*Nitrogen*

Article

# Effects of Multiple Global Change Factors on Symbiotic and Asymbiotic $N_2$ Fixation: Results Based on a Pot Experiment

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**Abstract:** Biological $N_2$ fixation, a major pathway for new nitrogen (N) input to terrestrial ecosystems, largely determines the dynamics of ecosystem structure and functions under global change. Nevertheless, the responses of $N_2$ fixation to multiple global change factors remain poorly understood. Here, saplings of two $N_2$-fixing plant species, *Alnus cremastogyne* and *Cajanus cajan*, were grown at rural and urban sites, respectively, with the latter representing an environment with changes in multiple factors occurring simultaneously. Symbiotic $N_2$ fixation per unit of nodule was significantly higher at the urban site than the rural site for *A. cremastogyne*, but the rates were comparable between the two sites for *C. cajan*. The nodule investments were significantly lower at the urban site relative to the rural site for both species. Symbiotic $N_2$ fixation per plant increased by 31.2 times for *A. cremastogyne*, while that decreased by 88.2% for *C. cajan* at the urban site compared to the rural site. Asymbiotic $N_2$ fixation rate in soil decreased by 46.2% at the urban site relative to the rural site. The decrease in symbiotic $N_2$ fixation per plant for *C. cajan* and asymbiotic $N_2$ fixation in soil was probably attributed to higher N deposition under the urban conditions, while the increase in symbiotic $N_2$ fixation per plant for *A. cremastogyne* was probably related to the higher levels of temperature, atmospheric $CO_2$, and phosphorus deposition at the urban site. The responses of $N_2$ fixation to multiple global change factors and the underlying mechanisms may be divergent either between symbiotic and asymbiotic forms or among $N_2$-fixing plant species. While causative evidence is urgently needed, we argue that these differences should be considered in Earth system models to improve the prediction of $N_2$ fixation under global change.

**Keywords:** actinorhizal plant; biological $N_2$ fixation; leguminous plant; rural–urban gradient; soil



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## 1. Introduction

Biological dinitrogen ($N_2$) fixation (BNF or $N_2$ fixation hereafter), a process by which diazotrophs convert atmospheric $N_2$ into ammonia catalyzed by nitrogenase, is a major pathway of external N input to terrestrial ecosystems [1]. As N is often the major limiting nutrient for net primary production, BNF is thus crucial in determining the structure and function of terrestrial ecosystems via its influence on soil N availability [2,3]. $N_2$ fixation can be divided into symbiotic $N_2$ fixation (SNF hereafter) and asymbiotic $N_2$ fixation (ANF hereafter). SNF is performed by diazotrophs residing in nodules of $N_2$-fixing plants, while ANF is conducted by diazotrophs freely distributed in ecosystem compartments such as soil [1]. Considering its key role in determining soil N availability, how $N_2$ fixation changes is tightly related to the responses of ecosystem structure, process, and function to multiple global change factors, including warming, altered precipitation, $CO_2$ enrichment, and atmospheric N or phosphorus (P) deposition.

Over the past few decades, a few studies have been conducted to explore the effects of global change factors on $N_2$ fixation [4,5]. Among these factors, warming, increased precipitation, and $CO_2$ enrichment generally benefit $N_2$ fixation, since $N_2$ fixation is an enzymatic and energetically expensive process [4,5]; however, drought and increased N deposition substantially inhibit $N_2$ fixation [4–6]. However, contrasting results have often been observed. For example, $CO_2$ enrichment suppressed nitrogenase activity in the nodules of Alfalfa (*Medicago sativa*) regardless of temperature and moisture conditions based on a pot experiment [7]. For another, ANF rates might not be suppressed by atmospheric N deposition in two subtropical forests [6,8]. Furthermore, very limited studies have explored the impacts of multiple global change factors on SNF or ANF, especially the former, probably owing to the difficulty in experimental layout with multiple factors [4]. Though quite a few studies include two factors, only one study has explored the response of SNF [7] or ANF [9] to three or more factors. Based on the limited studies, there are strong interactive effects of the global change factors on ANF rate or indices of SNF [5,7,9,10]. In a laboratory incubation experiment, the patterns of soil ANF response to moisture varied with temperature [5]. In a pot experiment with *Alnus hirsuta* and *Alnus maximowiczii*, the response of nitrogenase activity to $CO_2$ enrichment was insignificant, but the nodule biomass responses to $CO_2$ enrichment were divergent under high and low P availability [11]. Nasto et al. [12] reported that $CO_2$ enrichment significantly promoted SNF rates of four $N_2$-fixing plant species, but the positive effect was diminished or disappeared under N addition. Due to the complex interactive effects, results from studies with single or limited factors may not be suitable for predicting the responses of $N_2$ fixation to a global change in reality. As a matter of fact, the mechanistic representation of $N_2$ fixation is very weak in Earth system models [13,14], resulting in their poor performance in predicting the responses of $N_2$ fixation to global change in the boreal region [14]. It is hence urgently needed to investigate the responses of $N_2$ fixation to multiple global change factors using any suitable approaches.

The SNF of different $N_2$-fixing plant species may respond divergently to global change factors due to various $N_2$-fixation strategies or plant physiology. $N_2$-fixing plant species with a facultative $N_2$ fixation strategy down-regulate SNF as soil N availability increases, while those with an obligate $N_2$ fixation strategy would maintain SNF as soil N availability increases [15,16]. Menge et al. [16] explored the responses of SNF of eight $N_2$-fixing herbaceous species to N addition; they found that the SNF rates of six species decreased, and those of the other two species did not change under N addition. The response of SNF to $CO_2$ enrichment may also vary among $N_2$-fixing plant species. For example, West et al. [17] investigated the effects of $CO_2$ enrichment on SNF rates of four $N_2$-fixing plant species; they found that SNF rates of two species were stimulated, but those of the other two species were suppressed by $CO_2$ enrichment. Therefore, it is possible that SNF rates of different $N_2$-fixing plant species may respond divergently to multiple global change factors.

Rural–urban environmental gradients have often been used to simulate multiple global change factors, since cities experience higher levels of global change factors compared to the global average, including $CO_2$ concentration, temperature, N deposition and others [10,18]. Since actinorhizal and leguminous plants are the two main types of vascular plants that form symbioses with diazotrophs, two $N_2$-fixing plant species, i.e., *Alnus cremastogyne* (an actinorhizal species) and *Cajanus cajan* (a leguminous species), were included in the current study. The saplings of the two pant species were grown under rural and urban conditions, respectively. For comparison, SNF and soil ANF were determined. The major objectives are to address the following: (1) how would SNF and soil ANF respond to multiple global change factors, and (2) is there difference in the responses of SNF to multiple global change factors between the two $N_2$-fixing plant species?

## 2. Materials and Methods

### 2.1. Experimental Design

Huanjiang observation and research station for karst ecosystems in Huanjiang County (24°44′20″ N, 108°19′34″ E) and Guangxi University in Nanning City in Guangxi Zhuang Autonomous Region (22°51′19″ N, 108°17′13″ E) were used as rural and urban sites, respectively (Figure S1). For the rural site, mean annual temperature (MAT) is 20.1 °C with the lowest monthly temperature in January (9.4 °C) and the highest monthly temperature in August (27.1 °C); and mean annual precipitation (MAP) is 1603.3 mm with a wet season from April to September and a dry season from October to March. For the urban site, MAT is 21.9 °C with the lowest monthly temperature in January (15.5 °C) and the highest monthly temperature in September (28.1 °C); and annual average precipitation is 1548.7 mm with 80% contributed by wet season from April to September (Figure S2).

A pot experiment was conducted with saplings of two $N_2$-fixing plant species, i.e., *A. cremastogyne* and *C. cajan*. In March 2017, seeds of the two $N_2$-fixing plant species were sown separately in 0.3 L seedling bags. In April 2017, limestone soil (Luvisols) from the surface layer (0–30 cm) of a karst shrubland in Huanjiang observation and research station for karst ecosystems was collected and sieved to 1 cm, and then it was put into 20 pots with a volume of 13 L each after mixed thoroughly. Soil physicochemical properties are shown in Table 1. Specifically, soil pH was 7.80, and SOC, total N, and total P were 19.04, 1.68, and 0.81 g $kg^{-1}$, respectively. At the end of April, seedlings of the two $N_2$-fixing plant species were transplanted into the pots, keeping a single plant in each pot, with five replicates of each treatment. Each seedling was inoculated with a homogenate of nodules and rhizosphere soil of the corresponding plant species from the nearby forests to ensure inoculation, as performed by others [19,20]. At the beginning of May, five pots of each species were deployed to rural and urban sites, respectively. Plants were extirpate weed and desinsectization regularly during the experiment to ensure that their growth was not limited by other factors.

**Table 1.** Soil physicochemical properties before treatment. Values represent means ± standard errors (*n* = 3).

| Soil Parameters | Values |
|---|---|
| pH | 7.80 ± 0.04 |
| SOC (g C $kg^{-1}$) | 19.04 ± 3.32 |
| Total N (g N $kg^{-1}$) | 1.68 ± 0.21 |
| Total P (g P $kg^{-1}$) | 0.81 ± 0.08 |
| Exchange $Ca^{2+}$ (coml $kg^{-1}$) | 20.98 ± 0.61 |
| Exchange $Mg^{2+}$ (coml $kg^{-1}$) | 6.61 ± 0.07 |
| Available P (mg P $kg^{-1}$) | 6.80 ± 1.44 |
| Available Mo (μg $kg^{-1}$) | 24.96 ± 12.70 |

### 2.2. Sample Collection and Analysis

Rainwater and air samples were collected from May 2017 to April 2018. Rainwater was collected once per rainfall event for the measurement of nitrate ($NO_3^-$), ammonium ($NH_4^+$), dissolved organic N (DON), and dissolved P. The rainwater sample was filtered for the direct measurement of $NO_3^-$, $NH_4^+$, DON, and dissolved P using an auto-analyzer (Fiastar 5000; Foss Tecator AB, Höganäs, Sweden). Air samples were collected weekly for the determination of $CO_2$ concentration using an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermal conductivity detector.

All $N_2$-fixing plants were harvested in October 2017 after five months' growth, and they were then divided into shoots, roots, and nodules for each plant. A portion of the nodules with a few fibrous roots were used for the measurement of SNF rate in situ. The shoots, roots, and nodules were dried at 65 °C to constant weight to determine plant biomass and nodule biomass. Nodulation investment (mg nodule $g^{-1}$ plant) was calculated by the ratio of nodule biomass to whole-plant biomass. Fresh soils surrounding the roots were randomly collected and mixed thoroughly, and they were then divided into three parts. One part was used for the determination of ANF rates in situ, one part was stored at 4 °C for the measurement of soil available nutrients, and the third part was air dried for the analysis of soil physicochemical properties.

The fresh soil was sieved to 2 mm for the determination of soil $NO_3^-$ and $NH_4^+$, and the air-dried soil was sieved to 0.15 mm for the measurement of available P [21]. Soil $NO_3^-$ and $NH_4^+$ were extracted with 2 M KCl solution and analyzed by an auto-analyzer (Fiastar 5000; Foss Tecator AB, Höganäs, Sweden). Soil available P was extracted with 0.5 M $NaHCO_3$ at a pH of 8.5, and P contents were analyzed using the ascorbic acid molybdate method.

### *2.3. Determination of Biological $N_2$ Fixation Rate*

BNF rates were determined by the acetylene reduction method [22]. Fresh samples (10~15 nodules or 10 g soil) were put into 125 mL glass flasks with rubber stoppers. Then, 10% of the headspace in the flasks was replaced with high purity acetylene (99.99% purity). The flasks with sample only and acetylene only were also set up as references. All flasks were placed under the field conditions but away from direct sunlight. After incubation (0.5 h for nodule and 24 h for soil), 30 mL of gas sample from each flask was extracted with a syringe and subsequently injected into a pre-vacuumed 12 mL glass vial (Labco Exetainer, Labco Limited, Ceredigion, UK). The ethylene concentration in each gas sample was determined by an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector. The specific SNF (μmol $C_2H_4$ $g^{-1}$ nodule $h^{-1}$) or ANF (nmol $g^{-1}$ soil $d^{-1}$) rate was represented by acetylene reduction rate (ARA). The SNF rate per plant (or PNF hereafter; μmol $C_2H_4$ $plant^{-1}$ $h^{-1}$) was calculated by multiplying specific SNF rate by nodule biomass per plant. Since the current study aimed to investigate the response of BNF rate to multiple global change factors, the conversion factor which was used to transfer ARA to BNF was not determined.

### *2.4. Statistical Analysis*

All data were tested for normality and homogeneity of variances before the analysis. Two-way analysis of variance (ANOVA) was used to test the effects of site, species, and their interaction on indices of $N_2$ fixation and soil properties. *t*-test was used to examine the differences in atmospheric $CO_2$ concentration and deposition rates of N and P between the rural and urban sites. Pearson correlation analysis was adopted to analyze the relationship between $N_2$ fixation rates and soil physicochemical properties. The above analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

## 3. Results

### *3.1. Biological $N_2$ Fixation*

Specific SNF rate was significantly affected by site and its interaction with plant species; nodulation investment and PNF were significantly affected by site, plant species and their interaction, but specific soil ANF was only affected by site (Figures 1 and 2, Table 2). Specific SNF rate increased by 58.6 times at the urban site compared to the rural site for *A. cremastogyne*, but the rates were comparable between the two sites for *C. cajan*. Nodulation investment of *A. cremastogyne* and *C. cajan* decreased by 36.0% and 91.6% at the urban site relative to the rural site, respectively. The PNF of *A. cremastogyne* increased by 31.2 times, while that of *C. cajan* decreased by 88.2% at the urban site compared to the rural site. Specific soil ARA decreased by 46.2% at the urban site relative to the rural site.

### *3.2. Environmental Variables and Their Correlations with Biological $N_2$ Fixation*

Environmental variables differed significantly between the rural and urban sites (Table 3). MAT and atmospheric $CO_2$ concentration increased by 1.84 °C and 76 ppm, respectively, at the urban site relative to the rural site. The atmospheric deposition rates of $NO_3^-$, DON, total N, and P increased by 2.3, 1.4, 1.0, and 10.0 times at the urban site compared to the rural site, respectively, but $NH_4^+$ deposition was comparable between the two sites. Site had significant effects on soil $NO_3^-$, $NH_4^+$, and available P, but significant effects of plant species or the interaction between site and plant species were not found (Figure 3; Table S1). Soil $NO_3^-$, $NH_4^+$, and available P increased by 0.6, 0.6, and 1.4 times at the urban site compared to the rural site, respectively. Pearson correlation analysis showed that nodulation investment was significantly and negatively correlated with soil $NO_3^-$ (r = −0.48, $p$ = 0.03), $NH_4^+$ (r = −0.59, $p$ < 0.01), and available P (r = −0.60, $p$ < 0.01) (Table 4). Soil ARA was negatively correlated with $NH_4^+$ (r = −0.52, $p$ = 0.02) and available P (r = −0.49, $p$ = 0.03). Neither nodule ARA nor plant ARA was significantly correlated with soil physicochemical properties (Table 4).

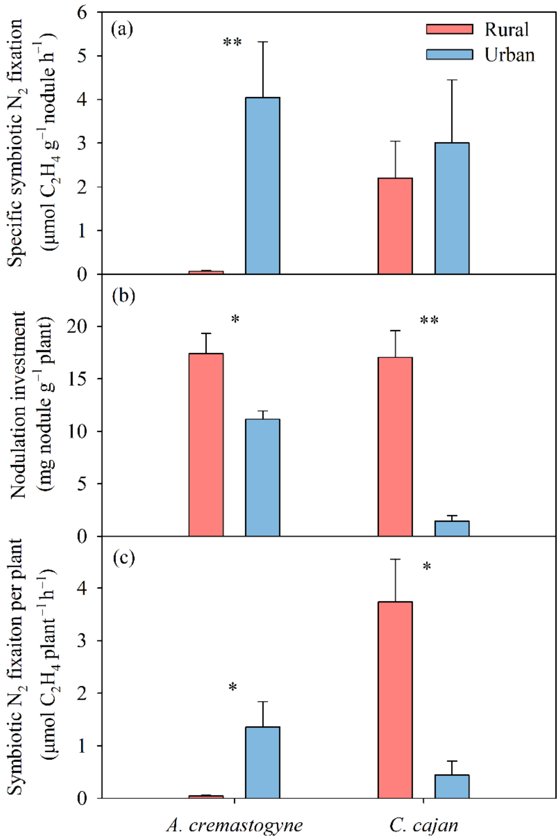


**Figure 1.** Indices of symbiotic $N_2$ fixation at the rural and urban sites: (**a**) specific rate of symbiotic $N_2$ fixation, (**b**) nodulation investment, and (**c**) symbiotic $N_2$ fixation for per plant (PNF). Bars represent mean values with standard errors (n = 5). * and ** denote significant difference at $p$ < 0.05 and $p$ < 0.01 between the rural and urban sites, respectively.

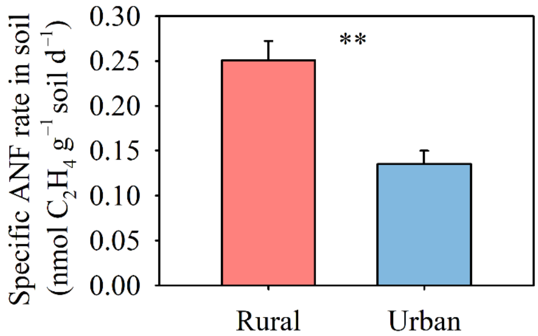


**Figure 2.** Specific rate of asymbiotic $N_2$ fixation in soil at the rural and urban sites. Bars represent mean values with standard errors (n = 10). ** denotes significant difference at $p$ < 0.01 between the rural and urban sites.

**Table 2.** Results from two-way ANOVA showing the effects of site (ST), species (SP), and their interaction on indices of biological $N_2$ fixation including specific rate of symbiotic $N_2$ fixation (SNF), nodulation investment, symbiotic $N_2$ fixation for per plant (PNF), and specific rate of asymbiotic $N_2$ fixation (ANF) in soil.

| Factors | Specific SNF Rate | | Nodulation Investment | | PNF | | Specific Soil ANF Rate | |
|---|---|---|---|---|---|---|---|---|
| | F Value | *p* Value | F Value | *p* Value | F Value | *p* Value | F Value | *p* Value |
| ST | 5.15 | 0.04 | 43.36 | <0.01 | 4.38 | 0.05 | 18.07 | <0.01 |
| SP | 0.27 | 0.61 | 9.18 | <0.01 | 6.77 | 0.02 | 0.42 | 0.53 |
| ST × SP | 4.54 | 0.04 | 7.94 | 0.01 | 14.25 | <0.01 | 0.21 | 0.65 |

**Table 3.** Atmospheric environmental variables at the rural and urban sites. Values represent means ± standard errors (n = 20 for $CO_2$ concentration and 49 for precipitation properties) except for annual average temperature and precipitation.

| Environmental Variables | Rural | Urban | *p* Value |
|---|---|---|---|
| Annual average temperature (°C) | 20.10 | 21.94 | – |
| Annual precipitation (mm) | 1603.3 | 1548.70 | – |
| Atmosphere $CO_2$ (ppm) | 499.5 ± 10.4 | 575.5 ± 22.1 | <0.01 |
| $NO_3^-$ deposition (kg N $ha^{-1}$ $yr^{-1}$) | 4.16 ± 0.50 | 13.89 ± 1.09 | <0.01 |
| $NH_4^+$ deposition (kg N $ha^{-1}$ $yr^{-1}$) | 15.92 ± 2.92 | 17.55 ± 1.91 | 0.64 |
| DON deposition (kg N $ha^{-1}$ $yr^{-1}$) | 10.17 ± 2.10 | 24.75 ± 2.79 | <0.01 |
| Total N deposition (kg N $ha^{-1}$ $yr^{-1}$) | 30.24 ± 4.58 | 59.86 ± 4.54 | <0.01 |
| P deposition (kg P $ha^{-1}$ $yr^{-1}$) | 0.33 ± 0.10 | 3.64 ± 1.05 | 0.01 |

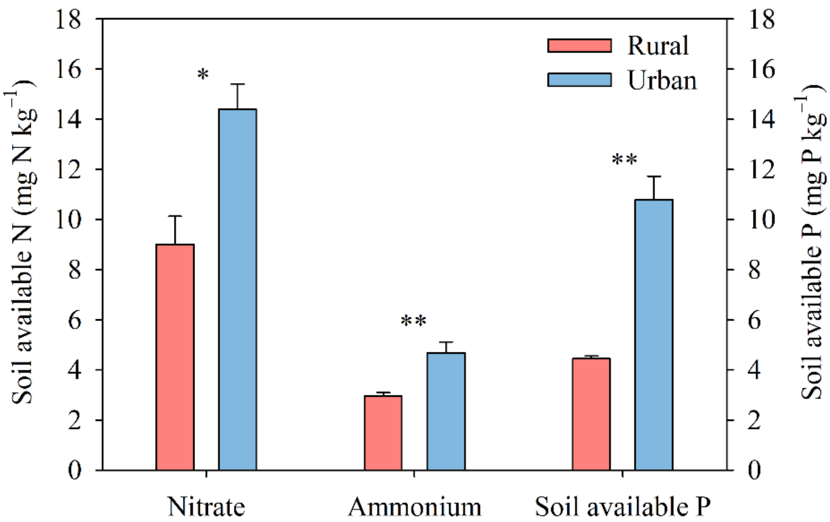


**Figure 3.** Soil N and P availability at the rural and urban sites. Bars represent mean values with standard errors (n = 10). * and ** denote significant difference at $p < 0.05$ and $p < 0.01$ between the rural and urban sites, respectively.

**Table 4.** Relationships between soil available nutrients and indices of biological $N_2$ fixation including specific rate of symbiotic $N_2$ fixation (SNF), nodulation investment, symbiotic $N_2$ fixation for per plant (PNF), and specific rate of asymbiotic $N_2$ fixation (ANF) in soil.

| Soil Nutrients | Specific SNF Rate | | Nodulation Investment | | PNF | | Specific Soil ANF Rate | |
|---|---|---|---|---|---|---|---|---|
| | r | *p* Value | r | *p* Value | r | *p* Value | r | *p* Value |
| Soil $NO_3^-$ | 0.34 | 0.14 | −0.48 | 0.03 | −0.33 | 0.16 | −0.30 | 0.20 |
| Soil $NH_4^+$ | 0.08 | 0.73 | −0.59 | <0.01 | −0.25 | 0.28 | −0.52 | 0.02 |
| Soil available P | 0.31 | 0.19 | −0.60 | <0.01 | −0.30 | 0.19 | −0.49 | 0.03 |

## 4. Discussion

### *4.1. Response of Symbiotic $N_2$ Fixation to Multiple Global Change Factors Depends on $N_2$-Fixing Plant Species*

Our results show that the responses of PNF to multiple global change factors were divergent between the two plant species. Since PNF is determined by both specific SNF rate and nodulation investment, we would discuss the responses of the two indices separately. The specific SNF rate is influenced by a few factors, including N deposition, soil P availability, atmospheric $CO_2$ concentration, temperature, etc. [11,19,23,24]. Specific SNF rate is usually suppressed or not altered by N deposition depending on $N_2$ fixation strategies of the $N_2$-fixing plants [20,25]. Specific SNF rates of plants with a facultative $N_2$ fixation strategy are often suppressed by N deposition, whereas those with obligate $N_2$ fixation strategy are less sensitive to N addition [16]. Therefore, the higher N deposition at the urban site would theoretically not alter specific SNF rate of *A. cremastogyne*, but it would inhibit that of *C. cajan* in the current study, since actinorhizal plants are usually obligate, while leguminous plants are usually facultative in their $N_2$ fixation strategies [15]. The increase in specific SNF rate for *A. cremastogyne* or unaltered specific SNF rate for *C. cajan* at the urban site suggests that the effect of N deposition may have been overridden or offset by the positive effects of other factors.

Promotion of specific SNF rate by increased soil P availability or atmospheric $CO_2$ has been demonstrated in several studies [19,24,26]. P is a necessary element since $N_2$ fixation needs large amount of adenosine triphosphate (ATP), and P plays a key role in regulating $O_2$ near nitrogenase, which catalyzes $N_2$ fixation, but it depends on legume species [27,28]. P may also regulate SNF via its fertilization role in the growth of $N_2$-fixing plants, which supply carbon to diazotrophs residing in nodules in the exchange of N [26]. This mechanism is also applicable to the regulation of atmospheric $CO_2$ on SNF, since atmospheric $CO_2$ enrichment usually stimulates plant growth given that there is sufficient P availability [11,19]. In a pot experiment, the specific SNF rate of *Inga punctata* increased by 3.5-fold under P addition [19]. In the current study, the higher P deposition and atmospheric $CO_2$ level at the urban site was probably responsible for the higher specific SNF rate of *A. cremastogyne* relative to the rural site. Meanwhile, a recent study revealed that the optimum temperature ranged from 29.0 °C to 36.9 °C for SNF conducted by *Rhizobia*-type or *Frankia*-type diazotrophs [29]. Correspondingly, the higher MAT could be another reason for the higher specific SNF of *A. cremastogyne* at the urban site. Nevertheless, the aforementioned explanations are plausible, and further investigations are needed to unravel the underlying mechanisms.

The nodulation investment was decreased under environmental change for both plant species in the current study. Previous studies showed that the nodulation of $N_2$-fixing plants could be influenced by atmospheric $CO_2$ concentration, soil P availability, and N deposition [19,20,30]. Positive effect of $CO_2$ enrichment on nodulation has been demonstrated by several studies [7,11,30], which reported that $CO_2$ enrichment significantly improved the nodule number and nodule biomass. In the current study, the higher atmospheric $CO_2$ concentration at the urban site should theoretically enhance the nodulation investment of the two plant species. Therefore, the decrease in nodulation investment was probably caused by other factors. Many previous studies have reported the promotion of nodulation by P addition [19,28,31,32]. For example, Wurzburger and Hedin [19] found that nodule biomass of *Inga punctata* increased by 1.8-fold under P addition. However, nodulation investment decreased with soil available P in the current study. This negative relationship should be apparent, and other factors might have a stronger negative effect on nodulation. A few studies have reported the N addition suppressed nodulation investment [20,33–35]. For example, Batterman et al. [20] reported that N addition suppressed the nodulation investment *Inga punctata* by 85% based on a pot experiment. Lin et al. [35] revealed that high $NO_3^-$ levels suppressed the nodulation investment by inhibiting the biosynthesis of cytokinin required for nodulation. Similarly, the decreased nodulation investment was accompanied by higher soil $NO_3^-$ and $NH_4^+$ levels at the urban site of the current study. Therefore, the decreased nodulation investment of both $N_2$-fixing plant species was probably due to the higher N deposition at the urban site.

### *4.2. Decrease in Soil $N_2$ Fixation in Response to Multiple Global Change Factors*

In the current study, specific ANF rate in soil was reduced under the urban conditions. Specific ANF rate in soil can be influenced by temperature, soil available P, and N deposition [5,27]. A meta-analysis revealed that warming promoted soil ANF [4]. Our previous study showed that soil ANF rate increased with temperature with the optimum temperature being higher than 35 °C based on a laboratory experiment using soils collected at a nearby karst forest [5]. Therefore, the higher temperature may have stimulated specific soil ANF rate at the urban site of the current study. P usually benefits soil ANF via its key role in ATP synthesis and maintenance of nitrogenase activity [27]. The promotion of P on soil ANF has been shown in many previous studies [36–38]. Thus, higher P deposition may also have promoted specific soil ANF rate at the urban site in the current study. The decreased specific soil ANF rate at the urban site indicates that the positive effects of temperature and P deposition may have been overridden by the negative effects of other factors. The inhibition of N deposition on soil ANF has been frequently demonstrated [6,27,39,40]. For example, our previous study found that high N addition decreased soil ANF by 17.1% in a karst forest [6]. Zheng et al. [40] found that soil ANF decreased by 33.7% under N addition based on a global data synthesis. This is because $N_2$ fixation consumes large amount of C and energy, so that soil diazotrophs prefer to obtain N directly from soils and hence down-regulate $N_2$ fixation if there is an abundance of available N [41]. In the current study, specific soil ANF rate was negatively correlated with soil N availability, corroborating that higher N deposition at the urban site was probably responsible for the lower specific soil ANF rate.

## 5. Conclusions

Our results suggest that the responses of biological $N_2$ fixation to multiple global change factors may be divergent either between symbiotic and asymbiotic forms or among $N_2$-fixing plant species. It should be noted that uncertainties exist in the current study. First, our study was based on a short-term experiment, so we are not sure whether the patterns from long-term response of $N_2$ fixation to multiple global change factors are similar. Second, the findings were obtained from a pot experiment, so we are not sure whether they are applicable to field conditions. Third, altered precipitation pattern is one major aspect of global change, but it was not considered in the current study. Fourth, the patterns of global change or the combinations of multiple global change factors may vary among regions, so the rural–urban environmental gradient in the current study may only represent one scenario of global change. Considering the uncertainties, more investigations are urgently needed in order to obtain a better understanding of the responses of $N_2$ fixation to multiple global change factors.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nitrogen4010011/s1, Figure S1: Schematic showing the geographic locations of the rural and urban sites; Figure S2: Daily and monthly air temperature and cumulated precipitation at rural and urban sites in 2017. (a) Daily mean air temperature and daily precipitation at rural site; (b) daily mean air temperature and daily precipitation at urban site; (c) monthly air temperature and cumulated precipitation at rural site; (d) monthly air temperature and cumulated precipitation at urban site. Data were obtained from Chinese National Meteorological Centre (https://data.cma.cn/) in 20 May 2020; Table S1: Results of two-way ANOVA showing the effects of site (ST), species (SP), and their interaction on soil $NO_3^-$, $NH_4^+$, and available P.

**Author Contributions:** Conceptualization, D.L.; methodology, Z.W. and X.S.; software, Z.W. and X.S.; validation, D.L., Z.W., X.S. and H.C.; formal analysis, Z.W. and X.S.; investigation, Z.W. and X.S.; resources, D.L., Z.W., X.S. and H.C.; data curation, Z.W. and X.S.; writing—original draft preparation, D.L., Z.W. and X.S.; writing—review and editing, D.L., Z.W. and X.S.; visualization, Z.W. and X.S.; supervision, D.L.; project administration, D.L. and Z.W. All authors have read and agreed to the published version of the manuscript.

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