27.4	22.4	22.4	21.9

Note: LT: long-term mean climatic data for 1991–2020.

Table 5. Changes in the stem height, stem diameter, number of branches, and number of nodes according to the quantity of soybean nitrogen topdressing fertilizer applied.

Treatment		Stem height (cm)	Stem diameter (mm)	No. of branches (ea plant^{-1})	No. of nodes (ea plant^{-1})
`22	N0	70.0ns	4.6b	1.1ns	11.8c
	N20	79.3	5.1b	1.5	12.1bc
	N30	74.3	6.4a	1.1	13.5a
	N40	76.2	5.6ab	1.8	13.0ab
`23	N0	60.3c	4.2ns	1.8b	9.8ns
	N20	72.7a	4.8	2.5ab	10.6
	N30	67.2ab	4.5	2.7a	10.1
	N40	62.7bc	4.6	2.2ab	10.1
Av.	N0	65.2ns	4.4ns	1.5ns	10.8ns
	N20	76.0	5.0	1.9	11.8
	N30	70.8	5.5	1.9	11.6
	N40	69.5	5.1	2.0	11.4
Source of variance					
Treatment (T)		0.023*	0.022*	0.281^{ns}	0.066^{ns}
Year (Y)		<0.000***	<0.000***	<0.000***	<0.000***
T × Y		0.704^{ns}	0.042*	0.258^{ns}	0.052^{ns}

In `22 and `23 sections, NS: not significant; different letters indicate significant differences within the column at $P < 0.05$ by DMRT. In source of variance section, NS: not significant; *: significant at $P < 0.05$; **: significant at $P < 0.01$; ***: significant at $P < 0.001$.

The effects on the yield components of the different topdressing treatments during the soybean pre-flowering (R1) and post-flowering (R2) stages were investigated (Table 6). NP was highest in the N20 treatment in both the first and second years, with no significant difference between the other treatments. NSP was highest in the N20 and N30 treatments and lowest in the N0 treatment in both years, although there were no statistically significant differences between N topdressing treatments. Overall, however, NSP was higher in the topdressing treatments than in the N0 treatment. In the first year, HSW was highest in the N40 treatment, but there were no differences between the treatments in the second year. HSW ranged from 27.3 to 28.2 g when averaged over the two years, however, with the N40 treatment showing the highest value, followed by the N30, N20, and N0 treatments. The total yield was highest in the N20 treatment in both years, and the N0 treatment produced lower yields. In particular, the yield (3,600 kg ha^{-1}) of the N20 treatment was increased by 44% over that of the N0 control treatment (2,400 kg ha^{-1}). Yields were highest in the N20 treatment, while other fertilizer treatments yielded slightly lower yields.

Table 6. Changes in the number of pods and number of seeds per pod, hundred-seed weight, and yield according to the quantity of soybean nitrogen topdressing fertilizer applied.

Treatment		No. of pods (ea $plant^{-1}$)	No. of seeds per pod (ea $plant^{-1}$)	Hundred seed weight (g)	Yield (Mg ha^{-1})
`22	N0	36.1b	1.5b	28.1c	2.5c
	N20	41.0a	1.9a	28.8b	3.9a
	N30	37.1b	1.9a	28.8b	3.4ab
	N40	36.3b	1.7ab	29.6a	3.1bc
`23	N0	30.0b	1.7b	26.4ns	2.3c
	N20	33.6a	2.2a	26.5	3.3a
	N30	28.6b	2.3a	26.7	2.9b
	N40	26.2c	2.3a	26.8	2.7b
AV.	N0	33.1ns	1.6b	27.3b	2.4a
	N20	37.3	2.1a	27.7a	3.6ab
	N30	32.9	2.1a	27.8a	3.0ab
	N40	31.3	2.0a	28.2a	2.9b
Source of variance					
Treatment (T)		<0.000***	<0.000***	<0.000***	<0.000***
Year (Y)		<0.000***	<0.000***	<0.000***	<0.001***
T × Y		0.207^{ns}	0.181^{ns}	0.004**	0.704^{ns}

In `22 and `23 sections, NS: not significant; different letters indicate significant differences within the column at $P < 0.05$ by DMRT. In source of variance section, NS: not significant; *: significant at $P < 0.05$; **: significant at $P < 0.01$; ***: significant at $P < 0.001$.

Analysis of the correlations between soybean yield components and the quantity of N topdressing applied showed that AE_N was moderately correlated with NP and NSP. In particular, there was a very strong correlation (r = 0.81) between AE_N and Y. By contrast, AE_N and HSW were poorly correlated (Figure 1A). The analysis also showed that HSW had nearly no correlation with NSP but a positive correlation (r = 0.67) with NP. There was a moderate negative correlation between NP and NSP. There were no significant differences between N topdressing treatments in the yield components NSP and

HSW; however, NP and Y were highest in the N20 treatment (Figure 1B).

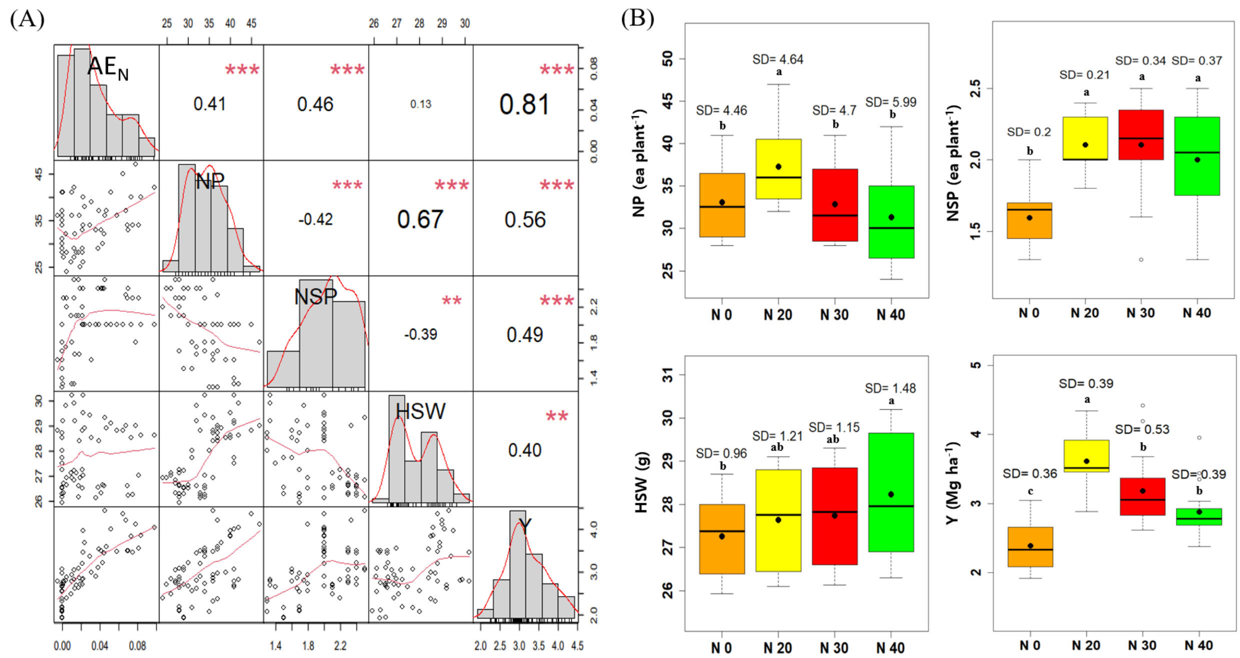

Figure 1. Correlation matrix plot (A) and boxplot (B) for yield components in soybean according to the quantity of nitrogen fertilizer topdressing applied during the 2022 and 2023 growing seasons. *: significant at P < 0.05; **: significant at P < 0.01; ***: significant at P < 0.001. The black circles in boxes in B indicate the means; SD indicates the standard deviation; different letters indicate significant differences within the column at P < 0.05 by DMRT. NP: number of pods; NSP: number of seeds per pod; HSW: hundred-seed weight; Y: yield; AE_N: Agronomic efficiency of nitrogen.

3.3. Effects of nitrogen topdressing on protein and isoflavone content in soybean

The effects of the different topdressing treatments on the protein and isoflavone content were investigated (Figure 2). Protein content was high, at 32.1%, in the N20 and N30 treatments but lower, at 31.4 % and 30.7% in the N0 and N40 treatments in the first year (Figure 2A). The N20 treatment tended to have the highest protein content in the second year, while the N0 treatment had the lowest. The mean protein content of soybean seeds was highest in the N20 treatment and then showed a tendency to decrease, such that N30 > N40 > N0.

The isoflavones daidzin, genistin, and glycitin are abundant in soybean seeds; their contents after the different topdressing treatments were therefore determined (Figure 2B). In the first year, the total isoflavone content ranged from 490 to 620 μg g^{-1}, the daidzin content ranged from 155 to 199 μg g^{-1}, the glycitin content from 60 to 73 μg g^{-1}, and the genistin content from 265 to 358 μg g^{-1}. The daidzin and genistin contents tended to be highest in the N30 treatment, whereas the glycitin content tended to be greatest in the N20 treatment. The total isoflavone content was lowest in the N0 treatment, whereas isoflavone content rose with the quantity of topdressing applied up to the N30 treatment and subsequently declined in the N40 treatment. In the second year, the daidzin, glycitin, and genistin contents ranged from 157 to 182 μg g^{-1}, from 47 to 57 μg g^{-1}, and from 206 to 257 μg g^{-1}, respectively, with a total isoflavone content of 410–490 μg g^{-1}. In contrast with the first year, the daidzin and genistin contents were highest in the N40 treatment, whereas the glycitin content was highest in the N0 treatment. Although the total isoflavone content showed the same trend in both years, it was lower in the second year than the first, producing a 2-year average of 450–550 μg g^{-1}. When the mean isoflavone content of soybeans was considered over the 2 years, levels of daidzin and genistin tended to be highest in the N30 treatment, followed by N40 > N20 > N0, whereas the N0 treatment contained the highest level of glycitin. The total isoflavone content peaked in the N30 treatment and then showed a tendency to decrease as the quantity of topdressing increased.

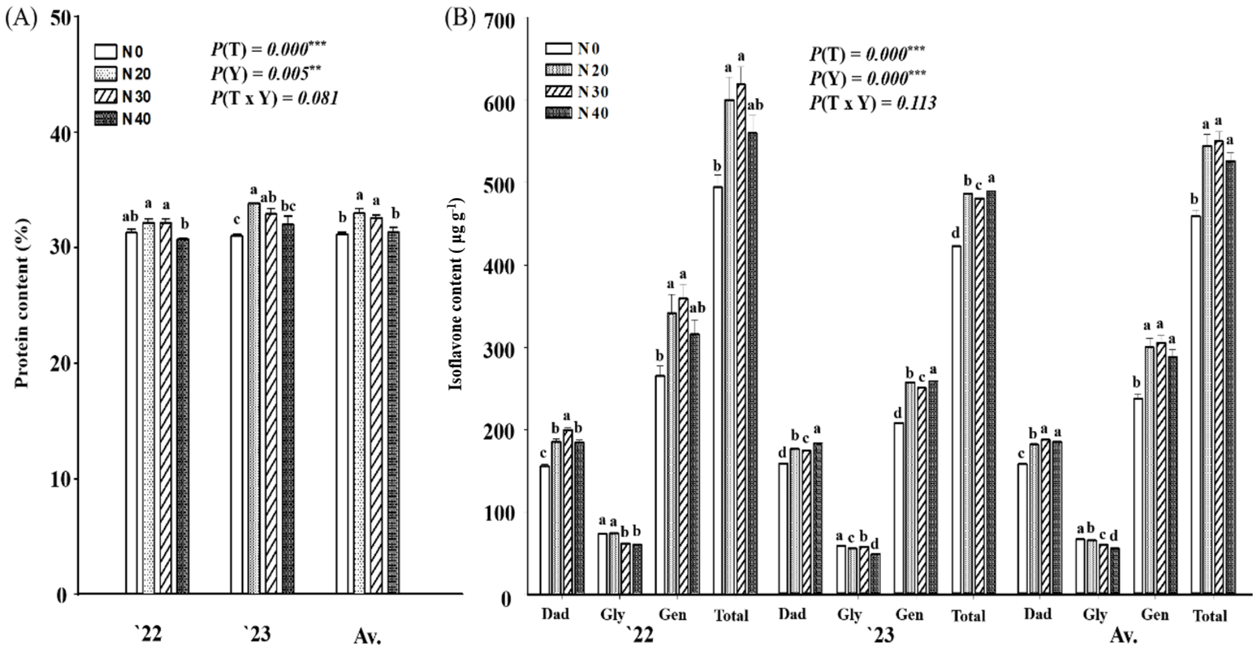

Figure 2. Protein (A) and isoflavone (B) content in soybean according to the amount of topdressing of nitrogen fertilizer applied. Dad, Gly, and Gen indicate daidzin, glycitin, and genistin, respectively. Vertical bars represent the standard error of the means (n = 3). Different letters indicate significant differences at 5% by Duncan's Multiple Range Test (DMRT, $P < 0.05$).

3.4. Effects of nitrogen topdressing on amino acid content of soybean

The amino acid content was investigated in the different topdressing treatments (Tables 7 and 8). In total, 18 of the 21 amino acids were detected in the first year and 17 in the second year. Tryptophan, asparagine, and glutamine were not detected in either year, and threonine was not detected in the second year. Rather than these absences being due to the amino acids not being present in the raw material, they may result from their decomposition during the acid hydrolysis step of sample preparation prior to amino acid determination.

Table 7. Changes in the essential amino acid composition of soybean according to the amount of nitrogen fertilizer topdressing applied.

Treatment		His*	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Total
		mg g^{-1}									
`22	N 0	9.7b	9.6b	22.3c	19.2c	1.3a	13.2c	16.7b	ND	8.5c	92.2b
	N 20	11.3a	11.4a	27.5a	22.3a	1.1a	16.7a	18.6a	ND	14.8a	109.1a
	N 30	7.0c	10.4ab	24.1b	20.7b	0.6b	14.3b	16.9b	ND	9.3b	94.4
	N 40	6.4c	9.5b	22.7c	16.1d	0.7b	13.4c	14.3c	ND	8.1c	83.0c
`23	N 0	20.6b	9.4b	17.8c	20.1ab	0.9b	20.1b	ND	ND	15.9a	105.2b
	N 20	23.1a	10.9a	19.9a	22.5a	1.0b	22.4a	ND	ND	17.1a	120.1a
	N 30	18.7b	6.1c	22.4b	20.9ab	2.3a	21.9a	ND	ND	9.2b	99.2b
	N 40	11.0c	5.5c	19.9b	17.1b	1.8a	19.0b	ND	ND	10.5b	85.1c
Av.	N 0	15.2ns	9.5ns	20.1ns	19.7b	1.1ns	16.7ns	ND	ND	12.2ns	98.7ab
	N 20	17.2	11.2	23.7	22.4c	1.1	19.6	ND	ND	16.0	114.6b
	N 30	12.9	8.3	23.3	20.8b	1.5	18.1	ND	ND	50.7	96.8ab
	N 40	8.7	7.5	21.3	16.6a	1.3	16.2	ND	ND	9.3	84.1a
Source of variance											
Treatment (T)		<0.000***	<0.000***	<0.000***	<0.000***	0.083 ns	<0.000***	<0.000***	-	<0.000***	<0.000***
Year (Y)		<0.000***	<0.000***	<0.000***	0.384 ns	<0.000***	<0.000***	<0.000***	-	<0.000***	<0.000***
T × Y		<0.000***	<0.000***	0.132 ns	0.953 ns	<0.000***	0.044*	<0.000***	-	0.008**	0.068 ns

* Essential amino acids. His: Histidine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Thr: Threonine; Trp: Tryptophan; Val: Valine. Different letters indicate significant differences within the column at $P < 0.05$ by DMRT. ND: not detected; NS: not significant; *: significant at $P < 0.05$; **: significant at $P < 0.01$; ***: significant at $P < 0.001$.

Table 8. Changes in non-essential amino acid composition of soybean according to the quantity of nitrogen fertilizer topdressing applied.

Treatment		Asn*	Arg	Asp	Cys	Gln	Glu	Gly	Ser	Ala	Tyr	Pro	Hyp	Total
		mg g^{-1}												
`22	N 0	ND*	22.8c	32.1b	0.9b	ND	55.4b	13.8b	1.9d	14.9b	10.2ab	22.2c	23.6b	198.2b
	N 20	ND	24.8b	36.4a	1.4a	ND	61.6a	12.9b	2.3a	19.5a	11.0ab	18.2d	29.5a	218.2a
	N 30	ND	25.7b	35.2a	1.1b	ND	52.6c	15.2a	2.1b	16.0b	11.4a	25.9b	27.2ab	212.9a
	N 40	ND	27.3a	30.4c	0.9b	ND	51.8c	13.8b	2.0c	14.9b	9.7b	28.8a	23.8b	203.7b
`23	N 0	ND	18.9ns	33.3bc	1.6b	ND	47.9bc	13.4b	16.6bc	7.7ab	0.9c	26.4c	2.6ab	169.8b
	N 20	ND	20.0	38.2a	2.2a	ND	53.6a	13.6b	18.5a	8.2a	0.9c	29.8bc	2.8a	188.4ab
	N 30	ND	20.1	36.0ab	1.7b	ND	50.5ab	19.8a	17.6ab	8.3a	1.2b	31.8ab	2.0ab	189.4a
	N 40	ND	18.4	32.4c	1.5b	ND	44.7c	21.5a	15.8c	7.3b	1.5a	35.5a	1.9b	181.0ab
Av.	N 0	ND	20.9ns	32.7ab	1.3ns	ND	51.7ns	13.6ns	9.3ns	11.3ns	5.6ns	24.3ns	13.1ns	184.0ns
	N 20	ND	22.4	37.1c	1.8	ND	57.6	13.3	10.4	13.9	6.0	24.0	16.2	203.3
	N 30	ND	22.9	35.6bc	1.4	ND	51.6	17.5	9.9	12.2	6.3	28.9	14.6	201.2
	N 40	ND	22.9	31.4a	1.2	ND	48.3	17.7	8.9	11.1	5.6	32.2	12.9	192.4
Source of variance														
Treatment (T)		-	<0.000***	<0.000***	<0.000***	-	<0.000***	<0.000***	0.001**	<0.000***	0.089 ns	<0.000***	0.009**	0.001**
Year (Y)		-	<0.000***	0.011**	<0.000***	-	<0.000***	<0.000***	<0.000***	<0.000***	<0.000***	<0.000***	<0.000***	<0.000***
T × Y		-	<0.000***	0.813 ns	0.066 ns	-	0.028*	0.004**	0.005**	<0.000***	0.020*	0.017*	0.023*	0.792 ns

* Non-essential amino acids. Asn: Asparagine; Arg: Arginine; Asp: Aspartic acid; Cys: Cysteine; Gln: Glutamine; Glu: Glutamic acid; Gly: Glycine; Ser: Serine; Ala: Alanine; Tyr: Tyrosine; Pro: Proline; Hyp: Hydroxy-proline. Different letters indicate significant differences within the column at $P < 0.05$ by DMRT. ND: not detected; NS: not significant; *: significant at $P < 0.05$; **: significant at $P < 0.01$; ***: significant at $P < 0.001$.

In the first year, the essential and non-essential amino acid content ranged from 83.0 to 109.1 mg g^{-1} and 198.2 to 218.2 mg g^{-1}, respectively, whereas in the second year, the essential amino acid content ranged from 85.1 to 120.1 mg g^{-1} and that of non-essential amino acids from 169.8 to 189.4 mg g^{-1}. The N20 treatment contained the highest essential amino acid content in both years. The non-essential amino acid content, however, tended to be highest in the N20 treatment in the first year but tended to be slightly higher in the N30 treatment in the second year, although there was no significant difference between the N20 and N30 treatments. The highest total amino acid content was in the N20 treatment in both years; total amino acid content in the other treatments followed the pattern N30 > N0 > N40. Overall, the total amino acid content tended to be higher in the first year than in the second. Leucine and lysine were the essential amino acids with the highest contents, and methionine the lowest, whereas, of the non-essential amino acids, significantly high glutamic acid and aspartic acid contents were present, but the cysteine content was the lowest.

Amino acid content was low in the N0 treatment, with the exception of a few amino acids. When the different topdressing treatments were compared, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, valine, aspartic acid, cysteine, glutamic acid, serine, alanine, and hydroxy-proline contents were highest in the N20 treatment but lower in the N40 treatment, as these amino acids tended to decrease as the amount of topdressing increased. By contrast, the proline and arginine contents tended to increase with the quantity of topdressing. There were no significant differences in the tyrosine and methionine contents between topdressing treatments.

The amino acid contents in the different topdressing treatments were compared. The total amino acid content was highest in the N20 treatment and tended to decrease in treatments involving greater quantities of topdressing. The amino acid content of soybeans was investigated over a two-year period and PCA was performed to determine which amino acids were most affected by nitrogen fertilizer (Figure 3A). PCA assigned the amino acids of soybean to 10 components across the different topdressing treatments. However, PC 1 (75.0%) and PC 2 (16.0%) comprehensively reflected 91% of the variation in the amino acids (Figure 3A). The proline, arginine, glycine, and methionine contents varied in the positive direction, whereas the other amino acids varied in the negative direction.

As this analysis did not explain the variation in amino acid content according to the quantity of topdressing applied, a sectional PCA analysis (N0 and N20, N0 and N30, N0 and N40) was performed using PC1, which had a strong explanatory power (Figure 3B). A sectional PCA analysis found that the contents of 10 of the 18 amino acids investigated in PC1 peaked in the N20 treatment and then decreased as the amount of topdressing increased, whereas the contents of four amino acids (glycine, tyrosine, leucine, and hydroxy-proline) were highest in the N30 treatment, and then decreased. The contents of some amino acids, including aspartic acid, glutamic acid, serine, histidine, threonine, alanine, tyrosine, cysteine, valine, methionine, isoleucine, phenylalanine, lysine, and hydroxy-proline, decreased when a large quantity of topdressing was applied (N40 treatment), and thus were negatively correlated with the quantity of topdressing. On the other hand, levels of proline and arginine rose consistently with the quantity of topdressing applied.

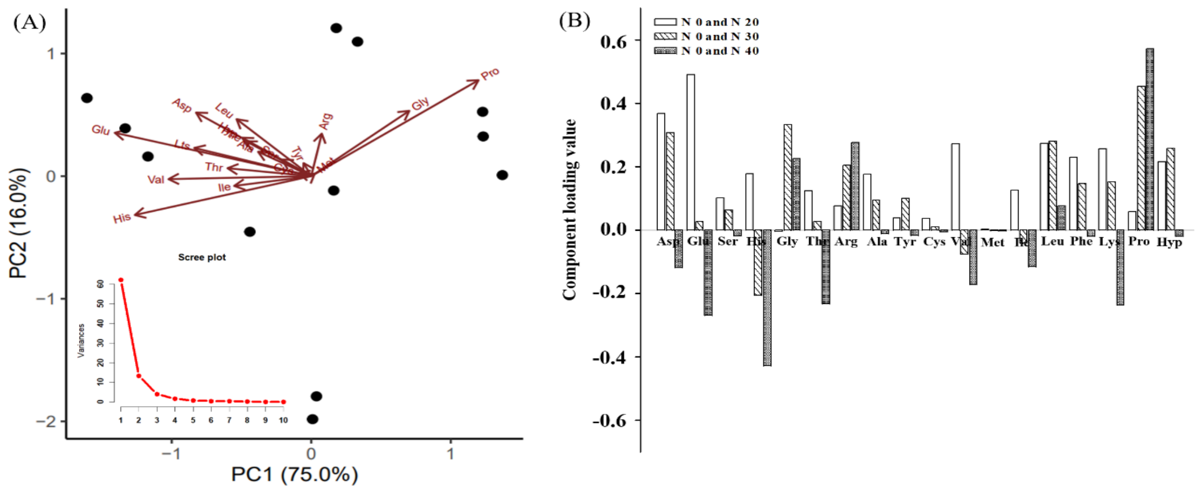

Figure 3. Analysis of the effect of the amount of nitrogen fertilizer topdressing on soybean amino acid components across two experimental years. (A) Principal component analysis (A). (B) Comparison of component loading value. Inset figure in A shows Scree plot. Asp: Aspartic acid; Glu: Glutamic acid; Ser: Serine; His: Histidine; Gly: Glycine; Thr: Threonine; Arg: Arginine, Ala: Alanine; Tyr: Tyrosine; Cys: Cysteine; Val: Valine; Met: Methionine; Ile: Isoleucine; Leu: Leucine; Phe: Phenylalanine; Lys: Lysine; Pro: Proline; Hyp: Hydroxy-proline.

3.5. Relationships between soybean AEN, yield, and quality

The relationships between AE_N, yield, and quality were analyzed according to the quantity of topdressing applied (Table 9). There was a high correlation between AE_N and yield (r = 0.81); given that the calculation to determine AE_N was itself based on yield, it is unsurprising that the correlation between these two factors (AE_N and yield) was very strong. AE_N correlated strongly with amino acid content (r = 0.744), but moderately with protein content (r = 0.525) and isoflavone content (r = 0.613). There was usually a strong correlation between yield and protein (r = 0.788), and between yield and amino acid (r = 0.780), but a moderate correlation between yield and isoflavone (r = 0.485). In the correlations between quality factors (protein, isoflavone, and amino acid), protein was strongly correlated with amino acid (r = 0.693), but not with isoflavone (r = 0.022). Furthermore, there was no correlation between amino acid and isoflavone (r = 0.298).

Table 9. Pearson's correlation between AE_N, quality, and yield according to the quantity of nitrogen topdressing of soybean.

Source of variation	AE_N	Yield	Protein	Isoflavone	Amino acid
AE_N	1	0.810***	0.525**	0.613***	0.744***
Yield		1	0.788***	0.485*	0.780***
Protein			1	0.022	0.693***
Isoflavone				1	0.298
Amino acid					1

*, **, *** indicate significant at $P < 0.05$, and significant at $P < 0.01$, significant at $P < 0.001$, respectively. AE_N: Agronomic efficiency of nitrogen.

4. Discussion

4.1. Growth and yield of soybean by nitrogen topdressing

In this experiment, although soybean growth was lowest in the N0 treatment, there were no statistically significant differences between the topdressing treatments (Table 5). Yield, however, was highest in the N20 treatment (Table 6; Figure 2B). Yield in soybean is comprised of various components, including NP, NSP, and HSW [34]. Previous research has shown that NP has the greatest impact on soybean yield, followed by NSP, and that HSW is poorly correlated with yield [35,36]. Teng et al. [37] stated that seed weight has a relatively low correlation with yield because it is an inherited trait closely associated with cultivar. In this study, however, yield had a moderate correlation with the yield components NP, NSP, and HSW because there was no linear relationship between the quantity of topdressing and yield: the yield components did not continuously increase with the quantity of topdressing but instead peaked at N20 (Figure 2B) because the plants did not absorb all of the fertilizer applied to them.

NSP and HSW differed significantly between the control and topdressing treatments. However, there was no significant difference between the topdressing treatments. This may be because there was a considerable hereditary influence on HWS, but the differences in NSP were due largely to environmental factors, such as the amount of light received during maturation [38]. Kantolic and Slafer [39] found a positive relationship between seed number and the timing and length of exposure to a long photoperiod during the R3–R6 stages. In this study, measurements of the growth components in the different topdressing treatments (Table 5) indicated that growth differed between the control and topdressing treatments, but not between the different topdressing treatments; this finding was consistent with the results for NSP, which showed no significant differences between topdressing treatments. By contrast, the NP increased with the NN [38,40]. Among the topdressing treatments, the highest NP was observed in the N20 treatment. Nitrogen supplied by topdressing allows a plant to transport nitrogen rapidly from leaves to seeds to sustain high growth rates during the reproductive stage [41]. Nitrogen applications in the 20–60 kg ha^{-1} range increase yield in soybeans, whereas applications above this amount reduce yield by inhibiting the activity of root rhizobia [42,43]. This reduces the proportion of nitrogen fixed and negatively affects nitrogen uptake due to antagonism between soil nitrate concentration and the nitrogen-fixing capacity of the rhizobia, limiting nitrogen uptake by the crop [6,44]. BNF activity is low in soil used to grow soybeans for the first time [45]. The application of nitrogen fertilizer, which acts as a supply of starter N to enhance BNF activity, plays a role in determining N availability and uptake in soil during the first cultivation of soybeans [46]. Applying the optimum amount of nitrogen fertilizer has significant effects on soybean yield because of these restrictions. Opinions on the optimal amount of nitrogen to apply vary between researchers; however, as the optimum amount depends not only on the effects of fertilizer on different cultivars with varying growth periods but also on environmental factors such as temperature, soil moisture content, and weather conditions in the field [44]. Under the environmental conditions of this study, applying a nitrogen topdressing of 20 kg ha^{-1} increased soybean yields, whereas applying higher amounts of topdressing reduced yield.

4.2. Protein and isoflavone contents in soybean vary according to the nitrogen topdressing treatments

The protein content of soybean seed varies depending on cultivation conditions but, in soybeans grown in Korea, the general range is from 32.5% to 43.7% [47,48]. The protein content in all treatments of the current study was slightly lower overall, at 30–35%. This may be because the soil moisture was held at about 16% under the rain shelter of the plastic house, as well as a maximum temperature over the experimental period that was higher than the long-term seasonal average (Table 4). Sobko et al. [49] reported that temperatures of 20–28 °C increased soybean protein content, but content decreased at higher temperatures. Carrera et al. [48] reported a negative correlation between precipitation and protein content. In the current study, the protein content of soybean seeds was highest in the N20 treatment but gradually decreased as the quantity of topdressing increased. Szostak et al. [49] and Ray et al. [50] report similar results; in their studies, the protein content increased with the quantity of nitrogen topdressing but decreased by 1.9–2.7% when excessive fertilizer was applied. La Menza et al. [51] report, however, that the protein content in soybean seeds significantly increased with the quantity of nitrogen applied, which contradicts the results of the current experiment. The protein content of soybean is strongly related to nitrogen accumulation in the seed during the seed-filling stage, suggesting that the accumulation of nitrogen in the seed is related to the nitrogen content of the soil, BNF activity, and translocation from the vegetative tissue to the seed [52]. It is widely accepted that topdressing treatments with excessive nitrogen reduce BNF activity [53]. Furthermore, protein synthesis is regulated by enzymes, including nitrate reductase (NR) and glutamine synthetase (GS), whose activities are inhibited by excessive nitrogen fertilizer [54]. Thus, it may be that, whereas excessive nitrogen topdressing reduces BNF, NR, and GS activities and consequently lowers protein content, topdressing with the appropriate quantity of nitrogen (N20) maintains these activities and increases protein content. Furthermore, soybean protein content is closely related not only to the quantity of nitrogen in the topdressing but also to environmental factors such as soil moisture and temperature, as discussed above. The precise relationship between the level of nitrogen fertilization and protein content in soybeans remains disputed and requires additional research, as its mechanisms are not yet well understood.

Isoflavones are the most highly represented functional components in soybeans. A total of 12 isoflavone compounds have been identified in soybeans, three of which, daidzein, genistein, and glycitein, have the basic skeleton of non-glycosides, whereas all of the rest, daidzin, genistin, glycitin, acetyl-daidzin, acetyl-genistin, acetyl-glycitin, malonyl-daidzin, malonyl-genistin, and malonyl-glycitin, are glycoside components [57]. The predominant isoflavones in soybeans, daidzin, genistin, and glycitin, are known glycosides, which contain sugars. These three compounds represent more than 80% of the total isoflavone content of soybeans grown in Korea [58]. In the present study, the total isoflavone content in soybean seeds ranged from 410 to 620 μg g^{-1}, although the total isoflavone content in the first year (490–620 μg g^{-1}) was higher than in the second year (410–490 μg g^{-1}). Isoflavone content increases rapidly under stress and is influenced by environmental and climatic factors, including temperature, precipitation, harvest time, sowing date, soil fertility, and post-harvest treatments [59]. In this study, the soil under the rain shelter plastic house was automatically irrigated, and the moisture content was maintained at 16% using a soil moisture sensor. The influence of rainfall was, therefore, likely to be negligible, but the effect of temperature was significant. As isoflavones accumulate when temperatures are lower as plants mature, but their contents are reduced at high temperatures, isoflavone content in this study may have been influenced by the higher temperatures encountered during the flowering and harvest periods of the second year, compared with the first year [60].

Isoflavone content was considered according to the quantity of nitrogen in the topdressing treatments. This analysis revealed that daidzin and genistin contents were higher in the topdressing treatments than in the N0 treatment; the glycitin content, however, was highest in the N0 treatment. Daidzin and genistin have been linked to nodule formation, suggesting that excessive nitrogen supply to soybean roots reduces the content of these isoflavones [61]. Furthermore, although daidzin and genistin share the same biochemical pathway, glycitin, which is produced from liquiritigenin, a precursor to daidzein, is in a different pathway [62]. Lee et al. [63] report that applying less than the standard amount of nitrogen fertilizer increases isoflavone content, whereas Macák and Candráková [64] report that applying 20 kg ha^{-1} of fertilizer increases isoflavone content but applying 40 kg ha^{-1} decreases isoflavone contents; this finding is similar to our results. Topdressing with the appropriate amount of nitrogen fertilizer therefore appears to be an effective means of increasing the protein and isoflavone content in soybean seed though excessive topdressing decreases protein and isoflavone content, reducing yields.

4.3. Amino acid contents in soybean vary according to nitrogen topdressing treatments

To date, only limited information is available concerning the effects of nitrogen topdressing on individual amino acid components. A sectional analysis of each of the amino acid components indicated that the amino acid contents decreased as the amount of N topdressing increased. This result was consistent with several previous studies showing that nitrogen fertilization does not improve soybean protein content [65–67]. As it is impossible to explain in detail all the changes seen in amino acid contents in response to topdressing, we will concentrate on describing the responses of a selected subset of amino acids. Previous studies on the responses of amino acids to the application of nitrogen fertilizer application vary greatly, but overall, amino acids play major roles in the regulation of nitrogen metabolism and as a source of nitrogen [68]. In this study, glutamic acid was the most abundant amino acid, although the contents of proline and arginine increased consistently with the quantity of topdressing applied. Glutamic acid is a product of initial amino acid synthesis and is a precursor for arginine, glutamine, and proline biosynthesis [69]. Our analysis confirmed that glutamic acid content decreased, but contents of arginine and proline increased at the highest amount of nitrogen topdressing (N40 treatment).

When nitrogen is available, the proline biosynthetic pathway (ornithine pathway) is predominant over the glutamine pathway, which is part of nitrogen metabolism [70]. Arginine acts as a nitrogen source as it is the amino acid with the highest nitrogen content in the form of organic nitrogen [71]. Additionally, in this study, glycine content tended to be higher in the N30 and N40 treatments than in the N20 treatment, possibly because glycine promotes nitrogen absorption and utilization and is involved in nitrogen metabolism [72]. Glutamine synthetase (GOGAT) and glutamate synthetase (GS) are enzymes that act in the major pathways regulating nitrogen accumulation and metabolism. Asparagine (and the enzyme asparagine synthetase) is also involved in nitrogen accumulation, although its role in nitrogen metabolism is less well-known than that of the glutamine pathway [73]. Glutamine was not identified in this experiment, but both glutamic acid and asparagine appeared to increase in the N20 and N30 treatments but decrease in the N40 treatment, suggesting that nitrogen metabolism was regulated. Furthermore, Zhao et al. [73] reported that contents of methionine and cysteine, sulfur-containing amino acids, decrease when high levels of nitrogen are applied due to nitrogen regulation; we also found lower methionine and cysteine contents when the quantity of topdressing increased. Therefore, application of an appropriate amount of nitrogen *via* topdressing increases soybean amino acid content, but excessive fertilizer has a negative effect on amino acid composition and content [74]. This suggests that excessive topdressing influenced not only the total amino acid content but also the spread of the different amino acid components. The sectional PCA analysis indicated, however, that arginine and proline contents increased in the positive direction up to the N40 treatment, but while levels of glycine and leucine also moved in the positive direction, they decreased in the N40 treatment. These amino acids are the primary storage materials, nitrogen sources, and osmotic stress regulators in soybean seeds, in which they are preferentially stored for germination [75,76].

4.4. Relationships between N topdressing and soybean quality

The relationship between nitrogen, yield, and quality factors has been described previously. When correlations between other factors are considered, protein synthesis in soybean seed appears to depend on N stored in vegetative structures mobilized from leaf senescence, and soybean yield is linked to the dilution in seed protein [5]. Also, many studies indicate a strong interaction between the synthesis of amino acids making up proteins [77,78]. The relationship between isoflavone and yield, protein, and amino acids is unclear, although the main precursor for isoflavone biosynthesis is known to be the amino acid L-phenylalanine [31]. However, Lee et al. [79] reported that the isoflavone and protein contents showed a low negative correlation, which is similar to our results. As a result, the improvement in AE_N resulting from an appropriate quantity of nitrogen topdressing, rather than excessive nitrogen topdressing, was found to have a positive effect on soybean yield and quality factors.

5. Conclusions

Our results showed that, in fields where soybeans were being grown for the first time, a moderate amount of nitrogen (N 20kg ha^{-1}) was more effective than a larger amount of nitrogen during the periods when soybeans had high nutrient requirements (R1 and R2). Excessive N topdressing application during these stages reduced AE_N, yield, and quality (protein, isoflavone, amino acids). Excessive nitrogen topdressing reduced the contents of most amino acids, leading to a decrease in protein content. It was confirmed, however, that some amino acid components increased regardless of nitrogen dose.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Acknowledgments

This work was financially supported by Rural Development Administration in the Republic of Korea (Grant/Award Number: RS-2021-RD009523).

Conflict of interest

The author declares no conflict of interest in this paper.

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