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Technical Note

Effects of different rhizobium strains on nitrogen fixation of mungbean using ureide and ^{15}N abundance methods

Chatprawee Dechjiraratthanasiri [1,3], Choochad Santasup [1], Martin Werth [2] and Jiraporn Inthasan [3,*]

[1] Department of Plant and Soil Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50000, Thailand

[2] Institute of Systematic Botany and Ecology, University of Ulm, 89069, Ulm, Germany

[3] Division of Soil Resources and Environment, Faculty of Agricultural Production, Maejo University, Chiang Mai, 50290, Thailand

* Corresponding author, e-mail: inthasan@mju.ac.th

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Abstract: The effects of different rhizobium strains on nitrogen fixation of mungbean were assessed by ureide and ^{15}N abundance methods. Three genotypes of mungbean (MJU3, KPS2 and CN72) were used as the main plot factor and three rhizobium strains, viz. *Bradyrhizobium* sp. (SB1), *Bradyrhizobium elkanii* (SB2) and *Rhizobium* sp. (SB3) plus an uninoculated control (without rhizobia), as the subplot factor. Nodule fresh and dry weights in all genotypes increased significantly upon inoculation with SB2 strain at the R7 stage. Inoculation with SB2 also resulted in all genotypes being highest in total nitrogen, whereas inoculation with SB3 caused the greatest gain in shoot fresh and dry weights at R3.5 and R7stages respectively. The highest percentage of nitrogen derived from the atmosphere both from the ureide and ^{15}N abundance techniques was found in MJU3 genotype combined with SB2 inoculation at R3.5 and R7 stages.

Keywords: mungbean, nitrogen fixation, rhizobia, ureide method, ^{15}N abundance method

INTRODUCTION

Rhizobium is one of the bacteria that can fix atmospheric nitrogen into a plant-available form of nitrogen. Nitrogen fixation by the legume-rhizobia symbiosis is influenced by the strains of native rhizobia present in the soil, soil moisture, temperature, soil pH, essential elements and light [1, 2]. In some cases native rhizobia can colonise the legume host more efficiently than commercial strains [3]. Koskey et al. [4] found that native strains of rhizobia are more effective compared with different commercial or released strains. The beneficial aspects of native rhizobia include nutrient uptake, promotion of sustainable management of agricultural ecosystems, promotion of plant

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growth, adaptability to soil and environmental stress, and increase in the resident soil microbiota [5, 6].

Currently, there are five methods of measurement of nitrogen fixation, viz. N balance, N difference, ^{15}N isotope dilution, ureide concentration and acetylene reduction [7]. Each method has its specific limitations and errors, depending on the situation and equipment. Unkovich et al. [8] suggested that the most suitable methods for quantifying biological N_2 fixation in crop legume are ureide and ^{15}N natural abundance. The advantage of the ureide method is its simplicity and low cost plus the possibility to use both the sample xylem sap and stem segments for sample colorimetric assays of ureides, amino-N and nitrate-N. On the other hand, the more technically-demanding ^{15}N natural abundance method can be applied at any location whether in the field or greenhouse where both N_2-fixing and non-N_2-fixing plants are involved.

The present experiment involves evaluation nitrogen fixation in mungbean varieties with native rhizobia using both ureide and ^{15}N natural abundance techniques.

MATERIALS AND METHODS

Experimental Design

Soil samples from the field at Ulm University were collected before planting for soil analysis with the following results: pH 7.53 measured in a 1:1 slurry with deionised water; 3.11% organic carbon and 0.35% total nitrogen by dry combustion with a LECO TruSpec analyser [9]; 0.15 mg kg^{-1} extractable phosphorus measured by calcium-acetate-lactate extraction method [10]; 287 mg kg^{-1} extractable potassium analysed by microwave plasma - atomic emission spectrometer (Agilent, Germany).

Field soils were mixed with sand at 1:1 ratio and sterilised by steaming at 90°C for 4-6 hr [11]. Plants were grown in plastic pots. Each pot contained 10.5 kg of soil : sand mixture with 0.035 kg organic fertiliser. The study consisted of 12 treatments and 4 replications with split-plot in a completely randomised design. Varieties of mungbean (*Vigna radiate* L.), i.e. Maejo 3 (MJU3), Khampangsan 2 (KPS2) and Chainat 72 (CN72), from Prince Chakrabandh Pensiri Center for Plant Development, Saraburi province (Thailand) were used as the main plot factor. Rhizobium strains, viz. *Bradyrhizobium* sp. (SB1), *Bradyrhizobium elkanii* (SB2) and *Rhizobium* sp. (SB3) isolated from nodules of MJU3, KPS2 and CN72 at Prince Chakrabandh Pensiri Center for Plant Development, Saraburi province, Thailand [12], were used as the subplot factor with no rhizobia (-Rh) as control. *Sorghum bicolor* (L.) Moench was used as reference plant. The plants were maintained in a phytotron chamber at the mean day/night temperatures of 35°/25°C and 65% relative humidity. Water was applied daily to maintain a moisture level of 40-60% maximum water-holding capacity. The seedlings were thinned to three plants per pot after planting for 10 days and powdered triple superphosphate was added (1.4 g/pot).

Sampling and Nitrogen Content

Plant biomass and nodules were collected to estimate the number of nodules, plant nitrogen content and fresh and dry weights at R3.5 (beginning seedfill) and R7 (maturity harvest) stages [13].

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All plant samples were oven-dried at 65°C for 48 hr and then ground into fine powder. The nitrogen content in the shoot was determined by dry combustion [9] using a LECO TruSpec analyser.

Root-bleeding xylem sap samples were collected at R3.5 and R7 stages for determination of relative ureide index (% RUI), calculated from the molar concentration of ureide [14], amino nitrogen [15] and nitrate [16] using the following equation [8]:

$$\% \text{ RUI} = (4 \times \text{ureide} / (4 \times \text{ureide} + \text{amino-N} + \text{nitrate})) \times 100$$

The percentage of nitrogen derived from the atmosphere (% Ndfa) was calculated from % RUI (% RUI = 8.6 + 0.75 x % Ndfa) at R3.5 and R7 stages [17]. Shoot samples were also collected at R3.5 and R7 for determining the natural abundance of the stable isotope ^{15}N using a stable isotope mass spectrometer (Thermo Scientific, Germany). The % Ndfa was then calculated using the following equation [8, 18] where *B* is the $\delta^{15}N$ (parts per thousand) of mungbean receiving all N from N_2 fixation:

$$\% \text{ Ndfa} = \frac{\delta^{15}N \text{ of reference plant} - \delta^{15}N \text{ of } N_2 \text{ fixing legume}}{\delta^{15}N \text{ of reference plant} - B} \times 100$$

Statistical Analysis

The data were analysed for statistical significance using Statistix 10 software, analysis of variance (ANOVA), mean separation, and least significant difference (LSD) test at significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Nodulation

The nodule assessment (number, fresh weight and dry weight) in Table 1 indicates that different varieties of mungbean are not significantly different for all parameters at the R3.5 stage. At the R7 stage, variety CN72 shows the highest fresh nodule weight at 0.83 g/plant but the result is not significant with KPS2 ($p < 0.05$). The strain of rhizobia has a significant impact on the parameters compared with control (-Rh) both in R3.5 and R7 stages. Generally, inoculation with strain SB2 results in the highest number of nodules (average of 93 nodules/plant) and the highest fresh and dry weights at the R7 stage (0.95 and 0.13 g/plant respectively) ($p < 0.05$) (Table 1).

The mungbean nodulation parameters and N_2 fixation are similar to other common legumes such as cowpea (*Vigna unguiculata*) and soybean (*Glycine max*) in glasshouse pot culture [19]. Not only inoculation with rhizobium strain, but the management of soil nitrite, compost fertiliser, variety of mungbean, and site or condition of growth also increase fresh and dry weights of legume nodules [17, 20-22]. Results from this study are similar to the inoculation of mungbean and mash bean [23-26] with rhizobium strains leading to enhanced nodulation, shoot and root dry weights and bean yields. Similarly, Dechjiraratthanasiri et al. [10] reported that SB2 could provide the highest number of nodules, highest nodule fresh and dry weights, highest chlorophyll content index and highest nitrogen content in the shoot and root of mungbean in the Leonard jar test.

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Table 1. Effects of different rhizobium strains on the number of nodules, fresh weight (g/plant) and dry weight (g/plant) of nodules at R3.5 and R7 stages

Treatment		R3.5			R7		
		No. of nodules	Fresh weight	Dry weight	No. of nodules	Fresh weight	Dry weight
Variety							
MJU3		40	0.31	0.043	68	0.73^{b}	0.096
KPS2		44	0.34	0.042	73	0.77^{ab}	0.118
CN72		46	0.35	0.047	73	0.83^{a}	0.118
F-test		ns	ns	ns	ns	*	ns
Rhizobium							
-Rh		18^{b}	0.13^{b}	0.017^{b}	30^{c}	0.44^{b}	0.078^{b}
SB1		48^{a}	0.36^{a}	0.049^{a}	76^{b}	0.87^{a}	0.121^{a}
SB2		56^{a}	0.40^{a}	0.059^{a}	93^{a}	0.95^{a}	0.127^{a}
SB3		50^{a}	0.44^{a}	0.052^{a}	88^{ab}	0.85^{a}	0.118^{a}
F-test		*	*	*	*	*	*
Variety x Rhizobium							
MJU3	-Rh	17	0.12	0.016	29	0.37	0.063
MJU3	SB1	44	0.39	0.051	59	0.84	0.107
MJU3	SB2	56	0.34	0.057	98	0.97	0.120
MJU3	SB3	44	0.41	0.047	87	0.73	0.093
KPS2	-Rh	26	0.18	0.020	30	0.45	0.087
KPS2	SB1	47	0.36	0.051	82	0.84	0.127
KPS2	SB2	52	0.46	0.052	92	0.92	0.127
KPS2	SB3	50	0.37	0.046	89	0.88	0.133
CN72	-Rh	13	0.10	0.014	30	0.49	0.083
CN72	SB1	53	0.34	0.045	85	0.93	0.130
CN72	SB2	61	0.52	0.066	90	0.95	0.133
CN72	SB3	56	0.42	0.062	87	0.94	0.127
F-test		ns	ns	ns	ns	ns	ns

* Different superscripts within each column denote significant ($p < 0.05$) differences between groups; ns = non-significant difference.

Plant Biomass and Total N

Results on the fresh weight of plant biomass indicate that at the R3.5 stage, the treatment with CN72 gives significantly greater shoot fresh weight (18.9 g/plant) than does MJU3 (15.7 g/plant) (Table 2). However, differences in shoot dry weight are not significant in this experiment in all stages among mungbean varieties. Inoculation with all rhizobium strains significantly increases the fresh and dry biomass and total N content of all genotypes. Inoculation with rhizobium strain SB2 results in the highest levels of total N for KPS2 in R3.5 stage and for MJU3 in R7 stage (4.65 and 3.40% respectively) ($p < 0.05$). At R3.5 stage, the highest shoot fresh and dry weights are found in all

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genotypes inoculated with strain SB3, but this is not significantly higher than the plants inoculated with SB2 or -Rh. However, there is no significant difference in total N and biomass among mungbean varieties and rhizobium strains (Table 2).

Table 2. Effects of different rhizobium strains on total N, fresh weight and dry weight of shoot (g/plant) at R3.5 and R7 stages

Treatment		R3.5			R7		
		Total N (%)	Shoot fresh weight	Shoot dry weight	Total N (%)	Shoot fresh weight	Shoot dry weight
Variety							
MJU3		3.25^{b*}	15.7^{b}	2.9	2.73	17.6	5.3
KPS2		3.66^{a}	17.4^{ab}	3.0	2.93	20.7	5.8
CN72		3.83^{a}	18.9^{a}	3.0	3.00	22.4	6.0
F-test		*	*	ns	ns	ns	ns
Rhizobium							
-Rh		3.22^{b}	18.2^{a}	3.1^{a}	2.55^{c}	19.3	5.6
SB1		3.33^{b}	15.9^{b}	2.7^{b}	2.89^{b}	18.9	5.3
SB2		4.20^{a}	17.1^{ab}	2.9^{ab}	3.30^{a}	21.0	5.8
SB3		3.57^{b}	18.2^{a}	3.2^{a}	2.81^{bc}	21.7	6.0
F-test		*	*	*	*	ns	ns
Variety x Rhizobium							
MJU3	-Rh	2.78	16.4	2.9	2.17	17.0	4.9
MJU3	SB1	3.22	14.8	2.7	2.75	17.2	5.0
MJU3	SB2	3.65	15.8	2.8	3.40	17.6	5.5
MJU3	SB3	3.36	15.9	3.1	2.60	20.6	5.8
KPS2	-Rh	3.05	19.0	3.4	2.83	21.9	5.7
KPS2	SB1	3.31	15.1	2.6	2.89	15.9	4.3
KPS2	SB2	4.65	17.1	2.9	3.27	20.9	6.1
KPS2	SB3	3.61	18.2	3.2	2.72	24.0	6.9
CN72	-Rh	3.83	19.1	3.0	2.63	21.2	6.0
CN72	SB1	3.44	17.7	2.7	3.04	23.6	6.6
CN72	SB2	4.30	18.4	2.8	3.24	24.5	6.0
CN72	SB3	3.75	20.4	3.3	3.11	20.4	5.3
F-test		ns	ns	ns	ns	ns	ns

*Different superscripts within each column denote significant ($p < 0.05$) differences between groups; ns = non-significant difference.

In particular, these results show that the native rhizobia SB2 (*Bradyrhizobium elkanii*) and SB3 inoculated in all mungbean varieties best increase the number of nodules and fresh and dry weights of shoot at R3.5 and R7 stages. This agrees with the previous experiment by Favero et al. [27] who selected native rhizobia from 10 samples of tropical soil at Seropédica, Rio de Janeiro,

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Brazil to increase mungbean yield and quality by examining their biological nitrogen fixation. They found that 99% of DNA of the most efficient nitrogen-fixing species showed similarity to *Bradyrhizobium elkanii* by 16S rRNA technique.

Percentage of Nitrogen Derived from Air (% Ndfa)

The % Ndfa values determined using the ureide method on the root-bleeding sap sampled at R3.5 and R7 stages are presented in Table 3. The % Ndfa (ureide) values are substantially higher at R7 than at R3.5 (average of 30% at R3.5 and 75% at R7). At R3.5 stage, the MJU3 variety has the highest % Ndfa (ureide) at 37% ($p < 0.05$). The % Ndfa (ureide) differs significantly for different rhizobium strains both in R3.5 and R7 stages. The SB2 strain fixes more N (43% at R3.5 and 82% at R7) than do SB3, SB1 and the uninoculated control.

Table 3. Comparisons of % Ndfa by ureide and ^{15}N abundance methods measured at R3.5 and R7 stages

Treatment		% Ndfa (ureide)		% Ndfa (^{15}N abundance)	
		R3.5	R7	R3.5	R7
Variety					
MJU3		37^{a}	76	37	74
KPS2		28^{b}	77	34	71
CN72		26^{b}	72	34	70
F-test		*	ns	ns	ns
Rhizobium					
-Rh		19^{c}	67^{c}	21^{c}	63^{c}
SB1		31^{b}	75^{b}	31^{bc}	70^{b}
SB2		43^{a}	82^{a}	51^{a}	79^{a}
SB3		27^{bc}	76^{b}	38^{b}	75^{ab}
F-test		*	*	*	*
Variety x Rhizobium					
MJU3	-Rh	28	68	24	65
MJU3	SB1	39	78	34	77
MJU3	SB2	48	83	52	80
MJU3	SB3	32	77	37	75
KPS2	-Rh	16	68	18	63
KPS2	SB1	28	79	30	68
KPS2	SB2	44	81	51	78
KPS2	SB3	22	79	39	75
CN72	-Rh	14	66	20	62
CN72	SB1	26	69	28	64
CN72	SB2	36	81	49	79
CN72	SB3	28	71	39	75
F-test		ns	ns	ns	ns

*different superscript letters within each column denote significant ($p < 0.05$) differences between groups; ns = non-significant difference.

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The % Ndfa values determined using ^{15}N abundance are not significantly different among varieties and for variety-rhizobium interactions at both R3.5 and R7. Strain SB2 again produces the highest % Ndfa (^{15}N abundance) values of 51% at R3.5 and 79% at R7. It has been previously reported that varieties of mungbean range in % Ndfa at maturity between 53- 68% [28]. Hayat et al. [29] using the ureide technique reported that mungbean and mash bean have average % Ndfa values of 40-71% and 41-71% respectively. There are limited studies comparing different methods of quantifying N_2 fixation, but those that do indicate that the ureide and ^{15}N natural abundance techniques have highly correlated % Ndfa in field experiments [23, 24]. Researchers have also found that relative ureide-N values tend to correspond with $\delta^{15}N$ in different site experiments and genotypes.

There are good correlations between % Ndfa (ureide) and % Ndfa (^{15}N abundance) at both R3.5 and R7 stages (R^2 = 0.72 and 0.77 respectively) (Figure 1) and an even stronger correlation between the two methods when the data for R3.5 and R7 samplings are combined (R^2 = 0.94) (Figure 2). However errors of ~10% are observed.

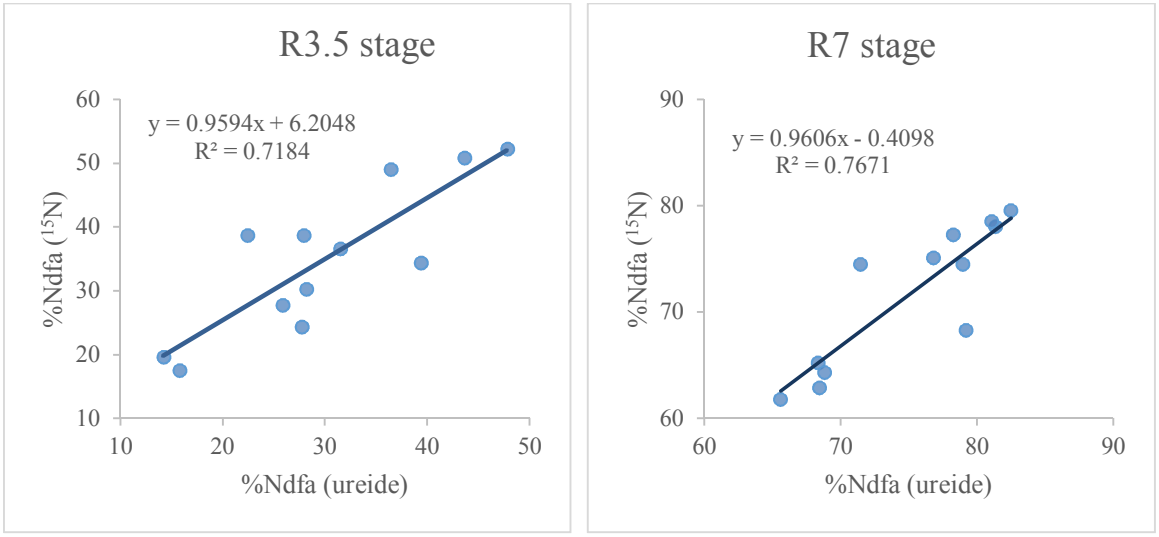

Figure 1. Regression of % Ndfa (^{15}N abundance and ureide) at R3.5 and R7 stages of mungbean growth

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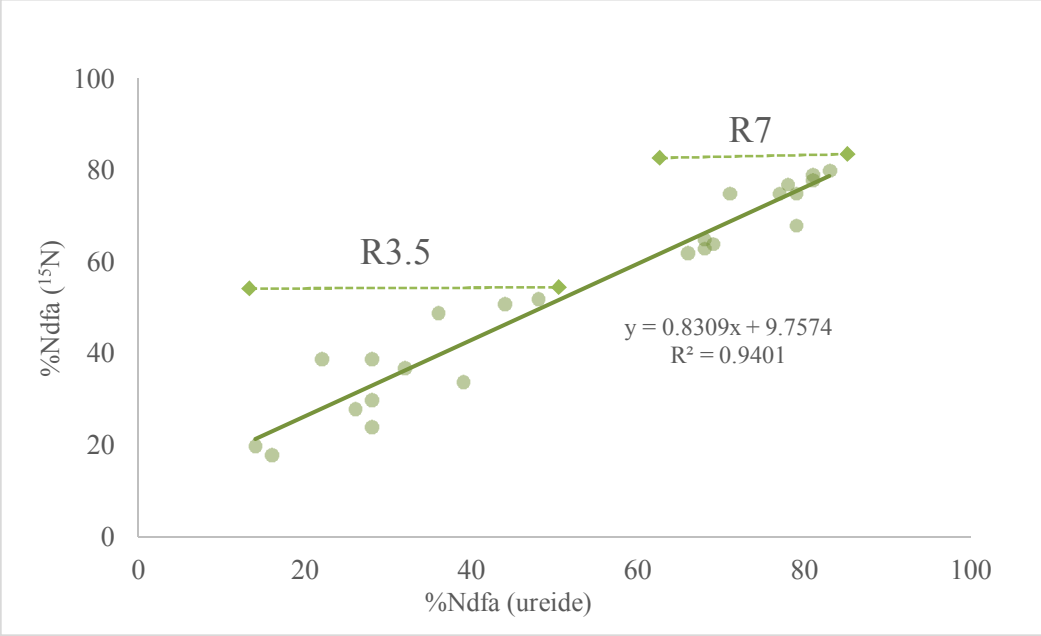

Figure 2. Regression of % Ndfa (^{15}N abundance) versus % Ndfa (ureide) sampled at R3.5 and R7

CONCLUSIONS

Inoculation of all mungbean varieties with rhizobium strain SB2 produces the greatest number of nodules, the highest fresh and dry weights of nodules and the highest % Ndfa. The ureide and ^{15}N natural abundance techniques produce similar estimates of % Ndfa for phytotron-chamber mungbean harvested at podfill (R3.5) and physiological maturity (R7) stages. Furthermore, the MJU3 variety has the highest nitrogen fixation compared with CN72 and KPS2 when measured by ^{15}N natural abundance. This study shows that ureide and in particular ^{15}N natural abundance are adequate methods for investigating nitrogen fixation of mungbean.

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