



RELATIONSHIP OF VARIOUS PARAMETERS TO *BRADYRHIZOBIUM JAPONICUM* IN SOYBEANS

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SUMMARY

Soybean is a dual-purpose crop, as it serves as pulse and fodder. Legumes like soybeans have a distinctive characteristic of nodule formation. Nitrogen fixation enhancement can succeed by inoculation of soybeans with specific strains of rhizobia, ensuring adequate levels of healthy bacteria near the seed. The presented study materialized during 2018–2020 at the University of Agriculture, Faisalabad, and Ayub Agricultural Research Institute, Faisalabad, Pakistan. Two strains of *Bradyrhizobium japonicum*—S377 and S379—were treatments for inoculating 80 soybean accessions collected from the National Agriculture Research Center, Islamabad. Sowing 10 inoculated seeds per genotype and two seeds for control continued in a randomized complete block design (RCBD). Fresh root and shoot weights, dried root and shoot weights, nodule dry weight, grain and biomass yield, protein, and oil content increased significantly after inoculating seeds with rhizobial strains. These traits also showed significant genotypic correlations. The protein content, followed by the fresh shoot weight, directly affected nodule formation, increasing nitrogen fixation.

Keywords: *Bradyrhizobium japonicum*, correlation, path analysis, nitrogen fixation, nodule formation, soybean

Key findings: Eighty accessions inoculated with two strains of *B. japonicum* showed significant improvement in fresh root and shoot weights, nodule dry weight, protein, and oil content. The protein content and fresh shoot weight directly affected nodule formation, predicting the opportunity to improve yield by precisely selecting these traits in future breeding programs.

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INTRODUCTION

Oilseed crops, grown worldwide, are vital due to their economic value (Miransari, 2016; Abiodun, 2017; Ahmad *et al.*, 2023). Recently, they have gained more attention because of their high demand for healthy vegetable oils, pharmaceuticals, livestock feeds, biofuels, and other industrial uses (Ahmad *et al.*, 2023). Among the oilseed crops, soybeans hold the maximum share of global production (Akram *et al.*, 2011). Soybean cultivation only covers 1000ha in Pakistan, with an average harvest of 800–1000 tons per 0.40 hectare. Meeting the population demand caused significant investment every year in seed oil importation.

A fundamental component of agriculture is nitrogen fixation and its efficient management. Soybean is a leguminous crop that requires nitrogen for significant production and better yield. Nodule formation is a primary legume characteristic, helping soil nitrogen fixation (Jin *et al.*, 2022). A minimum proportion of nitrogen comes from the soil's organic matter, but the chief proportion results in the symbiotic relationship of plant roots with rhizobia (Tiana *et al.*, 2012). Soybean inoculation of rhizobia can facilitate nitrogen fixation, ensuring an adequate level of healthy bacteria near the seed (Hungria *et al.*, 2017). Rhizobial inoculation increases the rate of nodulation, the number of seeds, growth performance, and yield. Seeds with a rhizobial inoculum are significantly superior to non-inoculated ones (Gebrehana and Dagnaw, 2020). Soybean yield can reach up to 50% when inoculated with rhizobia (Seneviratne *et al.*, 2000). The rhizobial group of soil contains soil α and β proteobacteria, belonging to different genera, and all are gram-negative rods ($0.5\text{--}1.0 \times 1.2\text{--}3.0 \mu\text{m}$), aerobic, and motile (Sawada *et al.*, 2003; Downie, 2010). Rhizobia are gram-negative bacteria that are highly crop-specific and do not form nodules other than the respective crops. However, a specific rhizobial strain can cause nodulation on more crops. Fast or slow-growing rhizobia can cause soybeans to nodulate (Hume and Blair, 1992).

For soybean, *Bradyrhizobium japonicum*, *B. elkanii*, *B. liaoningense*, and

Rhizobium fredii are effective, slow-growing rhizobia. The fast-growing rhizobium is *Sinorhizobium fredii*. The most widely used rhizobia for soybean is *B. japonicum* (Pastorino *et al.*, 2003). The soil environmental conditions, i.e., pH, organic carbon contents and available phosphorus, the latitude, and soybean genotype, determine rhizobial abundance, distribution, and N_2 fixing efficiency (Yan *et al.*, 2014). Symbiosis efficacy varies depending on the soybean cultivars, biotic and abiotic factors, and rhizobial species. Consequently, it is critical to comprehend the ecology of native soybean-nodulating rhizobia according to their genetic variation and the environmental elements contributing to their localization and dominance in the soil (Nakei *et al.*, 2022). In alkaline saline soil, *S. fredii* is the dominant rhizobia, while in acidic soils, *B. elkanii* and *B. japonicum* are more prevalent. The most biogeographic specificity emerges from *B. liaoningense* versus *B. elkanii* and *B. japonicum* (Habibi *et al.*, 2017). A better understanding of environmental conditions while inoculating is beneficial for soybean growers. The performance of inoculants is ineffective when improperly considering ecological factors.

The role of rhizobia strains in various legumes has existing documentation, while research on the role of *B. japonicum* strains S377 and S379 on genetic variability in soybean nodulation is rare. The study hypothesized that rhizobial strain applications may affect nodule formation, growth, yield, and yield-quality parameters, affecting genetic potentials in soybean accessions. However, the objective of this study was to evaluate the role of rhizobial strains in improving growth, yield, nodule formation, and quality parameters by affecting genetic variability in soybean accessions.

MATERIALS AND METHODS

The germplasm collection by screening 80 accessions (Table 1) came from the National Agriculture Research Centre (NARC), Islamabad, Pakistan. The germination test ensued in the Sunflower Lab of the Department

Table 1. Soybean accessions selected after screening for Rhizobial response.

1	PI 88297	17	PI 82184	33	PI 85014	49	PI 88296	65	PI 82278
2	PI 82315	18	PI 80488	34	PI 86032	50	PI 54854	66	PI 79848
3	PI 88288	19	PI 86150	35	PI 84671	51	PI 82218	67	PI 87167
4	PI 86024	20	PI 84581	36	PI 80470	52	PI 54583	68	PI 85625
5	PI 79846	21	PI 54859	37	PI 85342	53	PI 54809	69	PI 54865
6	PI 87968	22	PI 80468	38	PI 83836	54	PI 84660	70	PI 86116
7	PI 81038	23	PI 88292	39	PI 82588	55	PI 81042	71	PI 82307
8	PI 86425	24	PI 84979	40	PI 80822	56	PI 84965	72	PI 30600
9	PI 54609	25	PI 63362	41	PI 79863	57	PI 54619	73	PI 83881
10	PI 84939	26	PI 83940	42	PI 79874	58	PI 54608	74	PI 81775
11	PI 84757	27	PI 85519	43	PI 83925	59	PI 88282	75	PI 86071
12	PI 86004	28	PI 81785	44	PI 54615	60	PI 88294	76	FC 2108
13	PI 79761	29	PI 84637	45	PI 54857	61	PI 88287	77	FC 3981
14	PI 81037	30	PI 65342	46	PI 82296	62	PI 82264	78	FC 3548
15	PI 80485	31	PI 83874	47	PI 82286	63	PI 55089	79	FC3659
16	PI 85437	32	PI 81027	48	PI 80480	64	PI 81767	80	FC19976

of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan. Eighty soybean accessions reached sowing in sand-filled trays in a randomized complete block design (RCBD) included three replications. Recording observations daily was done for 15 days from sowing.

Two strains of *B. japonicum* (S377 and S379) specific to soybeans, collected from the Ayub Agricultural Research Institute, Faisalabad, Pakistan, received testing in a purity culture conducted for 28 days in the growth room. Soybean seeds inoculated with the tested strains of rhizobia transpired in the Pathology Lab, Ayub Agricultural Research Institute, Faisalabad. Dipping nodules for 30 s in a 0.1% mercuric chloride solution, then into 70% ethanol for 30 s, continued with a thorough rinsing of nodules with sterilized distilled water (Russell *et al.*, 1982). The prepared milky suspension application helped transfer and crush disinfected nodules to the test tubes with 5 mL sterilized distilled water. The yeast extract mannitol agar (YEMA) served as a medium plate using 0.1 mL aliquot of the suspension. Plates acquired Congo red for staining (Kenasa *et al.*, 2014). The YEMA medium contains 1 g/L yeast extract, 0.2 g/L MgSO₄ · 7H₂O, 10.0 g/L mannitol, 0.5 g/L K₂HPO₄, 20.0 g/L agar powder, 0.1 g/L NaCl, and 0.025 g/L Congo red (Kenasa *et al.*, 2014). The pH adjustment in the medium was at 25 °C to reach 6.8 (Pelczar *et al.*, 1977). Plates

sustained autoclaving, with the sterilized YEMA medium poured into Petri dishes containing rhizobium suspension. Seed sterilization used ethanol 96% for 30 s, HgCl₂ 2% for 3 min, and washing continued 6-8 times with distilled water. The strains' 1 ml suspension solutions, grown in the broth of a yeast extract mannitol (YEM), had a temperature of 28 °C for 72 hours. Tray filling with 2-3 mm fine-graded sand substrate attained plastic wrapping as covers and sterilized by autoclaving twice for two hours. Three treated seeds per chamber of each accession with two non-inoculated seeds (control) continued to sow with sterile pincers in RCBD with three replications. A laminar flow chamber helped carry out inoculation and planting.

Data recorded and analysis

Data recorded for traits of the soybean genotypes included fresh root and shoot weights (g) by collecting five seedling roots and shoots per replication per treatment, and then weighed in grams with a weighing balance (Setra-BL-410S). Later, those shoots and roots underwent oven drying (Fisher Scientific, Model 655 F) for 24 h at 52 °C. Dried roots, weighed in grams, using the weighing balance. Noting nodule dry weight consisted of collecting nodules from roots of five plants of each accession per replication per treatment and oven dried (Fisher Scientific, Model 655 F) for

24 h at 52 °C. Obtaining dried nodules' weight in grams engaged the weighing balance. The grain yield recorded in grams had five plants per replication per accession collected and sundried till complete dryness. Getting the seed weight collected from all the plants engaged the weighing balance. For the five plants per replication per accession, the weight of the whole plant, including stem and leaves, was measured with the weighing balance to determine the biomass yield in grams. Protein and oil content measurement employed the Near Infrared Reflectance Spectrometry (NIR). The recorded data underwent analysis of variance following Steel *et al.* (1997). The strength of a relationship among traits utilized correlation analysis, as proposed by Kwon and Torrie (1964). Path analysis helped determine the direct and indirect rhizobial strain effects on nodule formation (Dewey and Lu, 1959).

RESULTS AND DISCUSSION

Highly significant genetic variation was evident between genotypes for the traits studied, as shown in Table 2. Two strains of *Bradyrhizobium japonica* were specimens in the presented study. The results indicated significant genetic variability among the accessions for all observed traits. Effective selection resulted from the performance of the breeding material. Soybean seeds inoculated with the rhizobial strains enhanced the soybean's nitrogen-fixing ability (Ojo *et al.*, 2014; Yan *et al.*, 2014; Habibi *et al.*, 2017; Han *et al.*, 2020).

All traits, i.e., fresh root and shoot weights, dried root and shoot weights, nodule

dry weight, grain yield, biomass yield, protein content, and oil content, exhibited a significant increase after the seed inoculation with rhizobial strains. Strains of *B. japonicum* showed a high potential of N₂ fixation, as reported by Delić *et al.* (2010). Significant differences were prominent among fresh root and shoot weights, dried root and shoot weights, nodule dry weight, grain yield, biomass yield, protein, and oil content. Results are consistent with the research done by Solomon *et al.* (2012), which showed that *B. japonicum* inoculation improved the soybean's growth and yield by enhancing root nodulation and nitrogen fixation.

The bacterial strain may enhance the enzymatic production of pigments involved in photosynthetic processes, improving crop growth and photosynthetic rate (Prasad, 2021). Comparison of means depicted minimal differences among fresh root and shoot weights, dried root and shoot weights, and nodule dry weight; however, a significant difference occurred with biomass yield, grain yield, protein, and oil content (Figure 1). Bacterial strain injection enhanced photosynthate accumulation and translocation, enriching yield and yield characteristics (Prasad, 2021). Through various processes, including improved nutrient assimilation (biofertilizers) through biological nitrogen fixation, phosphorus solubilization, or iron acquisition, rhizobial treatment supplementation supports plant growth and development (Kuan *et al.*, 2016). This study demonstrated how crucial it is to inoculate soybean plants with *B. japonicum* bacterial strains. Maximum yield was remarkable under both strains, with PI 84757, PI 81027, and

Table 2. Analysis of variance of different accessions of soybean for quality and yield attributes.

Sources of variation	d.f.	Fresh root weight	Fresh shoot weight	Dried root weight	Dried shoot weight	Nodule dry weight	Biomass yield	Grain yield	Protein content	Oil content
Replications	2	0.237	0.282	0.220	29.887	1.675	4.254	188.82	8563.3	5.929
Treatments	179	1.122	1.796	0.731	0.737	0.638	182.566	366.37	452.0**	72.652
		**	**	**	**	**	**	**	**	**
Error	158	0.074	0.069	0.046	0.453	0.140	103.921	30.31	220.9	13.925

**= significant at a 0.01 probability level, *= significant at a 0.05 probability level.

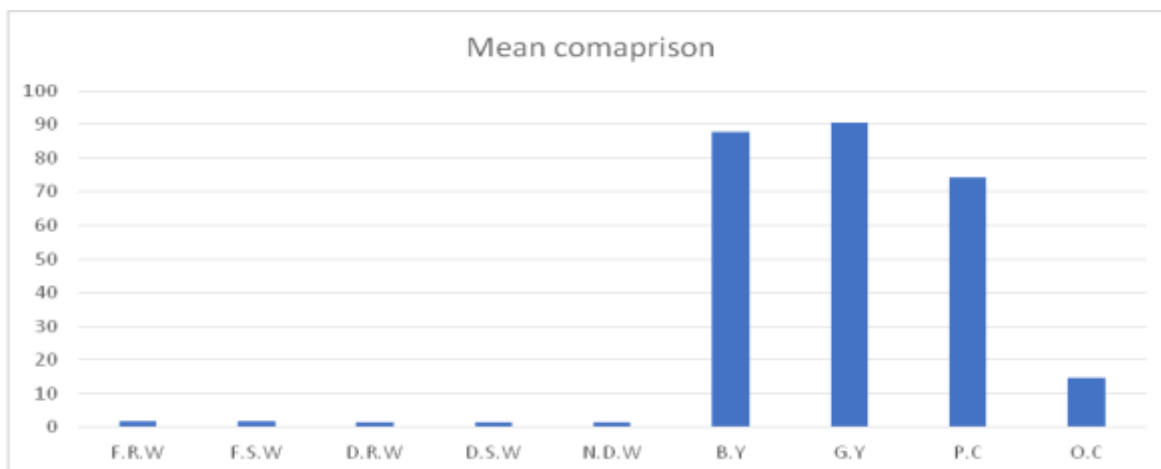


Figure 1. Mean comparison for effect of rhizobial strains on quality and yield traits. F.R.W=fresh root weight, F.S.W=fresh shoot weight, D.R.W=dried root weight, D.S.W=dried shoot weight, N.D.W= nodule dry weight, G.Y= grain yield, B.Y= biomass yield, PC= protein content, OC= oil content.

FC19976 accessions as superior genotypes provided better chlorophyll content and photosynthetic rates that may help the specific genotype grow and yield better (Ahmad *et al.*, 2021; Ahmad *et al.*, 2024). It indicated a substantial difference in growth, yield, and yield quality metrics between the soybean accessions under two bacterial strains in this investigation. It suggests that the bacterial strains had an even higher benefit. Teaching end users this strategy is crucial to increase agricultural yields and lower yearly costs associated with applying inorganic fertilizers, negatively changing the soil. Legume inoculation with bacterial strains is affordable and easy (Bitire *et al.*, 2022; Igiehon and Babalola, 2018).

Correlation analysis

Fresh root weight showed a significant positive genotypic correlation with dried root weight, dried shoot weight, nodule dry weight, and protein content, while a substantial negative correlation resulted in fresh shoot weight and oil content. Fresh shoot weight revealed a relevant positive genotypic correlation with oil content, while a negative significant correlation appeared with dried root and shoot weights, nodule dry weight, and protein content. Dried root weight indicated a meaningful positive

genotypic correlation with dried shoot weight, nodule dry weight, and protein content, whereas a negative significant correlation observed for oil content. Correlation analysis describes the relationship between two traits, allowing one to find the magnitude and direction of inter-relationships that help in effective and efficient crop improvement (Chandra *et al.*, 2012).

Dried shoot weight showed a significant positive genotypic correlation with the grain yield, biomass yield, and protein content; however, the oil content displayed a significant negative correlation. Nodule dry weight exhibited a considerable positive genotypic correlation with protein content, whereas biomass yield and oil content gave a notably negative correlation. Grain yield showed a significant positive genotypic correlation with biomass yield and protein content. Biomass yield indicated a significant positive genotypic correlation with the protein content. Protein content showed a meaningful negative genotypic correlation with the oil content. Fresh root and shoot weights, dried root and shoot weights, nodule dry weight, protein content, and oil content detailed a significant genotypic correlation, negative or positive, but non-significant phenotypic correlation (Table 3).

Table 3. Genotypic (upper) and phenotypic (lower) correlation in soybeans.

Variables	Fresh shoot weight	Dried root weight	Dried shoot weight	Nodule dry weight	Grain yield	Biomass yield	Protein content	Oil content
Fresh root weight	-0.435*	0.269*	0.607*	0.533*	0.020	-0.035	0.586*	-0.334*
Fresh shoot weight	-0.400	0.242	0.290	0.378	0.0113*	-0.028*	0.406*	-0.248
Dried root weight		-0.202*	-0.254*	-0.232*	-0.002	0.100	-0.464*	0.345*
Dried shoot weight		-0.188	-0.113	-0.139	0.004*	0.040*	-0.275	0.230
Nodule dry weight			0.850*	0.190*	0.057	-0.088	0.559*	-0.405*
Grain yield			0.302	0.113	0.030*	-0.031*	0.316	-0.295
Biomass yield				0.108	0.141*	0.436*	1.094*	-0.343*
Protein content				0.046	0.109*	0.060	0.296	-0.106
Oil content					0.008	-0.279*	0.399*	-0.182*
					0.013*	-0.087	0.161	-0.184
						0.448*	0.577*	-0.005
						0.189	0.200	0.011*
							0.808*	0.056
							0.076	0.076
								-0.616*
								-0.261

**= significant at a 0.01 probability level, *= significant at a 0.05 probability level.

According to the results reported by Yildirim *et al.* (2023), six soybean traits exhibited a positive correlation with grain yield, while the remaining traits gave a negative linkage with seed yield. The six positively correlated traits were pod per plant, days to 50% flowering, days to maturity, plant height, first pod height, and crude protein ratio. Other studies have also reported a significant positive correlation between pod per plant and grain yield, as noted by Akram *et al.* (2016), Guleria *et al.* (2019), and Belay *et al.* (2023). Similarly, Balla and Ibrahim (2017) found positive correlation coefficients for grain yield with days to 50% flowering and days to maturity, although Aditya *et al.* (2011) and Akram *et al.* (2016) reported negative correlations for these traits.

Grain yield and biomass yield were phenotypically significant but genotypically non-significant. Fresh shoot weight showed a negatively significant correlation with fresh root weight. Dried shoot weight displayed a significant positive correlation on a genotypic level with fresh root weight but a remarkable negative interrelation with fresh shoot weight. The genotypic correlation coefficient of dried shoot weight was positively significant for fresh root weight and dried root weight but was non-significant for fresh shoot weight. Nodule dry

weight showed a positive significant correlation coefficient for fresh root weight and dried root weight but a notable negative genotypic correlation with fresh shoot weight. Sulistyo *et al.* (2017) also analyzed the heritable agronomic traits contributing to soybean yield. The results revealed that most of the observed agronomic traits exhibited high heritability values, indicating a sturdy genetic component. However, the number of fertile nodes exhibited low heritability. Correlation analysis demonstrated that days to flowering, plant height, and number of fertile nodes positively correlated with seed yield per plot. Previous studies have consistently validated a significant positive correlation between plant height and seed yield. These researchers also identified remarkable positive correlations between seed yield and other agronomic traits, including the number of filled pods and a total number of nodes (Wirnas *et al.*, 2006), days to flowering, and total number of pods (Asadi, 2012), seed weight per plant (Hakim, 2012), and days to maturing (Balla and Ibrahim, 2017).

Furthermore, plant height has shown a positive correlation with yields in soybean vegetables (Li *et al.*, 2013). Biomass yield gave a positive genotypic significant correlation coefficient for dry shoot weight, nodule dry weight, and grain yield, whereas a positive

phenotypic correlation coefficient for fresh shoot weight but a negative phenotypic correlation for dried root weight. Machikowa and Laosuwan (2011) reported similar results by experimenting on 14 soybean lines using a randomized complete block design with three replications. Analysis of variance revealed significant variations among all genotypes for each characteristic, including yield and its components. Phenotypic correlation analysis indicated a positive correlation between seed yield and days of flowering. Furthermore, genotypic correlation analysis revealed positive associations between seed yield and all evaluated qualities, except for 100-seed weight. Protein content indicated a positive genotypic correlation for fresh root and shoot weights, dried root and shoot weights, nodule dry weight, grain yield, and biomass yield.

Path analysis

The direct and indirect effects of rhizobia strains on soybeans appear in Table 4. The observed highest direct effect emerged on fresh shoot weight, followed by protein content, grain yield, fresh root weight, nodule dry weight, biomass yield, dried root weight, and fresh shoot weight of both rhizobial strains. In the presented study, rhizobial strain inoculations had the highest direct effect on fresh shoot weight, followed by protein content, grain yield, fresh root weight, nodule dry weight, biomass yield, dried root weight, and fresh shoot weight, descendingly. Fresh

root weight showed the foremost indirect effect on protein content, biomass yield, grain yield, dried root weight, nodule dry weight, dried shoot weight, and fresh shoot weight, descendingly. Yildrimi *et al.* (2023) also reported that pods per plant, plant height, days to maturity, crude protein ratio, and days to 50% flowering directly and positively affected grain yield. Plants with more days to maturity have completed their life cycle and accumulated more sunlight, which helped them accumulate more dry matter and, ultimately, provided more yield and oil contents. Similar findings came from Akram *et al.* (2011), who reported the direct effect of 100-seed weight, days to flowering, and days to maturity with soybean yield.

Conversely, reports on grain yield revealed negative and direct effects from the 100-seed weight and crude oil ratio. The said research outcome disagreed with Ganesamurthy and Seshadri (2004) who reported that 100-seed weight showed a positive indirect effect on grain yield through fodder yield.

Fresh shoot weight had the highest indirect effect on dried root weight, dried shoot weight, nodule dry weight, fresh root weight, grain yield, protein content, and biomass yield, descendingly. Dried root weight showed the foremost indirect effect on biomass yield, protein content, grain yield, fresh root weight, nodule dry weight, fresh shoot weight, and dried shoot weight, descendingly. Dried shoot weight provided the optimum indirect

Table 4. Direct and indirect effects of rhizobia strains on soybeans.

Variables	Fresh root weight	Fresh shoot weight	Dried root weight	Dried shoot weight	Nodule dry weight	Grain yield	Biomass yield	Protein content
Fresh root weight	-0.094	-0.162	-0.091	-0.152	-0.126	-0.001	0.011	0.282
Fresh shoot weight	0.041	0.373	0.068	0.063	0.055	0.000	-0.032	-0.223
Dried root weight	-0.025	-0.075	-0.340	-0.213	-0.045	-0.004	0.028	0.270
Dried shoot weight	-0.057	-0.094	-0.289	-0.250	-0.025	-0.011	-0.141	0.528
Nodule dry weight	-0.050	-0.086	-0.064	-0.027	-0.237	0.000	0.090	0.192
Grain yield	-0.001	-0.000	-0.019	-0.035	0.001	-0.082	-0.145	0.278
Biomass yield	0.003	0.037	0.030	-0.109	0.066	-0.036	-0.324	0.390
Protein content	-0.055	-0.173	-0.190	-0.274	-0.094	-0.047	-0.262	0.482

effect on protein content, grain yield, nodule dry weight, fresh root weight, fresh shoot weight, biomass yield, and dried root weight, descendingly. Amogne *et al.* (2020) reported that the number of pods per plant had the most significant positive direct effect on grain yield, followed by the number of nodules per plant and the number of seeds per pod. Mesfin and Abush (2018) also reported a strong positive direct effect of the number of nodules on soybean grain yield.

On the other hand, the number of branches per plant had the maximum negative direct effect, with days to 50% flowering and plant height also contributing negatively to grain yield. The residual effect was 0.88, indicating that other unconsidered traits may influence the yield. Machikowa and Laosuwan (2011) also reported that pods per plant exhibited the highest positive direct effect on seed yield, followed by branches per plant. Additionally, most characteristics caused substantial indirect consequences on the seed yield through their influence on pods per plant. The effect of pods per plant (Ball *et al.*, 2001; Sudaric and Vratarić, 2002; Iqbal *et al.*, 2003; Arshad *et al.*, 2006) and branches per plant (Nakawuka and Adipala, 1999) on seed yield have several documentations. Iqbal *et al.* (2003) reported a positive direct effect of 100-seed weight on seed yield.

Conversely, unfavorable direct effects on seed yield were distinct for nodes per plant, seeds per plant, days to flowering, and days from flowering to maturity (Kurbanbaev *et al.*, 2023; Mansour *et al.*, 2023). Indirect effects through pods and branches per plant emerged when considering yield components and agronomic traits. Protein content showed a maximum direct influence, followed by fresh shoot weight.

CONCLUSIONS

Significant differences manifested among soybean genotypes for traits observed, revealing the role of both strains used. Fresh shoot and root weights, dried shoot and root weights, nodule dry weight, protein, and oil

content showed notable genotypic correlation. Results depicted that protein content, followed by fresh shoot weight, directly affected nodule formation, thus increasing the soybeans' nitrogen fixation ability. Hence, chances of improvement are high for nodule formation by directly selecting these characteristics.

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