Research Article

Effect of Nitrogen and Rhizobium Inoculation on Growth and Yield of Fenugreek (*Trigonella foenum-graecum*) Fresh Leaves

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Abstract | A study entitled effect of nitrogen and rhizobium inoculation on growth and yield of fenugreek (*Trigonella foenum-graecum*) fresh leaves was conducted at Peshawar, Pakistan in 28th of October 2023. The rhizobium inoculation significantly enhanced the growth and yield parameters of fenugreek. Seeds inoculated with *bradyrhizobium japonicum* resulted in the minimum days to emergence (5.92), more number of branches plant^{-1} (10.00), maximum number of leaves plant^{-1} (105.50), tallest plant (70.34 cm), highest nitrogen content in leaves (4.70 %), chlorophyll content (41.14) and maximum fresh leaves yield (7.51 tons ha^{-1}). However, nitrogen level at the rate of 60 kg ha^{-1} resulted in minimum days to emergence (6.42), more number of branches plant^{-1} (9.75), maximum number of leaves plant^{-1} (103.83), tallest plant (69.96 cm), highest nitrogen content in leaves (4.67%), chlorophyll content (39.43) and fresh leaves yield (7.49 tons ha^{-1}) and their interaction was found non-significant. Based on the obtained results, it is concluded that compared to uninoculated or untreated seeds, the inoculated seeds had excellent performance. Therefore, seeds inoculated with *bradyrhizobium japonicum* with the level of 60 kg ha^{-1} of nitrogen is advised for higher productivity and development of fenugreek for Peshawar.

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Introduction

Fenugreek *(Trigonella foenum-graecum*), is a member of the Fabaceae/Leguminosae family. Fenugreek, also referred to as methi, is a member of the *Trigonella* genus. West Asia, the subcontinent, Europe, and Australia are among the places where it is grown (Basu *et al.*, 2008). Additionally, it is grown throughout Pakistan, especially in the Sindh and Punjab regions, the production of fenugreek in Pakistan was recorded 494 tons cultivated on area 164 hectares. (Provincial crop reporting service centers 2021-2022). It is a herb that grows vertically, usually reaching a height of 10 to 50 cm (Suleiman *et al.*, 2008) having large, yellow grains produced by tall, semi-erect, moderately branching with the dark green, vitamin A, iron, and protein-rich leaves and greenish pods (Petropoulos, 2002). Fenugreek can be produced in sub-tropical climates with moderate winters and required temperature of 10-15 °C and requires pH of 6.0-7.0, relatively high temperatures increase seed formation (Singh *et al.*, 2014). Furthermore, Amin *et al.* (2005) discovered that fenugreek seed extract successfully prevents and inhibits the growth of breast cancer; this may be because flavonoids are present in the extract (Eidi *et al*., 2007) noted that fenugreek is also historically been used to cure a variety of illnesses, including rickets, depression, tumours, enlarged liver and spleen, heart disease, diabetes, fever and stomach disorders (Thorat *et al*., 2019). Its green leaves contains 1.1g fiber, 4.4 g proteins, 6 g carbohydrates, 86 g moisture, Vitamin B1, B2, B3, B6, and vitamin C (Wani *et al*., 2018). The seeds of fenugreek are frequently added as a spice to pickles, dal, and vegetable dishes. Curry recipes such as "aloo methi" curry frequently contain fresh fenugreek leaves. It has also been discovered that fenugreek has therapeutic qualities (Syeda *et al*., 2008; Vidyashankar, 2014). Legumes like fenugreek and Soybean, which are nodules forming plants, can fix atmospheric nitrogen into the soil by the action of a bacterial species known as "Rhizobia." These bacteria more especially, the rhizobium live in the root nodules of leguminous plants and establish a symbiotic connection with them. Rhizobiaum bacteria are able to suppress pathogens through a variety of strategies, including resource competition and antibiotic production (Essalmani and Lahlou, 2002). Additionally, they accelerate the process of biological nitrogen fixation, which supplies a large amount of nitrogen to plants, and encourage plant growth, Siddiqui *et al*., (2000) and Gendy (2013), conducted an experiment on fenugreek for selection of suitable rhizobium strains, results showed that fenugreek growth was enhanced by rhizobium inoculation in terms of leaf count, dry weight of the plant, and number of nodules per plant. In a different experiment, Ahmad *et al.* (2022) discovered five distinct bacterial strains from the rhizospheres of sesame, chickpea, lentil, berseem, and mungbean. The length of the shoot, root, and dry matter production of chickpea seedlings increased significantly in most isolates. In comparison to the control treatment, the experiment showed that isolates treated with rhizobium strain inoculation had higher plant heights, nodule counts and pod counts per plant. Being a leguminous plant, nitrogen is fixed by bacteria in root nodules of fenugreek. Rhizobium is a significant source of nitrogen for agricultural soils therefore more fresh leaves production, and improved soil quality have all been demonstrated benefits of using appropriate rhizobium strains. This experiment has been conducted to inoculate the fenugreek seed with different Rhizobium strains for yield improvement.

Materials and Methods

An experiment on "Effect of nitrogen and rhizobium inoculation on growth and yield of fenugreek (*Trigonella foenum-graecum*) fresh leaves" was conducted at Horticulture Research Farm, The University of Agriculture Peshawar, Pakistan during 2023. The research was arranged in Randomized Complete Block Design (RCBD) with split plot arrangement, with two factors and three replications, there were 16 treatments in each replication. A whole of 48 subplots and 12 main plots were used to prepare the field. Factor "A" was nitrogen levels (0, 20, 40 and 60 kg ha^{-1}) and factor "B" was different strains of rhizobium bacteria (uninoculated seeds, *rhizobium trifolium*, *mesorhizobium ciceri* and *bradyrhizobium japonicum*). Nitrogen levels were kept in main plots, while rhizobium inoculated seeds were kept in sub plots.

Laboratory analysis

Laboratory analysis were conducted at the laboratory of soil and environmental sciences division, Nu clear institute for food and Agriculture, (NIFA) Peshawar, for isolation of rhizobium strains Three different rhizobium strains were isolated from three different organic sources i,e berseem (*Rhizobium trifolium*), chickpea (*Mesorhizobium ciceri*) and soybean (*Bradyrhizobium japonicum*).

Table 1: *Isolation and purification of rhizobium strains.*

Ingredients	Quantity
K_2HPO_4 (5.0%)	10mL
$MgSO_4.7H_2O$ (0.2%)	10mL
NaCl (1.0%)	10mL
Yeast extract	1.0g
Mannite	10g
Agar	15g
pH	7.0

Rhizobium strains were isolated using Yeast Extract Mannitol Agar (YMA) media. The composition of the medium was (1000 mL) (Atlas 1993).

Using yeast extract mannitol agar medium (YMA), certain host plants such as berseem and chickpea were used to extract rhizobium species. 5% K_2HPO_4 (10 ml), 2% $MgSO_4.7H_2O$ (10 ml), 1% NaCl (10 ml), 10 g mannitol, 1 g yeast extract, and 15.0 g agar made up the YMA medium composition per liter. In order to sterilize the medium, it was autoclaved for 20 minutes at 121°C and 15-20 lb inch-2 pressure. Petri

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plates were aseptically filled with the sterilized medium inside laminar airflow cabinets. In the laminar flow cabinet, exposure to UV light further sterilized these petri plates. Berseem, chickpea and soybean plants were uprooted at flowering stage as the nodule number and health are expected to be maximum at this stage. The nodules from the plant roots were separated using knife and were washed with tap water. The nodule were surface sterilized with 95% ethanol and rinsed with DI water then again sterilized using 0.1% $HgCl_2$ and finally rinsed four to five times with sterilized distilled water. Using sterile forceps, the nodules were crushed, and the nodule juice was sprinkled onto the solidified medium by using sterilized micro pipette. The petri-dishes were then incubated for 48 hours at 28 °C in incubator. On recently produced plates, prolific single colonies of rhizobium species from berseem and chickpea were chosen and purified. Following purification, these cultures were stored at 5±1°C for further experimentation.

Preparation of broth culture media for seed inoculation

A broth medium for a 500 ml solution containing a variety of nutrients and chemicals 2.5 g of sucrose, 0.25 g of peptone, 0.1 g of $MgSO_4.7H_2O$, 0.5% NaCl (5 ml), 5 g mannitol, 0.25 g of KH_2PO_4, 0.25 g of Fe $(NH_2)_2SO_4$, and 5 g of yeast extract was prepared. After preparing and adding four distinct solutions to individual flasks, the autoclave was used to sterilize them for 15-20 minutes at 121 °C. After cooling and being exposed to UV light to avoid contamination, these liquid cultures were inoculated with three distinct isolated strains of rhizobium bacteria and the samples were incubated for 72 hours at a particular temperature of 30±2 °C in incubator. For comparison, an uninoculated control group was also prepared using same procedures and protocols. In the broth cultures, prolific colonies of rhizobium species were seen with naked eye as dense growths at the top. The produced broth was then used to inoculate fenugreek seeds.

Determination of nitrogen content by kjeldahl method

About 0.5 g grinded plant sample was taken in digestion tube and 3.5 g digestion mixture + 10 mL commercial sulphuric acid (H_2SO_4) added. Digested it at 420 °C for 2-3 hours and on cooling run in the auto-distillation apparatus using 10 ml of 4 % boric acid as a receiver in a conical flask and titrated against 0.1 N H_2SO_4 and the volume of acid used for titration was recorded. Following formula was used to get nitrogen content in leaves samples of fenugreek;

Calculations

$$N\,(\%) = \frac{(\text{Sample} - \text{blank}) \times \text{N of } H_2SO_4 \times 14 \times 100}{\text{Wt. of sample} \times 1000}$$

OR

$$N\,(\%) = (\text{Sample-blank}) \times 0.28$$

Inoculation of fenugreek seeds

After observing highest development of rhizobium in broth culture, the seeds of fenugreek were soaked for 6-8 hours in the prepared inoculum. After soaking the seeds were dried in the shade before sowing. Seeds of fenugreek Punjab local were sown after inoculation, on 28 October, 2023 in a four-row subplot, with six seeds per row. Line sowing was used, maintaining a distance of 10 cm between plant-plant and 30 cm between row-row. The studied parameters were, days to emergence, number of branches plant^{-1}, number of leaves plant^{-1}, plant height (cm), nitrogen content in leaves, chlorophyll content and fresh leaves yield (tons ha^{-1}).

Statistical analysis

The ANOVA was used to analyze the data in order to find whether there were any variations among the distinct treatments. The average difference was measured using the LSD test at the 0.05 % or 0.01 % significance level (Steel *et al*., 1997). The STATISTIX 8.1 software was used to execute the computations for both ANOVA and LSD.

Results and Discussion

Rhizobium inoculation and nitrogen levels had a pronounced effect on the growth and yield of fenugreek. The results obtained are discussed briefly as under.

Days to emergence

Rhizobium inoculation and nitrogen levels significantly effected days to emergence (Table 1) shows maximum days to emergence (11.92) was observed in uninoculated seeds while less days to emergence (5.92) was recorded in seeds inoculated with *Bradyrhizobium japonicum*. Rhizobium bacteria are capable of producing indole acetic acid (IAA) and gibberellic acid (GA), which are growth hormones crucial for germination and plant growth. Giri and

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Joshi (2010) also reported that rhizobium inoculation improved the seed quality and plant growth due to its easy handling and contemptibility. However, Hafeez *et al*. (2004) used different strains of rhizobium in their experiment and observed that *Bradyrhizobium japonicum* not only induced root elongation, shoot development and enhanced nutrient uptake, but also promoted early germination. Among nitrogen levels maximum days to emergence (10.00) was obtained in control while minimum days to emergence (6.42) was recorded at the level of 60 kg ha^{-1}. Increased levels of nitrogen not only play a significant role in improving maximum number of leaves and plant growth, but also enhanced germination (Monaco *et al*., 2003). Observations of Osuna *et al*. (2015) showed nitrogen is an essential nutrient and many plants in terrestrial ecosystems are adapted to conditions of low N availability, in addition to serving as a basic nutrient, N also promotes seed germination through function as a signaling molecule resulted in improved days to emergence (Table 2).

Number of branches plant^{-1}

The means of statistical analysis indicates that number of branches plant^{-1} was significantly affected by treatments (Table 1). More number of branches plant^{-1} (10.00) was recorded in *bradyrhizobium japonicum*, while les number of branches plant^{-1} (4.92) was observed in uninoculated seeds. Rhizobium inoculation can significantly enhance the number of branches plant^{-1} through improved nitrogen fixation, better nutrient uptake, hormonal effects, enhanced root development, and increased stress resistance. A study performed by Namvar *et al*. (2011) showed that rhizobium inoculation with *bradyrhizobium japonicum* and *mesorhizobium ciceri* significantly increase primary and secondary branches plant^{-1} over uninoculated plants. The mean values for nitrogen levels also displayed significant effect for number of branches plant^{-1} maximum number of branches plant^{-1} (9.75) was observed at the level of 60 kg ha^{-1} while minimum number of branches plant^{-1} (6.58) was calculated in control. Nitrogen is vital and key element that enhances number of branches plant^{-1} by promoting photosynthetic activities, improving protein synthesis and stimulate growth hormones such as Auxin (cell elongation and branching) and cytokinins (cell division and shoot initiation). Results of Kazemeini *et al*. (2010) showed that nitrogen application enhanced number of branches plant^{-1} by promoting several metabolic processes and increased cell division and expansion.

Number of leaves plant^{-1}

The number of leaves plant^{-1} was significantly affected by rhizobium inoculation and nitrogen levels (Table 1). While mor number of leaves plant^{-1} (105.50) was observed in *bradyrhizobium japonicum*, while les number of leaves plant^{-1} (62.25) was observed in uninoculated seeds. The symbiotic association between rhizobium and the roots of leguminous crops is responsible for the maximum number of leaves in inoculated plants (Sajid *et al*., 2011). However, Hassan *et al*. (2024) also concluded that *bradyrhizobium japonicum* greatly enhanced plant growth and number of leaves plant^{-1} as well as brought about positive developments in sustainable agriculture through processes such as phytohormone synthesis and nitrogen fixation.

Table 2: *Growth and yield characteristics as affected by rhizobium inoculation and nitrogen levels.*

Treatments	Parameters						
Rhizobium Strains	Days to emergence	No of branches plant^{-1}	No of leaves plant^{-1}	Plant height (cm)	nitrogen content in leaves	Chlorophyll content	Fresh leaves yield (tons ha^{-1})
Uninoculated seeds	11.92 A	4.92 D	62.25 D	54.83 C	3.79 C	29.18 D	5.96 D
Rhizobium trifolium	8.92 B	8.00 C	80.08 C	63.38 B	4.13 B	33.02 C	6.41 C
Mesorhizobium ciceri	7.25 C	9.00 B	93.92 B	67.19 AB	4.54 A	37.22 B	6.98 B
Bradyrhizobium japonicum	5.92 D	10.00 A	105.50 A	70.34 A	4.70 A	41.14 A	7.51 A
LSD	0.9027	0.7121	4.2373	5.1949	0.2567	1.7988	0.3064
Nitrogen levels (kg ha^{-1})							
0	10.0 A	6.58 B	73.17 D	56.84 c	3.95 B	31.43 B	6.18 C
20 kg ha^{-1}	9.42 A	7.50 B	79.92 C	62.60 bc	4.08 B	32.88 B	6.33 C
40 kg ha^{-1}	8.08 B	8.08 B	84.83 B	66.35 ab	4.47 A	36.83 A	6.67 B
60 kg ha^{-1}	6.42 C	9.75 A	103.83 A	69.96 a	4.67 A	39.43 A	7.49 A
LSD	0.9354	1.5344	4.6668	9.8452	0.2336	3.0475	0.3912

The data recorded for nitrogen levels show that maximum number of leaves plant^{-1} (103.83) was observed at the level of 60 kg ha^{-1} while minimum number of leaves plant^{-1} (73.17) was calculated in control. Nitrogen is vital for the development of new tissues, including leaves. Healthy nitrogen levels support the production of more leaves, which can enhance the plant's photosynthetic capacity and overall growth. Studies of El-Desuki *et al.* (2005) showed that application of nitrogen increased the vegetative growth. Petropolous *et al.* (2008) determined that higher nitrogen levels resulted in a greater number of leaves in the plant.

Plant height (cm)

The inoculation of rhizobium and nitarogen levels significantly affected plant height (cm), (Table 1). Tallest plants (70.34 cm) was given by the seeds which were inoculated with *bradyrhizobium japonicum* while small plant height (54.83 cm) was observed in uninoculated seeds. Maximum plant height in inoculated plants may be attributed due to the fact that rhizobium inoculation provides better soil environment for nutrients and water leading to taller plants (Tahir *et al*., 2009). These results are earlier confirmed by Sharar *et al*. (2000) who reported that seeds produced significantly taller plant height than uninoculated seeds. Data regarding nitrogen levels showed that maximum plant height (69.96 cm) was observed at the level of 60 kg ha^{-1} while minimum plant height (56.84 cm) was calculated in control. Nitrogen influences the production of growth hormones such as auxins and cytokinins which regulate cell division and elongation. Increased cell division and elongation contribute to the vertical growth of plants. Nitrogen fertilizer has significant effect on plant height, however, Argaw (2017) observed significant effect of nitrogen fertilizer on soybean plants.

Nitrogen content in leaves (%)

Rhizobium inoculation and nitrogen levels had a significant effect on nitrogen content in leaves (Table 1). However more nitrogen content (4.70) was recorded in *bradyrhizobium japonicum* while less nitrogen content (3.79) was observed in uninoculated seeds. Maximum nitrogen content in leaves may be due to maximum nutrients uptake by rhizobium bacteria in vegetative stage. The use of advantageous microbes greatly improved the quantity of nitrogen in the leaves of cotton (Khaliq *et al*., 2006). Based on the nutritional analysis of chickpea seeds, *bradyrhizobium japonicum* and *mesorhizobium ciceri* seed treatment with appropriate rhizobium strains promoted to raise P and N uptake in the leaves of plants. These nutrients are likewise necessary for the microorganisms, and their inoculation enhanced rhizobacterial colonization, which supply the crop with essential nutrient such as nitrogen. The findings of Sogut (2006) indicated that the inoculated plants tended to have a higher vegetative nitrogen content. Our conclusions support the earlier work of Koutroubas *et al*. (2023). They found that inoculation of seeds with *bradyrhizobium japonicum* not only improved nutrients uptake, but also increased nitrogen content in leaves and vegetative parts of the plant. Among nitrogen levels highest nitrogen content in leaves (4.67) was calculated at the level of 60 kg ha^{-1} while minimum nitrogen content (3.95) was noted in control. The amount of nitrogen content in leaves significantly affects several physiological and biochemical processes in plants such as photosynthesis, protein synthesis and most importantly it promotes vegetative growth, resulting in larger leaves and increased biomass. Petropolous *et al*. (2008) demonstrated that nitrogen fertilizer considerably raised the nitrogen concentration in leaves.

Chlorophyll content

The treatments had showed significant effect on chlorophyll content (Table 1). Maximum chlorophyll content (41.14) was produced in the inoculated plants, while minimum chlorophyll content (29.18) was produced in uninoculated seeds. Due to symbiotic association between rhizobium bacteria and root nodules more nitrogen is available to the plant resulting in more chlorophyll content. Nyoki and Ndakidemi (2014) examined the effects of *bradyrhizobium japonicum* inoculation and phosphorus supplementation on the uptake of macronutrients in cowpea. They discovered that inoculating *bradyrhizobium japonicum* significantly increased the total leaf chlorophyll content of the cowpea when measured five and seven weeks after planting. According to studies by Biswas *et al*. (2000) and Bambara and Ndakidemi (2010), soybeans inoculated with *Bradyrhizobium japonicum* showed a significant increase in the amount of chlorophyll content in their leaves. Among nitrogen levels maximum chlorophyll content (39.43) was observed at the level of 60 kg ha^{-1} while minimum chlorophyll content (31.43) was noted in control. Nitrogen is a structural component of protein and chlorophyll molecules, and it stimulates the development of chloroplasts and the formation of chlorophyll within them

Figure 1: *Research Glimpses.*

(Bojovic and Markovic, 2009). Results of an experiment on the impact of nitrogen fertilizer on chlorophyll content conducted by Hokmalipour and Darbandi (2011) showed that applying nitrogen fertilizer had a positive impact on chlorophyll content.

Fresh leaves yield (tons ha^{-1})

The treatments had a pronounced effect on fresh leaves yield (tons ha^{-1}), (Table 1). Highest fresh leaves yield (7.51 tons ha^{-1}) was obtained by the plants which were inoculated with *bradyrhizobium japonicum* while low fresh leaves yield (5.96 tons ha^{-1}) was given by uninoculated seeds. Rhizobium bacteria has the ability to improve fresh leaves yield by adapting several mechanisms such as, the symbiotic relationship between rhizobium bacteria and leguminous plants leads to improved nitrogen fixation, enhanced nutrient uptake, hormonal regulation, increased photosynthesis, better disease resistance, and overall soil health improvement. Findings of Wierzbowska and Zuk-Golaszewska (2014) revealed that inoculation with *bradyrhizobium japonicum* not only improved seed yield, but also increased fresh leaves yield. *Bradyrhizobium japonicum* contributed to the provision of additional vital nutrients, water, and growth-regulating phytohormones, which supported further plant development and yield (Rudresh *et al*., 2005). In case of nitrogen levels, maximum fresh leaves yield (7.49 tons ha^{-1}) was observed at the level of 60 kg ha^{-1} while minimum fresh leaves yield (6.18 tons ha^{-1}) was calculated in control. Applying N fertilizer boots fresh leaves yield, this strategy is only effective up to a certain amount of N fertilizer. Nitrogen application significantly impacts the fresh leaves yield, provided it is applied in the right amounts and under suitable conditions. Studies of Tehlan and Thakral (2011) showed that green leaves yield increased with the increasing levels of nitrogen. The results coincide with the findings of Mauyo *et al*. (2008).

Conclusions and Recommendations

Based on the above results, it is concluded that seeds inoculated with *bradyrhizobium japonicum* improved yield and other growth parameters of fenugreek. Seeds inoculated with *bradyrhizobium japonicum* also increased plant height and other yielding components. Seed inoculated with *bradyrhizobium japonicum* and nitrogen level at the rate of 60 kg ha^{-1} is recommended for better growth and yield of fresh leaves of fenugreek. Further study should be conducted to investigate the influence of nitrogen application at the higher level on fenugreek and other leguminous plants. In future, the performance of more rhizobium strains such as *Rhizobium leguminosarum* should be tested in fenugreek and other leguminous plants.

Novelty Statement

This study is the first of its kind in the Peshawar district to effectively evaluate the complementary effects of nitrogen fertilization and *bradyrhizobium japonicum* inoculation on fenugreek (*Trigonella foenum-graecum*) growth and yield of fresh leaves.

Author's Contribution

Aman Bibi: Did research, analysis and write-up of this manuscript.
Neelam Ara: Supervised the research work contributed in statistical analysis and interpretation of the results.
Haroon Shahzad: Co-supervisor for research, provided supervision for the laboratory analysis.
Faiza Aman: Helped in revision of the manuscript

Conflict of interest
The authors have declared no conflict of interest.

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