SHORT REPORT
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Evaluation of N_2 Fixation Traits in Thai and Korean Soybean Cultivars

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ABSTRACT: Thirteen Thai and 21 Korean soybean cultivars were investigated for N_2 fixing ability of 3 strains of *Bradyrhizobium japonicum*, using a factorial arrangement in a completely randomized experimental design (CRD). Data were collected on 5 traits, viz. nodule number per plant, nodule fresh weight per plant, nodule dry weight per plant, plant dry weight, and acetylene reduction activity (ARA). The variation in each trait was dependent of soybean cultivars, *B. japonicum* stains and interaction between cultivars and strains. Among the soybean cultivars, KKU35 had higher nodule number, but lower ARA than ST1, revealing that high nodule number may not correlate with the nitrogenase enzyme activity. For rhizobial strains, DASA 01026 gave high nodule number, nodule fresh weight, and nodule dry weight, while DASA 01054 gave high nodule number, plant dry weight, and ARA. Positive correlation coefficients (r) between ARA and nodule number, nodule fresh weight, nodule dry weight, and plant dry weight were all significant at the values of 0.438, 0.738, 0.703 and 0.635, respectively. Path coefficient analysis revealed that these N_2 fixation traits were inter-related and contributing directly and indirectly to the ARA, with the greatest contribution from nodule fresh weight.

KEYWORDS: Soybean, *Bradyrhizobium japonicum*, N_2 fixation.

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) originated in China, Manchuria, and Korea.[1] There is evidence that soybean was domesticated around the 11th century BC in the eastern half of northern China and extended to central and southern China, as well as the Korean peninsula, in the first century A.D. After the 15th to 16th century, soybean was brought into many countries, including northern India, Indonesia, Japan, Malaysia, Myanmar, Nepal, the Philippines, Thailand, and Vietnam.[2] Due to the expansion in the feed industry, Thailand has not been self-sufficient in soybean production during the past 20 years. In the crop year 2002-3, harvested area, production, and yield per ha were 0.175 Mha, 260 Kton, and 1.49 t/ha, respectively.[3] The factors responsible for low yield in Thailand are low quality of seed, biotic and abiotic problems, and low production efficiency. Fertilizer application in soybean is limited due to its high cost. However, like most legumes, soybean can fix N_2 from the air through symbiosis between soybean and the bacterium *Bradyrhizobium japonicum* associated in the nodules developed from root hairs. *B. japonicum* is slow growing, rod-shaped, gram negative, aerobic, 0.5-0.9 µm x 1.2-3.0 µm in size, non-spore forming, motile by flagella, forming white and opaque colonies.[4] N_2 gas is transformed from inorganic to organic compounds by the *B. japonicum* nitrogenase enzyme.[5] N_2 fixation in leguminous crops helps reduce the use of N fertilizer applied to the crops, minimizing ground water pollution, increasing seed protein, and giving the residual fixed nitrogen to the succeeding crops. Soybean can utilize N from atmosphere, soil and fertilizer.[6] People et al[7] suggested two strategies to enhance N_2 fixation, viz. through crop and soil management, and through plant breeding and selection. In the first strategy, the crop that can fix more N_2 should be planted first in a cropping system, especially at early wet season to maximize N_2 fixation. Other cultural practices that can enhance N_2 fixation are P-fertilization, irrigation, no or minimum tillage, and the use of rhizobial inoculation. In the second strategy, legume plants should be bred for more specificity to rhizobium strain to improve plant yield and tolerance to environmental stress. Thus, the traits related to N_2 fixation should be directly or indirectly evaluated to support breeding and selection processes. A direct method is to grow soybean in N-free medium and observe the N_2 fixation ability of the soybean genotypes. The alternative indirect measurement is to observe plant characters reflecting N_2 fixation potential, viz. nodule number per plant, plant dry weight, nodule fresh and dry weight, and acetylene reduction activity (ARA).[8] Nitrogen fixation activity increases sharply after flowering stage and gradually decreases after green pod stage.[9] Attewell and Bliss[10] suggested that the best soybean growth stage for determining ARA is at R_3 (early pod stage), since the sensitivity decreases rapidly after this stage.

The objective of this study was to evaluate nodulation response of major Thai and Korean soybean cultivars with strains of *B. japonicum*. The traits related to N_2 fixation among these diverse genotypes can be used as key traits for selection of soybean genotypes, rhizobial strains, and the combination between soybean and rhizobia.

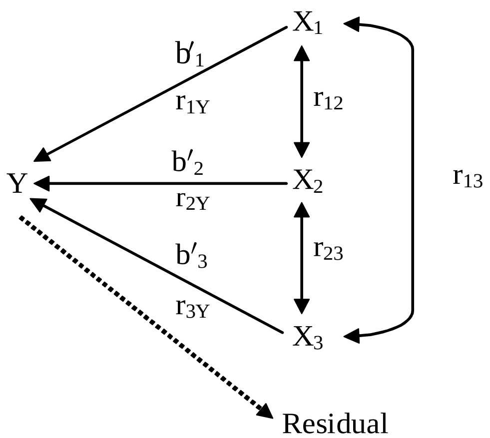
Materials and Methods

Thirteen Thai and 21 Korean soybean cultivars (name list shown in Table 2) were inoculated with 3 *B. japonicum* strains (DASA01026, DASA01042, and DASA01054) in a factorial manner (i.e. all possible combinations). A control treatment (uninoculated) from each cultivar was also included in the experiment. Completely randomized design (CRD) was used with 3 replicates.

The inoculation method was modified from that proposed by Somasegaran and Hoben.[11] Briefly, the soybean seeds from each genotype were surface-sterilized in 3% hydrogen peroxide in a beaker for 5 min, then the solution was drained off. The seeds were rinsed with sterile water for 4 - 5 times, more sterile water was added to submerge the seeds and they were let stand for 4 h. The seeds were rinsed with sterile water 2 - 3 times more, transferred onto a plate laid with wet sterile cotton wool, and left overnight at room temperature until the radicles were 0.5 - 1 cm long.

Four germinating seeds from each cultivar were sown in a plastic cup filled with sterilized sand pre-inoculated with *B. japonicum*. The cup was perforated at the bottom and placed on a Leonard jar containing N-free medium. The germinating seed was covered with autoclaved gravel. Five to 10 days after planting, 2 seedlings were cut with sterilized scissors and finally 2 plants were left in each cup. The N-free medium was added weekly into the jar thereafter. At the R_3 stage (pod size of around 0.5 cm in diameter), shoots of the 2 plants in each cup were collected in a paper bag and oven-dried at 70° C for 2 days and weighed for plant dry weight. The roots were collected in a flask with an air-tight rubber cap and the air was replaced with acetylene gas (C_2H_2). After one hour, the ethylene (C_2H_4) from the flask was collected to a tube by piercing the rubber lid with a syringe. The ethylene produced from reduction of the acetylene, as a result of rhizobial nitrogenase activity, was measured by a gas chromatograph to determine an acetylene reduction activity (ARA) value in µmole of C_2H_4 per plant per hour. The roots were then cleaned and determined for nodule number per plant, nodule fresh weight (g) per plant, and nodule dry weight (g) per plant.

Analysis of variance was performed in each trait using the Statistical Analysis System (SAS) program[12] to determine the significance of factors affecting N_2 fixation ability, viz. soybean cultivar, *B. japonicum* strain, and interaction between cultivar and strain. Once the F-test was significant, mean differences among cultivars and among rhizobium strains were declared by DMRT (Duncan's multiple-range test) at P = 0.05. The degree of direct association between the significant N_2 fixation traits was determined from their correlation coefficients (r). The indirect association was measured through path coefficient analysis of the ARA with the other fixation traits using the model proposed by Steel and Torrie.[13] Briefly, let Y = a dependent variable dictated by, say 3 independent variables X_1, X_2, and X_3. The relationship between them can be depicted as follows.



In this relationship b'_1, b'_2, and b'_3 are direct effects of X_i, as measured by standardized partial regression of Y on X_1, X_2, and X_3; r_{12}, r_{13}, and r_{23} are correlation coefficients between X_1 and X_2, X_1 and X_3, and X_2 and X_3; r_{1Y}, r_{2Y}, and r_{3Y} are total effects of X_i, as measured by correlation coefficients between X_1 and Y, X_2 and Y, and X_3 and Y, respectively.

The experiment was conducted in the laboratory of the Soil Microbiology Group, Soil Science Division, Department of Agriculture, Bangkok, Thailand.

Results and Discussion

Since the fixation data from the control jars were generally low with a number of zero values, they were not included in the analyses. Analysis of variance showed significant differences among soybeans, rhizobia, and their interaction in all N_2 fixation traits in this study (Table 1). It should be noted that there were 14 missing plants in this experiment, which caused a reduction in the degrees of freedom (df) of the Total and Error to 291 and 190, instead of 305 and 204, respectively. On the average, the Thai soybean cultivars were not different from the Korean cultivars in nodule number per plant (27.5 vs 26.6), but the mean of the Thai cultivars was higher than that of the Korean cultivars in nodule fresh weight (0.581 vs 0.482 g/plant), nodule dry weight (0.126 vs 0.096 g/plant), plant dry weight (1.913 vs 1.512 g/plant), and ARA (4.898 vs 3.845 µmole C_2H_4/pl/h). The interaction between Thai soybeans and rhizobial strains was significant in all fixation traits, revealing that there was a specificity between the soybean and the rhizobia. However, the interaction between Korean soybeans and rhizobial strains was significant only in nodule number per plant. The highest nodule number in Thai cultivars x strain was found in KKU35 inoculated with DASA01054 strain, and the lowest value was in CM60 inoculated with the DASA01026 (data not shown). CM1 with DASA01026 strain gave the highest nodule fresh and dry weight, while CM60 with DASA01042 gave the lowest value. The highest plant dry weight was found in CM1 with DASA01026, while the lowest value was in SJ2 with DASA01026. The highest ARA was obtained from ST1 with DASA01026 while the lowest was from SJ2 with DASA01054. The highest nodule number in Korean cultivars x strains was Jangkyung with DASA01026 and the lowest value was IT184222 with DASA01042 (data not shown).

The average values of the fixation components across 3 rhizobial strains in each cultivar are presented in Table 2. A rather high coefficient of variability (CV) was found associating with each trait. KKU35 gave the highest nodule number per plant, which was not significantly different from Songhak, followed by CM1, Keumkang, Sunheuk, Jangkyung, and SJ4. While IT184222, Jangyup, Dajang, and IT161471 from Korea and CM60 from Thailand had low nodule number. SJ4, ST1, RM1, Sunheuk, CM1, and SJ5 gave the highest nodule fresh weight per plant, while IT184222, IT161471, CM60, SJ2, and Milyang were among the lowest. In nodule dry weight, SJ4, ST1, Danwon, Sunheuk, SJ5, CM1, and RM1 were the highest, while several cultivars, including IT184222, CM60, SJ2, IT161471, and Milyang were the lowest. In plant dry weight, the Thai cultivars, RM1, CM1, SJ4, ST1, KKU35, and SJ5 grew more vigorously than most Korean cultivars. IT184222, IT161471, CM60, SJ2, and Milyang were low in plant dry weight. However, in ARA, the Thai and Korean cultivars fell into both high and low groups. ST1, RM1, Sunheuk, and Songhak were in the high ARA group, while IT184222, SJ2, IT161471, Jangyup, and Sowon were in the low group. It should be noted that IT184222 from Korea gave the lowest value in all fixation traits observed.

When the N_2 fixation components were averaged across all cutivars in each rhizobial strain, DASA01026 gave high nodule number, nodule fresh weight, and nodule dry weight. DASA01054 gave high nodule number, plant dry weight, and ARA, while DASA01042 gave only high plant dry weight (Table 3). This result is

Table 1. Mean squares from the analysis of variance of 5 traits related to N_2 fixation ability in 34 soybean cultivars inoculated with 3 rhizobial strains. All observations were determined by Leonard jar method. Data from the control jar were not included in the analysis.

Source of Variation	df	Nodules/plant	Fresh nodule wt/plant (g)	Dry nodule wt/plant (g)	Plant dry weight (g)	ARA (µmole C_2H_4/pl/hr)
Soybean cultivars	33	450**	0.235**	0.013**	2.12**	27.2**
Thai vs Korean	1	136^{ns}	0.881**	0.076**	11.82**	97.9**
Rhizobium strains	2	806**	1.248**	0.061**	1.34**	165.1**
Cultivars x Strains	66	116**	0.034**	0.003**	0.21^{ns}	7.4**
Thai cultivar x Strain	24	126**	0.056**	0.004**	0.32*	10.0**
Korean cultivar x Strain	40	81*	0.020^{ns}	0.002^{ns}	0.04^{ns}	5.3^{ns}
Thai vs Korean x Strain	2	699**	0.070*	0.004^{ns}	2.19**	15.7*
Error	**190**	**50**	**0.022**	**0.002**	**0.19**	**4.0**
Total	**291**					

*,** Significantly different at the 0.05 and 0.01 probability levels, respectively.
^{ns} non-significant (P > 0.05).

Table 2. Average number of nodules per plant, fresh nodule weight, dry nodule weight, dry plant weight, and ARA in 34 soybean cultivars averaged across 3 rhizobial strains.

Number*	Cultivar names	Nodules/plant	Fresh nodule wt/plant (g)	Dry nodule wt/plant (g)	Dry plant weight (g)	ARA µmole C_2H_4/pl/hr
1	SJ 1	24.56^{e-j}	0.544^{d-h}	0.129^{c-f}	1.79^{b-g}	4.75^{c-g}
2	SJ 2	19.22^{h-j}	0.213^{l-n}	0.046^{j-k}	1.00^{j-l}	1.90^{i-j}
3	SJ 4	33.22^{b-d}	0.834^{a}	0.189^{a}	2.28^{a-b}	5.71^{b-c}
4	SJ 5	27.00^{d-h}	0.681^{a-e}	0.152^{a-d}	2.11^{a-d}	4.62^{c-g}
5	ST 1	24.56^{e-j}	0.780^{a-b}	0.181^{a-b}	2.28^{a-b}	9.06^{a}
6	ST 2	23.00^{f-j}	0.576^{c-g}	0.121^{c-g}	1.65^{d-i}	5.25^{b-e}
7	CM 1	36.33^{b-c}	0.693^{a-d}	0.146^{a-e}	2.55^{a}	3.16^{e-j}
8	CM 60	16.57^{j-k}	0.204^{m-n}	0.035^{k}	0.93^{l-k}	3.10^{e-j}
9	NS 1	29.44^{c-g}	0.582^{c-g}	0.137^{b-f}	2.07^{b-d}	4.97^{b-g}
10	KUSL 20004	25.67^{d-i}	0.550^{d-h}	0.107^{d-h}	1.63^{d-i}	5.05^{b-f}
11	RM 1	27.33^{d-h}	0.767^{a-b}	0.146^{a-e}	2.55^{a}	7.73^{a}
12	KKU 35	43.78^{a}	0.630^{b-f}	0.142^{b-e}	2.16^{a-c}	4.06^{c-i}
13	CKP 1	26.89^{d-h}	0.495^{f-j}	0.110^{d-h}	1.87^{b-g}	4.31^{c-h}
14	Kumjung 2	32.00^{b-e}	0.515^{e-i}	0.090^{f-j}	1.76^{c-g}	4.71^{c-g}
15	Dajang	18.33^{i-j}	0.538^{d-h}	0.102^{e-i}	1.82^{b-g}	3.47^{c-i}
16	Sowon	24.67^{e-j}	0.346^{j-m}	0.070^{h-k}	1.23^{h-l}	2.25^{h-j}
17	Dukyu	26.22^{d-i}	0.640^{b-f}	0.114^{d-h}	1.70^{c-h}	3.50^{c-i}
18	Sunheuk	34.00^{b-d}	0.737^{a-c}	0.160^{a-c}	1.92^{b-f}	7.93^{a}
19	Jangkyung	34.00^{b-d}	0.454^{g-k}	0.090^{f-j}	1.38^{g-k}	2.79^{f-j}
20	Doremi	20.78^{h-j}	0.363^{i-m}	0.074^{g-k}	1.21^{i-l}	3.05^{e-j}
21	Kumjungol	27.29^{d-h}	0.595^{c-g}	0.114^{d-h}	1.85^{b-g}	4.76^{c-g}
22	Keumkang	36.00^{b-c}	0.553^{d-h}	0.107^{d-h}	1.77^{c-g}	5.54^{b-d}
23	Ilmi	29.22^{c-g}	0.428^{g-k}	0.091^{f-j}	1.44^{f-j}	5.61^{b-d}
24	IlpumKumjang	22.00^{g-j}	0.368^{i-l}	0.070^{h-k}	1.44^{f-j}	3.65^{c-i}
25	Danwon	31.89^{b-e}	0.635^{b-f}	0.164^{a-c}	2.04^{b-e}	3.85^{c-i}
26	Duyu	30.43^{c-f}	0.497^{f-j}	0.102^{e-i}	1.54^{e-i}	4.03^{c-i}
27	Milyang	31.88^{b-e}	0.343^{j-m}	0.060^{i-k}	1.02^{j-l}	2.61^{g-j}
28	Songhak	38.8^{a-b}	0.595^{c-g}	0.112^{d-h}	1.54^{e-i}	7.11^{a-b}
29	IT 161471	18.56^{i-j}	0.311^{k-n}	0.057^{i-k}	0.78^{l}	1.80^{i-j}
30	IT 184222	10.00^{k}	0.158^{n}	0.032^{k}	0.36^{m}	1.00^{j}
31	Danbaek	27.00^{d-h}	0.570^{c-h}	0.120^{c-g}	1.83^{b-g}	3.32^{d-i}
32	Taekwang	18.56^{i-j}	0.450^{g-k}	0.093^{f-i}	1.80^{b-g}	3.85^{c-i}
33	Jangyup	16.56^{j-k}	0.404^{h-k}	0.077^{g-k}	1.50^{f-i}	2.14^{h-j}
34	Suwon 157	30.78^{b-f}	0.631^{b-f}	0.127^{c-f}	1.83^{b-g}	3.78^{c-i}
	CV (%)	**26.26**	**28.46**	**37.08**	**26.22**	**47.67**

$^{a-n}$ Means followed by the same letters in each trait are not significantly different at the 0.05 probability level by DMRT.
* Cultivar numbers 1 – 13 are Thai cultivars, numbers 14-34 are Korean cultivars.

similar to that reported earlier by Somwang et al[14], who demonstrated that DASA01026 and DASA01054 exhibited ability to form more nodules, with higher nodule dry weight and nitogenase activity.

The N_2 fixation traits in each cultivar were not well correlated. For example, KKU35 was high in nodule number but low in the ARA assay. Aprison et al[15] demonstrated that soybean nodules with the diameter of less than 4 mm showed lower N_2 fixation ability than those with 5-6 mm in diameter. In mungbean, however, Tomooka et al[16] found that the genotypes with low ability to fix N_2 had larger nodules. Nodule size may not

Table 3. Average number of nodules per plant, fresh nodule weight, dry nodule weight, dry plant weight, and ARA in 3 rhizobial strains averaged across 34 soybean cultivars.

B. japonicum strains	Nodules/plant	Fresh nodule wt/plant (g)	Dry nodule wt/plant (g)	Dry plant weight (g)	ARA (µmole C_2H_4/pl/hr)
DASA01026 (T_1)	28.9^{a}	0.623^{a}	0.129^{a}	1.54^{b}	4.00^{b}
DASA01042 (T_2)	23.4^{b}	0.397^{c}	0.080^{c}	1.76^{a}	3.02^{c}
DASA01054 (T_3)	28.0^{a}	0.532^{b}	0.113^{b}	1.71^{a}	5.58^{a}

$^{a-c}$Means followed by the same letter in each trait are not significantly different at the 0.05 probability level by DMRT.

Table 4. Correlation between N_2 fixation components across 34 soybean cultivars and 3 rhizobium strains.

N_2 fixation components	Fresh nod. wt.	Dry nod. wt.	Dry pl. wt.	ARA
No. of nodules	0.606**	0.579**	0.557**	0.438*
Fresh nod. wt.		0.964**	0.914**	0.738**
Dry nod. wt.			0.899**	0.703**
Dry pl. wt.				0.635**

*,** Significant at the 0.05 and 0.01 probability levels, respectively.

be correlated with fixation activity but rather with the quality of leghaemoglobin in the nodules. The active nodules should have high leghaemoglobin which gives red or pink color inside the nodules, while the less active ones are green, white, or brown.[17] Leghaemoglobin acts as does haemoglobin in blood as an oxygen-carrying pigment for bacteriod in the nodules.

Table 4 shows significant association between ARA and nodule number, nodule fresh weight, nodule dry weight, and plant dry weight, with the correlation coefficients (r) of 0.438, 0.738, 0.703, and 0.635, respectively. This implies that an improvement of one fixation component results in improvement of the others, including the ARA value. A more detailed relationship between ARA and the other components can be demonstrated in a path coefficient relationship, as shown in Figure 1. It was obvious that ARA value was the result of the other fixation traits, plus undefined factors designated by the residual. In this study, nodule fresh weight expressed a high positive direct effect (b'_2 = 1.020) and total effect on ARA (r_{2y} = 0.738). While the

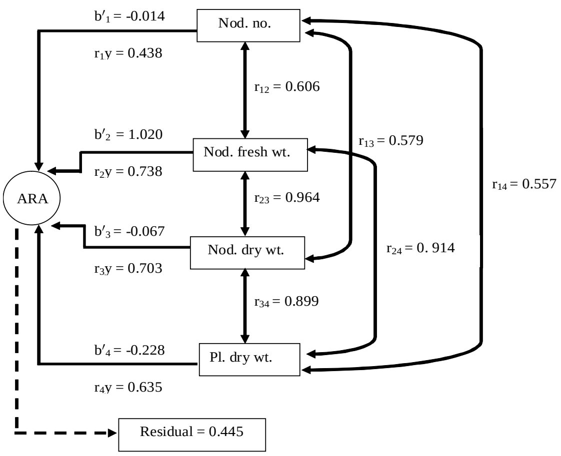


Fig 1. Path coefficient relationship between ARA and number of nodules per plant, fresh nodule weight per plant, dry nodule weight per plant, and dry plant weight across 34 soybean cultivars inoculated with 3 rhizobial strains.

other fixation traits had small direct effect (b'_i) on ARA, they also showed small indirect effect through each others. For example, the indirect effect of nodule fresh weight through plant dry weight was the highest among all the effects, with the value of –0.208 (b'_4 = -0.228 and r_{24} = 0.914). Nodule fresh weight contributed both directly and indirectly to the ARA, while nodule number, nodule dry weight, and plant dry weight had a little direct effect. Our finding is similar to that of Pazdernik et al[18] who reported a high positive correlation between nodule fresh weight and ARA (r = 0.86**), and a moderate correlation (r = 0.45*) between nodule number and ARA. DQbereiner[19] showed that nodule number did not always correlate with total N accumulated in shoots, while plant dry weight gave a more reliable correlation to N content. The total effect of all factors in our study upon ARA was moderate, because of the high indirect effect via nodule fresh weight. Thus, it can be concluded that N_2 fixation activity in this soybean population can be assessed through nodule fresh weight alone. A breeding program for improving N_2 fixation can be geared toward identifying the criteria for selection to improve nodule fresh weight. The criteria may be changed if the total N_2 fixed was used as the goal of N_2 fixation rather than the ARA.[20]

Conclusion

All the N_2 fixation traits in this study were positively correlated and this makes it rather easy to simultaneously improve them. Generally, the Thai soybean cultivars were superior to Korean soybeans in all the traits, except in nodule number per plant, which was not statistically significant. Nodule fresh weight was the most important trait contributing to ARA, and thus can be considered as the key trait for the improvement of N_2 fixation in this population.

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