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Summary

Soybeans [*Glycine max* (L.) Merr.] rely on large nutrient uptake, especially nitrogen (N), to produce seeds with high nutritional value. Biological N fixation (BNF) supplies most of the plant N demand and enhancement of this process might improve cropping systems' sustainability. Although seed inoculation with *Bradyrhizobium* spp. for soybean crop is a well-known management practice, co-inoculation with the free-living N-fixer *Azospirillum brasilense* has not been deeply investigated in the US, to our knowledge. Thus, this research explores the effect of co-inoculation with *A. brasilense* on soybean yield and seed nutritional quality (protein, oil, essential and sulfur (S) amino acids concentration) under contrasting fertilizer S rates. Two-way factorial experiments were conducted in Manhattan and Topeka (KS, US) during the 2019 growing season. Sulfur rates of 0 and 20 lb/a were combined with four inoculation strategies: 1) non-inoculated, 2) seed inoculation with *Bradyrhizobium japonicum*, 3) *A. brasilense*, and 4) co-inoculation using both bacteria. The proportion of BNF was estimated via the relative abundance of ureide-N (RAU) at the R5 stage (beginning seed filling). Shoot dry mass was also assessed at R5, as well as seed yield and seed size (1000-seed weight) at harvest time (R8 stage). Dry basis concentration of seed components was also determined (protein, oil, essential and sulfur amino acids). None of the treatment factors significantly ($P < 0.05$) influenced any observed trait. Overall, RAU averaged 80%, seed yield 65 bu/a, protein 42%, and oil 20%. Future research is necessary to eventually capture effects from co-inoculation and S fertilization in soybeans.

Introduction

Soybean [*Glycine max* (L.) Merr.] produces a great amount of protein and oil in the seeds, which highlights its worldwide importance for human nutrition. However, the high nutritional value depends on large nutrient uptake, especially N. Biological N fixation is a crucial process to enhance seed yield and protein, along with a relatively small contribution from soil N supply. However, the bacteria responsible for BNF (*Bradyrhizobium* spp.) must be introduced in agricultural soils (Albareda et al., 2009). In addition, *Bradyrhizobium* spp. is not the only organism capable of fixing N and benefiting the cropping system sustainability.

Azospirillum brasilense is not hosted in root nodules but can fix and release N close to the root surface. Moreover, this species is associated with root growth, which improves nutrient uptake in deeper soil layers; resistance to biotic and abiotic stresses; and potential increase in shoot dry mass and seed yield (Fukami et al., 2018). Therefore, *A. brasilense* is classified as a plant growth promoting rhizobacteria (PGPR). The process of

combining traditional *Bradyrhizobium* spp. inoculation with a PGPR is called co-inoculation and shows promising results in South America (Barbosa et al., 2021). However, little is known about the effect of co-inoculation on yield and seed composition in the US, or the influence on underlying processes such as BNF and S uptake. This research aims to investigate the effect of co-inoculation with *A. brasilense* on soybean yield and nutritional quality (seed protein, oil, essential and sulfur amino acids) under contrasting fertilizer S rates in Kansas.

Procedures

Sites and Measurements

Field experiments were conducted during 2019 at the Ashland Bottoms Agronomy Farm (39.14° North, 96.63° West, Manhattan) and Kansas River Valley Experimental Field (39.07° North, 95.77° West, Topeka). Sowing date was June 7, 2019, in Manhattan and May 17, 2019, in Topeka. Both locations received the genotype AG39X7 (maturity group 3.9) at 140,000 seeds/a. A characterization of the soil (prior to sowing) and weather parameters (from sowing to harvest) are presented in Table 1. At emergence (VE stage) (Fehr et al., 1971), two S fertilization rates were applied: 1) zero (unfertilized control); and 2) 83 lb/a of ammonium sulfate (AMS, with 21% N and 24% S), supplying a total of 20 lb S-SO_4 per acre. Before sowing, liquid inoculants (TerraMax, Eagan, MN) were applied to the seeds as: 1) non-inoculated (control); 2) *Bradyrhizobium japonicum*; 3) *A. brasilense*; and 4) both bacteria (co-inoculation).

The treatment structure was a two-way factorial in a randomized complete block design (RCBD) with four repetitions. Experimental units (plots) were composed of four rows of 40 feet length spaced 30 inches apart. During early seed filling (R5 stage), shoot fresh mass (lb) was sampled from a 25 ft^2 area, avoiding border rows at each plot. From the fresh sample, a 10-plant subsample was randomly selected in order to estimate water content (%), and thereafter dry mass in lb/a. Another subsample was collected, considering only 10 main stems. Whole plant and main stems were allowed to dry in a forced-air oven (150°F) until constant weight.

Main stems were ground in a micro mill (60-mesh screen) and subjected to ureide and nitrate (NO_3) analysis (Hungria and Araujo, 1994). Concentration of stem extracts (ureide and NO_3) was used to calculate the relative abundance of ureide-N (RAU), a proxy of BNF (Unkovich et al., 2008). At harvest time (R8 stage), the two central rows were combine harvested to estimate seed yield (13% moisture basis), excluding biomass sampling gaps. From the combine, a 2 lb sample was collected to measure seed size (1000-seed weight, lb) and seed nutritional quality (protein, oil, essential and sulfur amino acids) via near-infrared spectroscopy (NIR) (Pazdernik et al., 1997). The concentration of all seed components is expressed in dry mass basis (%).

Statistical Analysis

A linear mixed model for each observed variable was fitted with the lme4 package (Bates et al., 2015) in the R software (R Core Team, 2021). The model accounted for S fertilization (2 levels), inoculation strategy (4 levels), and its interaction, as fixed effect factors. Site (Manhattan and Topeka), block, and block nested in site, were included as random effect factors. Type III analyses of variance (ANOVA) were performed using the car package (Fox and Weisberg, 2018). Tukey test was performed for means comparison in case fixed effects were significant ($P < 0.05$). Finally, least square means

(LSMEAS) were extracted for all eight treatments and variables were subjected to a principal components analysis (PCA) with the factoextra package (Kassambara and Mundt, 2020). The PCA was intended to show the overall relationship among all observed variables (seed yield, size, dry mass, RAU, and seed components).

Results

Neither inoculation nor S fertilization influenced any observed variable (Table 2). Therefore, treatment LSMEANS are reported without means comparison (Figure 1). Yield averaged 65 bu/a, protein and oil concentration were ca. 42% and 20%, respectively. The RAU values reached ca. 80%, indicating BNF was the main N source in early seed filling (R5). Overall, seed yield was negatively correlated with oil concentration and positively associated with dry mass and seed size (Figure 2). The weak positive correlation between yield and protein is noteworthy. Protein concentration was strongly correlated with essential and sulfur amino acids. Future research should explore co-inoculation and S fertilization under contrasting growing conditions (e.g., low and high soil pH) and increase the number of treatment repetitions.

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Table 1. Site description for Manhattan and Topeka, KS. Soil parameters were measured prior to sowing from a 6-inch depth layer; except for soil SO_4 and NO_3, measured from a 24-inch depth layer. Weather data were summarized from sowing to harvest (140 days) at each location and obtained from DAYMET (Thornton et al., 2020).

Soil	Manhattan	Topeka	Weather	Manhattan	Topeka
Water pH	6.5	7.1	Radiation, MJ m^{-2} day^{-1}	4733	5343
SOM [a], %	1.4	1.7	Max. temperature, °C	28.4	27.7
Clay, %	8	22	Min. temperature, °C	16.6	16.9
Sand, %	47	11	Mean temperature, °C	22.5	22.3
Silt, %	45	67	Precipitation, mm	668	825
P[b], mg dm^{-3}	30	18	Precipitation SDI [d]	0.65	0.71
CEC [c], $cmol_c$ dm^{-3}	7.5	9.5	Evapotransp. [e], mm	650	683
NO_3, mg dm^{-3}	2.8	3.2	Relative humidity, %	72	76
SO_4, mg dm^{-3}	0.9	1.7	VPD [f], kPa	121	111

[a] Soil organic matter via loss-on-ignition (LOI).
[b] Phosphorus via Mehlich-3.
[c] Cation exchange capacity.
[d] Shannon diversity index of precipitation (Bronikowski and Webb, 1996); values range from zero to one, with one representing evenly distributed precipitation.
[e] Reference evapotranspiration (ET_0).
[f] Vapor pressure deficit.

Table 2. Analysis of variance (ANOVA) results for shoot dry mass (R5 stage), seed yield, seed size (1000-seed weight), relative abundance of ureide-N (RAU), seed protein, oil, essential and sulfur amino acids (%, dry basis). Sulfur fertilization, inoculation strategy, and their interaction were considered as fixed effects while site and block were random. Values between parentheses represent degrees of freedom, followed by the *P*-value (F-test).

Variable	Intercept	Inoculation	Sulfur	Interaction
Dry mass	(1) 1.99e-03 **	(3) 1.37e-01	(1) 8.33e-01	(3) 6.41e-01
Seed yield	(1) 2.84e-08 ***	(3) 8.09e-01	(1) 4.53e-01	(3) 4.32e-01
Seed size	(1) 3.13e-02 *	(3) 4.39e-01	(1) 1.41e-01	(3) 1.28e-01
RAU (R5)	(1) 4.27e-04 ***	(3) 3.65e-01	(1) 9.34e-01	(3) 6.38e-01
Protein	(1) 1.08e-02 *	(3) 6.44e-01	(1) 9.64e-01	(3) 9.43e-01
Oil	(1) 1.23e-02 *	(3) 6.11e-01	(1) 8.23e-01	(3) 2.34e-01
Amino acids				
Essential [a]	(1) 7.40e-03 **	(3) 3.37e-01	(1) 9.45e-01	(3) 9.57e-01
Sulfur [b]	(1) 3.82e-04 ***	(3) 7.33e-02	(1) 8.99e-01	(3) 6.84e-01

[a] Essential amino acids: isoleucine, leucine, histidine, phenylalanine, valine, lysine, cysteine, methionine, threonine, and tryptophan.
[b] Sulfur amino acids: cysteine and methionine. Amino acid groups were generated based on Pfarr et al. (2018).
* Significant at the 0.05 probability level. ** *P*-value < 0.01. *** *P*-value < 0.001.

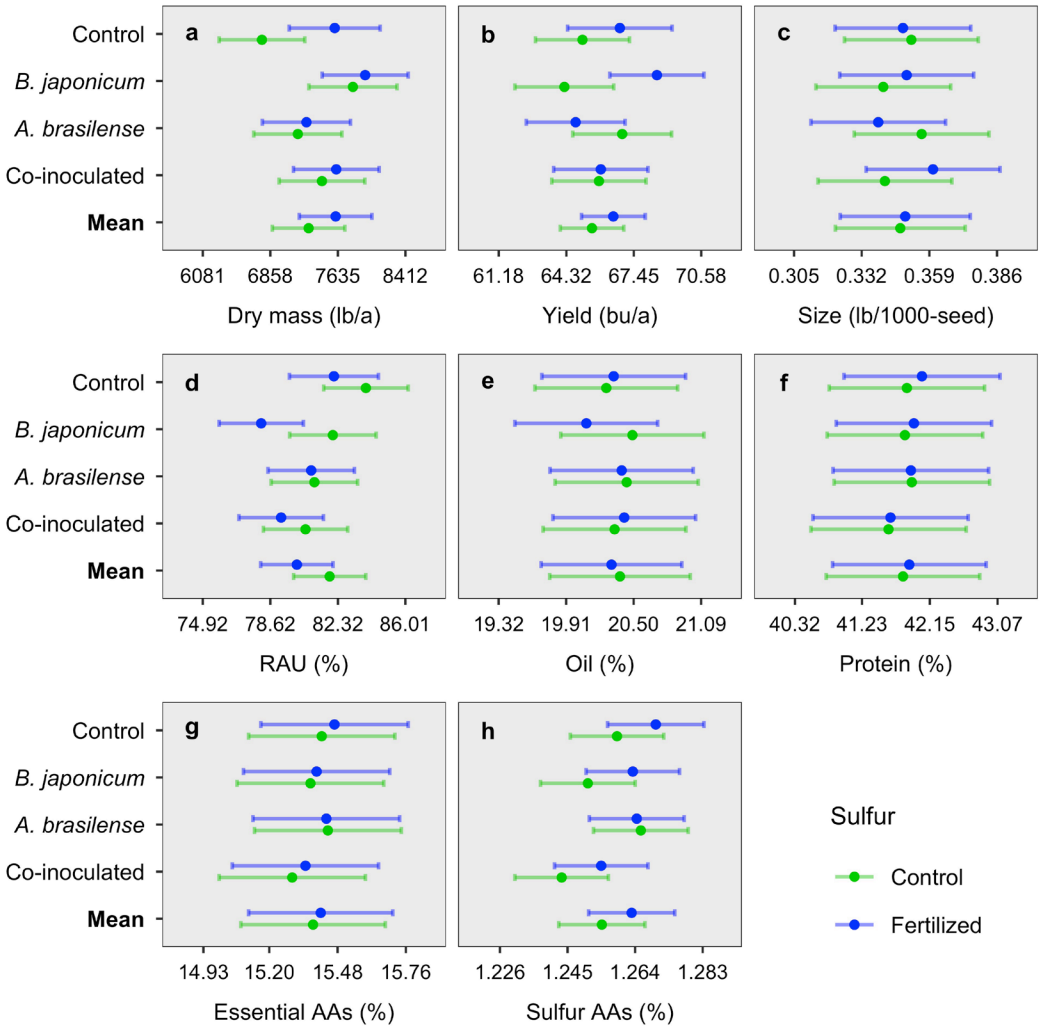

Figure 1. Least square means (LSMEANS) for R5 shoot dry mass (a); seed yield (b); seed size (c); relative abundance of ureide-N (RAU) (d); seed oil (e); protein (f); essential (g); and sulfur amino acids (h) concentration. No significant differences ($P < 0.05$) were observed among treatment factors; therefore, no means comparison was performed. Essential amino acids (AAs) correspond to the sum of isoleucine, leucine, histidine, phenylalanine, valine, lysine, cysteine, methionine, threonine, and tryptophan. Sulfur AAs correspond to cysteine and methionine. Error bars represent the standard error from the linear mixed models.

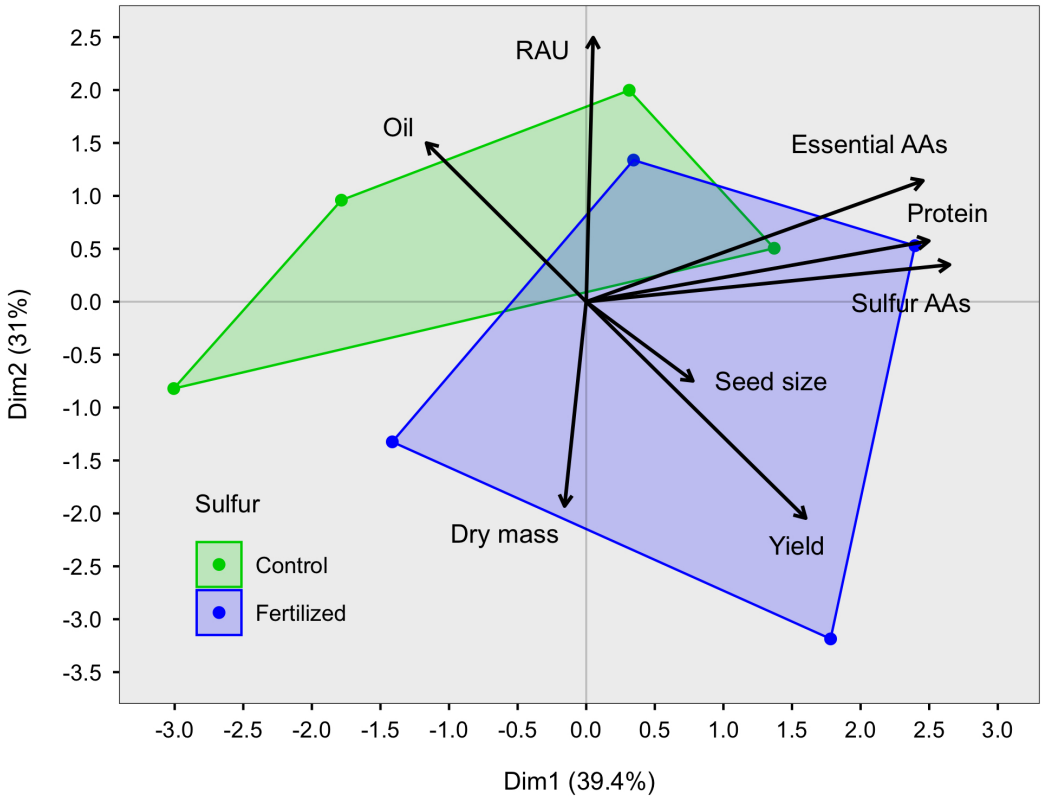

Figure 2. Principal component analysis (PCA) displaying the relationship among least square means (LSMEANS) of observed variables. With only eight variables, the first two dimensions (Dim) account for most of the treatment variation. Arrows pointing in the same directions indicate positive Pearson's correlation, otherwise negative correlation. Perpendicular arrows show no correlation between variables. The treatment LSMEANS were not significantly different in the analysis of variance (ANOVA), with $P < 0.05$.