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Biological N fixation but not mineral N fertilization enhances the accumulation of N in peanut soil in maize/peanut intercropping system

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ABSTRACT

Legume/cereal intercropping has the potential to maximize the use of resources to raise yields due to enhanced nitrogen (N) fixation by legume root nodules, while high N fertilization may inhibit the nodulation of legume. However, whether legume/cereal intercropping can promote the accumulation of soil N storage with N fertilization and its underlying mechanism are less clear. Here, we evaluated the long-term (5 years) effects of maize/peanut intercropping and mineral N fertilization on peanut soil total N content and soil N cycling functional genes. The experiment includes two planting patterns (peanut maize intercropping and peanut monocropping) with three N fertilization rates (0, 150, and 300 kg N ha^{-1}). Intercropping increased soil total N content (STN) by average 18.2%, and the positive effect of intercropping on STN decreased with N application rate. Highest N application decreased the nodule fresh weight (NFW) by 64.3% and 46.0% in intercropping and monocropping system, respectively. However, intercropping has no effect on NFW. Intercropping increased the *nif*H gene abundance by average 26.5%. SEM analysis indicated that NFW and *nif*H gene abundance combined can explain 46% of the variance of STN. Our results indicate that biological N fixation but not mineral N fertilization enhances the accumulation of N in soil planted with peanut in maize/peanut intercropping system.

1. Introduction

Soil total nitrogen is an important indicator of soil fertility and one of the essential elements for plant growth. In soils, the total N content is co-determined by several processes, such as biological N fixation, soil organic N mineralization, nitrification and denitrification, which are all driven by soil microorganisms [1,6,15,16]. For example, ammonia oxidation is catalyzed by ammonia-oxidizing microorganisms, including ammonia-oxidizing bacteria (AOB, archaea (AOA) and complete ammonia oxidizing microorganisms (Comammox), while nitrifier and denitrifiers carry out nitrification and denitrification, respectively [12, 22,27]. Thus, change of soil microbial activities are expected to have great impact on soil N content. Functional gene, such as *nif*H (for N fixation), *amo*A (for ammonia oxidation), *nir*K and *nir*S (for denitrification), are commonly used to quantify the potential microbial activities that are responsible for specific N transformation processes [12].

Intercropping is a common practice that involves the simultaneous cultivation of two or more crop species in the same field. Cereal/legume intercropping is the most common intercropping system in agriculture [11,18]. It is reported that cereal/legume intercropping may enhance symbiotic fixation of N by means of increasing biological N fixation (; [5, 8]. In addition [3], suggested that abundance of *nif*H was higher in intercropping than in monocropping system. The *nif*H gene harbored by free-living N-fixing bacteria encode the Fe-protein subunit of nitrogenase which catalyzes biological nitrogen fixation [2]. The fixed N may improve the availability of N in soil and N status of plant. However, less was known whether the increased N fixation derived from

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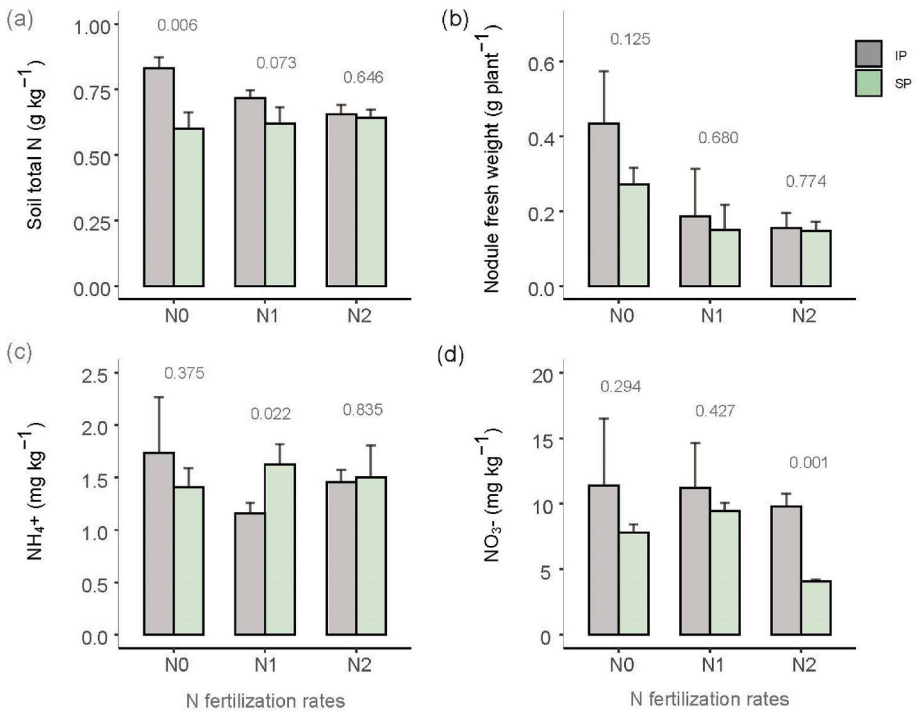

Fig. 1. Intercropping and N effects on soil total N (STN, **a**), nodule fresh weight (**b**), ammonium (NH_4+, **c**), and nitrate (NO_3-, **d**). IP, intercropping system; SP, monocropping system. Intercrop represents intercropping effect, and N represents mineral N effect. N0, N1 and N2 represent mineral N fertilization rates at 0, 150 and 300 kg N ha^{-1}, respectively. Error bars represent standard deviation (n = 3). Numbers above the bars represent the p values of pairwise comparison between intercropping and monocropping at each N fertilization level.

cereal/legume intercropping have an effect on soil total N pool. Long-term N fertilizer application has been widely proved can also increase the accumulation of N [10,19,31]. However, higher N application rate may depress biological N fixation by inhibiting nodulation of leguminous crop roots and the abundance of *nif*H gene [17,24]. It is still elusive how soil total N response to intercropping combined with N application, and its underlying microbial mechanisms.

Here, we evaluated the interactive effects of maize/peanut intercropping and mineral N fertilization on soil total N content, and evaluated which processes during N cycling are important for regulating total N content. We hypotheses that soil total N will increase under high N application in intercropping system, even if the nodulation of peanut root was inhibited.

2. Materials and methods

The experiment was conducted at the National Agricultural Experimental Station for Agricultural Environment, located on Liaoning Academy of Agricultural Sciences (41°49′N, 123°33′E), Shenyang, China. The climate is typically semiarid, with a mean annual precipitation of 700 mm and a mean annual temperature of 7.5 °C. The soil was classified as Brown Earth according to the Chinese Soil Taxonomy, with a soil bulk density of 1.38 g cm^{-3}, soil organic matter of 15.2 g kg^{-1} and soil total N of 0.62 g kg^{-1}. Two cropping systems were included: maize/peanut intercropping (IP) and peanut monocropping (SP). Each cropping system nested with three mineral N addition rates (same for maize): 0 (N0), 150 (N1), and 300 (N2) kg N ha^{-1}, 150 kg N ha^{-1} was recommended to get optimal peanut production in the experimental areas. All plots received 90 kg $ha^{-1}$of P and 105 kg $ha^{-1}$of K fertilizers, the N, P and K fertilizers were applied in furrow before sowing. Each treatment was replicated three times, thus, we totally have of 18 plots with length × width = 4 m × 2 m. All the plots were randomly distributed, and were separated by polypropylene with a depth of 100 cm into soils (see Supplementary Fig. 1 for the design of the experiment).

The experiment started in 2015. Maize and peanut were sown in May in each year. In both monocropping and intercropping systems, the sowing density for maize and peanut was 6 seeds m^{-2} and 24 seeds m^{-2}, respectively. The spacing between rows was 50 cm for all the cropping systems. The intercropping plots consisted of two rows of maize and two rows of peanut (see Supplementary Figs. 1b and 1c).

At 24th, August 2019 (the pod setting stage of peanut), 2 plants of peanut were sampled from each of the two inner rows in the monocropping plots (see Supplementary Figs. 1d and 1e). In intercropping plots, only the soils in peanut rows closed to maize were sampled, 4 plants of peanut were sampled from each row. Soil loosely adhering to the roots were shaken off. The remaining rhizosphere soils of the same plants of each plot were then homogeneously mixed and stored at −80 °C before DNA extraction as previously described by Ref. [14]. PCR protocols for N cycling marker genes were described in Ref. [3]. Fresh rhizosphere soil samples were immediately extracted with 1 M KCl for soil nitrate (NO_3-) and ammonium (NH_4+) measurements by an Auto-Analyser III Continuous Flow Analyzer (Bran & Luebbe, Norderstedt, Germany). Soil total N content (STN) was analyzed using elemental analyzer (Elementar III, Germany). For measuring peanut nodule fresh weight (NFW), 10 plants per row in intercropping plots and 5 plants per row in the inner two rows in monocropping plots were sampled, respectively. Nodules were removed by hand, and then weighed immediately. Peanut plants within a rectangle with length × width = 1.0 m × 0.5 m along the row were carefully dig up, and root biomass was measured after drying in an oven.

Linear regression model was conducted to detect the main effects of nitrogen and intercropping, and T-test was used to measure the difference between monocropping and intercropping. Pearson correlation was used to quantify the correlation between selected variables. The relationship between soil total C and root dry weight and NFW were stimulated using linear regression model. Structural equation model (SEM) was tested following a *d-sep* method employing 'piecewiseSEM' package (v2.1.0; [13]. Please see Supplementary Tables 1 and 2 for the detailed information how the SEM was constructed. All the statistics were conducted in R (v4.0.4; [25].

3. Results and discussions

Intercropping had significant effects on the abundances of *nif*H and *amoA*-AOB (Fig. 2a and c, and Supplementary Table 3). N fertilization significantly decreased the abundance of *amoA*-AOA (Fig. 2b, and

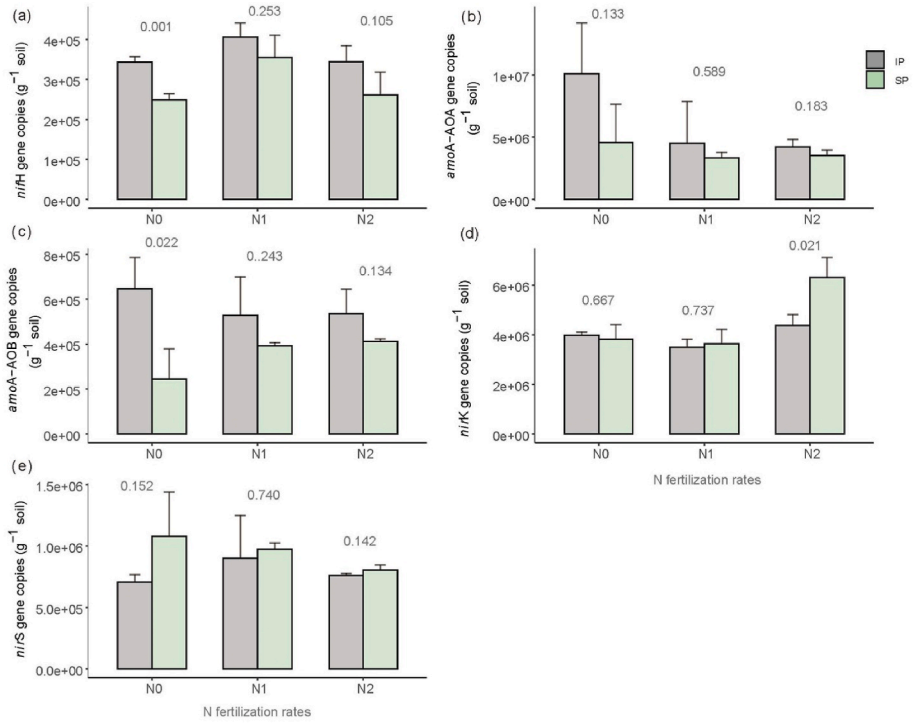

Fig. 2. Intercropping and N effects on *nif*H (**a**), *amo*A-AOA (**b**), *amo*A-AOB (**c**), *nir*K (d), and *nir*S (**e**). IP, intercropping system; SP, monocropping system. Intercrop represents intercropping effect, and N represents mineral N effect. N0, N1 and N2 represent mineral N fertilization rates at 0, 150 and 300 kg N ha^{-1}, respectively. Error bars represent standard deviation (n = 3). Numbers above the bars represent the *p* values of pairwise comparison between intercropping and monocropping at each N fertilization level.

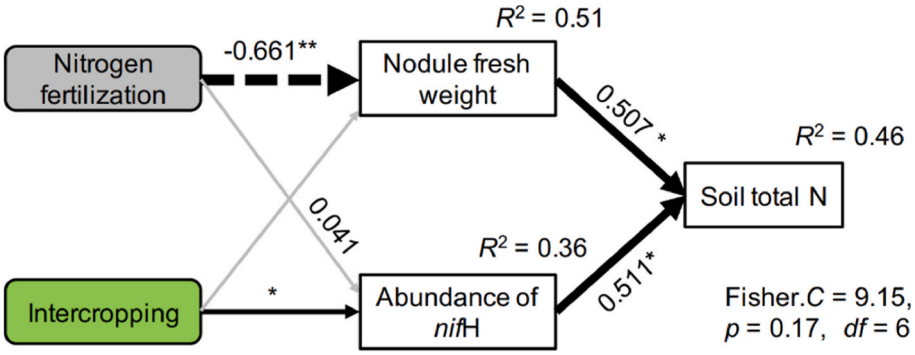

Fig. 3. (a) Structural equation modelling diagram representing connections between nitrogen cycling genes, nodule fresh weight (NFW), soil chemical properties and soil total N (STN). Soil TC, soil total carbon; soil C/N, the ratio of soil C to N. The width of the arrows represents estimates of the standardized path coefficients. Solid lines represent a positive relationship and dashed lines a negative relationship. Grey lines represent non-significant relationship. (b) Standardized total effect (direct plus indirect effects) of predictor variables on STN.

Supplementary Table 3), but highest N application increased the abundance of *nir*K (Fig. 2d). There was an intercropping and N interactive effect on the abundances of *amoA*-AOB. No intercropping, N and their interactive effect was observed for the abundance of *nir*S (Fig. 2e).

Intercropping significantly increased the soil total N content (STN) in peanut soils, while mineral N fertilization decreased the STN in the intercropping system (Fig. 1a and Supplementary Table 3). N fertilization showed a significant negative effect on NFW (Fig. 1b), NFW was significantly higher in intercropping compared with monocropping under the N0 treatment. Both N fertilization and intercropping had no effect on NH_4+ (Fig. 1c and Supplementary Table 3). Intercropping significantly increased the NO_3-, especially under high N rate (Fig. 1d). The decrease of NO_3- content under high N application rate may be due to the high denitrification rate (indicated as high *nir*K abundance), which enhance the loss of N from soil as gas [20].

The SEM indicated that 46% of the variation of STN were explained by the NFW, and abundance of *nif*H (Fig. 3). The NFW and *nif*H had positive effects on STN ($p < 0.05$) (Fig. 3). NFW (coefficient 0.507) and the abundance of *nif*H (coefficient 0.511) contribute almost equally to the variation of STN. NFW was only regulated by the N fertilization rate, while the abundance of *nif*H was mainly controlled by the cropping pattern. The contribution of NFW to STN may result from that N-fixing of nodules can increase the contents of macro-molecular and microbial-derived dissolved organic matter [28], resulting in the accumulation of soil organic N in the form of soil organic matter. In this study, both N application rates inhibited the nodulation of peanut (Fig. 1b), which can partially explain why long-term N application decreased the STN in intercropping system [5]. suggest that increase of STN may derive from plant root biomass. However, we found no relationship between STN and peanut root dry weight in this study (Supplementary Fig. 3). The abundance of *nif*H has been widely found positively related with soil biological N fixation [23,29,30], even if some of the *nif*H was recognized as pseudo-*nif*H sequences [21]. Our results indicated that intercropping can promote the *nif*H gene abundance in soils, which consistent with the result from Ref. [3]. The increase of *nif*H gene abundance in intercropping system may be induced by the growth of N fixation bacteria promoted by maize root exudates, such as flavonoids [9]. Negative effects of excessive N fertilization on the abundance of *nif*H are observed [4,26]. However, we found that no significant difference between the abundance of *nif*H with and without N fertilization, this is generally attributed to that N-fixers are often C limited due to lower C input from plant in soils without N fertilization [7].

In conclusion, this study shows that peanut soil total N significantly increased in peanut/maize intercropping system with low N input. The

accumulation of N in peanut soil is mainly regulated by biological N fixation. N fertilization has strong negative effect on legume nodulation, while intercropping induces asymbiotic biological N fixation. We therefore recommend adopting and optimizing the system of legume/cereal intercropping with reduced N application to improve soil fertility.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2022.100365.

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