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Research Article

Determination of Biological Nitrogen Fixation Induced N_2O Emission from Arable Soil by Using a Closed Chamber Technique

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Intensive use of mineral N fertilizers and organic amendments has resulted in higher N_2O emissions. A growing worldwide concern for these problems has motivated researchers, environmentalists, and policy makers to find alternatives to overcome such losses. Biological nitrogen fixation is one of many natural biological approaches to minimize the use of fertilizers and to possibly reduce N_2O emissions. A greenhouse study was performed by growing inoculated and noninoculated soybean seeds (*Glycine max (L.) Merr.*) in PVC columns. The objective was to measure the contribution of *Bradyrhizobium Japonicum* and mineral-N fertilizer to promoting N_2O emission. A closed chamber technique was used for gas sampling. N_2O measurements were carried out shortly after nodulation. *Bradyrhizobium Jopanicum* induced N_2O cumulative (121.8 $\mu g\,kg^{-1}$) fluxes of inoculated seeds was significantly ($\alpha = 0.05$) higher than those of mineral N fertilized treatment (NIS) and the control (bare soil). Total nitrogen content of the roots and seeds was not affected by inoculation. Total carbon (42.1 ± 0.1%), total nitrogen (3.1 ± 0.1%), and crude protein (19.9 ± 0.7%) contents of leaves of the inoculated seeds were significantly higher than those of noninoculated seed treatments. N_2O fluxes significantly increased with high dissolved organic carbon content (70.77 ± 3.99 mg L^{-1}) at R3 and at R8 stages when NO_3^- (39.60 ± 0.94 mg L^{-1}) concentrations were high.

1. Introduction

Nitrous oxide (N_2O) is an important anthropogenic greenhouse gas. It plays a key role in ozone depletion [1]. Agriculture is considered the largest N_2O resource [1–4]. Global N_2O emissions from agricultural soils are estimated to be 2.1 million tons nitrogen (N) per year [5] and will continue to increase annually at a rate of 0.25% [6]. N_2O is produced in soil by microbial transformations, that is, nitrification and denitrification, especially when N availability exceeds plants' requirements [7]. Application of N to soil, whether in an organic [8–10] or inorganic [11] form eventually leads to enhanced N_2O emissions. These emissions can be reduced by proper implementation and management by utilizing renewable resources.

Biological nitrogen fixation (BNF) through legumes is a renewable resource. Legumes are significant among other plants due to their symbiotic N fixation capability, transferring molecular N_2 into plant available form [12]. It is a microbial process whereby N_2 is reduced to NH_3 by nitrogenase enzyme [13], normally found in the soil bacteria of genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Azorhizobium* which are collectively called rhizobia [14]. Rhizobia infect root hairs of the leguminous plants and produce nodules [15]. This process requires adenosine triphosphate (ATP) as an energy source, resulting from oxidative degradation of sugars and related molecules for reduction of N_2 to NH_3 [16]. These sugars and related molecules are prepared by the host plant through photosynthesis and transferred to the nodules. Rhizobia receive energy from the plants and, in reply, make N available to the plants [17].

Globally, it is estimated that 150–200 million tons of mineral N are required yearly by the plants, out of which almost 100 million tons of N are fixed through the industrial

Haber-Bosch process [18] and 175 million tons of atmospheric N_2 are biologically fixed annually [19]. After photosynthesis, BNF is an important biological process. Both processes are closely linked, because BNF utilizes the energy captured by photosynthesis [20]. Undoubtedly, N_2 fixing ability of the legumes minimizes synthetic N input in soil [21]; conversely, there is also a question of minimization of negative environmental impacts.

Various reports suggest that N_2O emissions from the soils cultivated under legumes are higher than under fertilized legumes. Such N losses are directly comparable to manure or synthetic fertilizer-induced N_2O emissions [21]. Oppositely, Jensen et al. [22] and Crews and Peoples [23] suggested that there is a negligible difference between legume and fertilizer derived N_2O emissions. Zhong et al. [24] reported that BNF is not the source of N_2O emissions.

There is evidence that organic N inside the nodules is mineralized to NH_4^+, followed by nitrification and denitrification [25] by rhizobia due to their capability to denitrify [21, 26] and N can be lost as N_2O [27]. Philippot et al. [28] stated that N_2O production ratio decreased exponentially with decreasing pH because plant roots take up more cations than anions, during N_2 fixation, resulting in more (H^+) protons generating acidic conditions [5]; which suppressed nitrifiers' activity [29], thereby reducing N_2O emissions; but it can vary with acid-tolerant nitrifiers' population. Yet, Van Den Heuvel et al. [30] found no clear relationship between pH and N_2O emissions.

Few studies have underlined how BNF can control chemical and biological soil processes associated with C (carbon) and N cycles. Yang and Cai [31] revealed that N_2O emissions fluctuate with the growth phases of soybean, but they are controlled by the quantity and may be the quality of root exudates. The root exudates contain soluble C and N, stimulating microbial activity [32]. It results in reduction of O_2concentration in rhizosphere, invigorating N_2O emissions [33].

Some of the scientific data suggest that decomposition of inoculated leguminous crop residues results in higher N_2O emission [6, 33, 34] rather than during fixation [35]. Jeuffroy et al. [36] recently reported that N_2 fixation process itself may lead to N_2O emissions. Crews and Peoples [23] concluded that legume derived N has similar negative impacts on the environment as synthetic fertilizer. Nevertheless, reduction of fertilizer application leading to reliance on BNF is a worthwhile environmental goal [5].

Currently, there is insufficient knowledge about the origin of N_2O emissions during and after nodule establishment in legumes. A greenhouse experiment was designed to measure N_2O evolution from soil with soybean plants after nodule production by means of a closed-chamber technique. The specific objectives of this study were (1) to evaluate the N_2O fluxes during the biological nitrogen fixation process, (2) to observe the impact of pH, NO_3^-, and soil dissolved organic carbon (DOC) on the N_2O s, and (3) to compare C%, N%, and CP% contents of plant biomass under inoculated and noninoculated seeds treatments.

2. Materials and Methods

2.1. Soil Sampling. The soil selected for this study was sampled from "Heidfeldhof" research station of Hohenheim University, Stuttgart (48°43′00″N; 9°11′40″E), Baden Wurttemberg, Germany, where, the annual average temperature and total precipitation are 9.6°C and 689 mm, respectively [37]. The soil samples were taken from the 0–15 cm depth of recently harvested wheat plots after removing the upper vegetation. The soil contained the following properties: Haplic Luvisol [38], 19% sand, 52% silt, and 29% clay (silty clay loam) and NH_4^+ (5.6 mg kg^{-1}), NO_3^- (10.8 mg kg^{-1}), TC (1.2%), and pH 6.5 (1 : 2.5 soil/$CaCl_2$ suspension).

2.2. Experimental Setup. The soybean seeds (M 11) used in the study were commercially coated (preinoculated) with 4×10^9 cfu (colony farming units) of *Bradyrhizobium japonicum* (*B. japonicum*) with date of expiry and proper storage conditions under refrigerator below 15.5°C.

Three kg soil (20% water content by weight) was filled into polyvinyl chloride (PVC) columns (0.15 m diameter × 0.36 m length). Seeds were placed in the depth of 0.025 m [39]. The experiment consisted of three treatments: (1) soil with inoculated seeds (IS), (2) soil with noninoculated seeds (NIS), and (3) bare soil (control). NIS and control contained +6 replicates while IS had 9 replicates, respectively. Three extra replicates were kept for nodule examination. In total, 21 PVC columns were prepared and placed in a greenhouse with an average temperature (day/night) of 25/17°C and humidity (day/night) of approximately 55/80% (recorded by computerized Win Term programme) during the entire experimental period.

In total, 50 mg kg^{-1} as N calcium ammonium nitrate (CAN) was applied to NIS and control during entire experiment. 10 mg kg^{-1} N was applied at sowing to NIS and control and as a starter to IS [40]. NIS and control were fertilized at R3 (beginning of pod) (10 mg kg^{-1} N) and R6 (30 mg kg^{-1} N) (full seed) stages as well. Plants were harvested in the 15th week [39] (on the basis of three kg soil, CAN fertilizer was applied as 111.09 mg at sowing, 111.09 at R3 stage and 333 mg at R6 stage. Initially six seeds were sown. After emergence, three plants exhibiting consistent growth characteristics were retained in each pot and the others were manually discarded).

2.3. N_2O Measurements. Measurements of N_2O were started immediately, after nodule formation (which occurred at flowering stage) by means of the closed chamber technique [41]. In total, 16 measurements were taken at different growth stages. The manually operated closed chambers (0.11 m diameter × 0.15 m length) were prepared (containing 0.1424 m^2 area) by the same PVC cylinder material together with lid containing a hermetically fixed mounted rubber septum. The lid was fixed to the chamber with special clips. The closed chamber was penetrated to the soil up to 4 cm depth, prior to sampling. Gas samples were taken from the headspace at 0, 30, and 60 minutes time intervals after inserting the closed chambers through fixed mounted rubber septum in the lid

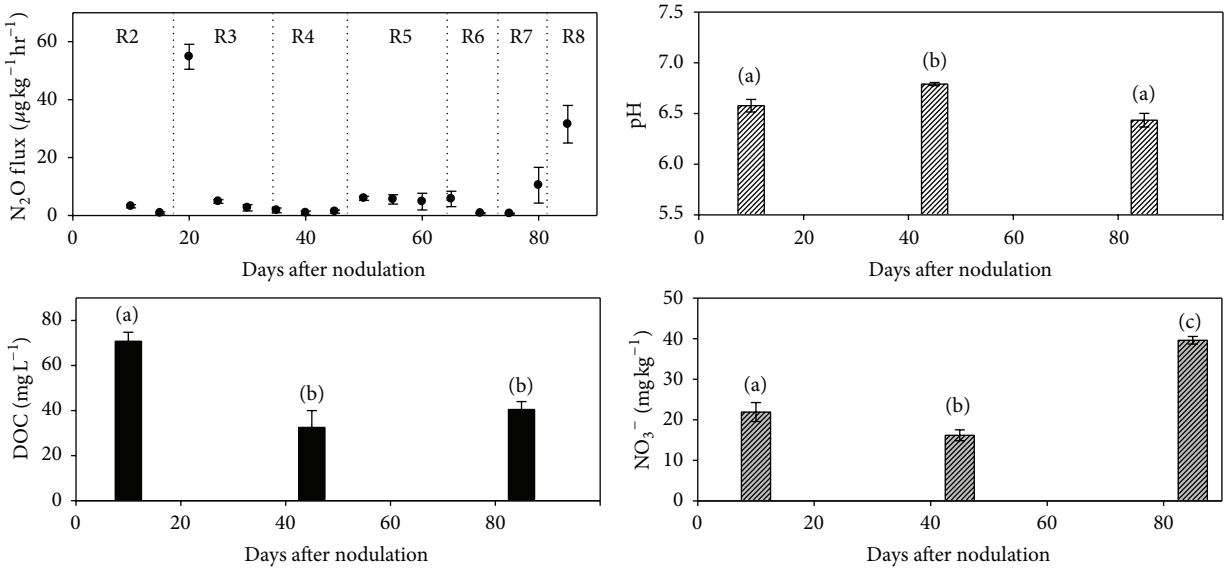

FIGURE 1: Temporal dynamics of N_2O flux ($\mu g\,kg^{-1}\,hr^{-1}$), soil pH, DOC ($mg\,L^{-1}$), and NO_3^--N ($mg\,kg^{-1}$) of IS treatment. Different lower case letters indicate a statistically significant difference at $\alpha = 0.05$. R2: full flowering; R3: beginning pod; R4: full pod; R5: beginning seed; R6: full seed; R7: beginning of maturity; R8: full maturity. Means of 3 replicates with standard error.

fixed to the chamber with special clips by using a multisample 100 cm^3 syringe.

To ensure the absence of N_2O, all gas-tight 0.25 L sampling tubes (vacutainers) were evacuated and rinsed with N_2 thrice before sampling. Gas samples were transferred to the vacutainers and immediately brought to the laboratory for analysis. During gas sampling, the bottom of PVC columns was tightly closed with PVC caps to ensure an airproof condition.

N_2O was analyzed by gas chromatography (AutoSystem XL Perkin Elmer) using an electron capture detector (ECD) coupled with an autosampler. The instrumental conditions were as follows: oven temperature 65°C: ECD operation temperature: 100–450°C (programmed), carrier gases for ECD: CH_4/Ar (10%/90%) and He (95%), respectively. Calibration was done with three external standards (0.3, 1.5, and 3.0 ppb). Gas rates were calculated linearly by measuring the change of gas concentration in the headspace of the microcosm. To estimate cumulative N_2O fluxes throughout measuring period, cumulative curves were created. It was done by multiplying mean rates of two sequential gas rates with the respective time periods. After wards, this time weighted means were summed out as described by Goldberg et al. [42].

2.4. Soil and Plant Sampling. Inoculation of soybean seeds with *B. japonicum* resulted in nodule formation at flowering stage (R2). The nodules were observed on tap roots [43]. The observation of nodules was done at R2, R4, and, R8 stages by removing each cylinder. All three plants were uprooted from each pipe to observe the nodule number. Soil from the root was removed carefully by gently digging out the plants with a small gardening trowel and by rinsing the roots in water. The nodules were separated, counted, and air dried. The nodules were then put in paper bags and oven dried for 48 hours at 70°C [44]. The nodules were weighed using an electric balance.

Soil, plant root, and shoot samples were preserved only at harvesting. Roots were separated from uprooted plants and then dried to 70°C and weighed.

2.5. Soil Analysis. Total carbon (TC%) and total nitrogen (TN%) were measured by a LECO 2000 CN analyzer. The particle size distribution was determined by the pipette method [45]. Soil pH was measured in 1 : 2.5 (soil/0.01 M $CaCl_2$) using glass electrode pH meter. Soil mineral N (NH_4^+ and NO_3^-) was measured by extracting soil with 1 M KCl solution (soil/liquid ratio 1 : 5 w/w) as referred to by Keeney and Nelson [46]. The filtrates were then analyzed on an automated injection analyser (Bran & Luebbe TrAAcs 800 Auto analyzer). DOC was analyzed by extracting soil with 0.5 M K_2SO_4 (1 : 4) and using a Multi N/C 2100s (Analytic Jena). Crudeprotein content was computed by multiplying N content in soybean seed determined by LECO 2000 CN analyzer by a factor of 6.25 as described by Jackson [47].

2.6. Statistical Analysis. The data were statistically evaluated by one-way analysis of variances (ANOVA) using IBM SPSS statistics version 19 at significance of $\alpha = 0.05$. Least significant difference (LSD) test was used to ascertain significant differences between the treatments.

3. Results

3.1. Temporal of N_2O. Figures 1, 2, and 3 illustrate the temporal patterns of N_2O fluxes ($\mu g\,kg^{-1}\,hr^1$) from the experimental soil columns planted with IS, NIS, and control

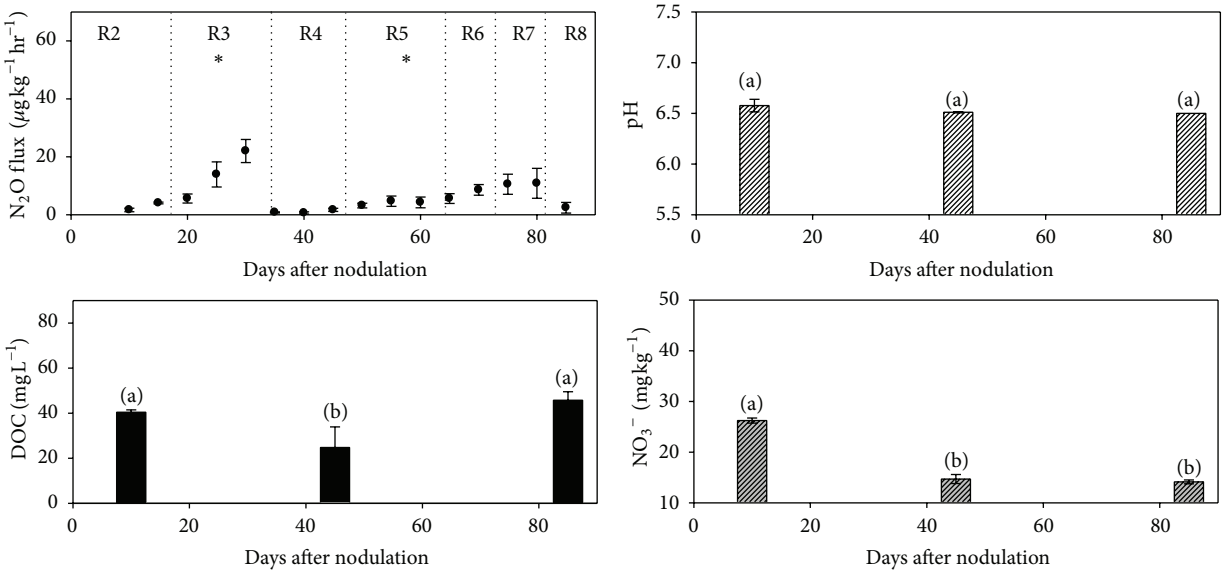

FIGURE 2: Temporal dynamics of N_2O flux ($\mu g\,kg^{-1}\,hr^{-1}$), soil pH, DOC ($mg\,L^{-1}$), and NO_3^--N ($mg\,kg^{-1}$) of NIS treatments. Different lower case letters indicate a statistically significant difference at $\alpha = 0.05$. R2: full flowering; R3: beginning pod; R4: full pod; R5: beginning seed; R6: full seed; R7: beginning of maturity; R8: full maturity. *Symbol indicates application of mineral N fertilizer. Means of 3 replicates with standard error.

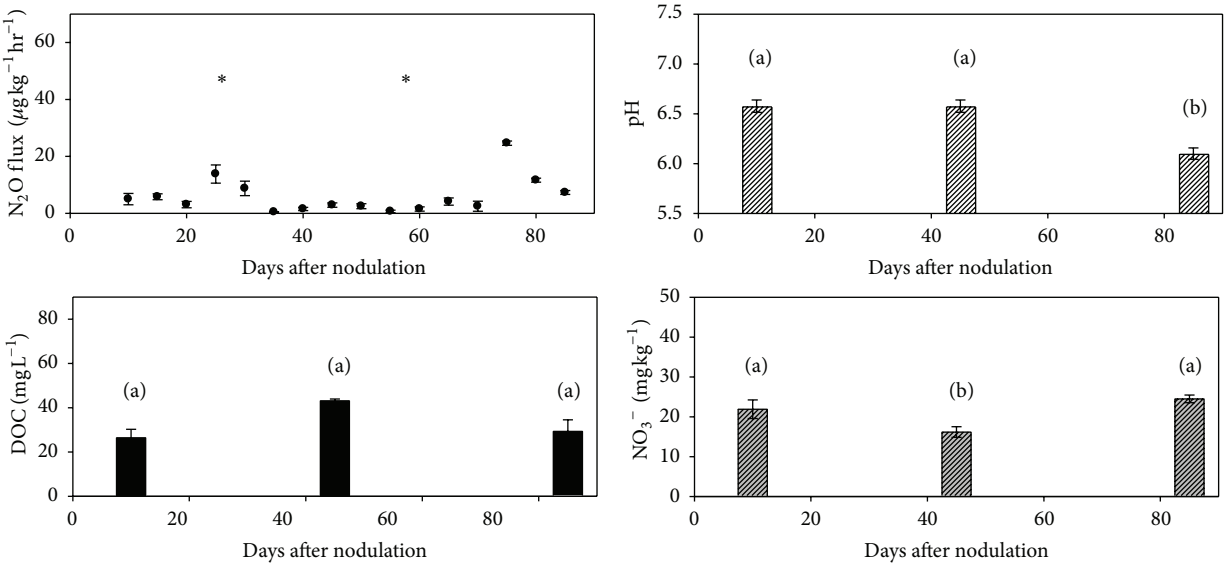

FIGURE 3: Temporal dynamics of N_2O flux ($\mu g\,kg^{-1}\,hr^{-1}$), soil pH, DOC ($mg\,L^{-1}$), and NO_3^--N ($mg\,kg^{-1}$) in the control treatment. Different lower case letters indicate a statistically significant difference at $\alpha = 0.05$. *Symbol indicates the application of mineral N fertilizer. Means of 3 replicates with standard error.

columns during a period of 90 days from nodule formation until maturity.

The N_2O fluxes of the IS treatment (Figure 1) are characterized by a low background flux rate ranging from 2.6 to 5.7 $N_2O\,\mu g\,kg^{-1}\,hr^{-1}$. However, on the 25th day after nodule formation, the N_2O flux suddenly peaked, reaching a maximum rate of 54.8 $\mu g\,kg^{-1}\,hr^{1}$. It is noteworthy that this peak flux is the highest flux in comparison with all other treatments. Five days later, however, the N_2O flux decreased again to a background flux rate of 5 $\mu g\,kg^{-1}\,hr^{1}$. Shortly before the onset of full maturity (R8) the flux increased again to 31.5 $\mu g\,kg^{-1}\,hr^{1}$.

The NIS treatment showed a slightly different emission pattern. N_2O flux increased exponentially, reaching a maximum value of 22 $\mu g\,kg^{-1}\,hr^{1}$ during the first 30 days (Figure 2). Five days later, the flux decreased to a

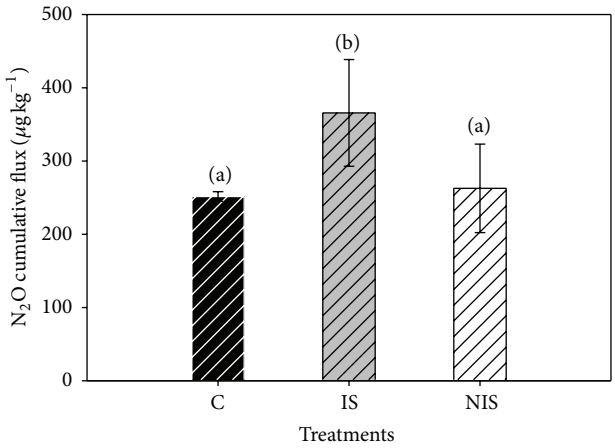

FIGURE 4: Cumulative N_2O flux ($\mu g\,kg^{-1}$) of different treatments. Means of 3 replicates per treatment with standard error. Different lower case letters indicate a statistically significant difference at $\alpha = 0.05$.

low background value of 1.67 $\mu g\,kg^{-1}\,hr^{-1}$. Subsequently, the fluxes increased gradually from 0.8 to 10.8 $\mu g\,kg^{-1}\,hr^{1}$ at the beginning of full maturity.

The background pattern in NIS treatment was interrupted twice by higher fluxes shortly after fertilizer application (Figure 2). The cumulative fluxes were 251.5, 365, and 262 $\mu g\,kg^{-1}$ (Figure 4) for C, IS, and NIS treatments, respectively. On average, the IS treatment was significantly ($\alpha = 0.05$) higher than those of NIS and control (Figure 4).

3.2. Effect of Soil pH, NO_3^-, and DOC on N_2O Emission. With regard to IS treatment, soil pH increased between R2 and R4, the time when the emissions were relatively lower and remained similar to the R2 and R7 stages (Figure 1). At R4 stage, NO_3^- significantly decreased to 16.1 $mg\,kg^{-1}$ and shot up to 39.6 $mg\,kg^{-1}$ at the full maturity stage (R8) in line with higher N_2O $\mu g\,kg^{-1}\,hr^{1}$ fluxes (Figure 1). DOC of IS remained significantly higher at R2 than R4 ($32.50 \pm 7.5\,mg\,L^{-1}$) and R7 ($40.50 \pm 3.5\,mg\,L^{-1}$).

However, in NIS treatment (Figure 2), pH remained unchanged and NO_3^- content decreased significantly with time. NO_3^- tended to decline with fertilizer application. On the other hand, DOC was significantly lower at R4 (Figure 2). In the control treatment (Figure 3), pH remained at 6.6 until the period corresponding to the stage R7 of planted treatments and significantly declined at R8. No significant change of DOC was observed. NO_3^- content significantly decreased at R4 (Figure 3) and significantly peaked at 24.5 $mg\,kg^{-1}$ (Figure 3) at R7. N_2O emission appeared to be unimpacted by these changes.

3.3. Total N, C, and CP% Contents of Plant Biomass after Harvest. The total N, C, and CP% contents of plant biomass after harvest are depicted in Figure 5. N, C, and CP% contents of leaves were significantly higher in IS treatments than in NIS treatments. TC% contents of the seeds and roots of both planted treatments were not significantly different. However, TN and CP% in seeds of IS were significantly (17%) higher than those of NIS.

3.4. Number of Nodules and Weight of Root. The number of nodules in IS (Table 1) increased at R4 and decreased at R8 stages significantly. Mass of roots of NIS and IS was not significantly different (Table 2).

4. Discussion

4.1. N_2O Fluxes from Nodulated Treatments. The DOC (Figure 1) was positively correlated with N_2O fluxes at R3 in IS treatment. Our results agree with Sehy et al. [48] and Kawabata et al. [49] in that the increase in soil DOC enhances N_2O emissions [50, 51] through denitrification. Significantly higher DOC availability at the onset of N_2 fixation (R3 stage) was observed. N_2 fixation process is nitrogenase enzyme mediated process that is the source of H_2 [52]. *B. japonicum,* being a facultative chemoautotroph, utilizes H_2 gas as an electron donor in a respiratory chain terminated by oxygen to provide energy for cellular processes and CO_2 assimilation [53]. Moore et al. [54] found a positive correlation between dissolved H_2 and rates of N_2 fixation.

Nonetheless, only one peak was detected. It might be either due to the short term availability of DOC [55] or the NO_3^- content in soil was low due to high demand of N at plant's high N demanding stages.

At R2, the N-fixation rate increases dramatically [56], thereby increasing N_2O fluxes (Figure 1). Cen et al. [57] reported that soil adjacent to nodules had significantly higher rates of N_2O fluxes than soil near roots lacking nodules, giving evidence of enhanced N_2O emissions related to the legume symbiosis. Bacteria normally start N_2 fixation after seven days of nodule formation [58]. Assumably, R3 was the peak stage of N_2 fixation. Zhu and Cheng [59] reported that fixation or nodulated roots produced a stronger rhizosphere priming effect on soil organic C decomposition.

Two significantly higher peaks of N_2O appeared at R3 and R8 stages in the IS treatment. The peak at R3 was higher than the peak at R8. Nevertheless, NO_3^- content at R8 was significantly higher than at R2. DOC was significantly higher at R2 (Figure 1) but not at R8 compared to R4. To sum up, N_2O emission was not always higher with higher DOC.

Nodule formation was detected at R2. Nodule formation is generally inhibited by higher mineral N content of soil in the root zone [60]. It appears that the mineral NO_3^- content (21.9 $mg\,kg^{-1}$) was not suppressive for nodule formation. It is, however, not clear from previous studies what exactly the favorable range of mineral N concentration for successive nodule formation is. The mechanisms for the inhibition of nodule formation are also not fully understood [61].

Surprisingly, higher fluxes were measured in the control and NIS treatments during the same period (Figure 2). One possible explanation could be the decomposition of already existing organic matter after watering to bring the soil back to the actual moisture content [62, 63]. These peaks

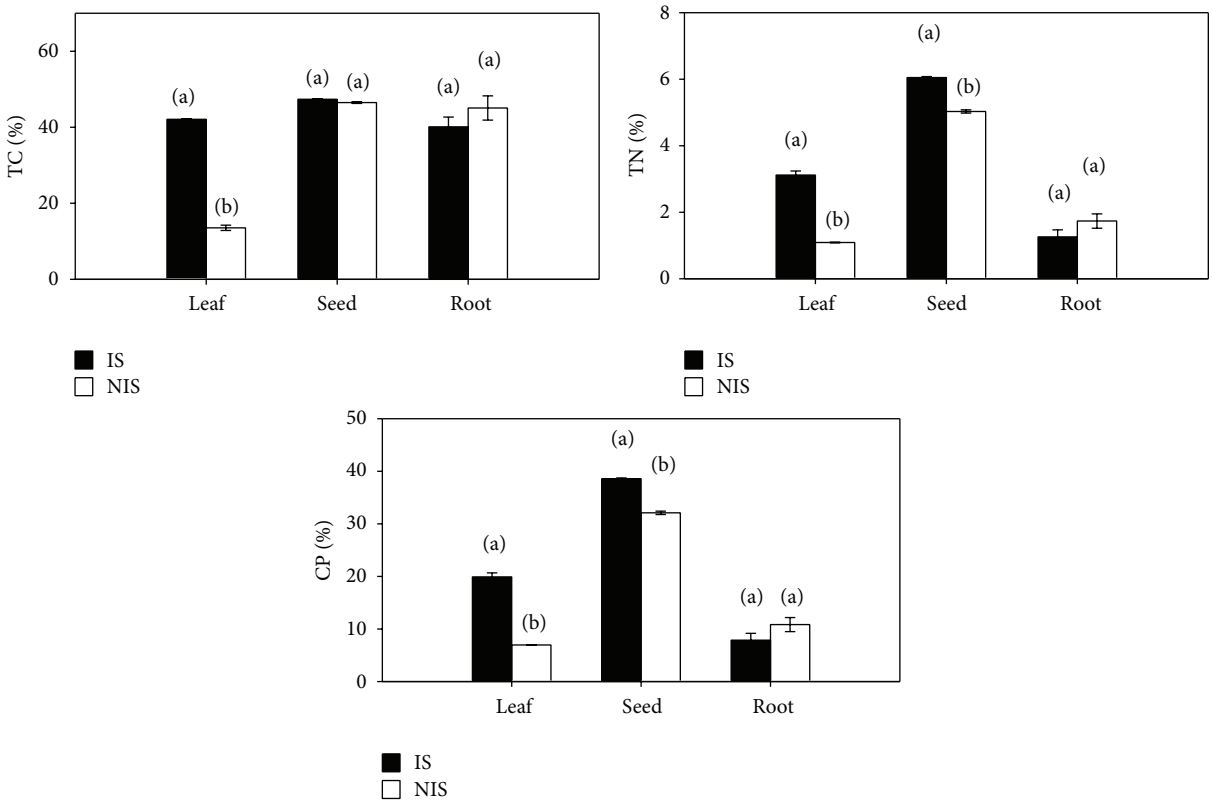

Figure 5: TN%, TC%, and CP% of leaves, seeds, and roots after harvest. Different lower case letters indicate a statistically significant difference at $\alpha = 0.05$.

Table 1: Number of nodules at different plant growth stages.

Number of nodules plant^{-1} (average of three plants)		
R2	R4	R8
10.67 ± 1.86^{a}	39.67 ± 2.72^{b}	16 ± 1.15^{c}

Table 2: Weight of root at harvest (average of three plants on dry matter basis).

	IS	NIS
Mass of root (mg)	108 ± 8.89^{a}	117 ± 12.57^{a}

are not due to temperature [64]. All measurements were recorded at 12 noon and the recorded data illustrate that all measurements were taken at 25–27°C (Figure 1). Soybean, being a subtropical legume, requires temperatures between 25 and 30°C to optimize symbiotic activity [65].

Low fluxes were measured at R4 to R6, when the N_2 fixation was presumably at its maximum [66]. After, NIS and IS treatments emitted continuously low background fluxes at pod (R4), seed filling (R5 and R6), and beginning of maturity (R7) (Figures 1 and 2). The fluxes decreased with increasing demand for N at R3–R7 stages. Fabre and Planchon [66] validated the enhancement in biological N_2 fixation rate during the pod filling period, especially between R5 and R6. Competition between growing plants and soil denitrifiers for available N results in less N availability for denitrifiers.

On cumulative basis, the rhizobia induced cumulative N_2O flux (IS) was 28.5% higher than the fertilized plants treatments. Our results are in an agreement with Jensen et al. [22]. In contrast, Crews and Peoples [23] observed minor differences between legume and fertilizer derived N_2O emissions.

N_2O emitted from fertilized (NIS) and nonfertilized (IS) treatments accounted for less than 1% of N fertilizer added. The second peak appeared at R8 stage (Figure 2) when the availability of NO_3^- appeared to be higher. Higher N_2O emissions from late growth stages of soybean have recently been reported [55]. The later N_2O fluxes were due to higher NO_3^- content (Figure 1). It was probably due to decrease in N uptake by the plant at the delayed stage of soybean growth [31] or nodular or root decomposition [67]. Root releasing C stirred the growth of heterotrophic microorganisms plus denitrifying bacteria [21, 26, 27, 33] for short time.

Generally, root senescence starts at 5–11 week old nodules [68] or when the size of nodule reaches a maximum of about 6-7 mm diameter; they eventually senescence and degrade [61]. It might be the main N source at late stages of plant growth. Some studies suggest that degraded nodules exclusively generate N_2O emissions at late stages of growth [33, 55]. The number of nodules at R8 (Table 2) was in the range of those observed by Chaudhry and Moiser [69].

The number of nodules decreased significantly with time (Table 1). The decline in nodule numbers clearly shows the senescence of nodules. Wisconsin [56] reported that N_2 fixation increases with the number of nodules. At about R5, the N-fixation rate peaks and drops rapidly thereafter.

At the beginning, NIS emitted 37% more N_2O than control. This was presumably due to the effect of plant roots on N mineralization [70]. At the late stages of plant growth, when the plants require less N soil microorganisms which may immobilize N [71, 72], fertilized treatments produced lower background N_2O emission patterns at pod filling and maturity stages (Figure 1) potentially due to a quick provision of mineral N and uptake by the growing plants. Mineral N fertilizer addition to the bare soil (control) yielded a moderate N_2O peak; thereafter NO_3^- became immediately available. On average, in the control treatment, 0.42% of total N applied was lost as N_2O. No significant release of N_2O was observed immediately after fertilization. Lower N_2O emissions from bare soil as compared to planted soils have already been reported by Linzmeier et al. [73].

We detected negative effects of pH and N_2O [30, 74] at R8 (Figure 1) in the IS treatment. Higher N_2O emissions were detected at low pH. Our results disagree with Philippot et al. [28], Jensen and Hauggaard-Nielsen [5], and Zasoski [29]. Thus, we cannot postulate that soil pH was the only factor related to the higher N_2O during biological N_2 fixation. DOC was slightly decreased in NIS and control treatments. On the contrary, it was higher with higher N_2O in IS treatment at R3 stage (Figure 2); organic carbon tends to increase the water-holding capacity of a soil that in turn may increase the rate of denitrification [75]. Secondly, the role of organic carbon (C) as an electron donor in the denitrification process has widely been acknowledged [76]. On the other hand, a significant reduction in NO_3^- content in IS was observed during N fixation (Figure 2). Interestingly, NO_3^- content declined with time which may be due to immobilization. On the contrary, the NIS treatment, despite having higher DOC and sufficient NO_3^- content, did not emit more N_2O. Addition of CAN fertilizer in the NIS treatment did not change the pH of soil. However pH fluctuated in bare soil (Figure 3). CAN fertilizer contains NH_4^+ and calcium; NH_4^+ generally decreases pH but due to calcium that increases pH resulted buffering effect to soil pH (Figure 2). Probably, plants growing on NO_3^- generally maintain electronic neutrality by releasing anions including OH^- [77].

4.2. Total N%, C%, and CP% Contents of Plant Biomass after Harvest. The significantly higher contents of TN% and CP% in leaves and seeds and TC% in the leaves of NIS treatment (Figure 5) are credited to B. japonicum efficiency, which aspires to rapidly fulfill the N requirements of growing plants. Biological nitrogen fixation increases plant nitrogen content. As a result, the vigorous plant increases photosynthetic activity [78]. To fix one gram of N_2 by Rhizobium, the host plant invests 1–20 grams C to rhizobia that becomes available through photosynthesis [15]. Legume residues are rich in carbon and after incorporating in soil they can sequester C. The N fixation in root is directly dependent on the translocation of carbohydrates from the leaves [79].

Our results contradict with previous findings [80]; we did not observe reduced growth of nodulated plants compared to fertilized plants. The more recent studies support the significant interacting effects of inoculation on N [81–84], chlorophyll, crude protein, fiber, and carbohydrate contents [85] in soybean shoot.

The plant mobilizes a large quantity of N from vegetative tissue to meet the demand for seed N at pod filling stage [86]. It is evident from the data that seeds in both treatments contain more N than leaves (Figure 5), though N content was higher in leaves than in roots in both treatments. Marenco and Lopes [87] found similar results. The nodule numbers and root mass were consistent with the results found by Mosharof Hossain et al. [88].

Inoculation of *B. japonicum* strain enhanced the nodule formation, vegetative growth, and N uptake in soybean plants. Crude protein content is in the range of those observed by Alam et al. [67]. They further described that BNF is the main nitrogen source for seed protein content. In our results, crude protein content was 20.28% higher than noninoculated seed. Egamberdiyeva et al. [81, 82] also found similar results.

The TC% content was also significantly higher in the host plant in spite of providing C sources derived from plant photosynthesis to endosymbiont [89]. The experiment confirms that seed N reserves of inoculated seeds were as high as the treatments where mineral fertilizer was supplied. This may be attributed to the efficient transfer of mobile N and C from the vegetative part (leaf) to the reproductive part (seed) [90]. Rhizobia have fulfilled the N demand of the plant at different growth stages. Thus, earlier findings cannot be confirmed where N at the pod filling stage is exclusively supplied only by the soil [91]. Increasing N_2 fixation rates during the pod fill period, especially between stages R5 and R6, leads to the possibility of simultaneously improving yield and protein content [66].

The total N% content of IS treatment was statistically nonsignificant, which reveals that nodule formation did not affect the absorption and transport patterns of NO_3^- absorbed in the roots [61].

4.3. BNF and Soil Carbon Sequestration. Significant reduction was observed at R4 and R8 stages than R2 (Figure 1). The TC% content of roots of IS and NIS treatments was also not significantly different than each other (Figure 1). It can not be concluded that BNF process can sequester C. Decomposition and C release from nodules depend on the C/N ratio of the nodules. The nodules generally contain C/N ratio less than 1, which is likely prone to decomposition and releasing rapid C and N. Such rapid release can be an energy source for heterotrophs and can enhance N_2O emissions later on. Besides nodules, leaves contain also low C/N ratio that can also increase N_2O emissions after incorporation. The roots, however, in our analysis contain C/N ratio greater than 20 (Figure 5) in both treatments [33].

5. Conclusion

It is concluded that N_2O emissions from the rhizobia inoculated treatment were significantly higher than rhizobia free soils. Clearly, the BNF process due to release of H_2 during fixation stimulated the heterotrophs including *B. japonicum*, accelerating organic matter decomposition. Besides this, the denitrifying capability of *B. japonicum* also enhances N_2O emissions. Nodule senescence and decomposition during late growing stages and residues decomposition after harvest can also produce substantial N_2O. The C/N ratio of soybean residues, either inoculated or noninoculated seeds, can sequester carbon but enhance N_2O emissions. Similar studies with different rhizobial strains are suggested to investigate their denitrifying capabilities to enhance N_2O emissions during BNF process.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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