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Combined Inoculation of Rhizobium and Nitrogen Fertiliser Application on Nitrogen Fixation in Soybean (*Glycine max* L.) at Central Vietnam

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Abstract

Background: The use of soybeans (*Glycine max* L.) as a food and animal feed has grown dramatically throughout Vietnam in recent years. Relatively few studies have examined the relationships between rhizobium inoculation and nitrogen application on soybeans in Central Vietnam. The study aimed to evaluate the responses of rhizobium inoculation and split N fertiliser application on nodule and N_2 fixation in coastal sandy soil at Hue city, Central Vietnam.

Methods: Field experiments were conducted in coastal sandy soil in two cropping seasons of 2025. Six treatments with two factors including split times of N fertiliser application in combination with and without inoculation of rhizobium which were laid out in a split plot design with three replications. Data was collected as nodule number, fresh and dry weight and nitrogen fixation efficiency.

Result: The obtained results showed significant differences ($p<0.05$) in nodules characteristics, N uptake and N fixation between inoculated and no inoculated treatments combined with split times of N fertiliser application. The three times of N fertiliser application and rhizobium inoculation was shown the best in all parameters. Further study will be considered on how the N_2 fixation related with soybean yield and N use efficiency.

Key words: Coastal sandy soil, N fertiliser application, N fixation, Rhizobium, Soybean.

Introduction

As a major legume crop in the world, soybean (*Glycine max* L.) is considered for its high protein content, balanced amino acid profile and broad use in human and animal nutrition (Rizzo *et al*., 2018). Leguminous crops' ability to fix nitrogen biologically (BNF) has attracted attention as a nitrogen supply, thus it can reduce the need for N fertiliser to maintain crop yields. BNF from legumes, either alone or in conjunction with fertiliser, can be used as a sustainable farming method (Iannetta *et al*., 2021). Although soybean may symbiotically fix nitrogen (BNF) with *Bradyrhizobium* spp. to meet a large portion of their nitrogen needs, the effectiveness of this process depends on a number of agronomic and environmental conditions (Szpunar-Krok *et al*., 2023). Among these, root nodulation and nitrogen uptake are significantly influenced by soil temperature, water availability and nitrogen fertilisation (Zhang *et al*., 2023). Both native soil populations and commercial inoculants given to seeds during planting are the sources of rhizobia that colonise legume root nodules (Tamagno *et al.,* 2018). At present, inoculation with effective strains enables the soybean plant to get all of its nitrogen requirements from BNF (Getachew and Dagnaw, 2020). This is a natural process of major relevance in global agriculture and has enormous potential for smallholder farmers in Vietnam. Leguminous crops like soybean, common bean, chickpea and peanut also exhibit extraordinary growth and yield response to rhizobia inoculations in various agro-ecologies, according to many field demonstrations conducted in Vietnam (Doan *et al*., 2017). It is important to emphasise that there are still insufficient *B. japonicum* populations in coastal sandy soils, thus farmers or seed manufacturers must inoculate their own seed. Relatively few studies have examined the relationships between split N fertiliser application and rhizobium inoculation on nitrogen fixation in field-grown soybeans in Central Vietnam under coastal sandy soil conditions, most of studies were only conducted in the North and the South of Vietnam for a long time ago (Hiep *et al*., 2002; Doan *et al.,* 2017). Hence, this study was implemented to fill in existing knowledge gaps about the split N fertilisation and rhizobia inoculation combination and nodulation efficiency as well as N_2 fixation from soybean in this area.

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Materials and Methods

Description of the research area

Field experiments were carried out in Quang Loi commune (old) (16.60.34°N, 107.48.18°E), Hue city, during spring season (February to June) and summer season (June-August), 2025. The FAO/UNESCO soil classification indicates that Haplic Arenosols is the predominant soil type in the area which > 80% sandy content (light texture soil) and poor nutrients (OC <1%; N <0.05% and CEC <5 cmolc kg^{-1}) (Hoang *et al*., 2019).

Experimental set up

Rhizobium strain (10^9 CFU g^{-1}), a well-known commercial rhizobial inoculant provided by SIAMB (Vietnam), was employed in the experiment with the soybean variety namely DT90. Split times of N fertiliser application (1, 2, 3 application times in corresponding with 1 time (at basal), 2 times (at basal and top dressing at 2-3 fully leaves - 12 DAS) and 3 times (at basal, top dressing at 2-3 fully leaves (12 DAS), 5-6 fully leaves -35 DAS were evaluated as subplot treatments and two inoculation levels [inoculated (1) and non-inoculated (0)] were used as the main plot in the split-plot design with three replications. For every plot, two weeks before sowing, 500 kg ha^{-1} lime ($CaCO_3$-56% CaO) was broadcast and mixed into the soil to a depth of 20 cm. All plots received a basal application of cattle manure at 8 t ha^{-1} (1.05-1.15% N, 0.67-0,70% P_2O_5, 1.21-1.32% K_2O) and phosphorus (SSP, 16% P_2O_5) at 90 kg P_2O_5 ha^{-1}. Potassium (60 kg K_2O ha^{-1}) and nitrogen (40 kg N ha^{-1}) were utilised as potassium chloride (60% K_2O) and urea (46% N). Following row treatments, the K fertilizer was additionally applied at two times in the plant's growth cycle: 12 days after seeding, one-third of the total at the time the 2-3 fully leaves expanded and 35 days after seeding, with the remaining two-thirds immediately before blooming (5-6 fully leaves). The N fertiliser was applied following treatments as follows: In 1 time (100% as basal), 2 times (1/3 N at basal and 2/3 N at the 2-3 fully leaves expanded) and three times (1/3 N at basal; 1/3 N at the at the 2-3 fully leaves expanded and 1/3 N at the at the 5-6 fully leaves expanded). Six rows made up the 3 m by 5 m (15 m^2) plot, with two rows designated for nodulation and shoot dry weight characteristics and the remaining rows-aside from the borders-being harvestable rows. Plots were spaced 0.3 m apart, with blocks separated by 0.5 m. Each plot was made into a bed by preparing canals around it. The soybean seeds are soaked and mixed with the inoculant at the rate of 500 ml of the inoculant per one kilogram of the soybean seeds and air-dried for 30 minutes before planting. To ensure that every seed had a thin layer of the inoculant, the thick slurry of the inoculant was carefully combined with dried seed. To preserve the viability of the bacterial cells, all inoculations were carried out right before planting in a shaded area. The infected and un-inoculated seeds were then planted at a spacing of 10 cm between plants and 30 cm between rows making 33 plants m^{-2}. To identify the N_{fixed}, local maize variety as a reference crop was used, which was grown in the rows outside the experiment plots in an area of 50 m^2.

Fresh and dry weight of the nodulation

At full flowering stage, ten randomly selected plants were taken from each plot at border rows. To extract undamaged roots and nodules for nodulation parameters and plant dry weight, the entire plant was gently uprooted using a spade. To prevent nodule loss, uprooting was accomplished by exposing the entire root system. The roots with intact nodules were gently washed with water over a metal screen to remove the clinging soil. Nodulation, nodule dry weight and the number of nodules per plant were recorded using the same 10 plants from each plot. Three plants from each plot were cut at ground level to determine the shoot dry weight after nodules were removed. An electronic balance was used to determine the total weight of fresh shoots. Using brown envelopes, subsamples of shoots were oven-dried for 48 hours at 70°C. Weighing the dry materials allowed us to record the average shoot dry weight for each plant. N shoots and roots of soybean were also analysed following the digestion, distillation and titration processes of the Kjeldahl method.

N fixation of soybean

Biological nitrogen fixation (BNF) was calculated by formula as follows (Mogale *et al.,* 2023):

$$\%BNF = \frac{N_{fixed}}{N_{total}} \times 100$$

$$N_{fixed} = N_{uptake}\ (soybean) - N_{uptake}\ (reference)$$

$$\%N_{dfa} = \frac{N_{fixed}}{N_{uptake}\ (soybean)} \times 100$$

N_{uptake} = N concentration in shoot and root (mg kg^{-1}).

Data analysis

The 2 factors ANOVA analysis method offered by Statistix statistical software version 10 was used. The effects of treatment parameters (split times of N fertiliser application and inoculation levels) and their interactions on nodulation and N fixation were assessed by using analysis of variance. The LSD technique was used to compare means among treatments ($p<0.05$).

RESULTS AND DISCUSSION

Soybean nodulation characteristics

There was effects of rhizobium inoculation and split N fertiliser application on the number of nodules (Table 1); the highest number of nodules were found in treatment with inoculation and 3 times of N fertiliser application (32.40 and 28.43 nodules $plant^{-1}$); however, the highest number of nodules was found at non-inoculation and 2-3 times of N fertiliser application (23.59 and 22.70 nodules $plant^{-1}$). Number of effective nodules $plant^{-1}$ were recorded a significant higher in spring season than in summer season from 14-28% at 3 times of N fertiliser application both in inoculation and non-inoculation. At both cropping seasons, fresh and dry weight of nodules were exhibited the highest values on combination treatment of 3 times of N fertiliser application with inoculation with 1.69-1.94 and 0.73-0.74 mg $plant^{-1}$, respectively. There were significant differences between fresh and dry weight of nodules for the most treatments.

Therefore, N fertiliser application with rhizobium inoculation had an impact on nodulation quantity and weight. This outcome is consistent with prior research showing that co-inoculation increased the number of large nodules and the volume of nodules (Bais *et al*., 2023). Rhizobia seed inoculation outperformed uninoculated treatments by a wide margin. Both rhizobia inoculation and N rates administered separately and enhanced soybean nodulation and yield (Getachew and Dagnaw, 2020; Szpunar-Krok *et al*., 2023). Khaitov and Abdiev (2018) showed a significant advantage of rhizobium inoculation over uninoculated treatments at all N fertilisation rates (50, 75 and 100 kg N ha^{-1}). Jarecki and Bobrecka (2019) indicated an increase in nodulation on soybean roots after seed inoculation with *Bradyrhizobium japonicum*. The legume fixation at lower N rate may not be sufficient to meet its N demand, an additional high N rate as a starter to create a symbiosis with rhizobia, which could explain the split times of N fertiliser application reported enhanced nodule yield after inoculation (Ntambo *et al*., 2017; Budiastuti *et al.,* 2025). When soybeans were treated with 3 times of N application, the number of nodules at the was significantly increased, although we found that split times of N fertiliser application inhibits nodulation in our study, as evidenced by a decrease in nodule number and volume with the less times of N fertiliser application, the mechanism by which N inhibits nodulation is not well understood and may be due to a variety of factors including cultivation technology, soil and climate (Xia *et al*., 2017; Jarecki and Bobrecka, 2019). Several theories have been put out to explain how N inhibits nodulation, including lower O_2 diffusion into nodules that limits bacteroids' ability to breathe, feedback inhibition caused by a byproduct of nitrate metabolism and carbohydrate shortage in nodules (Bais *et al*., 2023). When process of nodulation formulation by native soil rhizobia is either nonexistent, very low, or restricted in soils lacking of homologous rhizobia, the number of nodules increases following inoculation (Thilakarathna and Raizada, 2017). Incompatibility between the host micro-symbiont and the function of both known and undiscovered biomolecules, such as flavonoids, polysaccharides and hormones, may be the cause of the absence or the development of inefficient nodules on soybean roots (Daayf *et al.,* 2012).

N content in shoots and roots and uptake of soybean

There were increasing of shoot and root N accumulation following the split times of N fertiliser application with inoculation of rhizobium in soybean (Table 2). Both non-inoculation and inoculation treatments had the highest values of shoot N (2.31-2.36% and 2.41-2.56%) and root N (2.23-2.40% and 2.50-2.62%) at 3 times of N fertiliser application in both cropping seasons. Omari *et al*. (2022) reported that under greenhouse experiment, shoot N levels were consistently greater after GMM36 and GEM96 inoculation. When compared to the non-inoculated control, the GMM36 and GEM96 inoculation dramatically raised the

Table 1: Response of inoculation of Rhizobium and N fertiliser application times on nodulation characteristics of soybean.

Inoculation treatment	Split N fertiliser application	Spring 2025: Total nodules plant^{-1}	Spring 2025: Effective nodules plant^{-1}	Spring 2025: Fresh weight of nodule (g plant^{-1})	Spring 2025: Dry weight of nodule (g plant^{-1})	Summer 2025: Total nodules plant^{-1}	Summer 2025: Effective nodules plant^{-1}	Summer 2025: Fresh weight of nodule (g plant^{-1})	Summer 2025: Dry weight of nodule (g plant^{-1})
Non inoculation	1	18.85^{c}	12.63^{c}	0.86^{d}	0.31^{c}	16.92^{d}	11.34^{d}	0.58^{e}	0.28^{d}
	2	23.59^{bc}	15.80^{bc}	0.79^{d}	0.35^{c}	17.55^{cd}	11.76^{cd}	0.67^{de}	0.32^{cd}
	3	22.59^{bc}	15.13^{bc}	1.23^{bc}	0.58^{ab}	22.70^{bc}	15.21^{bc}	1.03^{bc}	0.52^{b}
Inoculation	1	23.93^{b}	16.03^{b}	1.02^{cd}	0.30^{c}	21.59^{bcd}	14.46^{bc}	0.93^{cd}	0.27^{d}
	2	26.99^{b}	18.09^{b}	1.46^{b}	0.55^{b}	25.17^{ab}	16.87^{ab}	1.29^{b}	0.49^{bc}
	3	32.40^{a}	21.70^{a}	1.94^{a}	0.74^{a}	28.43^{a}	19.05^{a}	1.69^{a}	0.73^{a}
$LSD_{0.05}$		4.57	3.06	0.32	0.36	4.91	3.29	0.33	0.39

Values with a common letter in the same column are not significantly different using LSD at 5% level.

Table 2. Response of inoculation of Rhizobium and N fertiliser application times on N content in shoot and root and N uptake of soybean.

Inoculation treatment	Split N fertiliser application	Spring 2025: Shoot N (%)	Spring 2025: root N (%)	Spring 2025: N_{uptake} (kg ha^{-1})	Summer 2025: shoot N (%)	Summer 2025: Root N (%)	Summer 2025: N_{uptake} (kg ha^{-1})
Non inoculation	1	2.25^{d}	2.17^{d}	185.42^{c}	2.17^{c}	2.20^{e}	151.87^{d}
	2	2.33^{cd}	2.27^{cd}	187.18^{c}	2.23^{c}	2.30^{d}	156.46^{cd}
	3	2.36^{c}	2.23^{bc}	196.12^{c}	2.31^{b}	2.40^{c}	161.40^{c}
Inoculation	1	2.46^{bc}	2.43^{bc}	228.93^{b}	2.35^{ab}	2.48^{c}	185.56^{b}
	2	2.52^{ab}	2.60^{a}	240.55^{ab}	2.37^{ab}	2.53^{b}	189.07^{b}
	3	2.56^{a}	2.50^{ab}	247.34^{a}	2.41^{a}	2.62^{a}	200.02^{a}
$LSD_{0.05}$		0.09	0.15	12.45	0.07	0.04	5.66

Values with a common letter in the same column are not significantly different using LSD at 5% level.

Table 3: Response of inoculation of Rhizobium and N fertiliser application times on N fixation of soybean.

Inoculation treatment	Split N fertiliser application	Spring 2025			Summer 2025		
		BNF (%)	$N_{2\ fixed}$ (kg ha^{-1})	N_{dfa} (%)	BNF (%)	$N_{2\ fixed}$ (kg ha^{-1})	N_{dfa} (%)
Non inoculation	1	43.18^{d}	55.92^{d}	30.06^{d}	32.64^{c}	37.37^{d}	24.51^{c}
	2	44.54^{cd}	57.68^{d}	30.78^{cd}	36.65^{bc}	41.96^{cd}	26.81^{bc}
	3	51.44^{abc}	66.62^{cd}	33.95^{abc}	40.96^{ab}	49.89^{bc}	29.04^{b}
Inoculation	1	47.22^{bcd}	73.43^{bc}	31.98^{bcd}	36.84^{bc}	46.96^{cd}	26.89^{bc}
	2	54.69^{ab}	85.05^{ab}	35.22^{ab}	39.44^{bc}	53.47^{b}	28.20^{b}
	3	59.06^{a}	91.84^{a}	37.04^{a}	47.51^{a}	64.42^{a}	32.06^{a}
$LSD_{0.05}$		8.06	12.28	3.65	4.46	5.66	2.36

Values with a common letter in the same column are not significantly different using LSD at 5% level.

shoot N content by an average of 79%. Under field conditions, there was an average 39% increase in shoot N between the inoculated and non-inoculated control. N uptake was found the highest values at treatment of inoculation combined with 3 application times of N fertiliser in both crop seasons (200.02-247.34 kg ha^{-1}). Number of nodules and N uptake were noticeably higher in the greenhouse than in the field conditions, suggesting that abiotic factors have a substantial impact on nodulation and soybean growth (Goyal *et al*., 2021; Abiyot *et al*., 2022).

N fixation ability of soybean

Across all data sets, BNF had a close relationship with N fixation, N_{fda} and it tended to increase as N fertiliser application time together with inoculation (Table 3). BNF data was ranging from 43.18 to 59.06% in spring season and from 32.64 to 47.51% in summer season. It was found higher than 9.3-14.8% in spring season and 12.9-15.9% in summer season at inoculation treatment compared to non-inoculation at 3 times of N fertiliser application. Generally, BNF was the highest values at 3 application times of N fertiliser combined with inoculation in both cropping seasons. Similarly with result of Martins *et al.* (2022) who reported that inoculated soybean exhibited higher levels of nodulation, plant biomass, BNF and yield components compared to non-inoculated by preserving BNF at subsequent growth stages, further inoculation in the V3 growth stage demonstrated many benefits. When non-inoculation was added, the maximum amount of N_2 fixation reached at 3 times of N fertiliser application (66.62 kg ha^{-1} and 49.89 kg ha^{-1} in both cropping seasons). N_2 fixation was higher than 29.1-37.8% in inoculation compared to non-inoculation. Similarly, it was also found that N_{dfa} was 29.04-33.95% at non-inoculation treatments in both seasons and 32.06-37.04% at inoculation in both seasons. These results suggested that N uptake, BNF, N_2 fixation and N_{dfa} were the highest at treatment of N fertiliser application at basal, 12 and 35 days after emergence combined with rhizobium inoculation, though they were not significantly differences from that of inoculated and uninoculated rhizobium on soybean. Plant growth and dry matter production are the main factors influencing how much N_2 legumes fix. However, at both sample intervals, plant %N_{dfa} and N accumulation did not react to further inoculation (Martins *et al*., 2022). If no new inoculations are produced, less effective strains with high saprophytic capacity may eventually dominate the soil population, reducing the benefits of BNF, even though the soybean crop responses to inoculation which is demonstrated successful in the first few years (Zilli *et al*., 2021). Since symbiotic N_2 fixation requires nodulation, soybeans may have stimulated free-living N_2-fixers, or any type of biological N_2 fixation that does not involve a clearly defined symbiotic relationship between plants and microorganisms, which is typical in undisturbed soil (Reed *et al*., 2011). Methodological issues may also be the cause of the BNF activity that we saw in non-inoculated on soybeans (Martins *et al*., 2022). The soil used in this experiment was coastal sandy soil with high sand content (>80%), implying that the soil was well aerated, low water and nutrient holding capacity, then the number of nodules were low in less times of N fertiliser application and non-inoculation of rhizobium resulting in low BNF (Thanni *et al*., 2017). N_2-fixing systems can thrive in soils poor in N that they are a source of proteins and they provide N for soil fertility (Cordeiro and Echer, 2019).

CONCLUSION

Fertilisation of N fertiliser application up to 40 kg ha^{-1} at three times (as basal, top dressing at 12 and 35 days after sowing) may be the ideal time for efficient nodulation and better N uptake, which would increase soybean BNF, N_2 fixation and N_{dfa}. The current findings show that employing rhizobia inoculant in conjunction with split N fertiliser application into 3 times at 40 kg N ha^{-1} has the potential to improve soybean nodulatio and N_2 fixation. Further research will consider on how the N_2 fixation related with soybean yield and N use efficiency.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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